SCHEDULE

Brucellosis 2014 International Research Conference

Including the 67th Annual Brucellosis Research Meeting

Berlin, 9-12 September 2014

FRIEDRICH-LOEFFLER-INSTITUT
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Bundesforschungsinstitut für Tiergesundheit
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Brucellosis 2014
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Brucellosis 2014 International Research Conference

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Imprint

Abstracts

Brucellosis 2014 International Research Conference

All authors are responsible for the content of their respective abstracts.

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Welcoming Addresses

Andreas Hensel

President of the Federal Institute for Risk Assessment (BfR)

Welcoming address by the President of the Federal Institute for Risk Assessment

More than 100 years after the first description of *Micrococcus melitensis* by Sir David Bruce, brucellosis is still of major public health concern both in endemic and non-endemic countries all over the world. The impact on animal and human health is tremendous and eradication and control of this zoonotic disease remains a global and interdisciplinary challenge. Although eradicated in German livestock brucellosis is still a matter of interest because it has emerged as a disease among immigrants associated with diagnostic delays, possibly resulting in treatment failures, relapses, chronic courses, focal complications, and a high case-fatality rate. The identification of health risks is the guiding principle for our work in the field of food safety and consumer protection. A total of 800 employees spare no effort to prove that *no risk is more fun*.
I am delighted to host the Brucellosis 2014 International Research Conference at the Federal Institute for Risk Assessment, here in Berlin, the capital city of Germany, which was home and domain of so many Nobel laureates. Emil von Behring got the first Nobel Prize in Medicine for his work on serum therapy, especially its application against diphtheria; Robert Koch first identified pathogenic microorganisms as the cause of infectious diseases such as tuberculosis, anthrax and cholera; and Paul Ehrlich developed the first antibiotic drug to treat syphilis and is regarded as the founder of chemotherapy. By the way, Robert Koch is probably the most famous staff member of the Imperial Health Agency which is the predecessor of our institution, the Federal Institute for Risk Assessment.
Berlin is a breakthrough city combining tradition and modernity and you have to pass the Brandenburger Tor to enjoy its spirit of freedom and creativity. From 9 to 12 September 2014 my team and I will do everything for you to ensure that you can feel like ‘Ich bin ein Berliner’.

It is a great honor for us to welcome the *Brucella* research community and I wish you an inspiring meeting with fruitful discussions.

Karin Schwabenbauer
Head of Directorate *Animal Health, Animal Welfare* of the Federal Ministry of Food and Agriculture (BMEL)
President of the World Organisation for Animal Health (OIE)

Welcoming address by the German Federal Ministry of Food and Agriculture

The control of zoonotic diseases has been a focus of the Federal Ministry's scientific support for many years. For some time we have known that a one-sided view of animal populations, public health, food safety and the security of global trade cannot provide the large-scale success in the fight against zoonotic diseases which we wish to achieve, and which citizens expect us to achieve. It is only if health authorities, veterinary authorities, the agricultural sector, the food industry, the scientific community and an informed public work together that we will succeed, in the sense of 'One Health', in attaining our goal of successfully controlling zoonotic diseases. In Germany we therefore launched the 'National Zoonotic Research' platform in 2006 which incorporates all necessary scientific disciplines. One distinctive feature of this programme is that it only supports projects that are multidisciplinary. Germany is in the happy position of its cattle, sheep and goat stocks having been officially free of brucellosis since 2000 - only a few sporadic outbreaks have been reported since. This success has been the result of hard work. It is only possible to maintain this status through an ongoing, legally binding national control procedure, substantial funds and a nationwide monitoring system that is still in place. This successful example of disease control can act as encouragement for, and a basis for control procedures in, those countries in which brucellosis still causes considerable difficulties for animals and people. However, account must be taken of the specific socio-economic and socio-cultural situation in each country. Germany, for instance, cannot be compared with countries in which the pastoralism of sheep and goats represents an important means of livelihood. Or in which the health services do not have sufficient funds at their disposal. It is our duty as the international community to provide help and assistance in , one of the most prevalent food-related zoonotic diseases in the world, with the consumption of raw milk and raw-milk cheese representing the most common means of transmission. Although pasteurisation of milk is consequently a very effective measure for minimising cases of brucellosis in mankind, the best way of eradicating brucellosis in animals and mankind is to monitor animals stocks.Although Germany is deemed officially free of brucellosis, it is still particularly important, in developing more efficient methods of detection, and in particular in assessing individual animals, to achieve success at international level and thus contribute to containing these zoonotic diseases. As false positive diagnoses still lead to considerable losses in international trade, I hope - also in my capacity as President of the OIE - that this conference will provide corresponding proposals that can then be incorporated into the OIE Manual.

I wish you all the best for this conference; I hope that you will have stimulating discussions that result in new ideas for successfully fighting zoonotic diseases.

Thomas Mettenleiter
President of the Friedrich-Loeffler-Institut (FLI)

Welcoming address by the President of the Friedrich-Loeffler-Institut

Brucellosis accompanied mankind as a life threatening zoonosis and burden for livestock since the domestication of sheep, goats, cattle and pigs to improve quality of human life. Proof of high disease prevalence in humans can already be found in the skeletal remains of individuals from ancient Egypt or Roman Pompeii. The importance of brucellosis for modern human and veterinary public health is demonstrated by approx. 500 000 new human infections worldwide every year, its rapid re-emergence in developing countries and countries struck by civil disruption, and its continuing presence around the Mediterranean basin and the Levant despite enormous efforts for control presently supported by the EU. Therefore, research on brucellosis epidemiology, prophylaxis and control is well within the mission of the Friedrich-Loeffler-Institut as Federal Research Institute for Animal Health and OIE Collaborating Centre for Zoonoses in Europe. The OIE reference laboratory for brucellosis at FLI currently is engaged in projects in Africa, Southern America, Europe and Asia. With our new research center on the island of Riems, best equipped and modern P3 laboratories and experimental animal facilities are available for cutting-edge research projects. The work of our brucellosis laboratory will also benefit from new facilities to be built at the FLI site in Jena. Personally, I am very glad that FLI can and will continue to make important contributions to worldwide control and eradication of brucellosis inspired by the idea of ‘One world – one health’. Eradication of brucellosis is feasible as demonstrated by the experiences of countries like Germany where brucellosis was eradicated successfully from domestic livestock during the last century by applying consequent veterinary measures. I am also very glad that we have the pleasure to host the Brucellosis 2014 International Research Conference in Germany for the first time.

I wish you an inspiring meeting, a fruitful exchange of ideas and pleasant days in Berlin, the lively capital of Germany.

Sascha Al Dahouk, Chair
Federal Institute for Risk Assessment

Heinrich Neubauer, Co-Chair
Friedrich-Loeffler-Institut

Herzlich Willkommen

Қош келдіңіздер خوش دی‌آمد Soo dhawaada

에 오신 것을 환영합니다 Welkom Bine ai venit

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欢迎 Karibu Καλώς ήλθατε ברכה Bienvenido

Bem-vindo Mirë se erdhët Vitajte Velkommen

Tervetuloa आपका स्वागत है أَهْلاً وَسَهْلاً Bienvenue

Witamy Üdvözöljük բարի գալուստ Velkommen til

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Andreas Hensel
Federal Institute for Risk Assessment, Berlin, Germany

Karin Schwabenbauer
Federal Ministry of Food and Agriculture, Berlin, Germany

FAO works to curb the burden of brucellosis in endemic countries
Ahmed El Idrissi

Global research on *Brucella* species 1950-2013
Mahmoud Abo-Shehada

10:15 a.m. – 11:00 a.m. Coffee Break

11:00 a.m. – 12:15 a.m. **Session 1: Taxonomy, Evolution, Emerging Species**

Chair: David O'Callaghan

Keynote Lecture: **Erko Stackebrandt**
Leibniz Institute DSMZ, Braunschweig, Germany

Full taxonomic description of the proposed eleventh *Brucella* species, *Brucella papionis sp. nov*., isolated from baboons
Adrian M. Whatmore

Round-Table

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Chair: Jean-Jaques Letesson

Keynote Lecture: **Stephan Köhler**
Centre d'études d'agents Pathogènes et Biotechnologie pour la Santé, Montpellier, France

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Chair: Renato de Lima Santos

Keynote Lecture: **Sean Crosson**
University of Chicago, Department of Biochemistry and Molecular Biology, Chicago (IL), USA

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Chair: Thomas A. Ficht

Keynote Lecture: **Stefan H.E. Kaufmann**
Max Planck Institute for Infection Biology, Berlin, Germany

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Chair: David W. Pascual

Keynote Lecture: **Steven C. Olsen**
National Animal Disease Center, United States Department of Agriculture, Ames (IA), USA

Cloning, expression and immune response against protective antigens of *M. bovis* in *B. abortus* vaccine strain RB51
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National Health Service and Agri-Food Quality (SENASA), Buenos Aires, Argentina

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Chair: Rebekah Tiller

Keynote Lecture: **Assimoula Economopoulou**
European Centre for Disease Prevention and Control (ECDC), Stockholm, Sweden

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Swiss Tropical and Public Health Institute (TPH), Basel, Switzerland

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Chair: Philip H. Elzer

Keynote Lecture: **Jacques Godfroid**
University of Tromsø, Department of Arctic and Marine Biology, Tromsø, Norway

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03:30 p.m. – 04:00 p.m. Poster Award/Closing Remarks and Farewell

Abstracts

Opening Lectures

FAO works to curb the burden of brucellosis in endemic countries

Ahmed El Idrissi and Katinka de Balogh

Food and Agriculture Organization of United Nations, Rome, Italy

Brucellosis is recognized as a significant public health challenge, with major economic and financial burdens in countries where the disease remains endemic. To address the global threat of brucellosis for both animal health and public health, the Food and Agriculture Organization of the United Nations (FAO) has been advancing practical knowledge and experience of brucellosis in various countries and assisting the development of sound strategies and policies for sustainable control programmes. Technical support has been provided to selected countries where brucellosis has significant impacts on human health and livestock on which households depend for income and food security.
The FAO brucellosis programme promotes capacity building; provides technical support, cutting-edge knowledge and practical experience in laboratory diagnostics and surveillance; and assists the development and implementation of sound strategies for sustainable control programmes against brucellosis in livestock. The control strategies promoted by FAO in endemically infected countries aim to reduce prevalence and disease in susceptible species, therefore limiting spread within and among flocks and herds, using long-term vaccination as the main tool. The FAO brucellosis control programme in Tajikistan is one of the most successful in Central Asia and is a model for other countries in the region.As part of ongoing efforts to assist member countries in launching and improving programmes aimed at controlling and eradicating brucellosis in animals and humans, FAO has developed a roadmap with a stepwise approach for progressive control of the disease. This roadmap is based on information from extensive research and practical experience in the field in many countries and over many years. The greatest challenge facing FAO's programme is how to tackle the huge brucellosis disease burden in countries where the disease is endemic or re-emerging. Achieving effective control of brucellosis in these countries will require long-term commitment from all parties, with application of appropriate policies and control strategies. Country programmes must be based on reliable, science-based data and information. Estimates of initial prevalence in both humans and livestock and of disease distribution are essential to ensure that the country strategies planned have a high likelihood of success. To accomplish this, realistic impact analyses and cost-benefit studies will be needed to support selection and planning. Pilot studies in selected regions would also be beneficial.

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Global research on *Brucella* species 1950-2013

Mahmoud Abo-Shehada

Faculty of Epidemiology and Population Health, London School of Hygiene & Tropical Medicine, London, England

Systematic search for *Brucella* research output produced worldwide (190 countries) between 1950 and 2003, and 2004 to 2013 was carried out using the Web of Science (WoS) database of Thomson Scientific's Essential Science Indicators database. *Brucella* research was compared and ranked based on the number of articles, average citation per article and Hirsh-Index. For the time period of 63 years 12,036 articles on *Brucella* are listed in WoS database, 81 % were produced by 20 countries and 46 % in the last 10 years. During 1950 to 2003, 55.3 % (105 of 190) produced at least one article. The highest number of articles was 1,959, and the quartiles were Q1: 3, Q2: 7, Q3: 45. The maximum H-Index for the period was 87, Q1: 2, Q2: 4, Q3: 13. During the last ten years 60.5 % (115 of 190) of countries produced at least one article, maximum 1,072, Q1: 2, Q2: 9.5, Q3: 34.75. USA, Spain, France and India were consistently in the top 10. Turkey and Argentina improved their ranks from 10th to 2nd and from 8th to the 5th, respectively. China, Brazil, Germany and Italy joined the top ten. The maximum H-Index was 56, Q1: 1, Q2: 4, Q3: 10. USA, France and Spain topped the list of the H-Index for the two periods. Germany and Argentina improved their rank from 7th to 4th and from 9th to 6th,respectively. England, Belgium and Canada were consistently in the top ten, and Italy and Turkey joined. When normalized to population using growth domestic product per capita the output of most high income countries lagged behind those with middle income. The last 10 years witnessed 5 % increase in *Brucella* active research countries and an increase in the number of articles produced. In the developing countries, brucellosis research strengths and needs were identified.

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Session 1: Taxonomy, Evolution, Emerging Species

Keynote Lecture: Erko Stackebrandt
Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH, Braunschweig, Germany

Against the wind: The taxonomy of the genus *Brucella*

The epoch-making work of Carl Woese on the discovery of the three domains of life and the use of ribosomal chronometers marked a paradigm shift in microbial taxonomy in that semantides instead of superficial phenotypic properties were used to position a microbial strain next to its phylogenetic neighbour. Most taxonomic schemes in use today include measures of evolutionary relationships based on gene sequence similarities (most notably the 16S rRNA gene and genes used in Multi-Locus Sequence Analysis [MLSA]), in order to determine the phylogenetic position of an isolate. Knowing its place in the hierarchic scheme a strain can be either affiliated to a known species or described as a novel species by determination of novelty by the so called polyphasic approach. Here, a broad range of chemotaxonomic, physiological and cultural properties are investigated in order to assess novelty and to measure genomic uniqueness by DNA-DNA hybridization (DDH) similarities between the new strain and type strains of neighbouring species which share higher than 98.5 % 16S rRNA similarities. Though only the naming of a prokaryotic taxon is governed by the Bacteriological Code over time also the methods to be applied to describe a novel species and their interpretation became more and more regulated as laid down in individual minimal standards. This lead to the uncomfortable position of taxonomy to be recognized as an orthodox field, meaning that the description of taxa (and here especially the species) follows fixed rules for defining taxonomic units which are artificial and arbitrary. Granted, the present application of such rules provided bacteriology with a hitherto unknown stability but prevented progress made recently in ecology and methodological advances.
A convincing species concept has not yet replaced the present species definition because due to intrinsic properties of the prokaryotic cell different major branches and twigs of the phylogenetic tree underwent different mode and tempo of evolution and were subject to different degrees of horizontal gene transfer; hence, more than a single species concept may encompass the prokaryotic domain. As at present the pragmatic species definition cannot be replaced by a theory-based species concept, it continues to adopt novel approaches only hesitantly and so far ignores advances in ecology and genomic information. MLSA has the discriminating resolution power to look into the intraspecific structure (mainly of strains with clinical relevance) and has in many cases demonstrated the existence of discrete subspecific clusters which correlate with their ecological niche. On the other hand, ecologically distinct populations are able to coexist in the same region and in the absence of phenotypic differences used in the classification of the traditional taxonomy the concept of the ecotypes was not further developed though some authors advocate the use of ecotypes as an alternative for describing microbial diversity.
At the methodological level the substitution of the slow and ‘black box’ DDH approach by the average amino acid identity method has been proposed but in the absence of mandatory generation of at least draft genome sequences this worthy suggestion was ignored by taxonomist. It was not considered timely as no minimal standard included this or other genomic based approaches, e.g. supertree analysis genome distance, genomic signatures codon usage bias, metabolic pathway content, core and pan genome analysis, and *in silico* proteome analysis in the list of mandatory requirements for species description.
One of the few examples for the deviation from the traditional circumscription of the taxon ‘species‘ is the genus *Brucella* (*B.*), class Alphaproteobacteria. The genus is the type genus of *Brucellaceae*, having *Ochrobactrum, Pseudochrobactrum* and *Paenochrobactrum* (www.arb-silva.de/projects/living-tree/) as neighbours. Six *Brucella* species were described until 2006, all of which exhibit distinct host preferences, though cross-species infections do occur and other hosts may be infected. However, the fact that type strains were highly relat-

ed among each other lead to the notion that they are all members of the same species for which *B. melitensis* has priority. The other species were considered synonyms and named biovars. This opinion was published in the subcommittee meeting in 1986, published by the International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of *Brucella* (1988). Interestingly, the subspecies concept was never used. However, host specificity, serology, phenotypic traits and biovar specific molecular identification techniques such as identifying IS711 and OMPs, and applying ERIC, APCR, MVNTR, MLST, MSLA, SNPs, PFGE, microarray, chromosome size allowed differentiation among the biovars, reinforcing the view that the biovars should be considered species. It was the *Brucella* Subcommittee that advocated a return to pre-1986 *Brucella* taxonomy with the consequence that six *Brucella* nomenspecies were re-approved. In contrast to current thinking, these authors and the *Brucella* subcommittee members stated that the DDH method is not discriminating enough to solve all problems of bacterial species delineation. It shows that the community of taxonomists can change orthodox taxonomic thinking to make better sense of scientific knowledge. In July 2014, the Gold database of genome sequences lists 414 entries for draft, incomplete and complete *Brucella* genome sequences, sufficient material for discussing similarities and differences at the genetic and epigenetic level.

Co-Authors: Carmen Scheuner and Markus Göker

Oral Presentations (O1)

O1: Full taxonomic description of the proposed eleventh *Brucella* species, *Brucella papionis* sp. nov., isolated from baboons

Adrian M. Whatmore[1], Nicholas Davison[2], Axel Cloeckaert[3,4], Sascha Al Dahouk[5], Michel S. Zygmunt[3,4], Simon D. Brew[1], Lorraine L. Perrett[1], Mark S. Koylass[1], Gilles Vergnaud[6,7,8], Christine Quance[9], Holger C. Scholz[10], Edward J. Dick Jr[11], Gene Hubbard[12] and Natalia E. Schlabritz-Loutsevitch[13]

[1]OIE/WHO/FAO Brucellosis Reference Laboratory, Department of Bacteriology, Animal Health and Veterinary Laboratories Agency (AHVLA), Addlestone, United Kingdom; [2]Animal Health and Veterinary Laboratories Agency (AHVLA), Polwhele, Truro, United Kingdom; [3]INRA, UMR1282 Infectiologie et Santé Publique, Nouzilly, France; [4]Université François Rabelais de Tours, UMR1282 Infectiologie et Santé Publique, Tours, France; [5]Federal Institute for Risk Assessment (BfR), Berlin, Germany; [6]Univ Paris-Sud, Institut de Génétique et Microbiologie, Orsay, France; [7]CNRS, Orsay, France; [8]DGA/MRIS, Mission pour la Recherche et l'Innovation Scientifique, Bagneux, France; [9]Mycobacteria and Brucella Section, National Veterinary Services Laboratories, USDA-APHIS, Ames, IA, USA; [10]Bundeswehr Institute of Microbiology, Munich, Germany; [11]Southwest National Primate Research Center, Texas Biomedical Research Institute, San Antonio, TX, USA; [12]University of Texas Health Science Center at San Antonio, Department of Pathology, San Antonio, TX, USA; [13]University of Texas Health Science Center at San Antonio (UTHSC), Department of Obstetrics and Gynaecology, San Antonio, TX, USA

Two Gram-negative, non-motile, non-spore-forming coccoid bacteria isolated from baboons (*Papio* spp.) that had delivered stillborn offspring were subjected to a polyphasic taxonomic study. On the basis of 16S rRNA sequence similarities both strains, which possessed identical sequences, were assigned to the genus *Brucella*. This placement was confirmed by extended multilocus sequence analysis (MLSA), where both strains possessed identical sequences, and whole genome sequencing of a representative isolate. All the above analyses suggested that the two strains represent a novel lineage within the genus *Brucella*. The strains also possessed a unique profile when subjected to the phenotyping approach classically used to separate *Brucella* species reacting only with *Brucella* A monospecific antiserum, being sensitive to the dyes thionin and fuchsin, being lysed by bacteriophage Wb, Bk_2, and Fi phage at routine test dilution (RTD) but only partially sensitive to bacteriophage Tb and with no requirement for carbon dioxide, no production of hydrogen sulphide, but strong urease activity. Biochemical profiling revealed a pattern of enzyme activity and metabolic capabilities distinct from existing *Brucella* species. Further molecular analysis of the *omp2* locus genes showed that both strains had a novel combination of two highly similar *omp2b* gene copies. Both of the strains shared a unique multiple copy IS711 fingerprint profile of this *Brucella* specific element. Like MLSA, a multilocus variable number of tandem repeat analysis (MLVA) showed that the isolates clustered very closely together, but represent a separate cluster within the genus *Brucella*. The two isolates could clearly be distinguished from all existing known *Brucella* species and their biovars by both phenotypic and molecular properties. Therefore, by applying the *Brucella* species concept suggested by the ICSP Subcommittee on the Taxonomy of *Brucella,* we conclude that they represent a novel species within the genus *Brucella* for which the name *Brucella papionis* sp. nov. is proposed.

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Poster Presentations (P1-P7)

P1: Characterisation of a novel *Brucella* from *Litoria caerulea* (White's tree frog) in the United Kingdom

Jakub Muchowski, Mark Koylass, Krishna Gopaul, Lorraine Perrett, Emma Jane Dale and Adrian Whatmore

OIE/WHO/FAO Brucellosis Reference Laboratory, Department of Bacteriology, Animal Health and Veterinary Laboratories Agency (AHVLA), Addlestone, United Kingdom

In the last ten years the genus *Brucella* (*B.*) has expanded thanks to several new isolations. These include newly recognized species such as *B. microti*, *B. inopinata* and isolations that are yet to be formally named from several sources. The application of multilocus sequence analysis (MLSA) has played a key role in characterisation of such isolates allowing rapid phylogenetic placement relative to extant diversity of the genus.
A pure bacterial isolation from a White's Tree Frog *Litoria caerulea* was submitted to AHVLA for further characterisation following preliminary identification by MALDI-TOF as *B. melitensis*. Upon submission to AHVLA Weybridge, although this isolate did not agglutinate with A, M or R monospecific sera, and would be excluded as *Brucella* by conventional biotyping, the sample was identified as *Brucella* on the basis of positive reactions in real-time PCR assays targeting the genus specific *bcsp31* and IS711 markers.
Sequence analysis of 16S rDNA revealed over 99 % identity with other members of the genus with closest match being to *Brucella inopinata*. To reveal further diversity and establish the position of the isolate within the genus, MLSA targeting 9 distinct loci was applied. Although divergent from both classical and atypical species, this isolate clearly belongs to the genus when compared to the closest phylogenetic neighbours, *Ochrobactrum* spp. The tree frog isolate generated a profile clearly distant from 'core' *Brucella* species by this assay, closely related to, but distinct from, two isolates recently described from amphibians in a quarantine facility in Germany and representing so called 'atypical' *Brucella.*
This report represents the first isolation of *Brucella* spp. from amphibians in the UK and, to our knowledge, only the third globally and may indicate that these emerging organisms are widespread.

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P2: Development of a pan-*Brucellaceae* multi-locus sequence analysis scheme (MLSA) facilitating accurate taxonomic and phylogenetic placement of emerging members of the group

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Brucellosis is a disease caused by organisms of the genus *Brucella*, and is a zoonosis of great socio-economic importance. Historically, identification of *Brucella* isolates was based on a combination of host specificities and phenotypic characteristics. However, this methodology requires expensive handling facilities requiring highly specialised and experienced staff. The results can sometimes be subjective which can lead to ambiguity between laboratories. This has led to the development of a number of DNA based identification and characterisation tools.

To overcome the problem of reliability and reproducibility associated with phenotypic approaches we have developed and employed a multi-locus sequence analysis (MLSA) scheme as a molecular typing tool for the *Brucella* genus that has been applied to around 1,000 isolates in recent years. However, the scope of this method is limited when describing the increasing number of novel *Brucella*-like isolates that are emerging. This reflects the fact that some loci from non-*Brucella* members of the family cannot be amplified under the existing scheme and thus isolates cannot be placed in context with the whole family including closely related genera such as *Ochrobactrum, Paenochrobactrum, Pseudochrobactrum,* and *Falsochrobactrum*.

To address this problem we describe the development of a 6 locus pan-*Brucellaceae* MLSA scheme using degenerated primers to facilitate amplification of equivalent loci already included in the existing *Brucella* scheme. The scheme was tested successfully against 38 type strains representing the entire *Brucellaceae* family and used to place 52 *Brucellaceae* field strains. This pan-family MLST scheme will be a valuable tool for characterising novel/newly emerging *Brucella* organisms by placing them within the context of the wider *Brucellaceae* family with applications in diagnostics, taxonomy and understanding phylogeny and evolution of the group.

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P3: Extended multilocus sequence analysis and whole genome sequencing are consistent with the division of the current paraphyletic species *Brucella ceti* into two distinct species

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Since the first isolation of *Brucella* (*B.*) from marine mammals in the early 1990s strains have been associated with a wide range of cetaceans and pinnipeds. While the isolates were formally described some as *B. pinnipedialis* (associated with pinnipeds) and *B. ceti* (associated with cetaceans) some years later, it has been suspected for some time that the taxonomic descriptions are not consistent with phylogenetic divisions. Evidence from a number of studies based on a variety of molecular approaches has suggested that *B. ceti* may represent two distinct groups preferentially associated with dolphins and porpoises. Here we describe the application of extended multilocus sequence analysis to a large collection of marine mammal *Brucella* isolates, along with whole genome sequence analysis of a subset of isolates, to confirm the relationships within the group. In combination these analyses confirm the existence of three major groupings in the marine mammal *Brucella* two of which correspond to the existing *B. ceti*. We suggest that these data, in conjunction with a full polyphasic taxonomic study, may be consistent with a future proposal to subdivide the paraphyletic isolates currently described as *B. ceti* into *B. ceti* (isolates preferentially associated with porpoises) and *B. delphini* sp. nov. (preferentially associated with dolphins).

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P4: *Brucella ceti* in the Mediterranean Sea shows a specific clade

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In 1994 *Brucella* sp. was isolated for the first time from a marine mammal. Different studies have been conducted to elucidate the biology of the bacterium and now two species with different tropism are recognized: *Brucella* (*B.) ceti* (affecting cetaceans) and *B. pinnipedialis* (infecting pinnipeds). Extensive studies using MLVA-16 and MLST methodologies have been conducted to understand the interspecies evolution among the marine mammals.
In Italy, surveillance activity on strandings has largely improved in the last years. During the period 2012-2013 three cases were reported from the Ionian Sea. The aim of this study was to clarify the genetic relationship among the three Ionian *B. ceti* strains and other *B. ceti* isolates by a whole genomics approach.
Single-pair reads were generated using an Ion Torrent™ PGM (about 40x coverage) and de-novo assembled. A SNPs matrix was generated by a kmer approach for the three Mediterranean strains and for another five *B. ceti* genomes and one *B. pinnipedialis* available in Genbank including the *B. abortus* genome NC_006932, NC_006933 as outgroup. A Maximum Parsimony tree was constructed on the basis of core-SNPs only (approximately 2,719 informative SNPs).
The Maximum Parsimony tree revealed three distinct clades, i.e. clade 1 associated with strains belonging to the sequence type 23 (ST23), clade 2 characterized by the *B. ceti* strain *Cudo*, and clade 3 comprising the strains of sequence type 26 (ST26). The Italian strains fell into the ST26 clade together with two Scottish isolates, but formed a distinct sister sub clade with 46 SNPs of distance from the Atlantic strains. Moreover, two Italian strains isolated in the same area but with a gap of eight months, showed nine common derived SNPs, suggesting that probably they were epidemiologically linked. On the contrary, the third strain was found to be ancestral for those SNPs, suggesting that this case is not strictly related to the previous ones. It is interesting to note that the MLVA profile was identical for the first two strains but diverged for two hyper-variable loci (bruce 07 and bruce 16) in the third one.
These findings suggest that the analysis of WGS SNPs is a more robust phylogenetic method than the MLVA assay, and that it is reliable in understanding the genetic relatedness. By using the MLVA assay, it is very difficult to measure the actual distance among different strains due to the homoplasy of the VNTRs.
This study is based on the molecular characterization of *B. ceti* isolated in Italian seas and highlights the importance of the WGS approach to elucidate the genetic relationships among *Brucella* isolates. It is known that dolphins from the Mediterranean Sea rarely migrate to the ocean and vice versa, which is demonstrated by the genetic structure of the dolphin population. Further studies are needed to verify the presence of other *B. ceti* clones in the Mediterranean Sea.

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P5: MLVA-16 genotyping of *Brucella ceti* isolates from stranded striped dolphins on the Costa Rica Pacific coast

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Brucellosis in marine mammals was first described in 1994. Since then, the number of cases distributed in oceans worldwide has been increasing. Within *Brucella* (*B.*) *ceti* five different subgroups have been recognized according to their preferred host, bacteriological properties and genetic traits. Two subgroups named A1 and A2 represent *B. ceti* isolated from dolphins inhabiting the Atlantic. Another subgroup represents those isolated from dolphins of the Mediterranean Sea. Cluster B comprises *B. ceti* isolated from porpoises. The *B. ceti* isolate originating from a human case in New Zealand stands alone. Twenty-three isolates of stranded striped dolphins from the Pacific coast of Costa Rica had been collected from 2006 to 2013. Genotyping was carried out using multilocus variable number of tandem repeats analysis (MLVA-16) which showed that these isolates represent a new subgroup that we named *B. ceti* P1 (Pacific). This new subgroup confirms that spatial distribution of *B. ceti* genotypes is in agreement with its host's geographical location and suggests close evolution of the host and the bacteria.

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P6: Identification of Mongolian *Brucella abortus* isolates from various animals and humans by biochemical and molecular typing

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At present, *Brucella* (*B.*) *abortus* consists of seven biovars (bvs 1, 2, 3, 4, 5, 6 and 9). Generally, biotyping and molecular detection methods are considered to discriminate most *Brucella* species and biovars. In a collaborative research project of South Korea and Mongolia, many *Brucella* isolates were obtained from humans and various animal sources such as sheep, goats, cattle and camel. In this study, we focused on and characterized the Mongolian *B. abortus* isolates, especially those which did not agree with any of the currently known biovars using well-established biochemical and molecular typing methods.
A total of sixteen Mongolian *Brucella* isolates identified as *B. abortus* by species biotyping and PCR analysis were investigated. For biotyping, classical biochemical tests such as oxidase test, urea hydrolysis, CO_2 requirement, H_2S production, anti-A, -M, -R-monospecific sera agglutination test, growth on dyes, and phage lysis were applied. For molecular typing, BASS-PCR, *omp2a*-PCR and advanced multiplex PCR were performed to discriminate species and biovars. In addition, molecular epidemiological techniques such as MLVA (Multi-Locus Variable-number tandem repeat Analysis) were included to analyze the *B.* abortus isolates primarily identified as untypable.
Putting biochemical and molecular typing results together, nine out of the sixteen *B. abortus* isolates from Mongolia revealed to be bv 3, whereas seven isolates remained untypable according to the existing scheme. The bv 3 isolates showed the specific traits of *B. abortus* bv 3: agglutination with monospecific sera A(+), M(-) and R(-), growth on dyes (thionin +, basic fuchsin +), and lysis for all phages except R/C. In contrast, the other seven isolates showed agglutination with the monospecific sera A(+), M(+) and R(-), growth on both dyes (thionin +, basic fuchsin +), and lysis by 3 phages, i.e. Tb, Wb, Iz. Biovars 1, 2, and 4 isolates were not identified using BASS-PCR and *omp2a*-PCR. Advanced multiplex PCR results revealed that all Mongolian isolates were *Brucella* spp. MLVA-16 grouped the bv 3 isolates closely together with Chinese *B. abortus* isolates, and the untypable isolates were grouped with the bv 7 Mongolian isolates from 1999.
In summary, we assume that bv 3 is the most prevalent *B. abortus* type in Mongolia. For further characterization of the untypable *B. abortus* isolates, deeper analyses are required.

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P7: Identification of single nucleotide polymorphisms specific for *Brucella* strains isolated in the country of Georgia

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Brucellosis is one of the most globally widespread zoonotic diseases and is endemic in Georgia where it causes substantial human morbidity and significant agricultural economic loss. Because of its high infectivity in mammals, *Brucella* (*B.*) *abortus* and *B. melitensis* are classified as Category B biological threat agents. The lack of genetic resolution with available methods has made it challenging to understand how this pathogen has spread across the globe. Whole genome sequencing (WGS) allows for a deeper understanding of phylogenetic relationships among bacterial strains. In order to study *Brucella* genetic variation within Georgia, 10 *Brucella* strains, *B. melitensis* (n=3) and *B. abortus* (n=7), were chosen for 454 whole genome pyrosequencing and subsequent SNP discovery. These strains were chosen as representatives of major genetic clusters, determined by MLVA-15 as part of DTRA Cooperative Biological Research project GG-17. Whole genome sequences were assembled using the Newbler method. These assemblies, along with existing complete genomes of *B. abortus* and *B. melitensis* from GenBank were aligned using numeric MUMmer 0.7.5a to either the reference genomes of *B. abortus* 2308 or *B. melitensis* 16M, as appropriate. SNPs were called with MUMmer show-snps 0.7.5a, organized into a matrix and exported with custom java scripts. Based on whole genome comparisons a phylogenetic tree was constructed using PAUP* 4.0 beta 10. A comparison of Georgian *Brucella* whole genome sequences to a worldwide collection of genomes showed that Georgian strains of *B. abortus* largely form a unique clade basal to the most common radiation of strains from biovars 1, 2, and 4, and are most similar to strains from Central Asia. Georgian *B. melitensis* isolates are less distinct and appear to mostly fall into the East Mediterranean lineage but also group with isolates found worldwide. Based on these WGS data, new SNPs specific for Georgian strains were discovered and incorporated in real-time PCR assays. These assays were added to the established SNP genotyping assay panel at the NCDC Lugar Center. This panel will allow the screening of not only archival, but also newly isolated *Brucella* strains in Georgia and neighbouring countries, allowing for a rapid understanding of their global phylogenetic context.

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Session 2: Genomics, Proteomics and Metabolomics

Keynote Lecture: Stephan Köhler
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Exploring the landscape of genomics, transcriptomics and proteomics in *Brucella*

Brucella (*B.*) spp. are Gram-negative, facultative intracellular bacteria responsible for brucellosis, a major bacterial zoonosis worldwide. Until 2007, six species were recognized within the genus: *B. abortus*, *B. melitensis*, *B. suis*, *B. canis*, *B. ovis*, and *B. neotomae*, of which the first four have been associated with human disease. Since 2007, four additional species have been added to the genus: *B. ceti*, *B. pinnipedialis*, *B. microti*, and *B. inopinata*. More *Brucella* species will likely be described in the near future, as additional strains have been isolated from a human patient, baboons, rodents and frogs, the first non-mammal host described for *Brucella*. Although it has been proposed in the past to group *Brucella* species as biovars of a single species based on DNA hybridization studies, the current classification in species according to differences in host preferences and in pathogenicity is preferred.
Following the entry into humans or animals, the pathogens replicate in macrophages and dendritic cells. Intracellular growth has also been reported *in vitro* in non-professional phagocytes and in trophoblasts. In fact, virulence of *Brucella* relies on its capacity to survive and to replicate in the *Brucella*-containing vacuoles of the phagocytes. In the past 15 years, lipopolysaccharide (LPS), the type-IV secretion system VirB, and the BvR/BvrS two-component system have been described as central in *Brucella* virulence, in addition to an important number of genes more generally involved in bacterial metabolism, transcriptional regulation or adaptation to stress.
The age of 'Omics' for *Brucella* started with the publications of the genome sequences of *B. melitensis* and *B. suis* in the year 2002, representing the real 'kick-off' of genomics and systematic approaches to the identification of virulence factors and mechanisms.

The first entire and assembled genomes of *Brucella* to be published were those of *B. melitensis* 16M and of *B. suis* 1330 in 2002, followed by *B. abortus* in 2005. The availability of these whole genome sequences allowed scientists to look deeper into genetic organization, genome comparison between species and virulence mechanisms of the pathogens. However, there is a constant need to correct and re-annotate these first whole genome sequence versions; this is especially true for *B. melitensis.* A high degree of similarity between *Brucella* species was noted at both the gene and nucleotide level, confirming previous DNA hybridization data. Publication of the *B. suis* genome revealed extensive homologies with plant pathogens (*Agrobacterium tumefaciens*) and symbionts (*Mesorhizobium loti*). On May 20, 2014 a total of 431 *Brucella* genomes at various levels of assembly, including 17 complete genome sequences of multiple species and strains, were accessible at the 'Pathosystems Resource Integration Center' (PATRIC; http://patricbrc.org/portal/portal/patric/Taxon?cType=taxon&cId=234), as compared to only 39 genomes in October 2011. Development of less expensive and much faster DNA-sequencing techniques such as pyrosequencing has contributed to this significant increase in the number of available *Brucella* genomes, resulting in part from a dedicated genome sequencing program at the Broad Institute (http://www.broadinstitute.org/). The possibility of extensive sequence comparison between *Brucella* strains, together with the identification of strain-specific genetic markers and single nucleotide polymorphisms (SNPs), will be a major asset in the development of new vaccines and of new strain identification methods, useful for diagnostics and in molecular epidemiology.
The first complete genome sequences of *Brucella* species also made clear that this pathogen lacks classical bacterial virulence factors such as capsules, toxins or lysins. In parallel, the first large-scale transposon mutagenesis of *Brucella*, describing the intramacrophagic virulome of *B. suis* 1330, confirmed this finding and postulated the assembly of a pathogen-specific intracellular niche, where the bacteria behaved furtively, resisted to stress conditions and low oxygen

tension, and synthesized all the metabolites necessary for survival and replication. The first genome analysis of a non-zoonotic species was performed in 2009 for *B. ovis*, describing genome degradation by accumulation of pseudogenes and transposable elements, as well as host range narrowing, possibly due to inactivation of genes involved in nutrient acquisition and utilization, cell envelope structure and urease activity. More recently, whole genomes of a non-classical and of a marine mammals-associated *Brucella* species have been reported: *B. microti* and *B. pinnipedialis* B2/94. *B. microti* is the first representative of fast-growing *Brucella* and, as a biochemically highly active bacterium, shares more phenotypic traits with *Ochrobactrum* than with other brucellae. However, genome sequence comparisons showed little homology to *Ochrobactrum*, but very high sequence similarity to *B. pinnipedialis* and to *B. suis* 1330. A more fine-tuned comparative analysis of the available genomes then led to the proposal of a branching order for the main groups of host-associated *Brucella* species, thus shedding light on the evolutionary history of the genus and the explosive radiation following adaptation to a host-associated lifestyle. Later, comparative genomics of 40 *Brucella* genomes with reference to *Ochrobactrum* split the genus into two groups: the recently identified, early-dividing 'atypical' strains, and a highly conserved core clade with the classical strains and major pathogenic species comprising *B. microti* as the most basal lineage. Lateral gene transfer differentiated *Brucella* strains from *Ochrobactrum* and resulted in the stepwise acquisition of known virulence factors such as the type IV secretion system and perosamine-based O-antigen. Subsequent radiation of the core clade resulted in adaptation to their preferred mammalian hosts and in virulence restriction, allowing chronic infections. In addition, bioinformatic genome analysis of the new strains *B. inopinata* BO1, BO2, and Australian rodent strains, as well as experimental data, revealed the existence of atypical LPS molecules.
It is noteworthy to state that in the 'early' period of *Brucella* genomics 10 years ago, scientists focused on the differences in DNA sequences of known species as possible explanations for distinct virulence mechanisms and host specificity. It has now become clear that vastly different phenotypes, such as those observed for *B. suis* and *B. microti*, cannot be easily explained at the genome level, shown to be often highly similar. To conclude, over the past decade, *Brucella* genomics contributed significantly to the identification and localization of virulence factors and strain-specific markers on the different genomes, and to a better characterization of the evolutionary relationship between *Brucella* species and strains.

Based on the genome analysis and annotations performed for the three species *B. melitensis*, *B. suis* and *B. abortus* during the period 2002-2005, extensive work has been published since 2010 using techniques of transcriptomics for global gene expression studies. At least four different approaches have been used: (1) Transcriptome analysis of brucellae under various conditions in liquid medium; (2) transcriptome analysis of intracellular brucellae; (3) transcriptome analysis of host cells infected by brucellae *in vitro*; (4) transcriptome analysis of host tissues infected by brucellae *in vivo*. Microarrays containing spotted oligonucleotides or PCR products of predicted *Brucella* ORFs in variable numbers were either 'home-made' or commercially available. The power of transcriptomics applied to *Brucella* resides in the capacity (1) to identify the target genes of a given transcriptional regulator, hence opening additional perspectives on characterization of previously unrecognized factors in host-pathogen interaction, (2) to follow global adaptation to changing environments, such as extra- and intracellular localization, by differential gene expression in a given strain, and (3) to study the host side in the response to *Brucella* infection.
Differential gene expression under the control of the transcriptional regulators BvrR, VjbR, BabR and MucR was studied in *B. abortus* and *B. melitensis*. These regulators are essential for virulence of the pathogen and participate in cell envelope modulation, quorum sensing, and adaptation to various stresses, LPS synthesis, and mutual cross-regulation. Hence, the powerful tool of microarray analysis using comparison between wild-type and regulator mutant strains allowed significant progress in the global characterization of the biological functions of these important regulators. The *Brucella* general stress response and the stringent response were also studied by transcriptome analysis under oxidative or nutrient stress conditions, focalized on the roles of *rpoE1* and *rsh*, respectively, encoding sigma factor RpoE1 and the enzyme responsible for

(p)ppGpp-synthesis. Microarray data for stringent response confirmed that known *Brucella* virulence determinants were among the regulator target genes. 12 % of the genome of *B. suis* were regulated in a stringent response-dependent manner, and transcriptomics contributed to a better understanding of the correlation between stringent response and *Brucella* virulence. Interestingly, (p)ppGpp-dependent cross-talk between at least three stress responses playing a central role in *Brucella* adaptation to the host, namely nutrient, oxidative, and low-oxygen stress, was evidenced. Finally, very recent work made advantage of transcriptome analysis in the characterization of the biological roles of the two small regulatory RNAs (sRNAs) AbcR1 and AbcR2, and of Hfq, which is a RNA-binding protein mediating interactions between mRNAs and sRNAs and acting as as a key regulator in environmental stress adaptation and intracellular survival. In parallel, global comparative gene expression studies were performed with intramacrophagic *B. melitensis* and *B. canis*, contributing to the elucidation of different intracellular survival strategies. On the host side of interactions with brucellae, also called the interactome, one of the first microarray analyses of murine macrophages gene expression confirmed previously observed inhibition of apoptosis and innate immune mechanisms. Systems biology approaches, linking transcriptomics with pathogenesis and host response by means of bioinformatics, have been developed for *Brucella* and are promising in the identification and modeling of the dynamics of host-pathogen interactions. Recent *in vivo* systems biology pioneer work on *Brucella*-infected bovine Peyer's Patches showed both subversion of the mucosal epithelial barrier function and immune response mechanisms during early infection.

The availability of the first annotated *Brucella* genomes in 2002 opened up the way for global proteome analysis, together with the development of the corresponding techniques, in some cases coupled to transcriptome analysis. The first publications in the field applied traditional 2-D gel electrophoresis with normalization of quantification across replicate gels using a defined protein spot and its volume, followed by MS-based identification of picked protein spots. A later study used liquid-phase chromatography separation of peptides analyzed by quantitative, label-free tandem mass spectrometry (MS/MS) prior to searching of the obtained spectra against protein databases to quantify the impact of BvrR/BvrS on cell envelope proteins. Our group described the first quantitative proteome analyses of intramacrophagic *Brucella* and of *B. suis* under oxygen deficiency, as well as under conditions of extreme long-term nutrient starvation applying multiplexed 2-D DIGE technology and sample labeling with 3 different fluorescent dyes, including internal standard, coupled to MALDI-TOF for protein identification. The high reproducibility and accuracy of this technique allowed efficient in-gel discrimination between host and bacterial proteins and reliable quantification. In the late stage of macrophage infection, we proved an adaptive response by quantitative reduction of bacterial energy processes, protein, and nucleic acid metabolism, whereas under restricted oxygenation, we showed a rather unexpected variability of highly flexible respiration systems for this strictly aerobic pathogen. *B. suis* is also capable to adapt to long-term, severe nutrient deficiency by the combination of several strategies, allowing reduction of metabolism and of energy consumption to the strict minimum necessary for survival, and resulting in a state of persistence. A different approach was performed for a temporal analysis of the intramacrophagic proteomes of *B. abortus* 2308 and attenuated S19 at 3, 20 and 44h post infection, compared to extracellular bacteria in the late log phase. Bacterial proteins were digested and the peptide mix was directly analyzed by Liquid Chromatography (LC)-MS/MS, followed by protein identification with appropriate software and database screening. The virulent strain initially reduced most biosynthesis and adapted respiration, reverting to preinfection levels in the late phase. S19, in contrast, was unable to match the virulent strain’s level of metabolic adjustments, giving possible clues to the understanding of its intracellular attenuation.
Combined transcriptomic and proteomic approaches using microarrays and 2-D DIGE coupled to MALDI-TOF or iTRAQ (isobaric tag for relative and absolute quantification) coupled to LC-MS/MS analysis were performed in another two studies, allowing to obtain complementary data and a more complete characterization of the VjbR/BabR or sRNAs regulator targets. Recently, our group started analysis of the role of the RegB/RegA-two-component-system regulator RegA in the establishment of the non-replicative state of *B. suis*, an important feature of chronic infec-

tion. To identify RegA-regulated genes, we developed an *in vitro* model of persistence. In this model, a *regA* mutant is strongly affected in the non-replicative phase, and a comparative microarray-based transcriptome analysis of the wild-type versus a *regA* mutant was performed at the onset of persistence. A total of 12 % of the genome is under the control of RegA, with up-regulation of genes encoding transcriptional regulators, whereas genes coding for envelope biogenesis, the VirB secretion system, and energy metabolism, are down-regulated. In parallel, quantitative 2-D DIGE and multiplexed isotope-coded protein label (ICPL) analysis, allowing high-throughput proteome profiling, yielded data complementing transcriptomics.
In summary, the different *Brucella* transcriptomics and proteomics approaches focused on: (1) Definition of the role of specific transcriptional regulators in global gene expression regulation (identification of target genes); (2) gobal adaptation of a strain to defined environmental conditions (growth phase, stress, intracellular state); (3) comparison between strains/species of global adaptation to a given environmental condition; (4) host cell reaction to infection.

Prospects and Conclusions. In the field of **genomics**, continuing increase in high-throughput genome sequencing efficiency is expected. This will allow further fine-tuning of the evolutionary history of the genus *Brucella* and of the relationship between species or specific genes/groups of genes. It must be stressed that the impressive accumulation of sequencing data, confirming very high identity at genome levels, strengthens arguments in favor of differential gene expression as a major player in the origin of species-specific phenotypes (metabolism, virulence, adaptation to environmental stress) and host specificity profiles, and/or in favor of epigenetic modifications. In the ongoing next-generation sequencing era, increasing interest in the exploration of bacterial epigenomics reflects the awareness that DNA modifications such as methylation facilitate adaptation to varying environmental conditions. In the future, single molecule real time (SMRT) DNA sequencing technology, allowing simultaneous acquisition of genomic and epigenomic data at the nucleotide level, coupled to analytical chemistry tools identifying the type of epigenetic modification, will most certainly contribute to breakthroughs in understanding molecular mechanisms including variable events not encoded in the genome, and which may influence on metabolic shifts, persistence, or host adaptation.
In *Brucella* **transcriptomics**, a marked shift from microarray technology to high-throughput RNA-Seq approaches will certainly occur in the near future, and has already been observed in the study of other pathogens. RNA-Seq is different from hybridization techniques, since data are matched to genes by sequence alignment, and has several advantages: (1) unbiased study of all transcription, including non-coding RNAs (ncRNAs); (2) higher resolution by sequence data mapping; (3) much greater dynamic range for measuring variability in expression levels; making RNA-Seq one of the most powerful tools in analysis of differential gene expression. Saturation of sequence data by abundant transcripts such as rRNAs remains an issue, requiring efficient depletion prior to cDNA library construction, and the existing sequencing platforms offer different compromises between length of reads and depth of coverage. The use of strand-specific libraries resulting in directional RNA-Seq helps to define operons and ncRNAs, contributing lately to the highly interesting concept of the ‘excludon’, where asRNAs can inhibit expression of one operon, while functioning as mRNA for the adjacent operon. Analysis of a combined sense/antisense bacterial transcription under defined conditions will definitely contribute to a better understanding of the discrepancy between *Brucella* phenotypes, including host specificity, and genome conservation. RNA-Seq costs still remain high, especially when subcontracting is necessary, and expert bioinformatic assistance is generally required for data analysis.
After a decade of 2-D gels quantification techniques such as 2-D DIGE with spot-picking coupled to MS, the future of *Brucella* **proteomics** will be dedicated to the application of high-throughput proteomics based on LC separation of peptides, followed by peptide identification and quantification by mass spectrometry. Multiplexed quantitative proteomics with previously differentially labeled and digested bacterial or host cell protein samples can be performed using the stable isotope labeling techniques iTRAQ or ICPL. Despite their advantages and the more precise data generated, these methods are in the process of being outstripped by label-free approaches allowing identification of higher numbers of proteins. Furthermore, iTRAQ has been described to result in underestimation of protein fold changes. On the other hand, due to the

impossibility to multiplex samples, the label-free approaches suffer from variability in the quantitative domain and the lack of reproducible quantification, especially for low-abundance proteins. Next-generation proteomics technology is expected to significantly increase the coverage of peptide detection in expressed proteins and to further develop 'top-down' approaches, where intact proteins may be accurately resolved by MS.

The current explosion of genomic data and the constant progress in mass spectrometry technologies for protein analysis allows shotgun proteomic approaches that are more rapid and less expensive than traditional transcriptomic approaches. Proteogenomics, consisting in mapping high-throughput proteomic data obtained by MS/MS onto genome sequences, is becoming a powerful strategy in correcting or refining automatic genome annotations, especially identification of translation initiation codons. In the field of *Brucella* research, one publication on proteogenomics of *B. abortus* compiled 621 proteins from independent experiments, confirming expression of proteins previously considered as putative and correcting several annotation errors.

The most obvious current limitations of high-throughput 'Omics' are (1) yet underdeveloped bioinformatic tools dedicated to (very) large datasets, (2) imperfect protein databases used for the interpretation of MS/MS profiles, and (3) the dynamic range of mass spectrometers, which has to be improved to identify compounds of very low abundance. Additional restraints for the microbiologists are the often limited competences in bioinformatics to analyze big data sets and the very time-intensive biological validation and interpretation of the obtained data. In addition, only few colleagues work on aspects of 'Omics', and strict application of laws regulating the handling of this 'restricted agent' in various European countries and the U.S. puts an additional brake on the study of this exciting pathogen.

Oral Presentations (O2-O6)

O2: A robust WGS pipeline for identifying and genotyping *Brucella* species

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Whole genome sequencing (WGS) has become a more viable option in the diagnostic bacteriology laboratory for routine identification as well as genotyping. Many laboratories rely on biochemical testing to augment molecular diagnostics for characterization of *Brucella* spp. and biovars. Here, we propose that a PCR assay to identify *Brucella* spp., followed by WGS with a flexible bioinformatics pipeline, can eliminate biochemical testing and allow for high resolution identification and genotyping directly from a primary culture.
DNA from isolates identified as *Brucella* spp. is prepared using the Illumina Nextera XT library prep kit and sequenced using the Illumina MiSeq. The resulting FASTQ files are used to screen for a 30-mer deletion unique to each species and/or biovar. If a match is found, the most closely related reference genome is used for resequencing. BWA, Picard and Broad Institute's GATK program are used to output BAM and VCF files. If a match is not found, the reads are assembled *de novo* and contigs are used in a comparison that does not require a reference genome. BAM and VCF stats, along with unmapped reads assembled into contigs, are used to verify sequence contents. SNPs contained in VCFs are then placed through a pipeline which uses standard Unix programs to output easy to interpret SNP comparisons of isolates in the form of SNP tables and fasta files which are used to generate phylogenetic trees. Defining SNP positions, which identify clusters of closely related isolates, are used to automatically sort isolates into groups, subgroups and clades depending on the resolution needed.
For sequences where no 30-mer deletion match is found, contigs are processed via kSNP, a phylogenetics analysis program that compares isolates using the k-mer concept. kSNP generates phylogenetic trees and matrix tables which characterize the unknown isolate in relation to the known *Brucella* spp.
Using this pipeline, we are able to efficiently characterize *Brucella* spp. and generate high resolution genotyping reports quickly, providing timely information to assist field epidemiological investigations. Pipeline available at: https://github.com/stuber/SNP_analysis.

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O3: Whole genome comparative study on *Brucella suis* biovar 2

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B. suis biovar 2 is the most commonly isolated biovar in Europe in pigs, wild boars (*Sus scrofa*) and hares (*Lepus europeaeus*). The prevalence in wild boars appears to be high throughout continental Europe and this species has been identified as the potential source of transmission of biovar 2 to extensively reared pigs. In Portugal and Spain, this biovar is the unique biovar isolated from domestic pigs and wild boars and the majority of strains share specific molecular characteristics establishing an Iberian clone (haplotype 2d, 2e). Though, in the North-East region of Spain (above Ebro river) strains have been isolated from wild boars similar to those isolated in various Central European countries, both in pigs and wild boars (haplotype 2c). In contrast to other *B. suis* biovars, biovar 2 has been rarely isolated from humans, and its zoonotic potential is doubtful. Currently, it is unknown which molecular or physiological mechanisms are responsible for the loss of virulence of this biovar. Whole-genome comparison, at greater resolution, of closely related *Brucella* spp. expressing different pathogenicity may provide insights into their lifestyle, virulence determinants and evolution. This work aims to disclose the genomic and structural differences between *B. suis* biovar 2 Iberian and Central European strains and to better understand the low pathogenicity of biovar 2 for humans.

We performed whole-genome sequencing of five *B. suis* biovar 2 strains isolated from wild boars, including three Iberian and two Central European strains of haplotypes 2c, 2d and 2e. Genomic sequences were obtained by Illumina HiSeq 2000 technology using a paired-end 35-bp protocol and reads were *de novo* assembled using de Bruijn graph method. Additionally, high-resolution single-molecule restriction maps of the whole genomes were produced through optical mapping technology, allowing high confidence and prompt disclose of typical sequence assembly problems. A comprehensive analysis and comparison of the genomic structure and allelic variation enabled a comparative functional and metabolic analysis of the five strains investigated with other *Brucella* spp. genomes available.

The five *B. suis* biovar 2 genomes under study had similar sizes and were composed of two circular chromosomes with approximately 1.93 and 1.40 Mb, with an overall G+C content of 57.2 %. Three copies of 5S, 16S, and 23S rRNA genes were identified and a set of 54 copies of tRNA genes were predicted. The genome sequences of Iberian type strains PT09143, PT09172 and Bs143CITA presented 72 % and 71 % of similarity when compared with *B. suis* ATCC 23445 (biovar 2; CP000911, CP000912) and *B. suis* 1330 (biovar 1; AE014291, AE014292) reference genomes, respectively; the Central European strains Bs364CITA and Bs396CITA showed 99 % and 98 % similarity in comparison with those reference genomes. Extended synteny was evident; the relatively low level of similarity in Iberian clone is due to the presence of a 944 Kbp inversion in chromosome I carrying >88 % of all missense mutations in comparison to all other *B. suis* strains in this study. Functional, metabolic and evolutionary analyses of these genomic variants will be discussed.

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O4: Revisiting the erythritol catabolic pathway 40 years later

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Erythritol, a four carbon polyol, has been associated for more than half a century with the virulence of *Brucella.* This preferential carbon source is found in substantial quantities in the reproductive tract of natural hosts for which the bacteria have a particular tropism. It was therefore postulated that erythritol, at least partially, may account for this specific distribution and the local massive proliferation of the pathogen leading to the classical symptoms of sterility and abortion.
Erythritol catabolism was first described about 40 years ago and involves 5 enzymatic reactions, three having been assigned to gene products from the Ery operon. It starts with a phosphorylation to L-erythritol-4-phosphate catalyzed by EryA, followed by an oxidation to L-3-tetrulose-4-phosphate by EryB. Further steps were not clearly defined, but are thought to involve oxidation and decarboxylation reactions yielding triose-phosphates, which may then enter the glycolytic/gluconeogenesis pathway.
In contrast to this acknowledged pathway, we provide genetic, biochemical and isotopic evide nce that L-3-tetrulose-4-phosphate is converted to a metabolite of the pentose phosphate pathway, D-erythrose-4-phosphate, by three isomerisation reactions catalyzed by EryC, TpiA2 and RpiB. Contrary to previous assignments, EryC is a tetrulose-4-phosphate racemase, whereas TpiA2 (renamed EryI) catalyzes the isomerisation of L-3-tetrulose-4-phosphate with D-erythrulose-4-phosphate. The latter is isomerized with D-erythrose-4-phosphate by RpiB (renamed EryJ). Erythrose-4-phosphate can then be converted to fructose-6-P and glyceraldehyde-3-P by enzymes of the pentose phosphate pathway.
This revision of the erythritol pathway may provide new clues about the preferential metabolism of *Brucella* and its role in pathogenicity. Interestingly, this pathway is not restricted to the pathogenic lifestyle of *Brucella spp.* because it can be found in phylogenetically close bacteria such as *Rhizobium* spp. (plant symbionts), *Ochrobactrum anthropi* (environmental bacteria).

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O5: sRNAs and Hfq regulation in *Brucella abortus*

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Brucella spp. are intracellular pathogens able to survive and replicate in the hostile environment of macrophages, and the etiological agent of brucellosis, the most prevalent zoonosis worldwide. Although there is an increasing knowledge of the virulence factors present in this genus, very little is known about the post-transcriptional regulation of these virulence factors at the RNA level. An *hfq* mutant in *B. abortus* is profoundly affected in its virulence and in several stress responses, suggesting that such a control exists. However, the targets for Hfq, either mRNAs or small noncoding RNAs (sRNAs), are largely unknown for *Brucella*, and only two sRNAs regulated by Hfq have been published so far.
Aiming at sRNA discovery and identification of new regulatory mechanisms, directional mRNA enriched libraries have been constructed from strain *B. abortus* 2308 and an *hfq* isogenic mutant derivative. The libraries were tagged and sequenced in an Illumina HiSeq2000 machine. Filtered reads were aligned to the reference *B. abortus* 2308 genome with bowtie2 and normalized read counts, expressed in RPKMs, were determined for specific features from the alignment *sam* or *bam* files with Artemis or Htseq. A strand specific plot of the reads starting at each genome position was also used to visualize the transcription along the genome. From this plot non annotated regions producing transcripts were selected as putative sRNAs. Secondary structure analysis with RNAfold, promotor and terminator searches and comparison with previous results, were used to filter the candidates and to elaborate a list containing 170 strong sRNA candidates. A subset of them has been selected for further biochemical characterization by Northern blot, RT-PCR and cloning of their 5' and 3' ends. The analysis performed also identified genes regulated by Hfq, and several prediction programs were used to determine the putative targets of the sRNAs. Comparison of the target list with the regulated genes allowed the identification of sRNAs-target pairs.

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O6: Role of the transcriptional regulator RegA in establishment of *Brucella suis* persistence in an original *in vitro* model

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Low oxygen tension is one of the environmental conditions encountered by *Brucella* during intramacrophagic replication and chronic infection of the host. At chronic stage of brucellosis, the bacteria can be located in granulomas that tend to evolve into abscesses where anoxic conditions predominate. Therefore, tackling the capacity of this facultative aerobic microorganism to adapt to and persist under oxygen deficiency is of major interest. In the past few years, we demonstrated the high metabolic flexibility of *Brucella* (*B.*) *suis* with respect to oxygen deprivation (micro- and anaerobiosis). We proved the central role of the two-component system RegB/RegA in the coordinated control of oxidative respiration and denitrification respiratory systems, which are crucial for virulence and/or persistence *in vivo*. More importantly, RegA was found to be essential for *B. suis* persistence in mice. Recently, we developed an original *in vitro* model, characterized by progressive oxygen deprivation in minimal medium, which allowed to show that RegA is essential for optimal long-lasting *in vitro* persistence. To identify RegA-dependent genes and proteins in this model, global transcriptomic analysis (microarrays) and whole proteome quantifications (2-D DIGE) were performed comparing wild-type *B. suis* to a Δ*regA* mutant strain. Analyses were conducted when anaerobic conditions had been established, corresponding to the cessation of wild-type strain multiplication. Genetic validation by quantitative PCR (RT-qPCR) indicated that RegA potentially regulates 12 % of the *B. suis* genes, and its role in bacterial adaptation to oxygen deficiency was confirmed.The down-regulation of genes or proteins involved in cell envelope biogenesis and in cellular division suggests that RegA could be involved in the establishment of a non-replicative state. In addition, RegA-dependent repression of various genes involved in energy production may provide evidence of a RegA-associated slowing-down of central metabolism as it enters into the persistence phase. This was substantiated by the finding that two-thirds of the differentially produced proteins belonging to this functional class were also found repressed, amongst others isocitrate lyase, the first enzyme of the glyoxylate shunt. Several genes of the *virB* operon were also found repressed by RegA e.g. its regulator VjbR. In addition, biological validations were performed to confirm results of the transcriptomic and proteomic analyses. In conclusion, RegA was found to regulate genes that encode proteins of all functional groups. This makes the two-component system RegB/RegA a main regulatory system required for adaptation of *B. suis* to oxygen depletion, which can contribute to the constraint of bacterial growth, characteristic of chronic infection.

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Poster Presentations (P8-P20)

P8: Characterization of genetic loci which may contribute to the host specificity of diagnostic brucellaphages

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Brucellosis is one of the world's most widespread bacterial zoonotic diseases and is of great public health concern. Members of the genus *Brucella* (*B.*) are facultative intracellular pathogens which may cause reproductive failure or abortion in animals and a feverish multiorgan disease in humans. The bacteria are transmitted by wildlife and domestic animals. The history of brucellaphages and their use as a diagnostic tool for the identification and differentiation of brucellae began with the discovery of the phage Tb (Tbilisi) in the 1960s. On the basis of their host ranges, the currently known virulent brucellaphages are allocated to seven groups (prototype phages: Tb, Fi, Wb, Bk2, R/C, Iz and Np). Due to the high similarity (podoviral morphology, restriction profile, physicochemical properties) of the phages, it has been suggested to classify them as a single virus species comprising different host-range variants. Recently, whole genome sequencing and genomic comparison of several diagnostic brucellaphages (Tb-Mexico, Pr, F1, Tb-Russia, Fz, Wb, S708, Bk and R/C) revealed high sequence similarity but also distinct differences among the genomes. From these analyses, brucellaphages have been assigned to different genetic groups. Several genetic loci exhibiting high diversity have been identified which may contribute to a varying host specificity due to adaptive selection by phage/host-interaction.
Here, we present the general characteristics of 30 diagnostic brucellaphages which are routinely used for *Brucella*-typing in our laboratory. Electron micrographs revealed that virions of all currently known brucellaphages were composed of an isometric head and a short, non-contractile tail. Thus the phages belong to the virus family *Podoviridae*. According to their host specificity, these diagnostic brucellaphages were allocated to different host range groups. All phages were investigated in terms of their genetic diversity in selected regions of their genomes. Sequence alignments showed a high level of identity of these loci with only minor nucleotide exchanges. The identified divergences may contribute to the different host specificities supporting the hypothesis that all virulent brucellaphages are host range mutants originating from a common ancestor.

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P9: Differences in primary and tertiary structure of Gp21 protein of five brucellaphages

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The high specificity that brucellaphages (B-phages) display for their host is an important matter. The analysis of putative proteins related to host attachment and adhesion is crucial to determine differential host specificity of B-phages. Recent studies comparing the whole genome of B-phages Tbilisi (Tb) and Perote (Pr) have shown evidence that protein Gp21 is involved in the specific interaction between phages and brucellae. This study was focused on the analysis of nucleotide variations in the *gp*21 gene of B-phages BFM1, Izatnagar (Iz), Berkeley (Bk), Pr and Tb, and their effect on the putative protein. Initially, the *gp*21 gene was amplified from each B-phage for subsequent sequencing. Prediction of the 3D protein structure and function were performed with the I-TASSER program; each model was evaluated with the programs PROSA and PROCHECK. Nucleotide differences were found in the *gp*21 gene sequence of the B-phages BFM1, Iz, Bk, Pr and Tb, which resulted in amino acid substitutions affecting the tertiary structure of the Gp21 protein. It was observed that the primary structure of the Gp21 protein has similarities with the meso hypothetical proteins 0234 of *Chelativorans* sp. BNC1, the Rpdx 2528 of *Rhodopseudomonas palistris* DX-1 and *Mesorhizobium ciceri* biovar *biserrulae* WSM127. Furthermore, the tertiary structure resembles proteins with catalytic activity in the metabolism of xyloglucans reported in *Geotrichum* sp. M128 and *Clostridium thermocellum.* In conclusion, our comparative analysis of five B-phages led to the identification of a putative xyloglucanase with differences in its tertiary structure.

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P10: Insights into the genetic lineages and current spread of *B. abortus* and *B. melitensis* in Italy

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Brucellosis is a recurring problem in animal husbandry that results in enormous economic losses and human infections. The disease has been endemic to the Mediterranean basin for centuries, but its phylogeography in Italy has not been extensively investigated. The burden of this zoonosis remains relevant in the southern part of the country and drawing the right risk maps is a priority for achieving its control. Determining which lineages are present throughout Italy is paramount for risk assessment, providing data to prevent the spread of the disease and protect public health. The aims of this study were to evaluate the genetic diversity of *Brucella* (*B.*) *melitensis* and *B. abortus* populations in Italy, and to establish their phylogenetic place in the global diversity of *Brucella*. Our previous MLVA analysis revealed several clades, from which 12 representative *B. abortus* and 16 *B. melitensis* strains from different geographical regions in Italy were selected for whole genome sequencing. Libraries were sequenced with both paired-end Illumina and Ion Torrent sequencing; our analysis also included 450 *B. abortus* and 300 *B. melitensis* publicly available complete genomes. Single nucleotide polymorphisms (SNPs) were detected in read alignments and whole genomes, but SNPs from duplicated regions were removed from the analysis, as were any SNPs including missing or ambiguous base calls, read coverage less than 10x, and a proportion of SNP calls at a given position less than 90 %. Maximum parsimony was used to determine the SNP-based phylogeny. Approximately 22,000 putative SNPs were identified among the *B. melitensis* samples. The Italian isolates formed one clade with three sub-clades, which in some instances demonstrate a specific geographical spread. The *B. abortus* analysis revealed approximately 9,000 putative SNPs. The Italian strains fell into one of three clades. The first clustered with the biovar 1/2/4 lineage from northern Europe and the U.S., while the other two were part of a basal Eurasian lineage. Our data demonstrate that *B. melitensis* in Italy belongs to a specific clade suspected to be present in northern Africa and Italy (central Mediterranean), but distinct from all other global clades. *B. abortus*, however, belong to three different clades in Italy, and are most similar to Asian and northern European isolates. This study is a step forward in understanding of *Brucella* evolution within the Mediterranean area, and demonstrates the utility of WGS SNP analysis paired with extensive epidemiologic data for analyzing the distribution of *Brucella* isolates throughout endemic regions.

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P11: Scaffolding and validation of *Brucella suis* genome assemblies using whole-genome optical maps

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Optical mapping is a new tool that creates single molecule high-resolution, *in situ* ordered restriction maps of a bacterial genome. These maps are used to determine genomic organization and perform comparative genomics to identify genomic rearrangements, such as insertions, deletions, duplications, and inversions. The main goal of this study was to assess the use of optical mapping, which had never been applied to *Brucella*, to complement and validate genome sequencing projects.
We performed whole-genome sequencing (Illumina HiSeq 2000 technology) of five *B. suis* biovar 2 strains isolated from wild boars, including three Iberian (one of haplotype 2d and two of haplotype 2e) and two Central European (haplotype 2c) strains. Additionally, we constructed the whole-genome optical maps of abovementioned strains as well as *B. suis* ATCC 23445 using the restriction enzyme *Bam*HI (OpGen Technologies, Inc.), and later compared with those generated *in silico* from the sequence data. The whole-genome sequencing generated between 8.408.102 and 12.172.794 high-quality reads (Phred score >30) that were *de novo* assembled into contigs using de Bruijn graph method (Velvet v. 1.2.09), resulting in depth coverage ranging from 89x to 129x. The assembly yielded between 55 to 116 contigs and the genome expected sizes ranged from 3.315.423 to 3.319.511 Mbp. Optical mapping produces a barcode-like genetic map of restriction sites with the depicted sites arranged in the order they occur in the genome. The consensus map was built within a minimal coverage of 50x. Overall, the six *Bam*HI whole-genome optical maps were very similar, generating 228 to 232 fragments, with sizes ranging from 1.5 to 85 kb. The estimated chromosome sizes for the five sequenced strains ranged from 3,328,344 to 3,337,024 bp. The optical maps defined unique genome landmarks in each of the strains and demonstrated the ability of this method to determine the relative placement and orientation of sequence fragments produced during the assembly process. Chromosome I optical maps revealed the presence of one insertion event with approximately 3.5 kb in all strains, in relation to ATCC 23445 *in silico* and optical maps. Moreover, some inconsistencies in the cut sizes between the ATCC 23445 optical and *in silico* maps were found. Considering chromosome II optical maps, there were no relevant inserts/deletions or other genomic rearrangements. Unexpectedly, this technology permitted the identification and location of a genetic inversion of ~944 kb in chromosome I of the three Iberian strains in comparison to *B. suis* 1330 (biovar 1) and *B. suis* ATCC 23445 (biovar 2) reference strains and both Central European strains. The inversion was checked by PCRs at the suspected inversion junction sites on 113 Iberian and 50 Central European strains. Indeed, the inversion was exclusively present in the Iberian strains (haplotype 2d and 2e).

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P12: Comparison of *Brucella canis* genomes isolated from different countries shows multiple variable regions

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Brucella (*B.*) *canis* is a pathogenic bacterium that mainly affects dogs but occasionally also infects humans. The aim of our study was to sequence, annotate and analyze the complete genome of the *B. canis* strain Oliveri isolated from a dog of a kennel in Medellín, Colombia, South America.
Whole genome shotgun sequencing was carried out using the ROCHE 454 GS FLX TITANIUM technology. The assembly procedure was performed using and comparing both a MIRA assembler 3.4 and a Newbler 2.6. In the genome annotation process, each contig was analyzed independently. *Brucella suis* GenBank Accession NC_004310.3 was used as the reference genome. Afterwards, annotation was manually carried out with the ARTEMIS software. The GC content of the genome was 57 %. A total of 2,132 and 1,158 coding sequences were annotated in chromosome 1 and 2, respectively. The genome of *B. canis* str. Oliveri showed perfect synteny with other *B. canis* and *B. suis* strains when compared with other genomes completely accessible from GenBank.
Predicted peptide comparison showed that 82 % of the proteins were identical with the *B. canis* reference strain ATCC 23365, while only 63 % were identical with *B. canis* HSKA 52141 (isolated in Hwasung, Geyonggi, Republic of Korea), but 71 % when compared to *B. suis* 1330. The low protein identity in comparison to HSK A52141 is noteworthy.
Single nucleotide polymorphisms between the genome of *B. canis* str. Oliveri and the other three genomes in our study were calculated. The sequences of chromosome I and II are deposited in EMBL with accession number HG803175.1 and HG803176.1, respectively.
The complete and annotated genome sequence of *B. canis* strain Oliveri shows unique genomic characteristics indicating that there are differences in the genome structure of different isolates within a species depending on their geographic origin. This genome may be useful in the development of diagnostic tools and vaccines helping to reduce health complications of canine brucellosis in dogs and humans.

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P13: Differentially expressed host proteins in *Brucella abortus*-infected bovine chorioallantoic membrane explants

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Brucella (*B.*) *abortus* is the most important etiological agent of bovine brucellosis, which causes great economic losses worldwide especially due to abortion. *B. abortus* is also an important zoonotic agent. Proteomic studies of the interaction between *Brucella* spp. and their target cells have become more common in recent years mainly because of improvements in proteomics techniques and protein identification. However, studies about the interaction of trophoblastic cells with *B. abortus* are scarce. Trophoblastic cells are the primary target for *B. abortus* infection in the bovine placenta. Thus, evaluation of the proteomic profile of bovine trophoblastic cells infected with *B. abortus* is extremely relevant, since the interaction of these cells with *B. abortus* is a key event in the pathogenesis and transmission of the disease. The aim of this study was to identify, by mass spectrometry, proteins differentially expressed in bovine trophoblastic cells infected with *B. abortus* 2308 (MOI 1:1000) using a previously described chorioallantoic membrane explant model. Protein spots that showed qualitative differences in the DIGE (Difference Gel Electrophoresis) analysis were analyzed by mass spectrometry. A total of 103 spots were analyzed and 74 (72 %, 74/103) could be identified, from which 73 (98.6 %, 73/74) belonged to *Bos taurus*, i.e. they were produced by the trophoblastic cells. Functional analysis indicated that the infected group had upregulated proteins with different functions when compared to the uninfected controls. Functionally, these proteins included maintenance of metabolic and structural homeostasis and also directed the host immune response to infection. Proteins related to recognition of PAMPs by TLR (HMGB1, TOLLIP), ROS production (AKR1B1, NDUFS8), intracellular trafficking (SCAMP2, RAB11A), and inflammation (AHCY, C1QBP, PDIA3, GALM) were identified only in *B. abortus*-infected trophoblastic cells. The role of some of these proteins during infection should be addressed in future studies about host-pathogen interaction during *B. abortus* infection.

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P14: Differentiation of *Brucella melitensis* Rev1 and field strains based on periplasmic protein analysis

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In our work we characterized candidate protein antigens useful for the differentiation of *Brucella* (*B.*) *melitensis* Rev1 from field strains. False-positive results of serologic testing for brucellosis can be attributed to cross-reactivity of antibodies directed towards *Brucella* spp., *Yersinia enterocolitica* O:9, *E. coli* O:157, *Salmonella* group N (O:30), *Pseudomonas* spp., *Francisella tularensis*, *Pasteurella* spp., *Moraxella phenylpyruvica* and *Ochrobactrum anthropi*. We tried to identify potential candidate antigens for serologic testing using bioinformatic analyses. After determining the cellular localization of 27.930 *Brucella* proteins, three subgroups, representing a total of 1.397 proteins, were investigated to identify candidate antigens that discriminate Rev1 vaccinated from infected animals and cross-reactive bacteria; 170 of these proteins were localized extracellularly, 308 were outer membrane proteins and 889 proteins were periplasmic. Presence and homology within the species of the genus *Brucella* and throughout the cross-reactive species were investigated for selected proteins and *in silico* antigenicity testing was performed. The following proteins were identified: Porin omp2a, TonB-dependent receptor protein, Leu/Ile/Val-binding protein homolog 2, nitrous-oxide reductase, periplasmic dipeptide transport protein, outer membrane protein assembly factor (BamD), competence lipoprotein (Coml), sugar ABC transporter and a probable sugar-binding periplasmic protein. Two candidate proteins were detected that can discriminate Rev1 vaccinated from non-vaccinated animals and additionally do not cross-react with antigens of non-*Brucella* species: the D0B7Y2 sugar ABC transporter and the Q8YI0 periplasmic dipeptide transport protein. Two proteins were identified as targets to discriminate *Brucella*-infected from non-infected animals: the Omp2a and the Q8YCE2, sugar binding periplasmic protein. Finally, four additional candidate antigens were identified to discriminate *Brucella*-infected animals from carriers of bacteria displaying cross-reactive antibodies: the DNA-dependent RNA polymerase beta chain (encoded by *rpoB*), the copper/zinc superoxide dismutase (*sodC*), the 50S ribomosal proteins L7/L12 (*rplL*) and the ATP synthase subunit. Periplasmic proteins including the D0B7Y2 sugar ABC transporter, the Q8YI0 periplasmic dipeptide transport protein, the Q8YCE2 sugar binding periplasmic protein, Q8YJ22 Leu/Ile/Val-binding protein homolog 2, Q8YBC6 nitrous-oxide reductase, Q8YI58 Coml competence lipoprotein were analyzed using 2-D electrophoresis.
Periplasmic proteins were extracted from *B. melitensis* cells using chloroform. 5-10μg of the extracted periplasmic proteins were analyzed by 2-D technique using the ZOOM® IPGRunner™ System (Life Technologies). First dimension IEF was performed on Zoom® Strip pH 3-10 and the second dimension was performed on a 10 % SDS PAGE gel. The 2-D gels were silver stained.
Field strains of *B. melitensis* had a common unique pattern different from that of Rev1 strain.

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P15: Identification of novel immunodominant proteins from *Brucella abortus*

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Bovine brucellosis has been reported from almost all countries where cattle are farmed. The disease is usually caused by *Brucella* (*B.*) *abortus*. It leads to severe economic losses on livestock farms infected. Diagnosis of brucellosis is based mainly on serology and isolation of the bacteria. Serological tests basically rely on *Brucella* lipopolysaccharide (LPS) antigens which are of insufficient specificity due to serological cross-reactions with other Gram-negative bacteria, e.g. *Yersinia enterocolitica*. These false positive reactions reduce the efficiency of monitoring programs. Thus, there is an urgent need for immunogenic proteins to be used in more specific serological tests. In the present work 12 immunodominant proteins were identified from whole cell lysates of a *B. abortus* field strain isolated from cow by 2D-immunoblotting with serum samples collected from cattle and buffaloes naturally infected with *B. abortus*. The proteins were identified by subsequent MALDI-TOF mass spectrometry. The identified proteins are: metal-dependent hydrolase, ADP/ATP translocase, putative DNA processing protein, lysine-arginine-ornithine-binding periplasmic protein, sugar ABC transporter substrate-binding protein, dihydrodipicolinate synthase, glyceraldehyde-3-phosphate dehydrogenase, lactate-malate dehydrogenase, 2-hydroxyhepta-2,4-diene-1,7-dioate isomerase, phosphopyruvate hydratase and a hypothetical protein. These proteins had no immunogenicity towards negative sera. Hence, the present study opens a window to discover novel protein antigens suitable for the serodiagnosis of brucellosis. The identified immunogenic proteins will be tested in serological assays to develop new sensitive and specific diagnostic tools.

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P16: Identification of immunogenic proteins of *Brucella canis*

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The diagnosis of *Brucella* (*B.*) *canis* infections in humans and dogs is hampered because of the low awareness of healthcare professionals and the lack of laboratory techniques allowing the specific detection. The aim of our study was to identify *B. canis* immunogenic proteins which induce humoral immune response in humans and dogs.
One and two-dimensional gels, Western blotting, MALDI TOF/TOF and LC MS/MS analyses were performed using the *B. canis* strain Oliveri (EMBL: HG803175-76) as well as human and dog sera which were seropositive for canine brucellosis in the 2ME-PARP assay to identify immuno-reactive proteins. Human serum samples positive in the Rose Bengal test and sera negative by the abovementioned two tests were used as specificity controls; dog samples were classified according to the results of 2ME-PARP, blood culture and PCR tests. Several proteins could be identified by means of mass spectrometry and bioinformatic analysis. Recombinant proteins were subsequently used as diagnostic antigens in ELISA and Western blots (WB).
By 2-D gel analysis and immunoblotting, 20 immunogenic proteins were detected. Five of these have not yet been reported for *Brucella* spp. These proteins showed strong immunogenicity: Inosine-5'-monophosphate dehydrogenase (52 kDa), pyruvate dehydrogenase subunit beta (49 kDa), elongation factor Tu (42.6 kDa), TetR family transcriptional regulator (27.4 kDa) and 50S ribosomal protein L10 (18 kDa). Currently, ELISA and WB assays are carried out to determine their usefulness as antigens in sensitive and specific diagnostic tests.
The identified proteins may allow the detection of *B. canis* infections at an early stage and the differentiation of acute and chronic cases in humans and dogs.

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P17: Immunogenic antigens based on infection periods with *Brucella abortus*

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Brucellosis is one of the major zoonotic diseases caused by pathogens belonging to the genus *Brucella*. The eradication of brucellosis in domestic animals can be supported by accurate diagnosis. Improvements in the eradication of animal brucellosis in stocks are directly related to the prevention of human infections. However, conventional serological tests may fail at diagnosing brucellosis during different stages of infection. The aim of this study is to determine reliable immunogenic proteins to detect a *Brucella* (*B.*) *abortus* infection according to the stage of infection which will help to manage brucellosis control.
Whole-cell protein extracts of *B. abortus* 544 were separated by two-dimensional electrophoresis (2-DE) within the pI range 4-7. The separated proteins were visualized by silver-staining of 2-D gels; simultaneously, proteins were transferred to nitrocellulose membranes for immunoblot analysis.
A total of 25 mice were divided into 3 groups, i.e. *B. abortus* infected (n=10), *Y. enterocoitica* O:9 infected (n=10) and uninfected controls (n=5). Sera were collected at three different stages of infection (10, 30, and 60 days post infection) and used for immunoblotting. The spots of gels and immunoblots were collated. After excluding both non-specific and cross-reacting spots, immunoreactive proteins with remarkable intensity more than mean were chosen and identified by MALDI-TOF MS.
On the silver-stained 2-D gels, a total of 1181 protein spots were found within the pI range 4-7. By immunoblotting, 13, 24, and 55 immunodominant proteins were identified by immunoblot profiles with *Brucella* antisera taken at days 10, 30, and 60 p.i., respectively. Excluding non-specific spots cross-reacting with antisera from *Y. enterocolitica* O:9-infected or non-infected mice, a total of 67 immunoreactive proteins were selected. Although there was no common immunogenic antigen found at all three stages of infection, there was a variety of immunodominant proteins with common spots in two different infection periods; 1, 5, and 8 spots were matched to correspondent spots at days 10 and 30, 10 and 60, and 30 and 60, respectively. Finally, 17 protein spots with remarkable intensity were selected and identified by MALDI-TOF MS and subsequently a NCBI BLAST search was conducted. Thus, 7 proteins (41.2 %) were predicted to be cytoplasmic, 2 (11.7 %) were outer membrane-bound and located in the periplasmic space, and 1 (5.9 %) was ribosomal, while the remaining 7 (41.2 %) were unknown. Immunogenic proteins identified at different stages of infection may be helpful for the improvement of diagnostic methods and vaccines.

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P18: Investigation on the carbon metabolism of the intracellular pathogen *Brucella suis* biovar 5

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As an intracellular pathogen, *Brucella* spp. have to adapt their metabolism to the new growth medium encountered during the infection i.e. the host. Little is known though on its metabolism even if it is closely linked to the virulence of the bacteria.
According to genomic data and biochemical information, *Brucella* possesses a complete pentose-phosphate pathway (PPP) and an incomplete classical glycolysis (EMP) as these bacteria seem to lack a phosphofructokinase. Moreover, brucellae have the two genes coding for the enzymes of the Entner-Doudoroff pathway (EDP) but no *in vitro* activity could be detected for the first enzyme. These information are useful to define the potential 'architecture' of the central metabolic network of brucellae but do not provide any evidence neither on how the network works nor on its adaptation potential.
In order to investigate the functionality of the metabolism of *Brucella,* stable isotope labeling experiments were performed. Such experiments rely on the culture of bacteria with a labeled substrate. In our study 1_^{13}C glucose was used, whose first C is ^{13}C which can be detected by mass spectrometry. The biomass derived from this culture consequently labels itself depending on the way the substrate has been metabolized. We investigated the metabolism of the 'fast growing' *B. suis* bv 5 grown in a chemically defined medium including 1_^{13}C glucose. The subsequent labeling of proteinogenic amino acids was analyzed by GC-MS.
EDP turned out to be the main glycolytic route, unlike the model usually considered in the literature describing the PPP as the major pathway. The relative significance of EDP and PPP for glucose metabolism can be confirmed by mutants deleted for the gene encoding the first enzyme specific of each pathway (i.e. *B. suis* bv 5 Δ*edd* and *B. suis* bv 5 Δ*gnd*). Indeed, the growth of *B. suis* bv 5 Δ*edd* mutant with glucose as sole carbon source is strongly impeded while the growth of *B. suis* bv 5 Δ*gnd* is also altered but to a lesser extent.
The relevancy of each pathway was also determined in an *in vivo* context. The ability of the two mutants to multiply within a host cell was assessed in RAW 264.7 macrophages. No attenuation could be observed for both mutants. Similarly, another mutant which is unable to catabolize glucose (*B. suis* bv 5 Δ*glk*) was also not attenuated.
These results suggest that the glucose catabolism pathways mainly found in *Brucella* are the EDP and to a lesser extent the PPP. These pathways and glucose, however, are not required for the bacteria to be virulent in a RAW 264.7 murine macrophage model of infection. Thus, *Brucella* seems to rely on other hitherto unknown carbohydrates to multiply during intracellular infection.

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P19: Link between PTS^{Ntr} and carbon metabolism in *Brucella abortus*

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Brucella is a Gram-negative intracellular bacterium belonging to α2-proteobacteria. It is the etiological agent of brucellosis which is one of the most widely spread zoonosis worldwide, causing abortion and infertility in animals. It can also infect humans causing a disease with flu-like symptoms called Malta fever. If not treated, it can lead to a chronic infection with severe focal complications.
To proliferate, bacteria need carbon sources which can be acquired from the environment with the participation of the phosphotransferase system (PTS). First discovered in *E. coli*, it forms a phospho-relay, ending with the entry and concomitant phosphorylation of a sugar. It possesses functions regulating the carbon metabolism and has, for instance, a well-known implication in carbon catabolism repression. *Brucella* (*B.*) *abortus* exhibits a paralogous system called Nitrogen PTS (PTS^{Ntr}), acting the same way except for the absent sugar entry. This suggests that it is only implicated in the metabolism regulation process. Starting from phosphoenolpyruvate, the phosphoryl group is successively transferred on histidine residues of Enzyme I (EI^{Ntr}) (encoded by the *ptsP* gene), then to the NPR protein (*ptsO*) and finally to $EIIA^{Ntr}$ (*ptsN*) or $EIIA^{MAN}$ (*ptsM*).
Since we were interested in the identification of a link between central metabolism and the *B. abortus* PTS^{Ntr} system, we have generated knockout mutants of the 4 abovementioned protagonists and studied their bacterial growth behavior on media containing different single carbon sources. *B. abortus* was mainly able to grow on sugar entering the pentose phosphate pathway. Interestingly, compared with the wild type strain, the *ptsN* ($EIIA^{MAN}$) mutant revealed a growth defect on those sugars while the *ptsM* ($EIIA^{Ntr}$) mutant showed better growth. These results suggest a potential role of the *Brucella* PTS^{Ntr} system in the regulation of central metabolism.

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P20: Biosynthesis of inositol in *Brucella*: Exploring its contribution in the bacterial virulence

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The biosynthesis of phospholipids is an area of considerable research attention, both in terms of the knowledge about host-pathogen interactions, as well as the understanding of phospholipid metabolism which may help to identify new therapeutic targets. One area of particular interest is to understand how facultative intracellular pathogens acquire basic molecules such as myo-inositol, serine, choline or ethanolamine for phospholipid synthesis. In *Brucella*, the inositol pathway and the implication of this pathway in virulence have not yet been studied. The predictive scheme of enzymes present in the genome of *B. abortus* 9-941 in the metabolic pathway of inositol phosphate from KEGG public database was used to address the study. These metabolic pathway enzymes predict two participants in inositol synthesis. The first enzyme is a phosphatase (EC 3.1.3.25) encoded by at least two genes (BruAb1_1696, BruAb2_0513), and the second enzyme is a dehydrogenase (EC 1.1.1.18) encoded by the gene BruAb2_0519. These genes were selected and mutated in *B. abortus* S2308 modified by integrative disruption (PTMm) generating the respective mutant *B. abortus* 1696, *B. abortus* 513 and *B. abortus* 519. Assessing the virulence in mice no attenuated phenotype was observed for the mutant *B. abortus* 519 with a disruption in the gene coding for the dehydrogenase. The other two mutants with disrupted genes encoding the phosphatase showed diametrically opposed forms of behaviour. The mutant *B. abortus* 513 was not attenuated, whereas *B. abortus* 1696 showed a highly attenuated phenotype in mice.

Based on the attenuated phenotype observed in *B. abortus* 1696, we decided to generate mutants in other *Brucella* species in order to determine whether it is conserved in the genus. By applying the method PTMm, we were only able to obtain mutants from *B. suis* 1330 in BruAb1_1696 and BruAb2_513 orthologous genes, named *B. suis* 1696 and *B. suis* 513, respectively. The phenotype of these mutants was similar to the parental strain *B. suis* 1330. We also investigated whether the strain *B. abortus* 1696 was able to generate a protective immune response in the mouse model of infection. After exposure with the mutant the mice were challenged with the virulent parental strain *B. abortus* S2308 and the vaccine strain *B. abortus* S19; then splenomegaly and splenic infiltration were measured. The *B. abortus* 1696 was able to generate a protective response both for the challenge with *B. abortus* S2308 and similarly with S19.

To the best of our knowledge, this is the first study analyzing the role of the inositol phosphate metabolism in the virulence of *Brucella* spp. Further studies should also take into account the possible implications of lipid molecules containing inositol (phosphatidylinositol, glycosylphosphatidylinositol) as well as inositol transporters or carriers with affinity to this compound, because reports on these topics are still missing.

The constructed mutants may help to get a deeper insight in the pathogenicity of *Brucella*. In the future, this can improve the design of new attenuated live vaccines.

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Session 3: Host-Pathogen Interaction

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Molecular and genetic analyses of environmental response systems in *Brucella abortus*

Bacteria often encounter stressful conditions over the course of their lifetime including nutrient deprivation, exposure to antibiotics and other toxic compounds, attack by bacteriophage, and changes in environmental conditions (e.g. temperature, osmolality, pH, etc.). To respond to these insults, numerous defense and adaptation mechanisms have evolved in the bacterial kingdom. Our group is investigating multiple classes of environmental response systems in *Brucella* (*B.*) *abortus* including type II toxin-antitoxin (TA) systems, two-component signal transduction systems, and alternative sigma factors. Our efforts and discoveries in these areas as they relate to *Brucella* physiology and infection are outlined briefly below.

Type II TA systems are protein-encoding genetic modules that have been implicated in persistence, biofilm development, and resistance to phage. These systems typically consist of a stable protein toxin and a labile protein antitoxin that is susceptible to proteolysis. TA systems are highly abundant on many bacterial and archaeal chromosomes; some species encode 100 or more, which can constitute up ~1.5 % of open reading frames. In contrast there are bacterial genomes that as of yet, have no predicted TA systems.
Using a combination of bioinformatic approaches, Makarova and colleagues predicted four type II TA systems in the *B. abortus* 2308 genome. Of these, two of the systems encoded toxins that were homologous to known type II toxins: RelE/ParE and Doc. Two other operons (*bab2_0933-932* and *bab1_0994-993*) were predicted to encode novel, uncharacterized toxins that were classified as part of sequence clusters COG5654 and COG2929, respectively. The RelE/ParE homolog is encoded by the gene *bab1_0436* and its cognate antitoxin is *bab1_0437.* The Doc toxin homolog is encoded by *bab1_0581* and the cognate antitoxin is *bab1_0582*. We have experimentally confirmed that the RelE/ParE and Phd/Doc systems are, indeed, functional type II TA systems in *Brucella.* Under our assayed conditions, BAB2_0933-932 had no genetic, biochemical, or physiological features of a type II TA system. However, we have demonstrated that *bab1_0994* encodes a novel ribonuclease toxin that is inhibited by a novel antitoxin, BAB1_0993. We have named this new toxin/anti-toxin system BrnT/BrnA and demonstrated that *B. abortus* BrnT is a ribonuclease.

Brucella abortus encounters many different stressors throughout its life cycle in the host, including low pH, oxidative stress, and nutrient deprivation. To test whether these and other stressors have an effect on transcription of *brnTA* we subjected *B. abortus* to heat shock (44°C), chloramphenicol treatment, 5 mM H_2O_2, and acidic pH (pH 4.0) to wild-type *B. abortus* and quantified *brnTA* transcript levels by RT-qPCR. Chloramphenicol, H_2O_2 stress, and low pH stress all induce *brnTA* transcription by 9- to 80-fold. We presume these stressors lead to the degradation of BrnA through an unknown protease and this degradation results in derepression of the *brnTA* operon. However, we cannot rule out other mechanisms of *brnTA* transcriptional activation.
Transcriptional activation of the *brnTA* during stress indicated that it may be playing a role in stress adaptation. To test this we measured growth of *B. abortus 2308* or *B. abortus 2308 ΔbrnTA* in various stress and non-stress conditions. When grown in complex medium (*Brucella* Broth), both wild-type and *ΔbrnTA* grow at identical rates and reach comparable densities in stationary phase. Similar results were obtained in Gerhardt's defined medium. There is also no significant difference in survival of wild-type *B. abortus* or *ΔbrnTA* upon exposure to

heat shock (44 °C), pH 4.0 (3 hour exposure), or 10 mM H_2O_2 (1 hour exposure). Although we did not observe a phenotype in the *Brucella ΔbrnTA* strain when treated *in vitro* with various stressors, it was still possible that this strain may exhibit differential survival and replication in macrophages. Peritoneal macrophages from BALB/c mice were activated with IFN-γ and infected with opsonized *B. abortus* 2308 or *B. abortus* 2308 *ΔbrnTA.* Both strains showed equivalent invasion and replication within these cells. Thus, to date, we have not identified a cellular/*in vivo* phenotype that is associated with genetic perturbation of this novel TA system. We are currently investigating other conditions *in vitro* and *in vivo* that result in differential growth or survival of *ΔbrnTA*.

The capacity of *Brucella* spp. to survive the harsh environmental stressors encountered *en route* to, and within, their replicative niche, including reactive oxygen species, acidic pH, and nutrient deprivation is a key virulence determinant. In collaboration with the group of Marty Roop, we have shown that the alphaproteobacteria general stress response (GSR) system consisting of the protein PhyR, NepR, and σ^{E1} is required for acute oxidative and acid stress *in vitro* and for maintenance of *B. abortus* within murine spleens during the chronic phase of infection (i.e. > 1 month post-infection).

Given our discovery that *B. abortus* strains lacking an intact GSR system are deficient in survival under acute oxidative and acid stress in vitro, we initially predicted that there would be some defect in the acute phase of animal infection where the *B. abortus* cell is exposed to oxidative burst and acidification of the early phagosome. However, our data indicate that *phyR* and *rpoE1* are dispensable in the context of initial colonization of both murine spleens and primary murine macrophages. These results provide evidence that other stress adaptation systems not under the control of PhyR-NepR-σ^{E1} are sufficient to mediate bacterial cell survival in the face of stresses encountered during the first month of infection; it is only during the chronic phase of infection that we observe a difference in the rate of clearance of *B. abortus* Δ*phyR* and Δ*rpoE1* strains relative to wild-type.
We envision two models by which the *B. abortus* GSR system mediates long-term cell survival in an animal host: 1) GSR is required for adaptation to a stress condition(s) that is unique to the chronic phase of infection or 2) the *B. abortus* GSR system regulates genes that are required for subversion or evasion of host immunity during the chronic stage. In regard to the latter model, our microarray analysis confirms that *rpoE1* controls expression of genes required for flagellum biosynthesis. It is known that expression of this classical pathogen-associated molecular pattern (PAMP) is required for *B. melitensis* host persistence and interacts with the host innate immune system. *rpoE1* also regulates genes involved in synthesis of the LPS O-chain, which has known immunomodulatory properties. Of course, neither of these models is mutually exclusive: regulation of chronic infection by the GSR system may involve changes in both *Brucella* stress physiology and modulation of its interactions with multiple arms of the host immune system, thus ensuring it remains weakly immunogenic throughout the course of infection. Future study of this regulatory system will provide insight into host side stresses and signals that are unique to the chronic phase of infection, and to which *Brucella* spp. must adapt in order to maintain long-term host colonization.

Oral Presentations (O7-O10)

O7: Marine mammal brucellae are attenuated in a mouse model of infection

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Brucella (*B.*) spp. were first isolated from marine mammals in 1994 and were validly published as the species *B. pinnipedialis* and *B. ceti* in 2007, with pinnipeds and cetaceans as preferred hosts, respectively. Marine mammal brucellae have, since then, been serologically proven in and isolated from pinnipeds and cetaceans around the world. Gross pathology associated with *Brucella* infection in marine mammals is reported almost exclusively in cetaceans.

The goal of this study was to evaluate the pathogenicity of *B. ceti* (NCTC 12891^T) from a harbour porpoise (*Phocoena phocoena*) and *B. pinnipedialis* (NCTC 12890^T) from a harbour seal (*Phoca vitulina*) in a BALB/c mouse model of infection. We also characterized the pathogenicity of the *B. pinnipedialis* hooded seal (*Cystophora cristata*) strain 22F1 in the same model. *B. suis* bv 1 strain 1330 was included as a classical brucellae positive control.

Spleen replication patterns showed that both *B. pinnipedialis* strains were strongly attenuated compared to *B. suis*. At day 35 p.i. approximately log 2 CFU were found in the spleen of the mice infected with *B. pinnipedialis*, and by day 84 p.i. the infection was cleared. The *B. pinnipedialis* reference strain yielded a slight spleen inflammation that peaked at day seven p.i., while the hooded seal strain yielded no increase in spleen weight. *B. ceti* was attenuated, but was still present in the spleen at day 84 p.i. (approximately log 2 CFU), and yielded a slight increase in spleen weight, peaking at day 14 p.i. By day 35 p.i. the spleen weights of the mice infected with the marine mammal brucellae were similar to that of the non-infected controls. The number of *Brucella* spp. in the liver and the liver weight showed the same kinetics as for the spleen. Histological findings were in accordance with these patterns of attenuation. *B. suis* and *B. ceti* were isolated from the brain until day 7 and 14 p.i., respectively. All the marine mammal brucellae were isolated from the blood, although not persistently, and the bacteraemia values were always less than those of *B. suis*. By day 21 p.i. *B. pinnipedialis* were not present in the blood, while *B. ceti* persisted until day 35 p.i. *B. ceti* was isolated from the faeces solely at day 14 p.i., while *B. suis* was isolated from the faeces until day 35 p.i.

The minimal pathology induced by both *B. pinnipedialis* strains in the mouse model is consistent with the lack of *Brucella*-associated gross pathology reported when *B. pinnipedialis* is isolated from true seals. *B. ceti* was also attenuated, but showed a more chronic pattern which is in line with the pathological findings found in porpoises. Whether other strains of *B. ceti*, e.g. from striped dolphin (*Stenella coeuleoalba*) where *B. ceti* have been isolated numerous times in conjunction with abortions, lesions in the reproductive organs and neurobrucellosis have a different pathogenic potential, warrants further investigation.

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O8: Defining the role of a LysR-type transcriptional regulator in the survival and pathogenesis of *Brucella abortus* 2308

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Small RNAs (sRNAs) play critical roles in both bacterial virulence and gene regulation. As a whole, bacterial sRNAs control the expression of a variety of genes by acting, most commonly, at the post-transcriptional level. In *Brucella abortus*, two sRNAs in particular, AbcR1 and AbcR2, are crucial for bacterial survival and replication in a mouse model of chronic *Brucella* infection. Although these findings are central to understanding the pathogenesis of *Brucella*, it was not known until recently how sRNA expression is regulated. Upon examination of the *Brucella* chromosomes, a LysR-type transcriptional regulator (LTTR), which we named VtlR, was found flanking *abcR2* on chromosome 1. It was demonstrated that VtlR controls the expression of *abcR2*, but not *abcR1*. To clarify the role of VtlR as an activator of *abcR2*, electrophoretic mobility shift assays (EMSAs) were employed, and these experiments showed that VtlR binds directly to the *abcR2* promoter, but not to the *abcR1* promoter. Furthermore, virulence assays with both macrophage and mouse models revealed a crucial role for VtlR in *Brucella* pathogenesis. The *vtlR* mutant strain exhibits a significant defect in its capacity to survive and replicate within macrophages, and additionally, the *vtlR* mutant is attenuated in experimentally infected mice compared to the parental strain 2308. Microarray analysis of the *vtlR* mutant strain also revealed three genes putatively encoding small proteins under the control of this regulator in addition to *abcR2*. Although we have defined the importance of VtlR in both gene regulation and virulence, the next steps include elucidating the signal/stimuli that VtlR is responding to within its host, and ultimately, to determine the mechanism by which VtlR is able to control gene expression in *Brucella*.
Interestingly, orthologs of VtlR are found amongst a wide range of other α-proteobacteria including *Sinorhizobium*, *Bartonella,* and *Agrobacterium*. In the long-term, our laboratory is interested in characterizing the evolutionary aspects of VtlR and its orthologs in terms of how these transcriptional regulators impact host-bacterium interactions.

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O9: Transcriptional profile of bovine chorioallantoic membrane explants in response to wild type, Δ*virB2* or Δ*btpB Brucella abortus* infection

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Brucellosis is one of the most important zoonoses worldwide. The pathogenesis of the *Brucella*-induced inflammatory response in the bovine placenta is not completely understood. Therefore, studies on the interaction between bovine trophoblastic cells and *Brucella* (*B.*) are extremely important. In this study the transcription profile of bovine trophoblastic cells infected with *Brucella* strains was evaluated. Transcription profiles of chorioallantoic membrane (CAM) explants inoculated with wild type (strain 2308), Δ*virB2* or Δ*btpB B. abortus* were compared by microarray analysis at 4 hours post infection. Genes with significant variation in levels of transcripts (fold change > 2 and $p<0.05$) were functionally classified, and transcripts related to defense and inflammation were assessed by quantitative real-time RT-PCR. Infection with wild type *B. abortus* resulted in more genes with decreased than increased transcription levels. Conversely, infection of trophoblastic cells with the Δ*virB2* or the Δ*btpB* mutant strains, that lack a functional T4SS or that has impaired inhibition of TLR signaling, respectively, induced more upregulated than downregulated genes. Importantly, wild type *B. abortus* impaired the host transcription of genes related to immune response when compared to Δ*virB* and Δ*btpB*. CAM explants infected with the Δ*virB2* or Δ*btpB B. abortus* had increased transcription of proinflammatory cytokines and chemokines, including chemokine (CC motif) ligand 5, chemokine (CC motif) receptor-like 2, chemokine (CXC motif) receptor 5, chemokine binding protein 2 (CCBP2) family with sequence similarity 19 (chemokine (CC motif)-like) member A4, IFN-alpha C, Alpha-omega interferon-like, interleukin 1 family member 6 (epsilon)-like, interleukin 15, interleukin 2 receptor alpha, tumor necrosis factor (ligand) superfamily member 10-like, tumor necrosis factor receptor superfamily member 13C, tumor necrosis factor receptor superfamily member 9, interferon alpha G and molecules of the complement cascade as complement component 3a receptor 1, complement factor H, complement factor I. Our findings suggest that proinflammatory genes can be negatively modulated in bovine trophoblastic cells at early stages of infection with *B. abortus*. The *virB* operon and *btpB* are directly or indirectly related to modulation of these host genes.

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O10: Cell cycle progression of *Brucella abortus* in culture and inside HeLa cells and RAW264.7 macrophages

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Brucella (*B.*) *abortus*, like several intracellular pathogens, displays a biphasic infection process starting with a non-proliferative stage. Whether this stage results from bacterial death, division defects or cell cycle arrest is unknown. Here, we study the cell cycle of *B. abortus* at the single-cell level, in culture and during infection of HeLa cells and macrophages. In order to monitor cell growth, we took advantage of its unipolar growth. The duplication of replication origins was detected by counting the number of fluorescent foci per cell, generated by the production of mCherry-ParB and YFP-RepB fusions. The insertion of plasmidic *parS* sequences recognized by a cognate plasmidic ParB fused to YFP or CFP, near the predicted replication origins validate the use of the fusions to ParB and RepB to monitor the number of replication origins. Moreover, the tagging of replication termination regions suggests that both chromosomes are oriented in *B. abortus*.
The localization of segregation and replication loci of the two bacterial chromosomes indicated that, immediately after being engulfed by endocytic vacuoles in HeLa cells, most bacterial cells are newborn cells. These bacteria do not initiate DNA replication for the subsequent 6 hours, indicating a G1 arrest. Moreover, growth is completely stopped during that time, reflecting a global cell cycle block. Growth and DNA replication resume later on although bacteria still reside within endosomal-like compartments, identified by the presence of the Lamp1 marker. Interestingly, a Δ*virB* mutant also initiates growth in Lamp1 positive compartments, but it is unable to complete its cell cycle afterwards. The cell cycle block of wild type *B. abortus* in early stages post-infection was also observed in macrophages, with a different timing since growth was detected earlier compared to HeLa cells.

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Poster Presentations (P21-P37)

P21: Adhesion, invasion and intracellular replication of *Brucella* in polarized epithelial cells

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Virulence of *Brucella* is completely dependent on the capacity of the bacteria to invade, survive and replicate in host cells, particularly in professional phagocytes such as macrophages and dendritic cells. Because of this, much has been done in order to understand the intracellular life cycle of the bacteria in these cell types and a good body of information has been generated regarding invasion and trafficking. Albeit that little is known about the intracellular behavior of *Brucella* in polarized epithelial cells, the first cell type encountered by the bacteria upon infection of the host. We identified an adhesin (*ilgA*), conserved in all *Brucella* species that harbors an intimin-like domain and mediates the adhesion and invasion of Madin-Darby canine kidney (MDCK) cells and other epithelial cells. Our results indicate that *Brucella* is able to invade and replicate in MDCK cells and that invasion occurs through the cell-cell interaction membrane in a process that depends, in part, on *ilgA*. *IlgA* knock out significantly decreased adhesion to host cells and over-expression of the gene dramatically increased both adhesion and invasion, indicating that this gene plays a central role in the initial interaction with the cells. Treatment of different cell lines with the recombinant intimin-like domain of IlgA showed that it localized to focal adhesion sites and induced complete detachment of the cells suggesting that it targets proteins in these structures. Additionally, we have performed a detailed microscopic analysis of the intracellular life cycle in these cells which showed that the bacteria achieve a complete cycle including a programmed exiting to re-infect other cells.

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P22: Investigating *Brucella abortus* cell cycle regulation in a HeLa cell infection model

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The intracellular trafficking of the facultative intracellular mammalian pathogen *Brucella* (*B.*) *abortus* has been often studied in host cells or animal models. However, the control of the cell cycle both in culture and during infection is poorly understood. Recent results obtained in our lab showed that, during the early non proliferative phase of a HeLa cell infection, *B. abortus* growth is arrested and its cell cycle is blocked in G1 phase. The goal of the present project is to investigate this cell cycle blockage by studying the phosphorelay involving the essential response regulator DivK and its potential kinases/phosphatases (PdhS, PleC and DivJ). This pathway is thought to control the central transcription factor CtrA that potentially regulates *B. abortus* cell cycle. Phosphotransfer assays done on purified recombinant proteins showed that the essential histidine kinase PdhS can phosphorylate DivK. Furthermore, we aimed to investigate CtrA regulon by fusing putative CtrA target promoters to a reporter gene coding for an unstable GFP. Hence, we were able to show that *pleC* promoter harbouring a CtrA binding box is not regulated in a cell cycle-dependent manner in culture; but its activity varies significantly during infection. Indeed, we detected a high activity of *pleC* promoter at 3h p.i. followed by a drastic drop in the fluorescence around 6-8h p.i., the time point at which the cell cycle is resumed in HeLa cells. These data suggest that the DivK-CtrA pathway could be differentially controlled along infection and that PleC might play a role in the cell cycle blockage of *B. abortus* at early time points p.i. We thus decided to monitor *B. abortus* growth during its intracellular trafficking by labelling the bacteria with TRSE (TexasRed succinimidyl ester). TRSE forms covalent bonds with amine groups present on the surface of bacteria and allows the staining of the bacterial envelop. Given the fact that *B. abortus* has a unipolar growth, the newly added material at the growing pole was not labelled with TRSE. The results showed that a strain deleted for *pleC* started its growth 2 to 4 hours before the WT strain; however, its growth was delayed at later points in time (10-12h p.i.). This suggests that PleC might contribute to the cell cycle blockage and that this blockage is important to allow an optimal growth of the bacteria at later stages post infection.

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P23: *Brucella suis* and *Brucella abortus* use distinct molecular components for adhesion to HeLa cells

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Little is known about the mechanisms allowing *Brucella* (*B.*) spp. to adhere to and invade host cells. So far, several studies have highlighted the importance of the interaction between *Brucella* and components of the host cell extracellular matrix for infection. More specifically, for *B. suis* it was shown that the deletion of two genes coding for the monomeric autotransporter *bmaC* and the trimeric autotransporter *btaE* had a severe impact on the adherence and thus infectiosity in HeLa cells.
Here, we report that the deletion of *bmaC* and *btaE*, separately or combined in a double deletion strain, does not alter the infectiosity of *B. abortus* 2308 in HeLa cells, thus implying that adherence mechanisms are not shared among these closely related bacterial species. We therefore assume that *B. abortus* 2308 uses other molecular components to stimulate adherence and/or invasion in HeLa cells.

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P24: The two-component system BvrRS of *Brucella abortus* senses intracellular acidic conditions and triggers signals required for replicationn

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The two-component system (TCS) BvrR/BvrS (BvrRS) is essential for the intracellular lifestyle of *Brucella* (*B.*) *abortus* and controls the expression of virulence determinants such as the transcriptional regulator VjbR and the Type Four Secretion System (T4SS) VirB. To understand the role of BvrRS we have analyzed the expression and phosphorylation of the system in bacteriological cultures and in intracellularly grown bacteria in cultured mammal cells. The TCS was highly expressed at early hours of the bacterial growth curve, and steadily declined at a later point in time. Incubation of log phase bacteria in minimal medium at pH 5.5 induced the phosphorylation of BvrR and increased the expression of VjbR and VirB. In contrast, stationary phase bacteria were unable to respond to these conditions. The BvrS was undetectable in the stationary phase. Consistently, cells infected with *B. abortus* at stationary phase achieved significantly lower levels of replication than those infected with bacteria at exponential phase. Bacteria extracted from infected cells demonstrated phosphorylated BvrR and an increase in VjbR and VirB8 proteins. Intracellular replication of *B. abortus* was affected by inhibition of the endosomal acidification of cells. However, the intracellular replication under these conditions could be reestablished if the bacteria were extracellularly incubated in acidic minimal medium before infection. Our results support a model where the TCS BvrRS senses the transition from an extracellular milieu to an intracellular niche triggering a transcriptional response required to guide the bacteria during the first hours of its intracellular journey.

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P25: The c-di-GMP phosphodiesterase BpdA regulates *Brucella melitensis* stealthy strategy

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Brucellosis is a worldwide zoonotic disease caused by facultative intracellular Gram-negative bacteria of *Brucella* (*B.*) species. A major hallmark of the infection process is the ability of the bacteria to evade both innate immune response and host apoptosis, thus leading to the tagging of *Brucella* spp. as 'stealthy' pathogens. Recently, *B. melitensis* deletion mutants for three of its c-di-GMP metabolizing enzymes [BpdA (BMEI1453), BpdB (BMEI1448) and CgsB (BMEI1520)] were found to affect the virulence of the bacterium in a mouse model of infection. Our aim was to use these mutants to study the c-di-GMP directed regulation of *B. melitensis* virulence and cognate stealthy strategy. We found that c-di-GMP phosphodiesterases attenuated mutants (*bpdA* and *bpdB*) as well as c-di-GMP synthase hyper virulent mutant (*cgsB*) presented a smaller colony size than wild-type bacteria, while the growth rate in liquid broth was not changed. The *bpdA* mutant was found to be most affected by its capacity to influence the host among the studied mutants. In accordance with low pathogenicity it showed lower bacterial counts in the spleen of infected mice compared to wild type infection. Moreover, using real-time PCR array for analyzing expression of type I interferon related genes in mouse bone marrow derived macrophages, we identified a higher than wild-type induction of this pathway by the *bpdA* mutant, as early as 4 hours post infection. For example, we showed increase in CCL4, IL10 and TYK2 expression. Nevertheless, 24 hours p.i. confocal analysis of the infected macrophages did not reveal differences between the mutants and the WT. Our results suggest that BpdA plays a key role in regulation of *B. melitensis* ability to avoid the innate immune response of the host. We propose that this c-di-GMP phosphodiesterase resides upstream to major cellular processes in *B. melitensis* affecting both the physical properties of the bacterium, and its communication with the environment.

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P26: Complementation of a natural mutation in the NtrB histidine kinase locus restores *Brucella suis* biovar 2 virulence *in vivo* in the mouse model of infection

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Brucella (*B.*) *suis* biovar 2 is rarely zoonotic in contrast to *B. suis* biovar 1, which is highly pathogenic to humans. To investigate genetic determinants responsible for the differential pathogenicity in humans between *B. suis* biovar 1 and biovar 2, we combined *in silico* approaches with *in vitro* and *in vivo* (mouse) studies. Here, we showed that the *ntrB* gene of *B. suis* biovar 2, encoding for the NtrB histidine kinase of the NtrBC two-component systems (TCS) carries a frameshift mutation and a deletion of 69 bp which renders it inactive (pseudogenisation). The mutations were conserved in all the tested *B. suis* biovar 2 strains (5/5) isolated from Belgian wild boars (*Sus scrofa*) over a period of 20 years. *B. suis* biovar 2 displayed *in vitro* a reduced proliferative capacity and persistence inside HeLa cells and THP1 human-derived macrophages, in comparison to *B. suis* biovar 1. The observed attenuated virulence of *B. suis* biovar 2 was restored by complementing *B. suis* biovar 2 with the wild type *B. suis* biovar 1-derived *ntrB* gene, cloned in a pBBR1 plasmid vector. The phenotype of the two biovars and the complemented strain was compared in the *in vivo* murine model of infection and it was found that the functional complementation of the *ntrB* gene in *B. suis* biovar 2 increased the persistence of the bacteria in spleen (5.1 log10 CFU at 8 week post-infection (8WPI)), inducing a phenotype similar to *B. suis* biovar 1 (4.9 log10 CFU at 8WPI), in comparison to *B. suis* biovar 2 wild type strain (3.4 log10 CFU at 8WPI). This is the first description of the functional complementation of a TCS pseudogene acquired through natural selection in wildtype *B. suis* biovar 2 and confirms that the NtrBC TCS proteins are necessary for displaying the full pathogenicity of *B. suis* as an intracellular pathogen.

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P27: Characterization of regulatory regions recognized by the two-component system BvrR/BvrS in *Brucella abortus*

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Two-component systems (TCS) are signal transduction systems commonly used by bacteria to regulate gene expression in response to environmental signals. The TCS BvrR/BvrS (TCS BvrRS) is one of the best characterized systems in *Brucella* (*B.*) *abortus* and has been related to *Brucella* virulence. Identification of genes under the control of TCS BvrRS will help to understand and control brucellosis. To date, many of these genes have been defined, nonetheless it is unknown if the exerted regulation is a direct or indirect mechanism. Three *B. abortus* genes that have been previously linked to the TCS BvrRS regulon and *Brucella* virulence, i.e. *virB*, *vjbr* and BAB10046 (*omp160*) were selected for further analysis. Using electrophoretic mobility shift assays (EMSA) we have demonstrated that BvrR-P binds directly to the regulatory regions of these genes, suggesting that the TCS BvrRS directly controls the expression of *virB*, *vjbr* and *omp160*. To narrow BvrR-P binding site at the *virB* regulatory region, 10 oligonucleotides of 40 bp each were submitted for EMSA analysis with BvrR-P. Only one of the 10 oligonucleotides bound to BvrR-P, confirming DNase footprinting analysis and denoting the specific binding site of BvrR-P within the *virB* regulatory region. Altogether, these data are contributing to dissect the intricate gene regulatory networks in *Brucella* and the role of TCS BvrRS in regulating virulence factors.

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P28: *In vivo* virulence and immunogenicity of the *Brucella melitensis* 133 *invA*-km strain.

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Brucellosis is a zoonotic disease distributed worldwide and caused by bacteria of the genus *Brucella* (*B.*). In animals, this disease is characterized by reproductive failure, and in humans, the disease is known to produce febrile episodes, general malaise and prostration. Out of the 10 recognized species of the genus, mainly *B. abortus, B. melitensis* and *B. suis* cause human infections. The invasion and intracellular survival mechanisms of *B. melitensis* are not fully understood. The complete genome sequence of *B. melitensis* shows an ORF with NUDIX hydrolase characteristics. This enzyme has been described in other bacterial pathogens as an invasin. In the present work, we generated a mutation in the BMEI0215 gene (*invA*) of *B. melitensis* 133 strain. The objective was to elucidate the role of the invA in the murine infection model. Mice were inoculated intraperitoneally with 10^4 CFU. Bacterial infection was determined by spleen colonization. Bacterial counts were performed per gram of tissue. Also, spleen weight was measured in order to evaluate splenomegaly. Antibody isotype switching in serum and IFN-γ transcription in blood was determined. Results of *in vivo* infection demonstrated that mutant spleen colonization was significantly lower than that of the parental strain ($p<0.001$). The most significant difference was observed at day 30 p.i., when the parental strain concentration was 6-fold higher than that of the mutant strain ($p<0.001$). Splenomegaly was constant in the mice which were inoculated with the parental strain, in contrast to the group inoculated with the mutant strain. The immune response tested by IFN-γ transcription and antibody production was similar for the Rev1 vaccine strain and the mutant strain. This response was less protracted than the immune response induced by the parental strain. Our results show that mutation of *invA* results in attenuation *in vivo*.

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P29: The *Brucella suis* effector protein BR1024 co-localizes with mitochondria in HeLa cells and interacts with the COP9 signalosome subunit CSN6

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The *Brucella* (*B.*) *suis* protein BR1024 is an ortholog of the previously identified *B. abortus* protein BPE043, which was shown to be translocated into mouse macrophage J774.A1 cells in a VirB-dependent manner, suggesting a role for this protein in host cells during infection. BR1024 is annotated as a 'kinesin-like' protein in the *B. suis* genome, but its function remains unknown. BR1024 is a conserved hypothetical protein of 1557 amino acids, with two predicted transmembrane domains in its N-terminal region and four apolipoprotein domains in the C-terminal region. The C-terminal of the protein also shows homology with eukaryotic myosin and kinesin. By qRT-PCR we found that the gene was co-expressed with the *virB* operon under acidic conditions, in agreement with a role for this protein in host cells. We were unable, however, to obtain an unmarked deletion of the gene encoding BR1024 after many attempts, suggesting this protein has an essential role in the bacteria, or possibly that other essential regulatory elements, such as sRNAs, may be located in the gene sequence. A GFP-BR1024 fusion protein ectopically expressed in HeLa cells was seen to co-localize with mitochondria. In a yeast two-hybrid screen we identified the CSN6 subunit of the COP9 signalosome as a putative interacting host protein. We will discuss the possible roles for this protein during *Brucella* infection.

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P30: Identification of human alpha-enolase (ENO1) as an interaction partner of a *Brucella abortus* VirB substrate

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Brucella (*B.*) *abortus*, the causative agent of bovine brucellosis, invades and replicates within cells inside a membrane-bound compartment known as the *Brucella* containing vacuole (BCV). After trafficking along the endocytic and secretory pathways, BCVs mature into endoplasmic reticulum (ER)-derived compartments permissive for bacterial replication. *Brucella* Type IV Secretion System (VirB) is a major virulence factor essential for the biogenesis of the replicative organelle. VirB system translocated effector proteins are thought to modulate host cell signaling pathways to favor intracellular survival and replication. Recently, many VirB substrates have been identified. Uncovering the targets and functions of these translocated effectors is essential to understand the role of VirB in pathogenesis.
BPE123 (YP_418361.1) is a *B. abortus* VirB-translocated effector protein identified by our group. It is a hypothetical protein whose function remains unknown. In an attempt to identify host cell proteins interacting with BPE123, a pull-down assay was performed and human alpha-enolase (ENO1) was identified by LC/MS-MS as a potential interaction partner of BPE123. These results were confirmed by immunoprecipitation assays. Microscopy studies further confirmed BPE123-ENO1 interaction: ENO1 relocalization was detected upon ectopic expression of BPE123 in HeLa cells, where both proteins localized to the ER. Furthermore, during macrophage infection we observed recruitment of ENO1 to the vicinities of BPE123 positive BCVs, indicating that interaction with translocated BPE123 might also occur during the intracellular phase of *B. abortus*. Taken together, these preliminary results suggest a direct interaction between BPE123 and ENO1, a multifunctional protein that has already been shown to be required for *Brucella* replication in host cells. Further experiments are underway to determine how BPE123-ENO1 interaction modulates the outcome of the infection process.

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P31: Interaction network and localization of *Brucella abortus* membrane proteins involved in the biosynthesis, transport and succinylation of cyclic β-1,2-glucans

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Cyclic β-1,2-glucans (CβG) are periplasmic homopolysaccharides that play an important role in several symbiotic and pathogenic relationships. *Brucella (B.) abortus* CβG synthase (Cgs) is a polytopic integral inner membrane (IIM) protein of 320 kDa (2,867 amino acid residues) with six transmembrane-spanning segments. Cgs itself acts as a protein intermediate and catalyzes four reactions, namely initiation, elongation, phosphorolysis and cyclization, required for the synthesis and control of the degree of polymerization of CβG. Once synthesized in the cytoplasm, CβG are transported to the periplasm by the CβG transporter (Cgt) and succinylated by the CβG modifier enzyme (Cgm). Cgt and Cgm as well as Cgs are polytopic IIM proteins. In this work, we used a bacterial two-hybrid system (BACTH) and co-immunoprecipitation techniques to study the interaction network between these three IIM proteins. Our results indicate that Cgs interacts with Cgt and Cgm, and that Cgt interacts with Cgm. We also observed that each one of these proteins form homotypic complexes. Analyses carried out with Cgs in-frame pentapeptide insertion mutants and Cgs deletion mutants revealed that a coiled-coil motif located in the N-terminal domain of the protein (Cgs-region 1 to 418) is required to sustain the interactions of Cgs with Cgt and Cgm as well as with itself, although other regions may also be involved. Finally, to analyze the subcellular localization of the protein complexes we performed fluorescence confocal microscopy studies fusing Cgs, Cgt and Cgm to mCherry, eYFP or eCFP and found that these proteins are localized at the cell poles in *B. abortus*. Taken together, these results demonstrate that Cgs, Cgt and Cgm interacts with itself (homotypic interactions) and with each other (heterotypic interactions) forming a membrane-associated biosynthetic complex located at the poles of the bacterial cell. Biosynthesis of CβG is predicted to require strict coordination of the initiation, elongation, phosphorolysis and cyclization reactions as well of its export to the periplasm and succinylation. We propose that the formation of the Cgs-Cgt-Cgm membrane complex at cell poles could serve as a mechanism to maintain the fidelity of cyclic β-1,2-glucans biosynthesis and coordinate the synthesis with its transport and modification.

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P32: Characterization of highly conserved hypothetical proteins in *Brucella abortus*

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Small RNAs (sRNAs) are important regulators of protein function and gene expression in bacteria. Their regulatory roles are crucial for a wide range of functions in bacteria, spanning from host-pathogen interactions in *Agrobacterium tumefaciens* to metabolism in *E. coli*. AbcR1 and AbcR2 are two sRNAs in *Brucella* (*B.*) *abortus* that are required for the survival and replication of the bacteria within macrophages and the host. Indeed, deletion of both *abcR1* and *abcR2* results in significant attenuation of *B. abortus* in experimentally infected mice.

Recently, we have identified a LysR-type transcriptional regulator that controls the expression of *abcR2*, but not *abcR1*. A *B. abortus lysR* mutant shows no transcription of AbcR2, and importantly, this mutant strain is attenuated in both macrophage and mouse models of *Brucella* infection. These experiments also revealed the transcriptional regulation of three genes putatively encoding hypothetical proteins in *B. abortus* by the LysR regulator. Genetic and amino acid analysis reveals that two of these hypothetical proteins share over 90 % protein similarity signifying that they may be serving redundant functions. Interestingly, there are over 100 orthologs of these hypothetical proteins found in other organisms, specifically in a wide variety of host-associated α-proteobacteria. Why are these hypothetical proteins so well conserved among highly related bacteria? This is one question that our lab is trying to answer by characterizing these proteins in *B. abortus.*

Our studies show that deletion of these hypothetical proteins does not affect the ability of *B. abortus* to survive and replicate in macrophages. Our on-going studies are assessing the capacity of these mutant strains to establish a chronic infection in the mouse model of brucellosis. The goals of this project are to 1) identify whether these hypothetical proteins are encoding proteins or sRNA, 2) determine the molecular function of each sRNA/hypothetical protein, and 3) assess the role of these hypothetical genes in the ability of *Brucella* to survive in and to colonize the host.

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P33: *Brucella abortus* mutant deficient in a cell envelope associated protein overexpressed during intracellular trafficking

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Intracellular pathogens are able to modify their protein composition to cope with different extracellular and intracellular conditions. During macrophage infection with *Brucella* (*B.*) *abortus* 2308, several bacterial cell envelope-associated proteins increased in time, as revealed by proteomic analysis. One protein corresponds to the Bab1_0046 gene product. After three hours of infection, this protein already accumulates and persists in high concentration up to 44 hours. It has been demonstrated that the inactivation of *B. melitensis* gene BMEI_1894 (orthologous to Bab1_0046) generates an attenuated phenotype. Bab1_0046 is supposed to be under control of the two component regulatory system BvrR/BvrS since BvrR binds to a sequence located upstream the promoter of this gene. Here, we report the generation of a *B. abortus* ΔBab1_0046 deletion mutant.

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P34: Differential contribution of OMP19 to the virulence in *Brucella* spp.

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The *omp19* gene of *Brucella* (*B.*) spp. encodes an outer membrane lipoprotein. The biological function of Omp19 remains undefined but its amino acid sequence shows conserved domains with *inh*, which is a family of protease inhibitors (Pfam accession no. PF02974). Omp19 orthologues can be identified in members of four families (Brucellaceae, Rhizobiaceae, Bartonellaceae and Phyllobacteriaceae) of the order *Rhizobiales*, appearing both in free-living microorganisms as well as in bacteria symbiotically associated with eukaryotic cells. Several reports have shown that purified recombinant Omp19 induces the secretion of proinflammatory mediators from antigen presenting cells such as endothelial cells. The inactivation of *omp19* in *B. abortus* S544 was postulated to alter their outer membrane properties, which suggests that Omp19 is important in the maintenance of the outer membrane integrity. This *omp19* null mutant was slightly attenuated in the model of splenic colonization in mouse, in comparison with their parental wild type S544.
We studied the contribution of Omp19 to the pathogenicity of the three more virulent species of *Brucella*. Therefore, we constructed *omp19* knock-out mutants of the reference strains *B. abortus* S2308, *B. melitensis* 16M and *B. suis* 1330. The mutants were evaluated in a mouse model of infection. The colony forming units (CFU) recovered from spleens at 4 weeks and 8 weeks *p.i.* were determined. We observed that the inactivation of *omp19* in *B. suis* 1330 and *B. melitensis* 16M did not alter its virulence in mice, whereas in the S2308 Δ*omp19* mutant the CFU counts obtained were almost 1 log lower in comparison to the parental strain, in both sampling points and in agreement with the results reported for S544 Δ*omp19*.
Comparable to all bacterial lipoproteins, the N-terminal cysteine residue of Omp19 is lipid modified. We investigated the role of Omp19 lipidation in the virulence of *B. abortus* S2308. We obtained an allele of *omp19* where the cysteine was replaced by a glycine residue, thus encoding a non-lipidated version of Omp19 (U-OMP19). The wild type *omp19* gene and the u-*omp19* version were cloned into pBBRMCS1 and were introduced by electroporation in the mutant S2308 Δ*omp19.* The complemented transformants were evaluated in mice. The results demonstrated that the lipidation of Omp19 was not essential to recover the full virulent phenotype of *B. abortus* S2308.

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P35: Organotypic brain cultures as a model for neurobrucellosis research

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Brucellosis is a zoonotic disease caused in humans by infection with different species of the genus *Brucella* (*B.*). The most characteristic symptoms are undulant fever, sweating, and joint and muscle pain, the classic brucellosis triad. One of the life-threatening complications of brucellosis is neurobrucellosis that can be diagnosed in up to one-third of the patients with confirmed *Brucella* infection. Studies of the mechanisms of infectious diseases in the brain classically rely on the use of animal models and two-dimensional (2D) cell cultures. However, 2D monolayers lack the structural complexity and the physiological relevance of *in vivo* tissue preparations. Hence, they are limited predictors of the infection process. Contrariwise, the use of animal models is time-consuming and depends on the availability of costly animal facilities. The introduction of the *ex-vivo* nervous system model (3D organotypic slice cultures) has introduced a fundamental experimental tool in this research field. Key features of the *ex vivo* model include well-defined cellular architecture, the presence of axonal projections, and the preservation of the *in vivo* 3D organization and long-term thickness of the preparation. Thus, the model mimics the morphological and functional features of the *in vivo* parental tissues.
Brain slice cultures have been used to study infections of the CNS with several pathogens, and we have tested their ability to support *Brucella* infection and to imitate some of the *in vivo* findings. Organotypic explants were obtained from newborn (7-10-day old) Sprague Dawley rats, and infected with *B. abortus* 2308 and several avirulent mutant strains. The kinetics of the bacterial infection was determined.

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P36: *Brucella* virulence in human trophoblasts

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The intracellular replication of brucellae has been extensively characterized in a variety of host cells. *Brucella* (*B.*) spp. have been shown to replicate in a vacuole derived from the endoplasmic reticulum (ER) in epithelial cells, macrophages and dendritic cells as well as in animal trophoblasts. We have investigated the ability of *Brucella* spp. to infect human trophoblasts, using immortalized and primary cultures of these specialized cells of the placenta. For the first time, we describe an atypical intracellular compartment where *Brucella* is able to survive and replicate. Furthermore, important differences were observed in the pathogenesis of *B. melitensis*, *B. abortus* and *B. suis*. These results will be presented and discussed, particularly with respect to the nature of the replication niche and its impact on *Brucella*-associated abortion in infected women.

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P37: Growth of *B. abortus* 2308 inside macrophages

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The characterization of the trafficking of intracellular *Brucella* (*B.*) strains inside host cells is incomplete yet and many interesting mechanisms still need to be studied and discovered. It is possible to follow the growth of single bacteria inside infected cells by labelling bacteria with Texas-Red succinimidyl ester (TRSE) since the unipolar growth generates new non-labelled parts on the surface of *Brucella*. In addition, the replication and segregation of replication origins of both chromosomes, named *ori*I and *ori*II, could be observed by using fluorescent fusions to ParB and RepB, putatively binding to *parS* and *repS* sequences located close to *ori*I and *ori*II, respectively. Besides, it was revealed that replication and segregation of *ori*I always occurs before *ori*II in tested conditions. Hence, a ParB fusion is sufficient to monitor G1 phase or the initiation of S phase. By using these methods and tools, in previous studies we have found that 80 % of the intracellular *B. abortus* block their growth and their cell cycle at the G1 stage during the first 6 hours p.i. and resume their growth and replication at 8 hours p.i. in HeLa cells. It would be important to continue to expand the discoveries of intracellular growth lifestyle of *B. abortus* in other cell types such as murine RAW264.7 macrophages, bone marrow-derived macrophages (BMDM) and trophoblasts which are more relevant to the natural host cell infections. Our results in RAW264.7 macrophages and BMDM show that about 70 % of the intracellular bacteria also arrest their growth during the first 4 hours p.i., and resume their growth after 4-6 hours p.i. In the same cells types, only a fraction of 21 % of the ΔvirB mutant is able to resume growth. This suggests that the more agressive behaviour of these macrophages against bacteria, compared to HeLa cells, is able to limit the resuming of growth of *B. abortus*.
We are interested in synchronizing the G1-arrested daughter cells from *B. abortus* 2308 because it would be interesting to prepare a bacterial population enriched with daughter cells, named 'newborns', to test their potential for host cell invasion and their pattern of gene expression. Following the method for synchronization of *Sinorihzobium meliloti* by using nutrient downshift, recently we tried to apply this method to synchronize G1 cells of *B. abortus*. In their method, they produced G1-arrested cells of *S. meliloti* by transfering early log phase cells to medium lacking preferred carbon and nitrogen sources. However, our results indicate that this is not an effective way to synchronize G1-arrested daughter cells from *B. abortus*. Nevertheless, using flow cytometry, we observed that a large fraction of the bacterial population being in the late stationary phase in rich medium is identified with a 1n genome content. Thus, we propose to develop new protocols starting from this culture condition to isolate strongly enriched in G1 bacteria, potentially able to be synchronized for cell cycle studies.

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Session 4: Immunology

Keynote Lecture: Stefan H.E. Kaufmann
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Tuberculosis research: Current status and future perspectives

Tuberculosis (TB) remains a major health threat. An estimated 8.6 million people develop active disease and 1.3 million die annually. Although this makes *Mycobacterium tuberculosis* (Mtb) one of the top infectious causes of morbidity and mortality of humankind, only 10 % of the more than 2 billion infected individuals develop active disease during their life. Control of TB is an active immunologic process mediated by T lymphocytes and effected by mononuclear phagocytes. The pathogen typically enters the host by the aerogenic route and hence, the lung is the main port of entry and main site of disease manifestation. At sites of Mtb implantation, granulomas are formed composed of lymphoid and myeloid cells, notably T lymphocytes, B lymphocytes, macrophages, dendritic cells and multinucleated giant cells. These cells are closely packed in an organized fashion, facilitating cognate interactions. The granuloma is typically surrounded by a fibrous wall. As long as the patient remains in a stage of latent TB infection (LTBI), the pathogen is successfully controlled in solid granulomas. As a consequence of a dysfunctional immune response, granulomas become disorganized resulting in necrosis due to massive cell death and later even caseation. In the necrotic and caseous material, Mtb can grow unrestrictedly. TB has become more threatening than ever before, mostly for two reasons. First, Mtb and the human immunodeficiency virus (HIV), the causative agent of acquired immunodeficiency syndrome (AIDS) have joined forces. Because HIV suppresses the immune response, the risk for individuals with LTBI to develop active TB is markedly increased. As a corollary, TB has become the major cause of death in HIV-infected individuals and HIV infection has become the driving force of the TB pandemic. Today, 15 million individuals are coinfected with HIV and Mtb of whom 1 million develop active TB annually. Second, TB control is facing major drawbacks due to the increasing incidences of multidrug-resistant (MDR) strains. Treatment of susceptible Mtb is already complicated by the need for three to four drugs given over a period of several months. MDR-Mtb has already infected 50 million individuals causing half a million of TB cases annually which are much harder to treat. More than 85 countries have reported occurrence of extensively drug-resistant TB and in some countries, totally drug-resistant TB that is almost impossible to cure has been notified.

General agreement exists that future control of TB will require development of better diagnostics, drugs and vaccines. The current vaccine, Bacille Calmette–Guerin (BCG) is an attenuated strain of *M. bovis*, a close relative of Mtb which typically causes TB in cattle. The strain was introduced into a human for the first time in 1921 and has proven to protect (at least partially) against severe forms of TB disease in newborn. However, it induces insufficient protection against the most prevalent form, pulmonary TB, in all age groups. Nevertheless, BCG vaccination has become part of the expanded program of immunization (EPI) for infants with approx. 4 billion children having been vaccinated thus far. BCG is safe, although adverse reactions can occur in rare incidences but poses a risk for HIV-positive newborn.

Through major research efforts over the last two decades, several new vaccine candidates against TB have been in development, which are now undergoing clinical assessment. These are first booster vaccines given on top of BCG to strengthen the immune response induced by BCG. A second type of candidate, BCG replacement vaccines are viable recombinant mycobacteria. We have developed a recombinant BCG which has already successfully completed a phase IIa trial in newborn. This rBCG expresses listeriolysin from *Listeria monocytogenes* and at the same time has a deletion in the urease C gene. While this vac-

cine progresses through the clinical trial pipeline, we have made major efforts to improve its safety and efficacy. To improve safety, we have deleted the *pdx* gene so that the resulting BCG Δ*ureC*::*hly* Δ*pdx1* is auxotrophic for vitamin B6 and fails to survive in the host over longer periods of time. The second vaccine candidate showing improved efficacy in preclinical models is rBCG Δ*ureC*::*hly* Δ*nuoG* with reduced antiapoptotic activity, thus causing strong apoptotic events in antigen-presenting cells to improve T cell stimulation (Gengenbacher et al. unpublished).

TB diagnosis is often performed microscopically to detect Mtb as acid-fast bacilli in sputum smears as developed more than 130 years ago. A second diagnostic test, the tuberculin skin test (TST), determines the immune response against Mtb infection and hence does not distinguish between active TB disease and LTBI. The TST has been further improved. These so-called interferon-gamma release assays (IGRA), determine production of type II interferon (IFN) involved in protective immunity. More recently, attempts have been made to harness global gene expression profiling of whole or selected blood cells for diagnostic purposes. Major efforts focus on transcriptome and metabolome analyses. Proof of principle has been obtained that gene expression profiles can discriminate between LTBI and active TB. Future research is aimed at developing prognostic signatures that allow identification of individuals with LTBI at highest risk of developing TB. Global gene expression profiles can also provide informative insights into mechanisms underlying protection and pathology in TB. One cogent part of the major characteristic signatures of TB is a type I IFN-driven response and mouse models have allowed elucidation of mechanisms underlying TB susceptibility. The microRNA (miR)-223 has been identified as a valid component of the diagnostic signature of TB and recent studies have revealed the regulatory role of miR-223 in control of inflammatory responses in TB. Biomarkers not only provide helpful tools for the development of novel diagnostic and prognostic assays but have also shed light on relevant host mechanisms underlying resistance and susceptibility in this disease. In sum, research on the response in TB patients can guide targeted basic research and a combination of both avenues are most appropriate for making a difference in TB control of the future.

Although the preferred host of Mtb is human, this pathogen can also infect other animals, including cattle. The close relative of Mtb, *M. bovis*, mostly targets cattle but can also infect humans, notably infants through unpasteurized contaminated milk. Brucellosis is a zoonotic disease, which can be acquired by humans, typically through discharges from infected animals, or unpasteurized milk. Both Mtb/*M. bovis* and *Brucella* sp. are intracellular pathogens that have chosen mononuclear phagocytes as main habitat. Accordingly, T cells play a critical role in protection and pathology and are natural targets of vaccination strategies. Vaccination approaches for brucellosis control are focused on livestock, whereas the main target of TB vaccines are humans, although in some countries efforts are being made to also improve vaccination against cattle TB. Accordingly, vaccination programs against TB and brucellosis follow similar avenues. Current vaccines in use for brucellosis are live-attenuated strains or killed vaccines. In addition, a number of vaccines are in development. The latter include recombinant proteins in adjuvants, viral and bacterial vectors expressing *Brucella* sp. protein antigens, DNA vaccines and outer membrane vesicles comprising proteins and lipoids of brucellae. Being targeted at animals, trials with brucellosis vaccines are less restrictive than TB vaccine trials in humans. In sum, TB vaccine development in the last decade has made major progress providing a blueprint for future brucellosis vaccination strategies. Learning from experiences with both strategies can be mutually beneficial, because of similar pathology and immunology. Experience with human TB trials could also facilitate the development of a human vaccine against brucellosis to protect individuals in close contact with livestock.

Oral Presentations (O11-O15)

O11: T Cell responses to *Brucella* in humanized mice

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Brucellosis is the most common zoonotic disease worldwide. To date, there are no effective vaccines for humans, although it is known that protection is cell-mediated immunity-dependent, involving TNF-α and IFN-γ. To address this void, we recently developed a live, attenuated double mutant *Brucella* (*B.*) vaccine protective against *B. abortus* and *B. melitensis*. This vaccine was found to be highly attenuated and effective in providing an average of four logs reduction in colonization by challenging wild-type brucellae, and in some cases, sterile immunity (no detectable brucellae in tissues). As new brucellosis vaccines become available for testing in humans, the development of suitable animal models is needed prior to any nonhuman primate or human testing. Thus, the goal of our work was to test whether human T cells are responsive to our vaccine. To meet these objectives, we took advantage of IL-2 receptor-γ chain$^{-/-}$ (IL-2Rγc$^{-/-}$) NOD mice on a severe combined immunodeficient (SCID) background (NSG mice) expressing HLA/MHC class I molecule. These mice were reconstituted with human hematopoietic stem (umbilical cord CD34^{+}) cells, and referred to as engrafted NSG-A2 mice. After successful engraftment was confirmed (by flow cytometry analysis of their peripheral blood mononuclear cells), groups of engrafted and non-engrafted NSG-A2 mice were vaccinated i.p. with 10^8 CFUs of double-mutant *B. abortus*, and colonization of spleens, livers, and lungs was evaluated 3 weeks post-vaccination. To determine the source of the cytokine-producing CD4^{+} and CD8^{+} T cells and CD56^{+} NK cells, flow cytometry analysis was performed on isolated NSG-A2 human lymphocytes co-cultured in absence or presence of heat-killed RB51. Our results showed similarly elevated colonization of examined tissues in both engrafted and non-engrafted NSG-A2 mice; however, splenic inflammation was only present in engrafted NSG-A2 mice. Evaluation of cytokines production revealed increased responsiveness by brucellae-specific CD4^{+} and CD8^{+} T cells responses. Interestingly, these immune human CD8^{+} T cells showed greater IFN-γ responses than their accompanied CD4^{+} T cells. Conversely, greater IL-17 production was observed by CD4^{+} T cells than CD8^{+} T cells. NK cells also showed increased IFN-γ production. Thus, these data show that *Brucella*-vaccinated humanized mice are capable of eliciting *Brucella*-specific human CD4^{+} and CD8^{+} T cell responses. Such model enables us to study *Brucella*-specific human T cell responses to learn about immune pathways responsible for protection.
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O12: CXCR2 is a critical mediator of *Brucella*-induced articular inflammation

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Osteoarticular complications are the most common focal manifestation of brucellosis in humans and *Brucella* (*B.*) is a common cause of infectious arthritis in countries in which the disease is endemic. Little is known about the pathology of osteoarticular brucellosis due to the lack of relevant murine models. However, we recently reported that genetic- or antibody-mediated ablation of IFN-γ in mice results in articular inflammation during infection with *B. abortus or B. melitensis*. Here, we report that adaptive immune cells are dispensable for *Brucella*-induced articular inflammation. Wild-type, $\mu MT^{-/-}$ (B cell deficient), and $Rag1^{-/-}$ (B and T cell deficient) mice all developed articular inflammation at a similar rate and severity when infected with *B. melitensis* 16M and depleted of IFN-γ. In addition, minimal levels of cytokines associated with T cell responses (IL-2, IL-4, IL-5, IL-13, and IL-17) were detected in arthritic joints. However, we did find increased numbers of neutrophils and macrophages in joints from mice with *Brucella*-induced arthritis. In addition, the concentration of chemokines, including CXCR2 ligands, was strikingly enhanced in *Brucella*-infected joints. Interestingly, $CXCR2^{-/-}$ mice displayed markedly lower clinical scores, joint swelling, and pro-inflammatory cytokine/chemokine levels than wild-type mice when infected with *B. melitensis* 16M and depleted of IFN-γ. Collectively, these results show that chemokines, such as CXCR2 ligands, are important mediators of *Brucella*-induced articular inflammation while adaptive immune cells are not required. Therefore, the targeting of CXCR2 and/or other chemokine receptors may have potential as a complementary antibiotic therapy for the resolution of *Brucella*-induced arthritis.

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O13: Bovine γδ T cells' unique WC1 pattern recognition receptor (PRR) directs cellular immune responses to bacterial pathogens

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Interferon (IFN)γ is perhaps the most important cytokine in control of brucellosis. We have shown that IFNγ-producing bovine γδ T cells bearing the WC1 co-receptor (SRCR family member) are a major cell population responding in recall responses to bacterial pathogens during the first month following vaccination or infection in two models. Annotation of the bovine genome showed there were 13 WC1 molecules coded for by individual genes. We evaluated the variegated gene expression of the individual WC1 molecules by specific subsets of γδ T cells; that is, individual cells express more than one WC1 gene product but only a restricted subset of the available 13 WC1 genes. The major subsets defined in this way are known as $WC1.1^+$, $WC1.2^+$ and $WC1.3^+$. Our data indicate that the WC1 molecules act as pattern recognition receptors interacting directly with bacteria or their components, and thus the expression of the WC1 molecules is likely to direct the response by the γδ T cells in conjunction with the TCR to respond to pathogens. Using RNA silencing we have shown that the WC1 co-receptor specifically contributes to the ability of γδ T cells to respond to bacteria and that the responsive γδ T cells are found within a subset of the $WC1.1^+$ γδ T cell subpopulation when leptospira is used. In contrast, in studies with another bacterial species we showed the γδ T cell response is composed of a heterogeneous mix of $WC1.1^+$ and $WC1.2^+$ subsets, e.g. responding *in vitro* to mycobacterial antigens in recall responses and accumulating in the lesions of *Mycobacterium bovis* infected animals. This recognition requires direct contact with APC and signaling through the T cell antigen receptor but in other studies we have shown that bacterial components interact with WC1 molecules. The results described herein evaluate the interaction with *Brucella abortus* to determine which WC1 molecules on bovine T cells act as pattern recognition receptors to activate γδ T cells to respond. Since these cells respond very early to infections and vaccinations knowing how to engage γδ T cells in next generation vaccines is of importance.

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O14: A *Brucella* spp. protease inhibitor is a useful adjuvant in oral vaccine formulations against infectious diseases

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Previous studies showed that a *Brucella* protein (BP) administered by the oral route and without adjuvants induced protection against oral *Brucella* challenge. BP is a broad spectrum protease inhibitor with a strong activity against serine proteases secreted by the pancreas to the intestine (elastase, trypsine and alpha chymotrypsin). These results prompted us to study its potential application as oral adjuvant for other antigens.
As BP is able to inhibit *in vitro* the activity of many proteases present in the gastrointestinal tract we directed all our efforts to study its potential application in oral vaccine delivery. We showed that in BALB/c or C57BL/6 mice oral delivery of OVA in the presence of BP resulted in a significantly increased OVA-specific T cell response (DTH assay; $p<0.05$, n=6) and OVA-specific IFN-γ producing $CD4^+$ and $CD8^+$ T cells determined by flow cytometry at the mucosal (mesenteric lymph nodes (MLNs) or Peyer´s patches; $p<0.05$, n=6) and systemic level (spleen; $p<0.05$, n=6). *Salmonella* (*S.*) Typhimurium in combination with the novel adjuvant BP is a strong candidate for an oral vaccine because it mimics the natural route of *Salmonella* infections and provokes a Th1-biased response. Therefore, we fed BALB/c mice (n=7) with i) heat-killed *Salmonella* (HKS), ii) HKS+BP or iii) HKS+CT and challenged them later with virulent *S.* Typhimurium by the oral route. HKS+BP-immunized mice induced an increased Th1 immune response in the spleen and MLNs ($p<0.01$) and an increase in HKS-specific IL-17 production. There was an increase in HKS-specific DTH response ($p<0.05$) and of IgA in faeces ($p<0.05$) as well as a slight increase of specific IgG in the serum of HKS+BP immunized mice compared to the control groups HKS and HKS+CT. Considering that *Toxoplasma gondii* infects the host through the gut mucosa, the efficacy of oral immunization with a recombinant protein from *Toxoplasma gondii* GRA4 plus BP was analyzed. C3H/HeN mice were orally immunized with i) PBS, ii) GRA4, iii) GRA4+BP or iv) GRA4+CT. Three weeks after the last boost, mice were challenged orally with Me49 tissue cysts and individual parasite levels were assessed 1 month later. A significant decrease in the brain cyst burden ($p<0.05$) together with a reduction of liver damage associated enzymes (AST, LDH and ALT) in serum and an increase ($p<0.05$) in GRA4-specific DTH and of IgA in faeces were observed in GRA4+BP immunized mice when compared to the control groups.
In summary, our results suggest that BP could be an ideal mucosal adjuvant. When co-delivered orally a vaccine can bypass the harsh environment of the gastrointestinal tract. Besides, it can enhance antibody production, Th1 and $CD8^+$ T Ag-specific immune response and thus, it would be a useful adjuvant in oral vaccine formulations against infectious diseases.

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O15: Protective immunity in intranasally infected mice with *Brucella melitensis*

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The α-proteobacterium *Brucella* (*B.*) is a Gram-negative intracellular bacterium responsible for brucellosis that causes abortion and infertility in animals and undulant fever in humans. If untreated, human brucellosis can become chronic with severe clinical complications. Although eradicated, in most of the developed countries brucellosis remains a worldwide public health problem.
The intraperitoneal (i.p.) inoculation of mice is the most common experimental model to study *Brucella* infection. However, natural infections are mainly transmitted by contaminated food or via aerosol. Bacteria have to cope with effectors of the mucosal immune system, which are bypassed by i.p. inoculation. Thus, we have developed an intranasal (i.n.) infectious model in mice, using a *B. melitensis* strain expressing the fluorescent mCherry protein.
In our study, we focused on the identification of essential signaling pathways and cells controlling the infection. IL-12 dependent IFNγ producing T $CD4^+$ (Th1) lymphocytes play a dominant role during the infection in the i.p. model. In contrast, we have shown an early role of IL-17 and γδ T cells in the control of *Brucella* infection in the lung. Immuno-histofluorescence highlighted differences in the localization of granuloma in the spleen between the i.p. and the i.n. route of infection. Finally, we analyzed the secondary immune response to *B. melitensis*. In order to determine the importance of specific antibodies, we compared the ability of vaccinated wild-type and B-lymphocytes-deficient mice to control an i.n. infection. In opposition to our observation in the i.p. model, circulating antibodies are not necessary to control a secondary i.n. infection.
Globally, our model shows that the route of infection has an impact on the immune response to *Brucella*. These results could push our efforts to generate a protective vaccine against brucellosis and others infectious diseases caused by intracellular bacteria.

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Poster Presentations (P38-P42)

P38: Characterization of splenic microarchitecture during chronic infection by wild type and mutant strains of *Brucella melitensis* in mouse experimental model

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The spleen is a sophisticated filter that manages blood cells and monitors immune functions. Micro-anatomically, the spleen is divided into white pulp and red pulp separated by the marginal zone (MZ) that forms the outer boundary of the white pulp in the mouse spleen. MZ plays a critical role in defense against pathogens that have entered the circulation. Two distinct macrophage populations are present in the MZ, namely marginal zone macrophages (MZM) and marginal metallophilic macrophages (MMM) that are localized at the outer and inner border of the MZ, respectively. Within the white pulp, T and B lymphocytes are also segregated into discrete compartments; B cells are situated in follicles, while T cells are located in an area around the central arteriole. It has been reported that viral and parasitic infections induced significant remodelling of splenic microarchitecture, including a loss of the MZM population and disappearance of T and B cells compartmentalization. The impact of bacterial infection on splenic microarchitecture has been poorly investigated. It would be interesting to characterize the modulation of splenic architecture during chronic bacterial infection.
Brucella (*B.*) is a facultative intracellular bacterium that chronically infects humans and animals causing brucellosis, a worldwide zoonosis. The protection against *B. melitensis* infections in mice models mainly requires $CD4^+$ T lymphocytes, Th1-type cytokines such as interferon (IFN)-γ that mediate activation of microbicidal functions of macrophages and inflammatory dendritic cells. Splenic microarchitecture of *B. melitensis* infected C57BL/6 mice has been examined *in situ* by immuno-histofluorescence. We observed that infection induced striking and long lasting changes in the MZ. Infection induced a reduction of MZM and marginal MMM, identified by ER-TR9 and MOMA-1 staining, respectively. This reduction appears dependent on Th1 response and lead to a decreased ability of MZ to sequestrate fluorescent beads. We will analyze the impact of this reduction on the ability of spleen to control infection by other pathogens and to mount a specific immune response against vaccine antigens.

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P39: Immunological diagnostics of poly-organ (concomitant) lesions in patients with acute brucellosis

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Acute brucellosis causes concomitant/poly-organ lesions, which can be explained by the highly invasive capabilities of brucellae. First, there are lesions of the musculoskeletal system: arthritis and polyarthritis, synovitis, and others. Typically, the reproductive system is also affected: orchitis and epididymitis may occur in men; a disordered menstrual cycle, endometritis, and/or spontaneous abortions in women. Symptoms of the nervous system can manifest as plexitis and ischio-radiculitis. In severe cases, there are signs of cardiac tissue damage. If a specific organ is involved in the infectious disease process, molecules or fragments of organ-specific structural proteins are disseminated within the body. In the presence of a tissue-specific antigen (TA), antigen-binding lymphocytes (ABL) capable of specifically binding to these TAs differentiate and circulate in the blood.
The aim of our study was to determine the significance of ABL/TA ratios and its correlation to the damage of brain, liver, kidney, and joint capsule tissues in acute brucellosis.
We investigated 90 patients suffering from acute brucellosis, aged 18 to 45. Brucellosis was diagnosed by clinical examination and laboratory tests (Huddleson and Wright reaction). The results showed that in patients with acute brucellosis the following organs were most frequently affected: liver and joint capsule tissues (in 100 % of the patients), kidney tissues (in 78.9 %), and brain tissues (in 47.8 %). The ABL/TA ratios of the aforementioned organs were: 5.14±0.16 %; 4.49±0.16 %; 3.39±0.13 % and 2.79±0.14 %, respectively (with up to 2 % in healthy people).
Immunological diagnostics led to the conclusion that acute brucellosis is characterized by the development of pathological processes in the liver, joint capsules, kidney and brain tissues. The extent of the pathological effects was more prominent in liver and joint capsule tissues than in kidney and brain.

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P40: *Brucella melitensis* outer membrane vesicles have no cytotoxic effect, induce bacterial internalization and IL-1β production in cultured human cells

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Gram-negative bacteria release outer membrane vesicles (OMVs) playing different roles from protein secretion to pathogenesis. Previously, we demonstrated the modulatory function of *Brucella* (*B.*) *melitensis* OMVs in the induction of Th1 cytokines in bone marrow dendritic cells; however, no others effects on the host cells have been explored so far. In this work, we assessed the potential of *B. melitensis* OMVs from rough (VTMR1) and smooth (16M) strains to promote adherence and internalization, induction of the pro-inflammatory cytokine IL-1β and cytotoxicity in human cell lines. Cytotoxicity was evaluated in HeLa cells as well as the ability to promote adhesion and internalization of bacteria by using different concentrations of OMVs. Human monocytes THP-1 were also stimulated with different concentrations of OMVs from both strains and the relative expression of the genes encoding components of the NLRP3 inflammasome complex (NLRP3, ASC, caspase-1 and IL-1β) was determined. Our results showed that OMVs from both strains, regardless of their concentration, had no cytotoxic effect in HeLa cells, and adhesion and internalization of *B. melitensis* only occurred in the presence of 10 μg OMVs originating from the smooth strain. OMVs from both strains induced a significant expression of IL-1β but no NLRP3, ASC or caspase-1 expression was observed. In contrast to previous observations made with regard to OMVs of other bacteria, *B. melitensis* OMVs revealed poor cytotoxic effects, but also promoted adhesion and internalization of the bacteria into the host cell and induced IL-1β production in a NLRP3 independent way.

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P41: Critical role of ASC inflammasomes and bacterial type IV secretion system in caspase-1 activation and host innate resistance to *Brucella abortus* infection

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Pathogens are detected by innate immune receptors that upon activation orchestrate an appropriate immune response. Recent studies have revealed the intracellular signaling cascades involved in the TLR-initiated immune response to *Brucella* (*B.*) *abortus* infection. However, no reports have claimed the role of inflammasome receptors in *Brucella* recognition. Therefore, we decided to investigate the function of NLRC4, NLRP3 and AIM2 in sensing *Brucella*. Herein, we have shown that NLRC4 is not required to induce caspase-1 activation and further secretion of IL-1β by *B. abortus* in macrophages. In contrast, we determined that AIM2 which senses *Brucella* DNA and NLRP3 is partially required for caspase-1 activation and IL-1β secretion. Additionally, mitochondrial reactive oxygen species (ROS) induced by *Brucella* were involved in IL-1β production. Furthermore, AIM2, NLRP3, ASC and caspase-1 KO mice were more susceptible to *B. abortus* infection than wild-type animals, suggesting that multiple ASC-dependent inflammasomes contribute to host protection against infection. This protective effect is due to inflammatory response caused by IL-1β and IL-18 rather than pyroptosis, since we observed augmented bacterial burden in IL-1R and IL-18 KO mice. Further, we determined that type I IFN regulates AIM2 expression in macrophages. Finally, bacterial type IV secretion system (T4SS) VirB and live but not heat-killed brucellae are required for full inflammasome activation in macrophages during infection. Taken together, our results indicate that *Brucella* is sensed by ASC inflammasomes that collectively orchestrate a robust caspase-1 activation and proinflammatory response.

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P42: Allergic asthma increases the susceptibility of mice to *Brucella melitensis* intranasal infection

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Brucella spp. are facultative intracellular bacterial pathogens responsible for brucellosis, a worldwide zoonosis that causes abortion in domestic animals and chronic febrile disease associated with serious complications in humans. There is currently no approved vaccine against human brucellosis and antibiotic therapy is long and costly.
The protection against *Brucella* (*B.*) *melitensis* in an experimental mice model requires IL-12 dependent IFN-γ-producing $CD4^+$ Th1 cells that mediate the activation of microbicidal functions of infected macrophages and dendritic cells.
Identification of key factors regulating host resistance to *Brucella* or other pathogens is classically investigated in immunologically healthy animals. This simplified approach ignores that host animals frequently suffer numerous diseases throughout their life. Asthma is considered to be a Th2 mediated immunopathology induced by various environmental factors such as dust mite allergens. Its occurrence has increased significantly since the 1970s. In 2011, 235–300 million people were diagnosed with asthma. In order to evaluate the impact of allergic asthma on the course of *Brucella* infection in mice, we induced asthma by repeated intranasal sensitization with house dust mites extract before intranasal infection with *B. melitensis*. Our results demonstrated that allergic asthma may decrease the ability of the lungs of wild type BALB/c mice to control *B. melitensis* growth. Asthma significantly increased the CFU bacterial count in lungs of astmatic mice but not in the liver and the spleen as compared with control mice. This suceptibility was not observed in STAT-6 deficient mice (deficient for Th2 response). Thus, we hypothesize that asthma induces a pulmonary Th2 response that locally inhibits the efficient Th1 immune response against *Brucella.* These results suggest better persistence of *B. melitensis* in the lungs of infected asthma patients and open up a new area of research on factors favoring *Brucella* infections.

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Session 5: Vaccination

Keynote Lecture: Steven C. Olsen
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Brucellosis Vaccines for Livestock: Current Status and New Approaches

As humans are essentially dead-end hosts for *Brucella* (*B.*), persistence of disease in livestock or wildlife reservoirs is required for maintenance of disease. Although brucellosis can cause significant economic losses to livestock producers, regulatory interest in control of brucellosis is primarily due its potential zoonotic risk. Addressing the disease in natural hosts of *Brucella* has been demonstrated in a number of numerous studies to be the most cost-effect mechanism to prevent human disease. Although regulatory efforts to control brucellosis have been active for decades in many countries, brucellosis remains a significant public health and livestock disease issue for many countries, and is re-emerging in others. Although identification of a human vaccine with acceptable safety and efficacy has remained elusive, live vaccines have been a critical part of regulatory programs addressing brucellosis in animal populations for decades. Historically, brucellosis vaccines for livestock have been composed of live attenuated strains because, in general, heat-killed or subcellular fractions have failed to provide protection equivalent to that induced by live vaccines. It can be argued that live attenuated strains were empirically developed in that the underlying protective mechanisms induced by the vaccine against the pathogen were not the impetus for the approach used in identification of the strains. The most commonly used live vaccines, *B. abortus* strain 19 and *B. melitensis* strain Rev1 were developed approximately 85 and 55 years ago, respectively. With the exception of the introduction of *B. abortus* strain RB51 in the United States in 1996, implementation of the use of *B. abortus* strain 82 in the Russian Federation in 1974, and *B. suis* strain 2 in China in the 1980's, it can be argued that no new brucellosis vaccines have been developed which have progressed to significant use under field conditions. It should be emphasized that current live livestock vaccines (attenuated *B. abortus*, *B. melitensis* or *B suis* strains) are highly effective in reducing production losses and disease transmission, but are less efficacious at preventing infection or seroconversion after exposure to virulent field strains. As seroconversion rather than infection is commonly used for evaluating disease prevalence, inability of vaccines to prevent infection and subsequent positive responses on diagnostic tests impair regulatory efforts. Most vaccines are administered parenterally and target stimulation of systemic immunity. Although there are practical benefits to deliver vaccines to livestock parenterally, natural infection by *Brucella* is generally across mucosal surfaces. Particularly with *B. melitensis* strain Rev1 in small ruminants, mucosal delivery (conjunctival) has been effectively utilized primarily to minimize post-vaccination seroconversion and confounding of diagnostic tests.

As compared to livestock vaccines, human vaccines against brucellosis need to meet a higher level of clinical safety that is highly unlikely to be achieved by use of live *Brucella* strains. Comparative vaccine development for humans and livestock may also be influenced by differences in pathogenesis of disease, tissue localization, and clinical signs between human and reservoir hosts, with *Brucella* preferentially localizing in lymph nodes, mammary gland, and reproductive tracts in natural hosts. Because of the author's veterinary research experience, the remainder of this manuscript will primarily focus on development of brucellosis vaccines for reservoir hosts (domestic livestock and wildlife). However, it should be noted that improvements in brucellosis vaccines for livestock may provide some benefits in development of human vaccines.

In addition to inability to prevent seroconversion and transient infections as discussed previously, current live vaccines for livestock also have limitations as be zoonotic in humans, have

limited shelf life in the absence of appropriate cold chain storage, and are potentially abortigenic in pregnant animals. As most regulatory programs target prepubescent animals for initial vaccination, inoculations can be administered when animals are not pregnant, and abortigenic effects are dramatically reduced if adult vaccination is administered to animals that have been previously inoculated, management actions are available which minimize potential abortigenic effects of live vaccines. Therefore, reducing cold chain storage requirements and/or potential zoonotic effects, while improving the ability to induce long-term protection after a single inoculation, are the most important criteria for development of new brucellosis vaccines for livestock or other reservoir hosts.
The ultimate goal of vaccination would be to induce protective immunity that, at minimum, mimics protection induced by natural infection with virulent field strains. Development of new vaccines needs to consider the interaction between the host immune system and live *Brucella* during infection. The complex interaction between live *Brucella* strains and the immune system leads to stimulation of aspects of innate immunity, activation of antigen-presenting cells and presentation of *Brucella* antigens, induction of Th1 type of CD4+ T cell responses, cross-presentation of *Brucella* antigens leading to activation/recruitment of CD8+ cells, and release of cytokines and expression of co-stimulatory molecules which ultimately lead to generation of effective memory cell populations and long-term protective immunity. Although a number of *Brucella* genes, and the proteins they encode, have been hypothesized to play a role in protective immunity, characterization of the role of these proteins in protective immunity in natural hosts remains incomplete. In addition to lack of knowledge on protective epitopes, use of recombinant proteins in development of subunit vaccines is impaired by the inability to achieve *in vivo* antigen expression in a manner that mimics the immunogenicity of natural infection. As proteins delivered alone or in adjuvant are frequently processed as exogenous antigens, they are not processed and expressed in a similar manner as antigens from live *Brucella* residing in its intracellular niche within phagocytes. By the cross-presentation pathway, antigens from internalized live *Brucella* gain access to the endoplasmic reticulum by fusion of phagosomes with endoplasmic reticulum-derived vesicles, leading to the loading of antigen onto MHC Class I molecules and presentation on the cell surface to CD8+ T-cells. Differences in immunity between live *Brucella* and nonliving *Brucella* products may be directly related to differences in antigen processing and presentation which would need to be overcome for successful development of non-living or subunit vaccines.

In this author's opinion, many efforts to develop new brucellosis vaccines are impaired by being primarily focused on attempts to attenuate *Brucella* strains. As pathogenicity and immunogenicity usually have a direct relationship, efforts to develop vaccines by reducing pathogenicity may result in reduced immune responses and protection. I believe it to be more appropriate for vaccine development to be driven by targeting improvements in immunogenicity, particularly stimulation of cellular immunity which provides long-term protection against intracellular bacteria, and focused on epitopes of most importance for protection. A similar concept was suggested by others who engineered a *Brucella* strain with a mutated lipopolysaccharide structure that made the strain more efficiently recognized immunologically. *Brucella*'s 'stealthy' behavior under *in vivo* conditions appears to be multilayered and is most likely an evolutionary development to assist in avoiding or circumventing immune detection and host bactericidal processes. The *Brucella* cell envelope has high hydrophobicity and its lipopolysaccharide (LPS) has a non-canonical structure that elicits a reduced and delayed inflammatory response when compared to other Gram-negative bacteria and has lower stimulatory activity on TLR4 receptors. The O side-chain on the LPS can form complexes with the MHC Class II molecules that interfere with the ability of macrophages to present exogenous proteins. *Brucella* ornithine-containing lipids and lipoproteins in the outer membrane are poor activators of innate immunity and may release outer membrane vesicles which down regulate innate immune responses. *Brucella* bacteria are also devoid of many classical structures involved in virulence, such as pilli, fimbria, capsules and plasmids that stimulate PRRs. In addition, *Brucella* prevents phagosome maturation and fusion with lysosomes; a mechanism that may interfere with innate and adaptive immune processes. As proteins have been

identified in *Brucella* that demonstrate significant homology with TLR adaptor molecules, these peptides may also be a mechanism that the bacteria use to subvert adaptive immunity by interfering with TLR signaling. Compared to other Gram-negative bacteria, *Brucella* induces a reduced innate immune response, and a lower rate of maturation and activation of dendritic cells. Modification or elimination of the mechanisms that *Brucella* exploit for avoiding immune detection should improve immunogenicity of live strains and may be the most promising approach for increasing protective responses.

Over the past several decades, technologic advances have provided new tools, technologies, or methods for developing new vaccines with improved safety and/or efficacy characteristics. Many of these technologies have been utilized in murine models using various *Brucella* antigens. Techniques or tools currently available include, but are not limited to, genetic engineering, plasmid overexpression of *Brucella* antigens in live strains, new adjuvants and co-stimulation molecules, and alternative vaccine formulations such as DNA vaccines, nanovaccines, liposomes, new delivery vectors, and synthetic vaccines. Assuming that protective antigens of *Brucella* are fully characterized, nanovaccines appear to be particularly attractive candidates for development of non-living vaccines as they may allow antigens and co-stimulatory molecules to be presented in a manner that targets antigen-presenting cells while synthetically mimicking the pathogen-immune cell interaction. Despite technologic advancement, the current status of knowledge on *Brucella* protective epitopes, and the need for a vaccine with high efficacy under field conditions after a single inoculation, it is likely that continuation of efforts to improve live vaccines for use in reservoir hosts will be required.

Development of new or novel vaccines is only one issue to be resolved in developing new vaccine tools for addressing brucellosis in reservoir hosts. Although *Brucella* spp. demonstrate high homology at the DNA level, the assumption that protective antigens will be similar across *Brucella* spp. and across natural hosts remains to be determined. In our laboratory, we have noted differences between host species in tissue colonization, clearance, and induction of immunologic responses after parenteral vaccination with *B. abortus* vaccine strains. We have also noted dramatic differences between ruminant hosts in responses to *Brucella* vaccines suggesting regulation of immune responses differs across reservoir hosts. In some reservoir hosts, immune responses after natural infection with virulent field strains fail to induce adequate levels of protective immunity. As some reservoir hosts are wildlife, alternative delivery methods need to be developed to effectively deliver inoculum in a manner that maintains immunogenicity. In addition, in many parts of the world livestock are at risk for infection with multiple *Brucella* spp. requiring development of vaccines capable of providing cross-protection. Because the highest prevalence of brucellosis is frequently in developing countries, new vaccines will need to be economical in cost, and capable of being maintained, distributed, and efficacious after inoculation in areas with limited infrastructure and minimal regulatory support.
The lack of dramatic improvements in brucellosis vaccines over the last several decades has not been due to a lack of active research efforts. Knowledge on the epidemiology of brucellosis has evolved with identification of new *Brucella* species and hosts, recognition of broader host ranges for particular *Brucella* species, increased understanding on intracellular infection processes, and greater awareness of the role of wildlife reservoirs in disease prevalence. In hindsight, the lack of breakthroughs in vaccine design might imply that easy improvements were achieved decades ago, and novel approaches or technologies will be required to develop the next-generation of *Brucella* vaccines for livestock or wildlife reservoir hosts. Because immunologic responses and efficacy of brucellosis vaccines can differ between natural hosts, this author's experience indicates that it is imperative that vaccine studies transition as quickly as possible to the relevant host species with efficacy characterized using standardized models.

Oral Presentations (O16-O21)

O16: Cloning, expression and immune response against protective antigens of *Mycobacterium tuberculosis/M. bovis* in *Brucella abortus* vaccine strain RB51

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Mycobacterium (*M.*) *bovis* is the causative agent of bovine tuberculosis, causing more than three billion dollars in annual agricultural losses worldwide. More importantly, *M. bovis* is considered a zoonotic pathogen that can be readily transmitted from animals to humans via droplet infection or by food intake of unpasteurized milk. Brucellosis is one of the most common zoonotic infections that affects agriculturally important animals. Creating a vaccine that provides protective immunity against bovine tuberculosis and brucellosis is an effective method of controlling the spread of these diseases and hence eradicating two major zoonotic diseases from animals and thereby from humans. Here, we report the development of four expression vectors that either express or secrete mycobacterial antigens using a live RB51 vaccine strain, and therefore have the potential to protect against brucellosis and tuberculosis challenges. In this study, two promoters TrcD and TrcD-*flgE* were designed for antigen expression. TrcD-*flgE* contains the putative signal sequence of *Brucella* (*B.*) flagellum E protein, which is used to secrete *M. tuberculosis (Mtb)* protective antigens. The two promoters were synthesized and cloned into the promoterless pNS4 plasmid to create pNS4TrcD and pNS4TrcD-*flgE* expression vectors. Genes encoding *Mtb* antigens Ag85B, ESAT6, and Rv2660c were synthesized using *Brucella* codons and cloned into the above vectors to create the four plasmids pNS4TrcD-*Ag85B,* pNS4TrcD-*flgE-Ag85B,* pNS4TrcD-*rv2660c-esat6,* pNS4TrcD-*flgE-rv2660c-esat6*. Expression and secretion of the antigens in *B. abortus* RB51 were confirmed by Western blotting. A clearance study in BALB/c mice indicated that the novel strains and the control RB51 cleared in 6 weeks post-vaccination. The bacteria isolated from the challenged mice maintained the expression vectors during the entire period of vaccine clearance. A lymphocyte proliferation assay on mice vaccinated with strain RB51 carrying mycobacterial antigens showed elevated levels of IFN-γ as well as TNF-α upon stimulation with Ag85B, ESAT6 and heat-killed *M. tuberculosis.* We hypothesize that RB51, used as a platform to secrete and present mycobacterial antigens, could induce a protective immune response against both *M. bovis* and *B. abortus* challenges. Future studies to make RB51 more appropriate for human use will involve delivering protective antigen in an irradiated RB51 potentially leading to a commercially viable immunization strategy in humans against *M. tuberculosis.*

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O17: Vaccination with recombinant *Brucella abortus* RB51 strain engineered to express increased levels of O-polysaccharide provides enhanced protection in murine brucellosis model

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Brucella (*B.*) *abortus* strain RB51 is a rough, attenuated mutant derived in the laboratory from the virulent *B. abortus* 2308. Animals vaccinated with strain RB51 do not produce antibodies to the O-polysaccharide (OPS) of the smooth lipopolysaccharide (LPS). Strain RB51 does not produce readily detectable levels of OPS. One reason for the absence of OPS synthesis in strain RB51 is the disruption of *wboA* gene, which encodes a glycosyltransferase required for the synthesis of the OPS, by an IS*711* element. Complementation of strain RB51 with a functional *wboA* gene (strain RB51WboA) results in low levels of cytoplasmic OPS synthesis, but it does not confer a smooth phenotype. Recent nucleotide sequence analysis of genes in the *wbo* and *wbk* loci did not reveal any other apparent mutation that could affect the smooth LPS synthesis in strain RB51. Therefore, in this study, we asked if increasing the amount of bactoprenol priming precursors in strain RB51WboA would result in smooth LPS synthesis. To achieve this, we cloned the *wbkF* gene, which encodes undecaprenyl-glycosyltransferase involved in bactoprenol priming for OPS polymerization, in plasmid pBB4*wbo*AB; the resulting plasmid was designated pBB4/*wbo*AKF. Strain RB51 was electroporated with pBB4/wboAKF to generate strain RB51WboAKF. In comparison with strain RB51WboA, strain RB51WboAKF expressed higher levels of OPS and its Western blot reactivity profile with the anti-OPS monoclonal antibody was similar to that of the smooth *Brucella* strains. However, this strain exhibited rough phenotypic characteristic in acriflavine agglutination test. *In vivo* bacterial clearance studies revealed no difference between strains RB51 and RB51WboAKF in their ability to persist in spleens of BALB/c mice. Mice immunized with strain RB51WboAKF developed increased levels of smooth LPS-specific serum antibodies, primarily of IgG2a type, when compared with those immunized with RB51WboA. Levels of serum IL-12p70, GM-CSF, and IL-10 were higher in mice vaccinated with strain RB51WboAKF when compared to the mice vaccinated with strain RB51. Also, splenocytes from the RB51WboAKF vaccinated group of mice secreted higher levels of antigen-specific IFN-γ, TNF-α, IL-2 and GM-CSF when compared to those of the RB51 or RB51WboA vaccinated groups. Increased numbers of antigen-specific IFN-γ secreting $CD4^+$ and $CD8^+$ T lymphocytes were detected in the strain RB51WboAKF vaccinated mice. Immunization of mice with strain RB51WboAKF induced enhanced protection against virulent *B. abortus* 2308 and *B. melitensis* 16M when compared with the RB51 and RB51WboA immunized groups of mice. These results suggest that, in addition to *wboA* gene, mutations affecting expression of several other genes involved in the synthesis of OPS and smooth LPS contribute to rough phenotype of strain RB51. Our results also suggest that strain RB51WboAKF could be a more efficacious vaccine than its parent strain in natural hosts.

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O18: The road towards the development of a safe and efficacious live attenuated vaccine for animal and human brucellosis: from the bench to the non-human primate model

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Given the ability of *Brucella* spp. to infect multiple host species including humans, brucellosis remains widespread and neglected in many areas of the world. The disease has a special link to poverty among the world's poor due to its impact not only on animal health but also on worker productivity, maternal health and perinatal outcomes. Yet despite its public health and economic importance, a safe and efficacious vaccine for human use is still unavailable. Ideally, a *Brucella* (*B.*) vaccine should be safe, confer cross-protective immunity against the different *Brucella* species and most importantly prevent from symptomology in the case of humans and reduced organism shedding from animals. In addition, it should be inexpensive and easily manufactured under resource-limited settings. For the past 12 years, we have focused our attention on the development of a safer but still highly protective live attenuated vaccine (*B. melitensis* 16MΔ*vjbR*) for its potential use in both animals and humans. We have demonstrated, not only the ability of the vaccine candidate to confer protection in multiple animal species, but also its anodyne properties using natural hosts and laboratory animal models. Here, we focus our attention on our most recent data demonstrating similar properties in the non-human primate model. Adult, Rhesus Macaques were vaccinated intramuscularly with a single dose of the 16MΔ*vjbR* vaccine candidate. None of the animals receiving the vaccine elicited any secondary effects associated with vaccination. At specific time points, animals were challenged using an aerosol dose of $1x10^6$ CFU of *B. melitensis* 16M/animal. The vaccine was capable of either preventing or reducing the severity of the disease manifestation such as hepatomegaly, splenomegaly and fever. In addition, animals receiving the vaccine had reduced numbers of bacterial burden in multiple organs including the spleen and liver. These results represent a significant advance in our road towards the development of a safe and efficacious brucellosis vaccine that could potentially be used in humans. The successful development of new vaccines against brucellosis also represents a potentially promising antipoverty intervention.

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O19: Preliminary evaluation of a recombinant immunocontraceptive brucellosis vaccine for swine

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Feral swine are a nuisance species across most of the United States costing around 1 billion USD each year in agricultural, environmental, and personal property damages. Despite extensive control efforts the population of wild pigs in the US continues to rapidly grow and novel control methods are needed. Furthermore, feral swine are known carriers of multiple zoonotic diseases such as brucellosis, pseudorabies, and leptospirosis, which threaten both livestock biosecurity and public health. Antigenic recombinant multimeric gonadotropin-releasing hormone (mGnRH) has been previously used as a subunit vaccine to induce immunocontraception in feral pigs, however, multiple doses are needed to elicit a robust anti-GnRH immune response and current delivery methods are limited. It is proposed that live bacterial antigen delivery using rough *Brucella* (*B.*) *suis* VTRS2 as a novel platform can be employed to deliver mGnRH without the use of antibiotic resistance markers, while simultaneously conferring protection against brucellosis in feral swine. VTRS2 was created by *cre-loxP* deletion of the LPS biosynthesis gene *wboA* as well as the *leuB* gene required for leucine biosynthesis inside the nutrient-scarce intracellular environment occupied by *Brucella*. Mutations in *wboA* are known to attenuate *Brucella* strains such as the vaccine strain *B. abortus* RB51, however, RB51 is rifampin resistant and has minimal efficacy in swine. Antigen overexpression in the live vaccine strain can be achieved using the plasmid pNS4*trc*D, which is maintained in a leucine-deficient environment by complementation of *leuB* and contains the hybrid constitutive overexpression promoter *trcD*. Additionally, pNS4 is stable for multiple generations in the absence of selection. VTRS2-mGnRH was designed to overexpress the immunogenic mGnRH multimer by use of mGnRH sequence codon-optimized for use in *Brucella* on pNS4*trc*D. VTRS2-mGnRH was tested in female BALB/c mice for clearance and challenge, as well as for specific IgG immune response against mGnRH. For clearance, 5 x 10^5 CFU VTRS2-mGnRH was administered intraperitoneal (IP) and CFU/ml were measured from homogenized mouse spleens at 4 and 6 weeks post infection (pi). Serum samples were also collected for anti-mGnRH ELISA. The vaccine strain cleared from mouse spleens by week 6 pi and anti-mGnRH specific IgG levels were significantly higher ($p<0.05$) at weeks 4 and 6 post-inoculation. For challenge studies, mice were vaccinated with either sterile saline or 5 x 10^5 CFU VTRS2-mGnRH IP and were challenged with 5 x 10^4 CFU virulent *B. suis* 1330 eight weeks post-vaccination. Spleens were collected 2 weeks post-challenge and CFU were determined using serial dilutions of spleen homogenates onto TSA plates. VTRS2-mGnRH vaccinated mice experienced 0.5 LOG reduction in splenic CFU ($P=0.011$) compared with saline controls. Future studies to evaluate the potential for VTRS2-mGnRH involve booster vaccination, challenge studies and breeding trials to measure the contraceptive effect of the vaccine in both male and female mice. Promising results in the mouse model warrant future vaccine trials in pigs as a novel control method for feral swine, while helping reduce the incidence of brucellosis in wildlife reservoirs. Additional use in domestic pigs could be considered where brucellosis is endemic and alternatives to surgical castration are desired.

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O20: Safety and protectiveness of a novel vector vaccine against *Brucella abortus* in first-calf heifers

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To establish a specific prophylaxis against *Brucella* (*B.*) *abortus* infections, we developed a novel vector vaccine based on the recombinant influenza A virus subtypes H5N1 or H1N1 expressing the *Brucella* proteins ribosomal L7/L12 or outer membrane protein-16 (OMP-16). The purpose of the present study was to confirm the effectiveness of these vaccines in first-calf heifers with respect to their safety and protectiveness.

Vaccine mixtures were prepared from the influenza viral vectors Flu-NS1-124-L7/L12-H5N1, Flu-NS1-124-Omp16-H5N1, Flu-NS1-124-L7/L12-H1N1 and Flu-NS1-124-Omp16-H1N1, which had been propagated in 10-day-old embryonated chicken eggs. The influenza viral vectors were mixed with stabilizing medium, aliquoted into 1 ml ampoules, lyophilized and stored at 2-8ºC. Immediately before use, the lyophilized vaccine was resuspended in a 20 % solution of Montanide Gel01 (Seppic, Puteaux, France) adjuvant in PBS. Fifty first-calf Kazakh white breed heifers (3-4 months pregnant) aged 16-18 months, that were seronegative for brucellosis, were divided into 5 groups (10 animals/group): two experimental groups vaccinated with the vector vaccine via the conjunctival or subcutaneous route of administration; two positive control groups vaccinated with *B. abortus* S19 or *B. abortus* RB51; and one negative control group (PBS + Montanide Gel01). To assess the protective effect of the vaccine, 60 days after initial vaccination (IV) the calf heifers (5-6 months pregnant) were challenged with the virulent strain *B. abortus* 544. Protection by the vaccine was evaluated using three main parameters: protection from abortion, effectiveness of vaccination and index of infection.

Immunization of first-calf heifers with the vector vaccine via the conjunctival or subcutaneous route did not have any negative impact on the overall clinical status (behavior, appetite, etc.) or the course of pregnancy in any animal throughout the observation period (2 months post-IV). The body temperature of the animals in the different experimental groups remained within the normal range (37.5-39.5ºC). After challenge, all tested vaccines provided significant protection against abortion and infection (including infection of fetuses and calves) compared to the negative control group, where the abortion and infection rate was 70 % and 100 %, respectively. Based on the tested parameters, the vector vaccine administered by either route provided slightly better results compared to animals vaccinated with *B. abortus* RB51, and comparable results (especially via the subcutaneous route) compared to animals vaccinated with *B. abortus* S19.

The proposed novel vector vaccine against *B. abortus* is completely safe for first-calf heifers and furthermore provides a high degree of protectiveness.

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O21: Testing of immunogenic characteristics of the vaccine *Brucella abortus* strain S19 applied in reduced doses on small ruminants

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Brucellosis is a zoonotic infection caused by bacteria of the genus *Brucella* (*B.*). Currently, the two vaccine strains *B. abortus* S19 and *B. melitensis* Rev1 are widely used for immune prophylaxis in animals. The use of Rev1 is restricted because the vaccine strain frequently causes abortions, and long-time persistence of agglutinins (up to 3-5 years) that result in positive serologic testing of serum samples. *B. abortus* S19 vaccination does not cause abortions or antibodies reactive in serological tests. In small ruminants, the vaccine is administered in full doses ($40x10^9$ bacteria) using the following scheme: first vaccination at the age of 3-5 months, before insemination, and re-vaccination every year. However, this scheme provokes high agglutinin titers that impede reliable diagnosis and cause sensitization. The aim of our study was to develop a vaccination method for small ruminants that does not stimulate long-time persistence of agglutinins in the blood. We, therefore, tested immunogenic characteristics of the *Brucella* strain S19 by applying reduced doses in small ruminants in comparison with full dose vaccination. Four groups of lambs were vaccinated with full doses and after 12 months, three of them were re-vaccinated by reduced doses; $500x10^6$, $1x10^9$ and $2x10^9$ bacteria, whereas the fourth group was re-vaccinated by full doses again ($40x10^9$ bacteria). A fifth group was not vaccinated and acted as control. Periodically, serum from each animal was tested to determine agglutinin titers. We were able to observe that agglutinin titers of animals vaccinated with reduced doses were gradually decreasing and were under the diagnostic threshold after 180-210 days. After 10 months, all animals were exposed to the virulent reference strain Novocherkassk-102 *B. melitensis*. Bacteriological results showed that the protective immunity of animals that were vaccinated using the reduced dose of $2x10^9$ bacteria was not weaker than the immunity of those animals vaccinated with a full dose. Hence, a reduced dose of the vaccine strain *B. abortus* S19 can reduce long-time persistence of agglutinins in the serum of small ruminants and provides immunity comparable protection against the infection as a full dose vaccination. Furthermore, the costs of vaccination can be reduced by applying the reduced dose and undesirable side effects of full dose vaccination can be avoided.

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Poster Presentations (P43-P52)

P43: Guinea pig (*Cavia porcellus*) – a model for the preliminary assessment of the efficacy of anti-*Brucella* vaccines

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Vaccination of domestic livestock still represents one of the most efficient ways to reduce brucellosis prevalence in animals and, hence, to prevent human infection. The guinea pig has been established as a model for preliminary testing of anti-*Brucella* vaccines and has been studied and adopted by the Weybridge reference laboratory. The high susceptibility of Guinea pigs revealed to be highly susceptible and can be effectively immunized using the vaccine strains *Brucella* (*B.*) *melitensis* Rev1 and *B. abortus* S19.
SENASA-DILAB conducted a controlled study on guinea pigs to evaluate and select variables and conditions that allow defining a lab model to assess the potency of anti-*Brucella* vaccines. *B. abortus* 2308 was used as challenge strain and *B. melitensis* Rev1 and *B. abortus* S19 as controls to monitor protection.
Fifty female American Short Hair guinea pigs weighing between 600 and 800 g were used. One group (A) of ten was vaccinated with *B. melitensis* Rev1, $2x10^7$ viable cells, 1 ml s.c.; the second group (B) of thirty animals with *B. abortus* S19, $5x10^7$ viable cells, 1 ml s.c. and the third group (C) of ten with 1 ml s.c. of PBS. Sixty days after vaccination the guinea pigs were challenged with strain *B. abortus* 2308, 10^3 viable cells, 1 ml i.m. inoculated into the right hind leg. The animals were sacrificed 60 days later.
The test results were in accordance with previous experiments using the same model. Parameters and ranges were within the normal variation recorded in biological studies. The control group was 100 % infected. Vaccine strain *B. melitensis* Rev1 revealed major protection (90 %) and *B. abortus* S19 showed a slightly lower protection rate of 83.3 %, expected to be 65 %. The median spleen weights in group A, B and C were 2.05 g, 1.9 g and 7.8 g, respectively. Spleen weights are well correlated with the infection status of individual animals which has already been described for infections with *B. abortus* 2308 Spleen weights may even replace bacteriology as an indicator for brucellosis in experimental infection models. The median body weight was significantly lower in the control group (100 % infected). Bacteriology, spleen weight and animal weight revealed a strong coherence within the respective groups. Hence, these parameters correlate with the infection status and can be used to forecast the outcome after infection or vaccination. In addition, in our procedure, the efficiency of a new vaccine can be monitored more precisely in comparison with the efficiency of two reference vaccine strains. However, further studies are needed to prove the usefulness of our infection model to assess anti-*Brucella* vaccines.

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P44: Immunogenic potential of an attenuated *Brucella ovis* strain lacking a species-specific ABC transporter in a murine model

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There is no specific vaccine available for controlling ovine brucellosis caused by *Brucella* (*B.*) *ovis*, one of the most important causes of infertility in rams. The aim of this study was to evaluate the immunogenic potential of a Δ*abcAB B. ovis* mutant strain (that lacks a *B. ovis*-specific ABC transporter) in a murine model of infection. Balb/c (n=20) and C57bl/6 (n=20) mice (6-7 weeks old) were divided into 4 groups (for each mouse strain) with 5 mice per group: a control group (sterile PBS inoculation), a group inoculated with sterile and empty alginate capsules, another group immunized with the Δ*abcAB* strain (10^8 CFU per mouse); and a group immunized with alginate encapsulated Δ*abcAB* (10^8 CFU per mouse). Sixty days later all mice were challenged with wild type *B. ovis* (10^6 CFU per mouse). Two weeks later, all mice were euthanized, and fragments of the spleen and liver were aseptically collected, weighed in falcon tubes, and homogenized in 2 mL of sterile PBS. Each sample was serially diluted (10-fold dilutions) and plated for CFU counting. Fragments from the spleen and liver were also collected for histopathological evaluation. Bacterial load was reduced (approximately one log) in the spleen of Balb/c mice immunized with encapsulated Δ*abcAB* strain when compared to other groups. In the liver, there was a reduced bacterial load in mice immunized with the encapsulated Δ*abcAB* strain when compared to groups inoculated with PBS or sterile alginate. There was a significantly reduced bacterial load in C57bl/6 mice immunized with encapsulated Δ*abcAB* strain compared to the controls in liver and spleen. Histopathological scores revealed significantly reduced inflammatory changes (histiocytic and neutrophilic infiltrate, necrosis, and trombosis) in the spleen and liver of Balb/c and C57bl/6 mice immunized with the encapsulated Δ*abcAB* strain when compared to other groups. These results support our notion that the *ΔabcAB B. ovis* strain has an immunogenic potential.

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P45: Safety, immunogenicity and genetic stability of recombinant influenza viruses that express *Brucella* genes *omp16* and L7/L12

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The epizootic persistence of brucellosis in farm animals in the republics of Central Asia and in Kazakhstan calls for a new generation of anti-*Brucella* vaccines. New candidate vaccines may be NS-gene-modified recombinant vector influenza viruses A expressing the *Brucella* genes *omp16* and L7/L12 constructed by reverse genetics. The objective of the study was to evaluate genetic stability, safety and immunogenicity of the recombinant influenza viruses Flu–NS1–80–Omp16 (H5N1) and Flu–NS1–80–L7/L12 (H5N1).

The recombinant viruses Flu–NS1–80–Omp16 (H5N1) and Flu–NS1–80–L7/L12 (H5N1) were constructed via co-transfection in Vero cell culture using plasmids encoding PB2, PB1, PA, NP, M and chimeric NS1 (NS1-Omp16, NS1-L7/L12; *Brucella* insert in position of amino acid 80) of influenza virus A/Puerto Rico/8/34 (H1N1) and HA, NA of vaccine influenza virus A/AstanaRG/6:2/2009 (H5N1), then adapted to 10-days chicken embryos. Safety of the recombinant vaccine was tested in a mouse model, and their protective ability was evaluated in guinea pigs by by challenging twice via conjunctival route with *B. abortus* 544 within a 21-day interval.

To assure genetic stability of NA, HA and NS genes five additional passages of recombinant viruses Flu–NS1–80–Omp16 (H5N1) and Flu–NS1–80–L7/L12 (H5N1) were performed. NA, HA and NS genes of passages 1 and 5 of the recombinant viruses were sequenced using the Big Dye Terminator Cycle Sequencing Kit v3.1 (ABI) with Genetic Analyser 3130 xl, Applied Biosystems and analyzed with Vector NTI and MEGA 4 software.

Comparative genetic analysis showed that the nucleotide sequence of the NS gene after five passages was 100 % identical to the original regions that contained chimeric viral genes NS1 with Omp16 and L7/L12 sequences of *B. abortus* included in pHW2000 plasmid DNA. Sequencing and analysis of HA, NA and NS genes of the recombinant viruses Flu–NS1–80–Omp16 (H5N1) and Flu–NS1–80–L7/L12 (H5N1) after 5 passages in 10-day old chicken embryos demonstrated absence of mutations Infection experiments proved safety of the influenza vectors. Neither weight loss nor death of mice was recorded during the observation period of 6 weeks. Moreover, the newly developed influenza vector vaccines protected mice from *B. abortus* infection.

A new candidate vaccine against *B. abortus* has been constructed on the basis of influenza vectors expressing *Brucella* proteins Omp16 and L7/L12 that revealed genetic stability, safety and demonstrated protective ability in the animal infection model.

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P46: Characterization of outer membrane vesicles of *Brucella abortus* and protection in mice

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Gram-negative bacteria release outer membrane vesicles commonly referred to as OMVs. These vesicles have previously been used as models for the development of acellular vaccines in Gram-negative bacteria. Recently, immunogenic proteins have been identified in purified OMVs from *Brucella* (*B.*) *melitensis*. The vesicles also serve as protection against virulent *B. melitensis* in infected mice. The aim of the present work was to identify the protein compositon of purified OMVs from *B. abortus* 2308 and to evaluate OMVs from *B. abortus* 2308 and *B. abortus* RB51 as vaccine *in vivo* as well. To accomplish this objective, OMVs from *B. abortus* 2308 and *B. abortus* RB51 growths on TSA plates were purified. The purified vesicles were observed by electron microscopy. Proteins were separated by one-dimensional SDS-PAGE and the bands were analyzed by LC-MS/MS. A total of 4 mice groups were evaluated, the first group was immunized with 5 µg OMVs from *B. abortus* RB51, the negative control with saline, and the positive control was vaccinated with $1.5x10^4$ UFC live *B. abortus* RB51. After 8 weeks, CFUs were obtained from the spleens of the mice. Results showed vesicles with double membranes measuring between 30-65 nm. Cu/Zn SOD, Omp19, Omp10, and chaperonin 60 kDa were found in the vesicles, all of them are related to immune response in mice. The mice vaccinated with OMVs were protected against virulent *B. abortus*. No significant difference was observed in mice vaccinated with OMVs compared with the *B. abortus* RB51 vaccine. This protection could be related with the fact that OMVs are composed by proteins that induce immune response.

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P47: Development of an inactivated vaccine against *Brucella ovis*-infection

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Brucella (*B.*) *ovis* infection produces a clinical or subclinical disease in sheep that is characterized by genital lesions in rams and placentitis in ewes. This disease is widespread and has caused significant economic losses in Ukraine. The primary and most effective method of preventing the disease is vaccination using an inactivated vaccine.
The aim of our investigations was to develop and study the protective and immunogenic properties of an inactivated vaccine against *B. ovis* infection. The vaccine was prepared from strain *B. ovis* 67/B (R-form) that was isolated from sheep in the Ukraine. *B. ovis* 67/B was adapted to cultivation on MPLGGA without the addition of animal serum and increased concentrations of CO_2.
The vaccine strain *B. ovis* 67/B was cultured on solid nutrient media. The bacterial mass was inactivated by boiling. Montanide ISA 70 was used as an adjuvant . Immunogenic and protective properties of the vaccine were studied in infection experiments using 170 guinea pigs and 52 rams. Experimental animals were immunized intramuscularly, guinea pigs were vaccinated with a dose of 0.5 ml (including $2.5x10^9$ CFU) and rams were vaccinated with a dose of 1.0 ml ($5.0x10^9$ CFU). Experimental animals were subsequently infected with a virulent strain of *B. ovis* with an intramuscular dose of $2.5x10^9$ CFU. Animals in control groups were only infected with the virulent strain but were not vaccinated. The animals were followed for 12 months. Serum samples were serologically tested using LCFT and AGIT (agar gel immunodiffusion test) along with microbiological examination of urine samples.
The inactivated vaccine strain *B. ovis* 67/B (R-form) with the addition of Montanide ISA 70 proved to be harmless and environmentally safe. There were neither local reactions nor general deterioration of health after immunization of guinea pigs or sheep.
All immunized animals could not be infected with the virulent *B. ovis* strain; clinical symptoms and asymptomatic carriers were not found. Protectivity of the vaccine in guinea pigs and rams was 100 %. Immunogenicity of the vaccine was also high. In vaccinated sheep anti-*B. ovis* antibody titers of 1.15±0.17 lg_{10} in LCFT and an average titer of 2.75±0.55 log_2 in AGIT has been detected for 12 months after vaccination. Antibodies to *Brucella* s-LPS antigens (*B. abortus, B. melitensis* and *B. suis*) were not found. Hence, the vaccine may not cross-react with the mandatory serological tests for sheep brucellosis.
It is shown that a heat inactivated vaccine based on strain *B. ovis* 67/B is harmless and environmental friendly and has high projective and immunogenic properties. The vaccine may be used for the prevention of *B. ovis* infection of industrial sheep.

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P48: Characterization of the immune responses of *Brucella abortus* S19 or RB51 vaccinated and RB51-revaccinated heifers

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The aim of this study was to characterize and compare the immune responses induced in the cattle prime immunized with *Brucella* (*B.*) *abortus* S19 or RB51 and boost vaccinated with RB51. Calves aged 4 to 8 months were immunized with either vaccine S19 or RB51 on day 0, and revaccinated with RB51 on day 365 of the experiment. The immune response was characterized using serum and peripheral blood mononuclear cells (PBMC). The culture supernatants were collected on days 0, 28, 210, 365, 393 and 575 post-immunization. They were evaluated, upon *in vitro* stimulation with γ-irradiated *B. abortus* 2308 for expression of cytokines (IL-4, IL-6 and INF-γ) in culture supernatant; expression of IL-4, IL-17A and IFN-γ in $CD4^+$, $CD8^+$ and $CD21^+$ subsets; mRNA transcript of cytokines (IL-10 and TGF-β); proliferation of $CD4^+$ and $CD8^+$ T-cells; expression of the phenotypes related to activation and immune-regulation events (MHC II, $FoxP3^+$ and CD25) in $CD4^+$, $CD8^+$ and $CD21^+$; induction of memory cells in subpopulations of T lymphocytes and in B lymphocytes; and cytotoxic activity (granzyme B and perforin) of $CD8^+$ T-cells. Results showed that S19 and RB51 vaccination induced a complex immune response characterized by IFN-γ expression, mainly by $CD4^+$ cells, IL-6 production, proliferation of antigen-specific $CD4^+$ and $CD8^+$ T-cells, $CD4^+$ memory cells, cytotoxic $CD8^+$ cells and overexpression of MHC class II in $CD4^+$ subset. However, S19 vaccination induced more persistent IFN-γ expression, $CD4^+$ memory cells and MHC class II expression in $CD4^+$ cells in comparison with RB51 vaccination, besides a significant higher IL-6 production, $CD4^+$ and $CD21^+$ memory cells and $CD8^+$ proliferation, whereas RB51 vaccinated calves showed higher $CD4^+$ regulatory profiles ($FoxP3^+CD25^{High}$) than S19 group. After RB51 revaccination, the immune response is chiefly characterized by increase in IFN-γ expression, proliferation of antigen-specific $CD4^+$ and $CD8^+$ T-cells, $CD4^+$ memory cells, cytotoxic $CD8^+$ cells, overexpression of MHC class II in $CD4^+$ subset and decrease in IL-6 production in both groups. Nevertheless, RB51 vaccinated cows induced more proliferation and effector $CD8^+$ cells than S19 vaccinated animals following RB51 revaccination. Comparison of S19 and RB51 groups after RB51 revaccination also showed that S19 vaccinated animals induced more Treg cells ($CD4^+FoxP3^+CD25^{High}$). Results of the present study indicate that after first vaccination both vaccine strains (S19 and RB51) induce a strong and complex immune response dominated by Th1 profile, though after RB51 revaccination the differences between immune profiles induced by prime vaccination with S19 and RB51 become more accentuated.

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P49: Validation of *Brucella* vaccines (S19 and Rev1) in Sudanese Camels (*Camelus dromedarius*)

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This study was designed to evaluate the serological response of camels after vaccination with *Brucella* vaccines. Vaccination trials were conducted in a camel herd housing camels of the Darfuri and the Rashydi types. Each type was divided into two groups whereby the age was carefully considered. All camels were serologically negative for anti-*Brucella* antibodies. The *Brucella* vaccines S19 and Rev1 were used in doses recommended for cattle vaccination using S19 or for sheep and goat vaccination using Rev1 (OIE manual, 2009). The induced serological reactions were observed.
Twenty-seven females were vaccinated out of 24 camels (39.4 %) of the Darfuri (D) type, and 37 camels (60.6 %) of the Rashydi (R) type. 15 D (62.5 %) and 12 (32.4 %) were females over one year, the remainders were aged between 3 and 12 months. All animals were vaccinated either with *Brucella* (*B.*) *abortus* S19 or *B. melitensis* Rev1 vaccine. The first group (5 D [20.8 %] and 3 R [8.1 %], aged more than one year) was vaccinated with S19 subcutaneously using the reduced recommended cattle dose. Another two camels (R) were vaccinated with the full dose usually used for heifers. As non-vaccinated controls 2 D (8.3 %) and 2 R (2.7 %) were used. The second group (8 D [33.3 %] and 4 R [10.8 %], aged more than one year) was vaccinated with Rev1 subcutaneously using the reduced dose recommended for sheep. Additionally, one R (2.7 %) aged three months to one year was inoculated subcutaneously with the full dose recommended for sheep.
Antibody titres were measured at the first, second, third and fourth week after vaccination and then monthly for one year and after two years. Both vaccines performed almost similarly well. S19 vaccinated camels: 8 (57.1 %) out of 14 revealed positive RBT agglutination results beginning in the first week, 2 (14.2 %) from the second week up to 5 months. One (7.1 %) showed seronegative results (no agglutination) with RBT starting from the third month, 2 (14.3 %) from the fourth month and 1 (7.1 %) from the fifth month and all were negative at the sixth month. Ten out of 14 (71.4 %) revealed SAT titres in the first month (ranging from 134 to 1280). SAT results started to become negative in the third month (one animal) and all animals were negative in the fifth month. Ten out of 14 (71.4 %) were seropositive using cELISA from the first week post vaccination up to six months. Rev1 vaccinated camels: 11 out of 13 (84.6 %) started to be seropositive in the RBT at the first week, 1 (7.6 %) at the second week and 1 (7.6 %) at the third week up to 5 months. Five animals (38.5 %) were RBT negative starting in the fifth month, 11 (84.6 %) showed negative results from the sixth month onwards. Eight camels (100 %) showed positive SAT results from the first month with titres between 106 and 1488 and were negative from the sixth month onwards. Thirteen (100 %) started to be positive in cELISA from the first week onwards up to six months and were seronegative afterwards. All animals included in the study stayed seronegative up to two years.

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P50: Assessment of brucellosis management measures and vaccination campaign in two districts of Buenos Aires, Argentina

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Bovine brucellosis is endemic in Argentina. Bovine brucellosis is endemic in Argentina. Approximately, 60 % of dairy farms (6,500 out of 11,000) are officially free certified whereas, as regards beef, 10 % of farms and 2 % of cattle are infected.
The National Control and Eradication Program establishes the compulsory sub-cutaneous vaccination of all 3 to 8 month-old females with 15-30 x 10^9 viable *Brucella* strain 19. In most cases vaccination is performed simultaneously with the Foot and Mouth Disease vaccination. The Argentine National Veterinary Service, SENASA, entrusts both vaccination campaigns execution to 310 Local Sanitary Entities (LSEs).
The objective of this study was to assess the vaccination campaign in two districts, Brandsen and Navarro, in Buenos Aires province, and to evaluate some farmers' and veterinarians' management measures concerning the disease.
Four different questionnaires were performed face to face to 105 farmers (52 dairy farms and 53 beef farms), 11 private veterinarians, 13 vaccinators and 2 people responsible for the LSEs. To evaluate the compliance between field practices of vaccination and the SENASA's regulation, serum samples of a subgroup of those studied farms were randomly taken from heifers vaccinated 21-50 days before. The buffered plate antigen test (BPA) was the diagnostic test chosen to verify the vaccine exposure.
As regards dairy farms, 11 (21 %) were not officially free certified and 7 of them (63 %) were in a cleaning up process. Only 7 beef farms (13 %) were officially free certified. Most veterinarians (80 %) suggested serologic testing when reproductive disorders were observed, which were mainly due to *Neospora caninum*. These animals were sold to slaughterhouses. 45 farmers (42 %) bought animals, of which 27 (57 %) came from not officially free certified farms. 77 (74 %) consulted a veterinarian when cattle showed reproductive disorders. None of them quarantined these animals and only 4 (15 %) ordered serological brucellosis tests. The individual identification of the vaccinated heifers was performed in 100 dairy farms (95 %) and only in 11 beef farms (10 %), so that some heifers might have remained unvaccinated. A high percentage of the vaccinators (77 %) calibrated the syringe before the vaccine injection, all of them temporally stored the vaccine in boxes with refrigerants and 40 % agitated the vaccine bottle during the vaccination process. If some vaccine dropped when vaccinating, 11 vaccinators (85 %) performed the vaccination again but none of them wrote it down in the vaccination records. The LSEs audited the performance of the vaccinators at least once per campaign. Preliminary results suggest that farmers and veterinarians applied management practices regarding brucellosis, though some of them should be improved. In addition, although the vaccination campaign was globally well performed, some improvements concerning the individual identification should be applied to beef farms, mainly in those with breading season throughout the year where the age of the heifers cannot be guaranteed.

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P51: Evaluation of the microbiological and serological patterns of female buffalo calves (*Bubalus bubalis*) vaccinated against brucellosis with B19 vaccine

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The objective of this study was to evaluate the serological reaction of buffalo (*Bubalus bubalis*) heifers vaccinated with the B19 vaccine. A total of 33 calves from two brucellosis free herds were used. The animals aged 3 to 8 months were immunized with the B19 vaccine. Blood, urine and vaginal swab samples were collected immediately before vaccination. These samples were also taken once a week for four weeks and then monthly to the age of 24 months. The sera were tested by Rose Bengal test (RBT), 2-mercaptoethanol test (2MET), complement fixation test (CFT) and fluorescence polarization assay (FPA). The cut-off value for the FPA was 93.4 mP. The urine and vaginal swab samples were submitted to microbiological culture and polymerase chain reaction analysis (PCR) to detect DNA of *Brucella* spp. A screening PCR was performed using a pair of primers directed to the interspace region of the ribosomal RNA operon of *Brucella* spp. (ITS-PCR). Samples with positive results in the ITS-PCR were tested with an additional PCR using primers directed to the *ery* of strain B19 (B19-PCR). All animals seroconverted in RBT, 2MET and CFT at 7, 14 and 126 days post-vaccination, respectively. In FPA, a higher number of animals showed positive results 28 days post-vaccination, and 16 animals did not seroconvert throughout the monitoring period. The 33 heifers remained positive until 17.62, 13.65, 11.56, and 8.17 months of age in average by RBT, 2MET test, CFT test and FPA, respectively. Seven animals aged over 24 months gave positive results with RBT, 2 with 2MET and 1 with CFT. All animals were negative by FPA after 14.33 months of age. The average duration of positive results detected by RBT, 2MET, CFT, and FPA was 11.43, 7.48, 5.35, and 1.80 months, respectively. All animals were negative by culture throughout the monitoring period. At least one urine and/or vaginal swab of 14 (42.42 %) heifers was tested positive using B19-PCR. The low number of B19 PCR positive samples points to the fact that there is a low risk that vaccinated heifers may shed the vaccine strain after inoculation. The RBT and 2MET are the routine serological tests used for brucellosis diagnosis in Brazil. However, the persistence of antibody titres in animals aged over 24 months may reduce the diagnostic specificity of these tests. The alternative use of FPA may increase the diagnostic specificity of brucellosis diagnosis and it might be applied in younger vaccinated animals as well.

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P52: Fluorescence polarization assay testing of Rev1 vaccinated goats

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Fluorescence Polarization Assay (FPA) is a serological easily to perform and low-cost test that has been used for many years for the detection of anti-*Brucella* antibodies. This test has primarily been developed for brucellosis surveillance in cattle where it allows differentiation between S19 vaccinated and infected animals. The FPA has also been applied as a diagnostic tool in other species, such as pigs, goats and wild animals. Other than cattle, goats are vaccinated against brucellosis using the live vaccine strain Rev1. In Argentina, Rev1 has been administered intra-conjunctively in areas where active brucellosis was detected. Because the entire flock was vaccinated, there were controversies whether titres observed post vaccination were induced by the vaccine or by the wild type strain.
The objective of this work was to show the value of FPA as a diagnostic test in goats conjunctively vaccinated with Rev1.Two groups of animals were studied: Group A comprised 105 female goats negative for caprine brucellosis; Group B comprised 74 female goats of a flock proven to be brucellosis (12 animals were tested positive). These animals were randomly selected from flocks where Rev1 vaccination has been conducted. In all goats, blood samples were collected before vaccination (T0), 30 (T30), 60 (T60) and 120 days (T120) post vaccination (PV). Sera were frozen until used for serological testing by buffer plate antigen (BPAT), indirect and competitive ELISA (iELISA and cELISA) and FPA. The following results were obtained: At T30 PV, all animals were positive in all tests. At T60, 45 animals of group A were negative (43 %) in all tests; and out of the other 60 goats, 10 were negatively tested by FPA and 5 by cELISA. In group B, 16 goats were negative in all tests, and 58 still were positive in BPA and iELISA, while 4 of them were negative in cELISA and FPA. At T120 PV, in group A, 36 animals were positively tested by BPA and iELISA, but 10 animals were positive using cELISA and FPA. In group B, 38 animals remained positive in BPA and iELISA; and 14 were negative in cELISA and FPA. Vaccination did not change the serological status of the 12 originally positive animals in group B. Four animals of group B that were negative at T60 became positive by BPA and FPA at T120 PV. The results of this experiment indicate that FPA is a test that can be used for diagnosis in caprine brucellosis; and for those circumstances where ELISAs cannot be used, the FPA gets similar results but is easier and faster to perform.

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Session 6: Diagnostics

Keynote Lecture: Ana M. Nicola
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The diagnosis of brucellosis – still a challenge?

Brucellosis is a major bacterial disease caused by *Brucella* spp., widely distributed in the world, and is a zoonosis with significant importance for public health and agricultural economy in most developing countries. The permanence of this disease in a herd limits the possibilities of the livestock sector and international trade. Due to economic losses in animal production, estimated at 20 % and 15 % for the production of milk and meat, respectively, and their impact on public health, various control programs and eradication of animal brucellosis have been developed in the world. For many Veterinary Services and diagnosticians it is difficult to determine which tests are appropriate for use in control, and ongoing diagnostic monitoring programs. These programs intend to reduce the prevalence or incidence of the disease in the population by detecting positive animals and to implement controlmeasures such as quarantine or slaughter, subsequently. These tests also have a high impact on implemented eradication and surveillance programs which are based on continuous surveys of populations to maintain the disease free status, and on the formulation of control policies.

The strategy selected for the control of brucellosis in an individual country or region is dependent of many factors. Areas with extensive range conditions of livestock, possibly with groups of people accompanied by their herds and flocks moving freely and with little control across national boundaries, will pose different problems than those areas where farms are well fenced, stock movement is well controlled, and animal identity is well recorded. All of these factors will have an influence on the decision which is the best strategy for controlling the disease. Due to the lack of pathognomonic signs useful for the clinical diagnosis of brucellosis in animals, laboratory confirmation of brucellosis is made by isolation of the pathogen or demonstration of specific antibodies.
Large scale, expensive centralized testing regimes, using sophisticated serological tests as has been used in some countries, may not be feasible elsewhere.
The only indisputable diagnostic test for brucellosis is isolation of the causative organism from fluids or tissues of suspected hosts. However, bacteriological isolation has some major drawbacks i.e. it is very time consuming, expensive, operator hazardous, level-3 facilities are required and mass testing is not applicable. Confirmation requires phage typing, testing of oxidative metabolism, or genotyping procedures. In addition, bacterial isolation depends on the type of sample, sample numbers, volume of inoculum, culture techniques used and the expertise of the operator. Indeed, bacterial isolation and biotyping can be more sensitive than serology in identifying individual infected animals in cattle herds. However, financial input and personnel effort required make this procedure impractical for use in large scale surveillance programs. Nevertheless, bacteriological isolation is still the only confirmed method for establishing the infection status. Isolation is also the Gold standard testfor defining sera to be used for sensitivity testing.
Presumptive tests based on determination of the antibody titre of the test matrix have been developed. These matrices can be serum, milk, vaginal mucus, and semen. A perfect serological test would be able to discriminate antibodies induced by vaccine and field strains, respectively, and would detect infection early in the incubation stage of the disease. It should also not be subject to false positive reactions due to nonspecific antibodies. Multiple testing will increase the confidence in the diagnosis and sequential testing over time will also provide a better insight in the disease status than single testing.
Serological tests are developed for two basic applications: screening and confirmation. Screening tests are the first line assays which are generally rapid and cost effective, and

have a high diagnostic sensitivity, usually at the expense of diagnostic specificity. Only the reactors, true and false positive alike, are subjected to confirmatory testing. The latter assays have a higher diagnostic specificity and a lower diagnostic sensitivity. Animals positive in confirmatory tests are usually culled. As the prevalence of disease falls to low levels, the majority of the reactions observed in screening will certainly be false positives. At that moment, the need for high diagnostic specificity is evident. To increase diagnostic specificity, two (or more) conventional confirmatory tests may be applied. Complete eradication will involve repeated testing for many years. Then herds and flocks will require monitoring to ensure that they remained free of disease.

Over the years, since the first serological test was described by Wright and Smith, a large number of tests has been developed. Until the 1970s, the test procedures relied on detection of antibodies by agglutination, fixation of guinea pig complement or precipitation.

A substantial number of antigenic components of *Brucella* have been characterized. However, the antigen that dominates the antibody response is the lipopolysaccharide (LPS). Initially, agglutination tests with whole cell antigen were used. It was soon realized that nonspecific reactions were frequent, and as a result numerous modifications of the agglutination test were developed. While agglutination tests have been successfully used for eradication of brucellosis in animals in several countries, these tests have major drawbacks in terms of low specificity and their inability to distinguish antibodies resulting from vaccination from that induced by field strains. A complement fixation test, using whole cell antigen and guinea pig serum as source of complement was also developed and has served as the diagnostic standard for many years. While the sensitivity and specificity of the complement fixation tests are excellent, major drawbacks are the technical difficulties during processing. Consequently, other tests have been developed like indirect ELISA, radioimmunoassay, competitive ELISA, particle-concentration counting fluorescence immunoassay, the fluorescence polarization assay (FPA), the time-resolved fluorescent resonance energy transfer (TR-FRET) and nanoparticle assays. The two lattertests are rapid, quantitative and homogeneous, so that the antigen-antibody reaction takes place in the liquid phase and is detected via fluorescent labels without subsequent washing or separation steps.

Devices using lateral flow immune chromatography (ICFL), for the detection of of antibodies at the pen or bedsite havebeen also established. The main reason for the acceptance of these tests is simplicity. The strip tests are compact and can be easily transported. Usually no external addition of reagents is required to obtain results, which are obtained within a few minutes and are easy to interpret without the need for an analytical instrument.

Multiplex assays that detect antibodies to antigens from several different pathogens have also been developed. The development of universal assays for detection of antibodies to *Brucella* spp. in sera from various hosts will allow better control of this disease and thereby eradication in shorter time. Although, most *Brucella* species are reasonably host specific, some cross infections may occur. In particular, unexpected infection with rough *Brucella* species may be seen in hosts that are usually only be infected with smooth *Brucella* species.These infections can therefore be missed easily. Rough LPS has been successfully used in the serodiagnosis of *B. ovis* and *B. canis* but has not been used for routine serological detection of infection with smooth *Brucella* species. .

Numerous outer and inner membrane, cytoplasmic, and periplasmic protein antigens have also been characterized. Some are recognized by the immune system during infection and are potentially useful in diagnostic tests; some examples are Lumazine synthase, copper/zinc superoxide dismutase (Cu/Zn SOD) L7/L12, malate dehydrogenase and BP26 – also known as outer membrane protein (OMP).

There are several problems concerning serological diagnosis. These problems include long turn-around times for results, subjectivity in the interpretation of the results of some tests, interference by antibodies from vaccination, and misdiagnosis due to the presence of antibodies to antigens not included in the test.

An alternative immunological test is the brucellin skin test, which can be used for screening animals of unvaccinated herds, provided that a purified (free of sLPS) and standardized antigen preparation is available. The brucellin skin test has a very high specificity, so that sero-

logically negative, unvaccinated animals that are positive reactors in the brucellin test should be regarded as infected animals.

With the advent of molecular techniques the high degree of DNA identity between classical *Brucella* species quickly became clear. DNA–DNA hybridization studies revealed >90 % homology between the six classical species. There are now several molecular techniques available to characterize the differences of members of this genus. These techniques are rapid, and reproducible. In the future they may also be applicable to clinical samples. The most promising candidates are also suitable for high throughput analysis. PCR-based methods proved to be more useful and practical than the goldstandard isolation. Most of these PCR-based methods were developed using *Brucella* spp. DNA prepared directly from cultured bacteria or extracted from culture. The quality and purity of *Brucella* spp. DNA is very important in performing these methods, especially for multiplex PCR methods. One of the first PCR assays to differentiate among *Brucella* species was the so-called *abortus–melitensis–ovis–suis* (AMOS) PCR, developed by Bricker and Halling in 1994. In 2006, a new conventional multiplex PCR (Bruce-ladder) using eight primer pairs in a single reaction was developed by García-Yoldi and colleagues. This PCR rapidly replaced the AMOS-PCR as a diagnostic tool and is used nowadays in many diagnostic laboratories. Later on, this PCR was ammended and novel species such as *B. microti* and *B. inopinata* can be detected as well. Also, PCR assays that allow discrimination among biovars within a given species were developed. The most recent multiplex PCR assay to differentiate among *B. suis* biovars 1 to 5 (*suis*-ladder) was developed by López-Goñi.

The sensitivity and specificity of most PCR-based methods are not well established and their real value for use with clinical samples and, hence, diagnosis has not been validated. There is still a great deal of work required for validation, establishment of standard positive and negative controls, internal and inhibition controls. The sensitivity of PCR can be affected by the deoxyribonucleic acid (DNA) extraction procedure as well. Quality assuranceguidelines have to be deloped before any of these methods may ever be used in routine laboratory testing for brucellosis. The ability of PCR to multiply DNA of living and dead bacteria is believed to increase sensitivity of detection when compared to isolation. Real-time PCR assays have also been developed, recently. The major advantages of real-time PCR are: results can be obtained real time in a very short time it does not require electrophoretic analysis, and avoids cross contamination. Matrices that were analsed by real-time PCR included culture, serum, blood, and paraffin-embedded tissues. For diagnosis for brucellosis in humans real-time PCRs can discriminateinactive, seropositive, and active state even if clinical findings are unknown.

Current data suggest that using PCR as the only screening technique to diagnose brucellosis is not sensitive enough for clinical use. New studies comparing the sensitivities of PCR technology and conventional isolation in the setting of routine diagnostic laboratories are needed. Combining the PCR with serological test may be an effective way to improve diagnostic accuracy.

New amplification techniques are emerging. One of them is LAMP (loop-mediated isothermal amplification).This technique combines amplification. and detection in a single step and there is no need for purchasing new and costly equipment. LAMP is also less demanding than PCR and it can be used in low setting laboratories or in the field.

There are a number of methods for genotyping *Brucella* isolates including. Multilocus sequence typing of single-nucleotide polymorphisms (MLST). This technique makes use of divergent evolution of housekeeping genes within the species. Seven, eight or nine housekeeping genes are commonly analyzed in order to obtain a reasonable balance between the acceptable resolution power, time and costs. Multilocus variable number of tandem repeats analysis (MLVA) assays take advantage of length variations within tandem repeats.Owing to the availability of whole genome sequences, tandem repeats can be readily identified, and tested for polymorphism. These typing tests are often not available in those veterinary laboratories that usually perform conventional testing in control and eradication programs.

The performance of all these tests depends on competent staff, the suitability of the reagents and equipment used. Some laboratories combine tests of lesser sensitivity and specificity but

do testing in series or in parallel instead. Series testing means that all samples tested give a positive result with all techniques applied, in contrast, parallel testing means that the samples give positive results with one or more technique or all are tested positive with all tests used. Parallel testing increases the sensitivity of diagnosis while series testing increases the specificity. Both, series and parallel testing may be useful approaches for control and eradication programs. The major drawback of the approaches, however, is that costs are increased and output of results is delayed.
In conclusion, diagnostic laboratories are a key to the study of the epidemiological situation of disease. The main mission is to discriminate between healthy and sick animals and infected and free herds, respectively An ideal diagnostic test should meet the following requirements: a high degree of validation concerning sensitivity and specificity, simplicity, quickness, inexpensivness, repeatability, applicability to a large number of individuals, and ability to differentiate vaccinated from infected animals. For international use the tests have to be recognized by World Organisation Animal Health (OIE). It is important to highlight that the laboratory diagnosis of animal brucellosis has to be interpreted always in the epidemiological context of the situation.

Oral Presentations (O22-O25)

O22: Oligosaccharide conjugates identify A and M epitopes and are superior ELISA antigens for presumptive diagnosis of brucellosis

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The control of brucellosis in endemic regions is only achievable by mass vaccination. Improved tools are necessary and a need for superior vaccines has been repeatedly identified (for example the recently published EU DISCONTOOLS Gap Analysis project http://www.discontools.eu/Diseases/Detail/42). The current vaccines (there are none available for humans and swine) are live vaccines which may be unstable, require extensive quality assurance, possess residual virulence, require the maintenance of a cold chain and interfere with diagnostic assays.

All effective serodiagnostic assays for brucellosis are dependent on the application of the O-polysaccharide (O-PS) component of the lipopolysaccharide for their effectiveness. To prevent confounding serology, vaccine research has focused on the development of products (strains, subunits and DNA) that are free from O-PS. However the O-PS is a major virulence factor and by removing it from a vaccine much of the protective efficacy is lost. Therefore the current paradigm is the paradox that the O-PS presents both the solution (sufficient protective efficacy) and the problem (serological cross reactivity).

We set out to design and synthesize *Brucella* (*B.*) A- and M-specific oligosaccharides. A-nonasaccharide showed identical binding to A- and M-specific mAbs while a pentasaccharide showed a preference for M-specific mAbs. Guided by these results, as well as unpublished data from Dr. John McGiven and recent data reporting the fine structure of *Brucella* O-PS, which proposed that O-PS is capped by a M-specific tetrasaccharide sequence, we designed and synthesized six oligosaccharides, a disaccharide, two trisaccharides, the terminal M capping tetrasaccharide sequence and two hexasaccharides.

Oligosaccharide-BSA conjugates were used to define the specificity of mAbs developed in our laboratory that bind A, M, and C/Y epitopes. The eight oligosaccharide conjugates were also tested against sera from a small group of human patients initially presumed to have brucellosis. The disaccharide antigen accurately identified a patient who was culture positive for *B. suis*.

Our data suggest that a simple test composed of disaccharide and hexasaccharide antigens could differentiate patients infected with *Brucella* strains expressing smooth LPS from those infected by Gram-negative bacteria known to elicit antibodies that are cross reactive in assays used for presumptive diagnosis of brucellosis.

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O23: Application of synthetic oligosaccharide conjugates (based on the structure of the native *Brucella* OPS) to the serodiagnosis of bovine brucellosis

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Members of the genus *Brucella* (*B.*) have a cell wall characteristic of Gram-negative bacteria which, in the most significant species, includes O-polysaccharide (OPS). Serology is the most cost-effective means of detecting brucellosis as infection with smooth strains of *Brucella* typically leads to the induction of high antibody titres against the OPS. This is an unbranched homopolymer of 4,6-dideoxy-4-formamido-D-mannopyranosyls (D-Rha4NFo) that are variably α-(1$\rightarrow$2) and α-(1$\rightarrow$3) linked. The proportion of linkage types is responsible for the classification of the OPS into 'A', 'M' or mixed 'A and M' types and many of the binding characteristics of anti-OPS mAbs.

We synthesised six BSA-conjugated D-Rha4NFo homo-oligosaccharides - each containing a single α-(1$\rightarrow$3) link but a varied number of α-(1$\rightarrow$2) links. Separate iELISAs were developed using these antigens as well as native OPS from *B. melitensis* 16M and *Yersinia* (*Y.*) *enterocolitica* O:9. These assays were applied to a panel of cattle sera to investigate their diagnostic capability. Five serum populations were evaluated: *a*) field sera from cattle confirmed to be infected with *B. abortus* by culture (n=45), *b*) randomly selected field sera from cattle from an officially brucellosis free country (n=125), *c*) field false-positive serological reactors (n=68), *d*) sera from four cattle experimentally infected with *B. abortus* strain 544 (n=16) and *e*) sera from four cattle experimentally infected with *Y. enterocolitica* O:9 (n=16).

The results from the field sera demonstrated that the antigens containing both 'A' and 'M' like properties had perfect diagnostic capability when comparing populations *a* and *b*. The 'minimal M' disaccharide was not as effective on this sample set as there was one false negative sample. When populations *a* and *c* were compared all the synthetic antigens had an improved ability to differentiate between the sample types compared to either native antigen. This capability increased as the number of α-(1$\rightarrow$2) links within the antigen decreased and was best when there was just the single α-(1$\rightarrow$3) linking the two D-Rha4NFo units in the disaccharide antigen. This was also the best antigen at discriminating between sera derived from the two groups of experimentally infected animals.

We conclude that the synthetic oligosaccharide conjugates are not only effective surrogates for naturally derived antigens but that the creation of single, discrete, epitope antigens has begun to unlock some of the previously untapped diagnostic potential that lies within the OPS.

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O24: Estimation of individual seroprevalence of brucellosis in large ruminants from bulk-milk-tank ELISA results

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Knowing the prevalence of brucellosis within livestock herds or village groups is critical for planning and assessing the technical and economic effectiveness of brucellosis control programs. Prevalence estimates are also a necessary input for simulating the effectiveness of different control programs such as vaccination or test-and-slaughter. However, obtaining unbiased estimates of brucellosis prevalence within livestock herds can be extremely challenging and costly in endemic areas.
Milk from individual cows is often combined into a bulk tank, either on large farms, or in village settings. In Great Britain, bulk milk tank testing is part of routine brucellosis surveillance. A standard interpretation of the tests is that the herd from which the milk samples came is either 'negative' or 'positive'. However it would be useful to estimate the seroprevalence of brucellosis within these herds, for the reasons discussed above. The aim of this study was to assess the usefulness of bulk milk tank brucellosis ELISA results to estimate brucellosis seroprevalence within herds.
151 milk samples were obtained from the same number of buffaloes and cows in one village in Menufia governorate, Egypt. Briefly, households were randomly selected, and all lactating cows and buffalos within the household were sampled. The following information was collected for each animal: species, breed, age, number of lactations, type of insemination, history of abortion and daily milk production. The individual milk samples were tested for *Brucella* antibodies using a commercial indirect ELISA (BRUCELISA, Veterinary Laboratories Agency, UK). In addition, in order to simulate the use of bulk milk tanks, 138 combinations of ten individual milk samples were created by mixing the milk samples in the laboratory. These combined milk samples were tested using the same indirect ELISA. Optical density values were recorded for each individual milk sample and each combined milk sample. For each combined milk sample, its optical density value was compared to the mean optical density value of its component individual milk samples, by plotting them on a scatterplot, and assessing the statistical correlation. Simulation was used to create a model to estimate seroprevalence within the herd based on the combined sample optical density results, as well as 80 %, 90 % and 95 % confidence intervals. The model was tested on a portion of the data that had been kept from the model-building stage.
There was good correlation between the mean of the individual milk optical density results and the combined milk sample results. Using the manufacturer's recommended cut-off values, of 53 tanks containing no positive samples, all 53 were correctly identified as negative. Of 85 tanks containing at least one positive sample, only 13 were identified as positive. The use of different cut-offs was explored. Estimation of seroprevalence of brucellosis within herds or village groups from bulk milk tanks may be useful for planning and monitoring brucellosis control programs where there are constraints to collecting individual samples. However this method should be validated in areas where the seroprevalence of brucellosis differs.

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O25: Use of bionumerics software and MALDI-TOF-MS data for the identification of *Brucella* species

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Members of the genus *Brucella* are Gram-negative, facultative intracellular pathogens that cause disease in humans and livestock in many parts of the world. *Brucella* (*B.*) *abortus*, *B. melitensis*, and *B. suis* being classified as Select Agents in the United States (US). Despite their potential for use in a bioterrorism incident, infections caused by *Brucella* spp. in the US are most commonly seen as a result consuming raw, unpasteurized dairy products. Safely handling and minimizing work with *Brucella* isolates is important since brucellosis is one of the most common laboratory acquired infections. Species specific identification of *Brucella* spp. using phenotypic methods can be time consuming. PCR assays, which are more rapid, have been described for species level identification but are not widely available in clinical laboratories. However, matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry has proven to be a powerful tool in clinical laboratories for the rapid identification of bacterial pathogens. Previous studies investigating the utility of MALDI-TOF analysis as a diagnostic for the detection of *Brucella* have shown that identification at the genus level is possible, but reliable identification at the species level remains problematic. Our laboratory has constructed a MALDI-TOF Biotyper library (Bruker Daltonics) of well characterized reference strains representative of *B. abortus*, *B. melitensis*, and *B. suis* isolates, as well as closely related *Brucella* species. We have interrogated our library using isolates taken from panels of phylogenetically diverse *B. abortus*, *B. melitensis*, and *B. suis* isolates. The protein profiles generated were analyzed using Bionumerics, which allowed for more detailed resolution of unique protein peaks that can be used to distinguish species. Preliminary data demonstrated that protein profiles generated with the Bruker Biotyper and analyzed using the Bionumerics software showed species specific clustering. We believe the combination of these two techniques could be an important step forward in using MALDI-TOF spectra to correctly identify *Brucella* spp. at the species level.

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Poster Presentations (P53-P84)

P53: Investigation of OPS oligosaccharides as candidate antigens for serodiagnosis of bovine brucellosis with improved specificity

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The most cost-effective means of monitoring the zoonotic disease brucellosis involves serology and depends on the detection of anti-*Brucella* antibodies in blood, but classical tests, which measure the serum response to the *Brucella* lipopolysaccharide (LPS) antigen, produce high numbers of false-positive serological reactions (FPSRs). These are due to the presence in the serum of antibodies against other Gram-negative bacteria which have O-polysaccharide (OPS) structures similar to that of *Brucella*. A more specific test might be based on antigenic determinants that are exclusive to *Brucella* and to this end, we have investigated whether short oligosaccharides derived from the *Brucella* OPS could provide this improved specificity.

The OPS from *Brucella* is an unbranched homopolymer of 4,6-dideoxy-4-formamido-D-mannopyranosyls (D-Rha4NFo) that are variably α-(1-2) and α-(1-3) linked. This structure contains some epitopes that are shared with the OPS from other organisms, notably *Yersinia* (*Y.*) *enterocolitica* O:9, and some that appear to be unique. Serum antibodies from *Brucella* infected cattle and from non-infected cattle presenting FPSR react differently to the OPS from *Brucella* (*B.)* and *Y. enterocolitica* O:9. To identify the structural source of this difference oligosaccharides were prepared from *B. abortus, B. melitensis* and *Y. enterocolitica* O:9 LPS and characterised by electrospray mass spectrometry. The oligosaccharides were obtained by the strong acid hydrolysis of OPS which had been obtained by mild acid hydrolysis of LPS following phenolic extraction of the bacterial culture. They were then separated by size exclusion chromatography and collected as a series of fractions. The oligosaccharide containing fractions were combined, concentrated and applied to antibody conjugated affinity columns containing M-epitope specific antibodies or polyclonal antibodies selected according to their affinity or otherwise, to *Y. enterocolitica* OPS. The oligosaccharides captured in this way were evaluated by chromatography on a graphitised carbon chip column linked via an electrospray interface to a triple quadrupole mass spectrometer. The results indicate the presence of oligosaccharides containing a specific linkage, not found in *Y. enterocolitica* O:9 OPS, that binds to a *Brucella* OPS specific mAb and to bovine polyclonal anti-*Brucella* OPS antibodies. We considered that harnessing such a structure in a diagnostic format might lead to an assay with superior diagnostic specificity than those currently in use and on the basis of the mass spectrometry evidence we commissioned the synthesis of a linear D-Rha4NFo tetrasaccharide which contained a single α-(1-3) link flanked on either side by single α-(1-2) links. This tetrasaccharide was used to develop an ELISA that improved the specificity of antibody detection.

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P54: False-positive reactions to serological tests for brucellosis: Analysis of antibody response to *Escherichia coli* O157:H7 and *Yersinia enterocolitica* O:9 in experimentally immunised sheep

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According to the European Union regulations, serological control of ovine and caprine brucellosis is based upon Rose Bengal test (RBT) or i-ELISA as flock screening test, and on complement fixation test (CFT) or c-ELISA, as confirmatory test. These tests are also recommended by the OIE for international trade, however, their major limitation is related to the use of either suspensions of smooth brucellae (s-brucellae) or LPS extracts. In fact, the LPS O-chains of s-brucellae are similar to the one present in other Gram-negative bacteria (*E. coli* O157:H7, *Y. enterocolitica* (Ye) O:9- Ye O:9, *Salmonella* group N) that, following infection of the animals, may lead to false-positive serological reactions (FPSR). As a part of research activities within the EU EMIDA ERA-NET project entitled '*Brucella melitensis*: biotyping and differential diagnostic - Brucmel', three sheep were immunized with 1.5 x 10^9 CFU/ml of live *E. coli* O157:H7; other three were immunized with 1.5 x 10^9 CFU/ml of viable Ye O:9; finally, two animals were included as controls. Antigens were administered weekly, initially by oral route (5 ml), then by subcutaneous (sc) route, doubling the dose (10 ml) in the last phase of the study. Sera were collected weekly throughout the entire experiment (381 days). To measure antibody response against *E. coli*, an indirect ELISA was developed using heat inactivated *E. coli* O157:H7 (10 μg/ml) as antigen and protein G-HRP conjugated as revealing system. Antibody response against Ye O:9 was detected by CFT using a commercial antigen and reference sera. Serological response to *Brucella* was assessed by RBT, CFT, i-ELISA and c-ELISA according to OIE Manual. Results showed that, following immunization with *E. coli* O157:H7, an increased production of antibodies against both *E. coli* and Ye O:9 was observed. In contrast, immunization with Ye O:9 determined a specific response against the homologue antigen only but produced no reactivity against *E. coli*. Sera from both *E. coli* and Ye O:9 immunized animals showed cross-reactivity against serological tests for brucellosis. *E. coli* immunized animals were positive in: RBT (24.2 %), CFT (24.2 %), c-ELISA (26.7 %), and i-ELISA (21.7). Ye O:9 immunized animals were positive in: RBT (30.9 %), CFT (37.2 %), c-ELISA (62.8 %), and i-ELISA (40.4 %). In both immunized groups, CFT Ye O:9 titres were higher than CFT titres for brucellosis. Although conducted on a limited number of animals, this study provided relevant information on the kinetics of antibody response against *E. coli* O157:H7 and Ye O:9 and on FPSR to brucellosis serological tests. Based on our preliminary results, the application of a diagnostic protocol that considers the use of serological tests for Ye O:9 (CFT), *E. coli* O157:H7 (i-ELISA) and *Brucella* (CFT) in parallel needs to be further evaluated on field sera suspected for FPSR. This protocol should be able to identify FPSR and it would represent an important tool in all cases where serological reactivity to official tests is not supported by epidemiological evidence of *Brucella* infection.

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P55: Development of a glycoconjugate, lipid and protein multiplex immunoassay for the serodiagnosis of porcine brucellosis

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Brucella (*B.*), a Gram-negative intracellular bacterium, is the causative agent of brucellosis, a worldwide zoonosis of significant economic and social importance. Serological surveillance of swine in Great Britain is not undertaken routinely and serology for swine brucellosis is used primarily to support the export of live animals. However, current serological tests produce a problem with false-positive serological reactors (FPSR) in areas of low prevalence. FPSR are caused by presence of antibodies raised against infection with bacteria possessing similar O-polysaccharide (OPS) structure, such as *Yersinia* (*Y.*) *enterocolitica* O:9. The serological tests currently used for the diagnosis of porcine brucellosis are not considered to be reliable for diagnosis. A multiplex serodiagnostic immunoassay offers potential to eliminate FPSR, by combining data from multiple antigens.
This multiplex immunoassay was developed to enable simultaneous detection of antibodies to *Brucella* OPS from *B. abortus* S99 and *B. melitensis* 16M OPS, included because they are used in current assays. Rough LPS from *B. abortus* RB51 was included as it has been previously shown to have no cross reaction with porcine FPSRs. Non-OPS antigens rough *B. melitensis* strain B115 (Brucellergene™) and two recombinant proteins BP26 and Lumazine synthase were also included. The inclusion of cross-reactive antigens with similar OPS structure to *Brucella* spp.: *Escherichia coli* O:157 OPS and *Y. enterocolitica* O:9 OPS provided more information about serum samples from different sample populations. A BioDot Inc. fluid dispensing system printed a 30 nl spot of each antigen into each well of a 96-well microwell plate. In order to bind the OPS to the microwell plate the OPS was conjugated to bovine serum albumin, to produce Ovalbumin, P62
a glycoconjugate, by oxidisation and reductive amination. Plates were read using a Q-view imager™. Signal intensity was calculated and was proportional to serum antibody binding. To demonstrate utility of the multiplex immunoassay a small panel of porcine serum samples were tested from three sample populations; including sera from culture-positive animals, *Brucella*-free serological negatives and FPSR.
In conclusion, a novel multiplex immunoassay using glycoconjugates, lipid and protein antigens has been developed for the detection of anti-*Brucella* antibodies and results may contribute to the elimination of FPSR from ongoing serological investigations.

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P56: Comparison of barbital veronal buffer and non-toxic MgCa buffer in the complement fixation test for the diagnosis of brucellosis in animals

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The complement fixation test (CFT) is an OIE approved serological test for the detection of antibodies against bovine, ovine/caprine or porcine brucellosis, mainly caused by *Brucella* (*B.*) *abortus, B. melitensis* and *B. suis,* respectively. The CFT is a semi-quantitative, highly sensitive and specific test. Its principle is based on the ability of heterologous complement to bind to antigen/antibodies complexes. The free complement is fixed on an indicator system consisting in antibody coated erythrocytes when specific immune complexes are not present. Thus, the degree of haemolysis inhibition allows quantifying the level of specific antibodies. Barbital compound buffer (diethylmalonyl urea, diethylbarbituric acid, barbitone and sodium barbital) or veronal buffer (VB) is used as a standard diluent buffer for sera and reagents in the CFT. This chemical product is considered as hazardous because of its toxicity and its drug properties, resulting in its gradual ban and complicating its import/export. The MgCa dilution buffer was previously recommended as another diluent to be used in the CFT. This study aimed at assessing this buffer as a non-toxic alternative, containing only magnesium and calcium diluted in NaCl, in comparison to VB.
VB and MgCa buffers were used in parallel CFT assays on international (OIEISS –anti-*B. abortus*–, ISaBmS –anti-*B. melitensis*–) and European standard sera (EUSBSS –Sheep–, EUGBSS –Goat–, EUPBSS –Pig–), as well as on field bovine, ovine and porcine serum samples covering a gradient of reaction intensity (from 0 to 100 % haemolysis inhibition). CFT obtained results using each dilution buffer were then compared.
CFT results of international and European standard sera were similar whatever the dilution buffer employed. The difference between results obtained with both buffers never exceeded a dilution factor (½) which is considered as not significant for this technique.
Of the 179 field sera from infected populations (bovine: n=60; ovine: n=59; porcine: n=60), 75.98 % of samples (bovine: 78.33 %; ovine: 77.97 %; porcine: 71.67 %) showed a similar haemolysis inhibition intensity whatever the dilution buffer used. In addition, 24.02 % (bovine: 21.67 %; ovine: 22.03 %; porcine: 28.33 %) showed a non-significant difference (less than one dilution factor). Only one bovine serum (0.56 %) was anti-complementary, and thus uninterpretable.
Of the 174 field sera from brucellosis-free populations (bovine: n=60; ovine: n=58; porcine: n=56), CFT results using VB and MgCa were identical for 98.28 % of samples (bovine: 100 %; ovine: 94.83 %; porcine: 100 %) or showed a non-significant difference (< 1 dilution factor) for one sheep serum (0.57 %). Two ovine sera (1.15 %) were anti-complementary in both buffers.
In conclusion, no significant differences were observed in CFT by using either one of the buffers for international or European standard sera as well as for field serum samples, whatever the host species (cattle, small ruminants or pigs).
These results highlight the non-toxic, easy to produce and cheap MgCa buffer as a suitable alternative to the barbital VB in the OIE approved CFT. The possible use of the MgCa buffer will be therefore proposed for inclusion in the next revision of the OIE Terrestrial Manual.

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P57: Investigation of recombinant immunogenic protein for alternative diagnosis of brucellosis

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Brucellosis is an important notifiable zoonotic disease in livestock and human which is caused by *Brucella* (*B.*) species. Herds are mainly tested by a variety of serological methods and positive animals are generally slaughtered for control purposes. Serological assays are mainly based on whole cell antigens or smooth lipopolysaccharide (LPS)/O-polysaccharide (OPS) antigens, however, they are often limited due to false-positive or -negative serological reactions. It is of great importance for future eradication programs to overcome this shortcoming. For this study, we tried to identify alternative antigens among proteins that are known to be immunogenic.
A total of 13 proteins were selected for further investigation: Omp28, Omp31b, SodC, DnaK, TRAP, L7/L12, Omp16, Omp31, Omp25, GroEL, HisD, L25 and AldA. Its respective genes were inserted into expression vectors pColdI, pETHis SMT3 or pET300/NT-DEST containing 6 x His Tags. Expressed proteins were purified on Ni-NTA columns and tested by SDS-PAGE and Western Blotting using ten pooled *B. abortus*-positive sera from naturally infected cattle. 0.1 µg of each purified protein was coated per ELISA plate well. Then, positive (n=109) and negative sera (n=70) were tested. Sera used were previously tested by RBT, STAT, FPA and C-ELISA with a positive or negative result. Cut-off values were set as mean value of negative sera ±2SD. The newly developed in-house ELISA based on recombinant proteins was validated using smooth LPS as test antigen. A NCBI BLAST was done to compare protein sequences used with homologous sequences of homologs of bacteria known to produce cross reactions in serology.
The κ-values for recombinant proteins were: *r*Omp28 (0.87), *r*SodC (0.75), *r*DnaK (0.71) *r*GroEL (0.69) and *r*L7/L12 (0.67), respectively. Conversely, *r*Omp16, *r*Omp31, *r*Omp25 and *r*HisD showed weak immunological reactions and κ-values were lower than 0.5. For *r*AldA, *r*TRAP, *r*L25 and *r*Omp31b, non-specific serological reactions using various negative sera were detected. Based on these results, five recombinant proteins were selected. While *r*SodC showed comparatively consistent results when compared to that of control smooth LPS, *r*OMP28 showed converse OD value. Alignments of the amino acid sequences of Omp28 and SodC showed similarity values higher than 98 % in comparison with other *Brucella* species but a similarity lower than 50 % when compared to other bacteria. Omp28 and SodC showed similarities of 84 % and 65 % to those of *Ochrobactrum* spp. In conclusion, recombinant Omp28 and SodC were specific and were comparable to smooth LPS antigen. Moreover, differences seen in the OD values of *r*SodC and *r*Omp28 may be related to the infection time or status. Thus, the appropriate combination or multiplexing of these two antigens (fractions) may help to develop an alternative or complementary serological method.

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P58: The study of modified Rose Bengal test for the diagnosis of bovine brucellosis

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The Rose Bengal test (RBT) is widely used for serological diagnosis of bovine brucellosis. However, it does not always provide reliable results. Such diagnostic errors need further follow-up investigation and may increase the costs for diagnosis.
The goal of our investigation was to study the effectiveness of the modified RBT for the diagnosis of bovine brucellosis under laboratory and field conditions. In this study, we investigated blood serum samples from 44 guinea pigs infected subcutaneously with *Brucella* (*B.*) *abortus* strains S19 and 104M with the modified RBT.
We examined blood serum samples from four heifers infected subcutaneously and over the conjunctival route with *B. abortus* strain S19, and samples from two bulls infected subcutaneously with *Yersinia* (*Y.*) *enterocolitica* serovar O:9. Furthermore, 45 field blood serum samples from cows were analyzed.
A commercial *Brucella* antigen (Kherson, Ukraine) was used for RBT (standard and modified method) and SAT, as well as common *Brucella* antigen for SAT (serum agglutination test), CFT (complement fixation test) and LCFT (long complement fixation test). Furthermore, a diagnostic kit for detection of *Y. enterocolitica* serovar O:3, O:6,30 and O:9 by means of SAT was included.
Brucella antibodies were detected in all sera of guinea pigs on day 7 with the modified RBT technique (RBT MT).In contrast with standard RBT and SAT only 79.17 % and 50 % of the sera were positive tested, respectively. On day 35, antibodies were detected in 100 % of animals with the RBT, SAT and CFT.
In serum samples from guinea pigs infected via the conjunctival route, antibodies were detected on day 14 in 80 %, while the unmodified RBT yield positive results only in 50 % of the tests. On day 35 antibodies were detected in all sera using RBT MT, RBT, SAT and CFT.
In heifers subcutaneously infected, antibodies were detected in all animals on day 7 with RBT MT, RBT, SAT and CFT. In sera from heifers infected via the conjunctival route antibodies were detected with RBT MT on day 14 and on day 35 with standard RBT, SAT and CFT.
In sera from bulls, infected with *Y. enterocolitica* serovar O:9, antibodies were detected on day 14 with standard RBT; SAT and CFT. Both animals showed positive results with SAT using *Y. enterocolitica* serovar O:9 antigen but were negative results with RBT MT.
Out of 45 field blood serum samples from cows100 % were positive with standard RBT and 11.11 % with SAT. If sera were tested by SAT based on *Y. enterocolitica* serovar O:9 antigen, 17.78 % of the samples were positive. Contrary, the modified RBT obtained negative results in all animals.
It has been shown that the RBT MT provides reliable results for brucellosis diagnosis in sera of guinea pigs and bovine field sera on days 7-14.

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P59: Identification of false-positive serological tests results within brucellosis investigations in cattle

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Bovine brucellosis is an important zoonotic disease, affecting cattle and humans. Diagnosis of bovine brucellosis is based on serological, microbiological and molecular investigations. The annual serological screening for brucellosis in livestock is a mandatory veterinary measure once a year in the Ukraine. The Rose Bengal test (RBT) is a fast, easy and inexpensive test for brucellosis. However, this test may be prone for false-positive results. In such cases, it is necessary to carry out additional investigations to confirm diagnosis.
In our investigation the number of false-positive test results for brucellosis in cattle was determined. Furthermore the nature of the cross reactive agents was elucidated. We investigated serum from cattle aged 1-5 years without clinical signs of infection. Cows in the last stage of pregnancy and during the two weeks after delivery were excluded from our investigation. Sera were tested with the RBT using commercial *Brucella* antigen for RBT (Kherson, Ukraine). In case of positive results, samples were re-tested on the same day and two week later. In this case, not only the positive animals, but also all animals of the contact group were examined. Retesting of serum was done using RBT, serum agglutination test (SAT) and complement fixation test (CFT) using commercial *Brucella* antigen for RBT, common *Brucella* antigen for SAT, CFT and LCFT (long complement fixation test), experimental RS-*Brucella* antigen for SAT, as well as diagnostic kits for *Yersinia enterocolitica* serovar O:3, O:6,30 and O:9, *Salmonella enterica* serovar Enteritidis antigen, and *S. enterica* serovar Typhimurium antigen. Serological tests were performed in accordance with standard methods.
A total of 3,293 cattle serum samples from seven regions of the Ukraine were investigated. 583 sera (17.7 %) were retested when a RBT-positive result was detected. After re-testing the same day, 2.57 % of serum samples were positive in RBT. All sera were positive in SAT using the common *Brucella* antigen and the *Y. enterocolitica* serovar O:9 antigen. The average titre in SAT was 1.81 $\log_{10}$ and 2.48±0.25 $\log_{10}$ for the Common *Brucella* antigen and the *Y. enterocolitica* serovar O:9 antigen. Additionally, all sera were also positive for the common *Brucella* antigen in CFT with an average titre of 1.38±0.15 $\log_{10}$.
Re-testing of sera showed 1.37 % positive results in RBT after 14 days. At this time point, all sera were negative in CFT. In SAT, the average titre was 1.74±0.55 $\log_{10}$, but no positive diagnostic titre (2.3±0.17 $\log_{10}$) was detected. All sera showed positive results in SAT using *Y. enterocolitica* serovar O:9 antigen again. The average titre increased to 2.56±0.15 $\log_{10}$.
Animals that were positive in RBT showed positive reaction neither in SAT using *Y. enterocolitica* serovar O:3, O:6,30 Antigen, nor *S. enterica* serovar Typhimurium, *S. enterica* serovar Enteritidis, and the experimental RS-*Brucella* antigen.
Culture of biological fluids from positive animals showed no growth of *Brucella*.
Y enterocolitica serovar O:9 isolates were recovered from six animals.
False-positive reactions in serological tests for brucellosis can occur due to infection of cattle with *Y. enterocolitica*. To simplify the differential diagnosis of brucellosis, diagnostic kits for serological detection of *Y enterocolitica* in SAT can be used.

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P60: Milk sample testing by indirect ELISA and Fluorescent Polarization Assay

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Brucellosis is a global health care and veterinary problem. Up to now the disease in humans and animals is not completely understood. It can cause significant losses in livestock farming because it affects several types of domestic animals, especially cattle, sheep, goats, and pigs. Brucellosis can be transmitted from animals to humans and causes severe disease which requires lengthy treatment. In Georgia, brucellosis is observed in all regions, but especially in the hyperendemic region of eastern-Georgia. The disease can be transmitted through the consumption of contaminated milk, raw cheese (new, without salt), sour cream and butter. In order to limit and restrict the spread of brucellosis, early detection and identification of infected animals, and isolation of the bacteria is important.
In this study, milk samples were tested by the Rose Bengal test (RBT). In addition, many modern techniques such as indirect ELISA and Fluorescent Polarization Assay (FPA) had been used to expand the diagnostic capabilities of the laboratory. Samples of previous animal studies were used to demonstrate the applicability of ELISA and FPA assays. The Rose Bengal test and the Plate Agglutination assay were used to monitor the antibody response to *Brucella* antigens within the samples. Positive bacteriological cultures were verified as *Brucella* using classical bacteriological typing tests like Gram staining, urease, catalase andoxidase activity, TSI, H_2S reaction, dye sensitivities, and rough or smooth reactions. Serum and milk samples from earlier studies were stored at -20°C until use.
For *Brucella* antibody testing, commercially available kits for indirect ELISA, competitive ELISA, and FPA were used. These tests were conducted on blood serum and on milk samples. A panel of 50 blood serum samples, which included cattle, sheep, goat, rabbit, cat, dog and human blood, were tested. Eight samples were positive-tested. These new techniques are now part of the standard algorithm used at the Laboratory for the Ministry of Agriculture in both research and in passive surveillance systems. The use of these methods for identification of brucellosis in animals will increase the ability of the laboratory to provide early detection of the disease in veterinary samples. This supports the long term goal to reduce the spread of brucellosis in the human population in Georgia.

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P61: Immunochromatographic test for detection of bovine brucellosis using unprocessed, non-serological milk samples

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Brucellosis is a neglected zoonotic disease that afflicts livestock and wildlife animals globally. The brucellae have a preference for reproductive organs of their hosts. Contact to infected animals and uptake of contaminated food is the main source for human infection. Diagnostics currently available for detection of brucellae suffer from a lack of specificity, accessibility to remote areas, the requirement of skilled personnel and expensive equipment for analysis. More notably is the fact that a result with conventional methods like culture is laborious and time-consuming. Point of care immunoassays can analyze various samples in a short time period. They are highly sensitive, specific and offer a great advantage in accurate and fast diagnosis of brucellosis. Using this technology, we have produced a point of care rapid diagnostic assay (Milk ICDT) that employs fluorescent silica nanosensors (FSNs) capable of specifically detecting anti-*Brucella* IgG antibodies in milk and serum samples of infected animals. Nanosensing is based on the ability of the particles to give fluorescence upon excitation by a UV light source. Additionally, this technique allows precise detection of antibodies with low sample volumes. FSNs were constructed by silica condensation around core self assembled micelles of a triblock co-polymer. Dynamic Light Scattering (DLS) data showed that these micelles are of approximately 50 nm in size. By silica condensation around the micellar structure more compact robust structures (FSNs) of approximately 30 nm with stable fluorescence signals over 6 months are obtained. A concentration dependent fluorescence quenching effect was observed upon incubation of FSNs with increasing concentrations of lipopolysaccharides. For concentrations beyond 1 mg/ml a constant fluorescence signal was obtained. Nitrocellulose membranes of a specific grade (pore size 10 µm) were incorporated in ICDT developed for milk samples comparable with conventional serum immunochromatographic strips (15 µm), as non-serological samples are less complex than serum or whole blood samples. PCR was used as a reference test to copare the results from the ICDT. The data show that ICDT had a high specificity (100 %) and a sensitivity (100 %) for milk samples. There was an excellent agreement between the results of PCR and ICDT for clinical samples. Also, there was complete agreement between ICDT and milk ELISA. Milk ELISA kits use antigens from reference strains as coating antigens and they showed a higher specificity than the milk ring test (90 %). ICDT developed for milk samples had high sensitivity, much higher than that of the serum tube agglutination test (STAT) (31 %) and comparable to that of milk ring tests (MRT) (98 %). We demonstrate for the first time recognition of *Brucella* (*B.*) *abortus* antibodies by capture by FSNs using non-serological milk samples validated by ELISA and PCR. The test results are reliable with high sensitivity, specificity and a short assay time of 15 min for antibody recognition. Well defined antigenic components and surface biomarkers of various disease causing microbes can be broadly incorporated within the purview of this technology for accurate and rapid detection of bovine diseases such as bovine brucellosis using non-serological matrices such as milk.

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P62: Serological diagnosis of canine brucellosis caused by *Brucella canis*

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The objective of this study was to evaluate the performance of a commercial immunocromathographic test (ICT) (Bioeasy, Animal Genetics, Suwon-si, South Korea) for canine brucellosis diagnosis in naturally infected dogs, by comparing its performance with the rapid slide agglutination test (RSAT), rapid slide agglutination test with 2-mercaptoethanol (2-ME) (Symbiotics Corporation, Kansas, USA), and agar gel immunodiffusion test (AGID) (Tecpar, Curitiba, Paraná, Brazil). A total of 102 dogs were used and according to the results of the clinical examination, blood culture and PCR in whole blood, semen and vaginal swabs were divided into three groups. Group 1 consists of 48 *B. canis* infected dogs (Group 1), which show positive results in blood culture and/or PCR applied to whole blood or genital (semen or vaginal swab) samples. Group 2 exhibit 33 *B. canis* non-infected dogs with negative results by microbiological examination and PCR. Dogs of this group show no clinical sign of brucellosis and belonging to kennels where clinical or laboratorial evidences of brucellosis were not observed. The third group (Group 3) comprise dogs suspected of having brucellosis with negative results in microbiological culture and PCR but belonging to the same kennels as the infected dogs. The diagnostic sensitivity and specificity of the serological tests were calculated using Group 1 and 2 as gold standard. The diagnostic sensitivity of RSAT, 2ME-RSAT, AGID, IDGA, and ICT was 75 %, 37.5 %, 27.08 %, and 89.58 %, respectively. The diagnostic specificity of the four serological tests was 100 %. In dogs with suspected brucellosis, the number of positive results by RSAT, 2ME-RSAT, AGID, and ICT was 9.67 %, 0 %, 3.22 % and 6.45 %, respectively. The ICT showed a higher diagnostic sensitivity when comparing to agglutination or precipitation tests for the diagnosis of bacteremic dogs, and may be a rapid screening test to be used for the diagnosis in breeding kennels.

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P63: Evaluation of indirect enzyme-linked immunosorbent assays using recombinant proteins to diagnose *Brucella canis* infection in humans

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Brucella (*B.*) *canis* infection in humans and dogs is rarely diagnosed due to the unavailability of highly specific and sensitive diagnostic assays in Colombia. The objective of this works was to develop and evaluate indirect enzyme-linked immunosorbent assays (iELISA) using two recombinant proteins for the diagnosis of *B. canis* in humans. Two cytoplasmic proteins of *B. canis* strain Oliveri, Pyruvate dehydrogenase E1 subunit beta (49 kDa) and Elongation factor Tu (42.6 kDa), were previously identified as immunogenic in humans using mass spectrometry analysis and bioinformatic tools. Genes encoding these proteins were PCR-amplified from DNA of *B. canis* strain Oliveri (AN HG803175.1 and HG803176.1 for chromosome 1 and 2, respectively). The genes were inserted in the pGEM-T easy vector or pTZ57R/T vector and transferred into *Escherichia* (*E.*) *coli* DH5α cells, subcloned in pRSET-A or pET-28a, and transformed into *E. coli* strain BL21. The expression of the proteins was induced and the proteins were purified, and then evaluated as diagnostic antigens in iELISAs to detect IgG antibodies in serum samples from humans and dogs. The conditions for the test were standardized.
In our tests PdhB and Tuf iELISAs showed 98 % sensitivity and 77 % specificity. Thus, these proteins have the potential of being used as target antigens for the identification of *B. canis* infections in humans. Thus, the use of recombinant antigens for the development of iELISAs to detect *B. canis*-specific antibodies in serum of humans could improve the specific serological diagnosis of this infection.

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P64: Active surveillance of human brucellosis in Uganda: Implications of low diagnostic test reliability

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Human *Brucella* (*B.*) infections are diagnosed on the basis of clinical findings backed by bacteriological culture and/or serological laboratory tests. Uganda has reported prevalence of up to 14 % in hospital patients, animal handlers and abattoir workers. However, brucellosis is not being a priority reportable disease in the weekly passive surveillance system. Only 4 % of the districts in Uganda report brucellosis. We conducted active surveillance to determine the prevalence of human *Brucella* infection and assess the reliability and accessibility of currently used diagnostic tests in Uganda.
The surveillance was conducted in Nakasongola, Kween, Kapchorwa and Kabale districts in March 2011. Multi-stage sampling of sub-counties, health centers and patients was done. Blood was sampled from every second patient with fever presenting at the outpatient department in selected health centers who consented to participate. Sample analysis with both *B. abortus* and *B. melitensis* microplate rapid agglutination (MAT) and indirect ELISA tests was done at Central Public Health Laboratories, Kampala for quality control. Data were analyzed in SPSS 17.0 to evaluate reliability of the two tests.
A total of 513 patients (58 % males) aged 18-56 years were sampled. *B. abortus* prevalence was 21.8 % and *B. melitensis* was 14.0 %; while overall prevalence by iELISA was 14.8 %. *B. abortus* MAT had 43.4 % sensitivity and 67.8 % specificity compared to iELISA. MAT had a positive predictive value (PPV) of 20.5 % and a negative predictive value (NPV) of86.8 %. *B. melitensis* MAT had 24.6 % sensitivity and 87 % specificity, thus giving 19.7 % PPV and 86.2 % NPV. The level of agreement between the tests was low; kappa statistic of 0.32 and 0.19 for *B. abortus* and *B. melitensis* rapid tests, respectively.
Health centers in Uganda rely on *Brucella* rapid tests for routine diagnosis with no access to ELISA and other advanced tests. Both *B. abortus* and *B. melitensis* MAT gave low sensitivity but good specificity values which undermines their wide use in Uganda. Urgent efforts to build reliable diagnostic capacity for *Brucella* testing are required to improve timely access to treatment.

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P65: Brucellosis in abattoir cattle in Algiers, Algeria

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During the last two decades, brucellosis has been a major problem of public and animal health in Algeria with thousands of human and animal cases reported annually. The disease is mostly endemic with occasional outbreaks, and represents an obstacle for the development of animal production. The aim of this study is to assess the presence of bovine brucellosis in areas close to the capital city (Algiers) where brucellosis vaccination is not implemented. A total of 402 cattle serum samples were collected from a slaughterhouse in Algiers. All serum samples were examined by the Rose Bengal test (RBT) using the official Spanish antigen according to the standard procedure. RBT-positive serum samples were then retested by complement fixation test (CFT), double gel diffusion with lipopolysaccharide (LPS) and native hapten (NH) polysaccharide (DDG) using two antigen concentrations: 1 mg/ml and 5 mg/ml according to the standard procedures, and home-made iELISA with polyclonal and protein G conjugates and smooth LPS (S-LPS) as the antigen. Twenty four serum samples were positive by RBT. By CFT, 18 had titres equal or higher 1/256; 2, 1/128; 1, 1/64; 1, 1/16; 1, 1/8 and 1, 1/4. By DDG with antigen concentration at 1 mg/ml, 21 serum samples were LPS+, NH+; 1 LPS+, NH-; 1, LPS-, NH+ and 1, LPS-, NH-. Using 5 mg/ml antigen, 20 serum samples were still LPS+, NH+, three were LPS+, NH- and one was LPS-, NH-. Similarly, all these sera were positive by ELISA when 20 % were selected as the cut-off. The results show that, in the absence of vaccination, RBT identifies the same animals as seropositive as CFT, DDG-NH or iELISA. Bovine brucellosis is strongly present in cattle in Algeria, despite efforts by the competent authorities. Further studies to determine the prevalence of this disease in herds and farms, and the isolation of the causative organism are necessary for the implementation of an adequate control.

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P66: A serological analysis by agglutination tests using *B. canis* M(-) as antigen for diagnosis of *Brucella canis* co-infection in Turkish patients with brucellosis

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Brucella (*B.*) *canis* transmitted to man by infected dogs or their secretions causes actually rarely human infections. However, considering the fact that epidemiological data are scarce to make correct evaluation for *B. canis* infections in humans, serological studies were conducted in both healthy population and in humans with brucellosis symptoms, previously. The present study was planned to reveal seroprevalence for *B. canis* infection in humans who had bacteriologically and/or serologically confirmed *Brucella* infection caused by smooth species of *Brucella* genus. A total of 566 patients from Eastern and South Eastern Anatolia who had anti-*Brucella* antibodies with different titers by serum agglutination test (SAT) using smooth *B. abortus* S99 strain as antigen were tested serologically by rapid slide agglutination test (RSAT), the 2-mercaptoethanol RSAT (2ME-RSAT) and microagglutination test (MAT) using *B. canis* (M-) strain as antigen in these tests. Of the samples tested, 142 (25.1 %), 55 (9.7 %), 49 (8.7 %) were positive by RSAT, MAT, 2ME-RSAT, respectively. According to the results, it was concluded that this relatively high seroprevalence may be depending on the region of Turkey or using a rough antigen that might indicate co-infection with smooth strains and *B. canis* or cross reaction with other rough organisms belonging to other bacterial genera, respectively. Regarding to the cross reaction, dissociation of smooth *Brucella* strains to rough strains during *Brucella* infection also might be a reason for high seroprevalence. Therefore, more specific antigens are needed to be used in serological tests to evaluate disease status serologically more precisely in brucellosis patients.

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P67: Development and evaluation of a new conventional multiplex PCR and real-time PCR to detect *Brucella* species

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Despite the fact that brucellosis is considered as one of the oldest diseases, little progress over the past 40 years has been achieved in the development of improved diagnostic tools for the detection of the infection in both humans and animals. Currently available diagnostic tests for brucellosis are not strain-specific, exhibit low specificity and sensitivity rates, are labor-intensive and expensive. Bacterial culture is the gold standard procedure to detect *Brucella* (*B.*), however it takes prolonged incubation periods, special growth media and multiple culture attempts, besides it can yield false-negative results and potential risk for exposure requiring biosafety level 3 facilities. Contrary to bacterial culture, the serum agglutination test (Rose Bengal test) is easy handling, fast, non-hazardous and sensible, nevertheless its results depend on the stage of the disease and may present cross reactivity with other bacteria strains. Recently, PCR has been suggested as an alternative to the conventional assays due to its high sensitivity and specificity, its rapid identification and its independency from bacterial viability. However, most of the detection assays are based on genomic fingerprinting techniques to discriminate among species. On the other hand, real-time PCR has recently been develop and is a refinement of the original PCR. Its advantages are that is rapid, does not require posterior steps (electrophoretic analysis), and reduced risk of cross-contamination. In the present study, we developed a new conventional multiplex PCR and a real-time PCR assays to detect *Brucella* genus and to differentiate among its species. The advantages include: single step, high specificity and sensitivity, it is based on genes rather than genome fingerprinting and the versatility to adapt the targets from the conventional PCR to the real-time PCR. The selected targets were the genes BMEI1056 (*amiC*), BMEII0036 (*vir12*) and BMEII1116 (*vjbR*). Additionally, the targets of the species specific assay were genes from several genomic islands: GI5, GI1, IncP and 26.5 kb. Probes for real-time PCR assays were designed accordingly. The culture growth strains used were *B. melitensis* 16M, *B. abortus* 2308, *B. ovis*, *B. suis* 1330, *B. canis* 23365, *B. ceti*. To test specificity, DNA from other proteobacteria that share high genomic homology with *Brucella* were used: *A. tumefaciens, O. anthropi, P. aereuginosa, Bordetella sp., E. coli, Y. enterocolitica* O:3 and O:9. To test sensitivity, serial DNA dilutions were prepared ranging from $2x10^4$-2 fg/µL for genus and species specific conventional multiplex PCR and ranging from $1x10^6$-0.1 fg/µL for real-time PCR. The results showed that the targets used in conventional multiplex PCR can be used to perform real-time PCR assay. The conventional multiplex PCR DNA detection limit varied among species ranging from 20-200 fg/µL, which represents 5-40 bacterial cells. The RT-PCR sensitivity varied among species ranging from 10-1,000 fg/µL, which represents 2-200 bacterial cells. Both techniques showed high sensitivity and specificity. Our next goal is to apply this system in the field and evaluate their performances using serum, blood, urine, milk and tissue samples. Our results show an advance in the molecular diagnostic tools to detect *Brucella* and represent a promising tool to use for the control of brucellosis.

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P68: Real-time PCR for diagnosis of brucellosis in water buffalo (*Bubalus bubalis*) of Bangladesh

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The water buffalo (*Bubalus bubalis*) is a large bovine animal, frequently used as livestock in southern Asia. Asia is the native home of the water buffalo, with 95 % of the world population, with about half of the total in India and Bangladesh. It is valuable for its meat and milk, as well as the labor it performs. It is often referred to as 'the living tractor of the East', as it is relied upon for plowing and transportation in many parts of Asia including Bangladesh. Brucellosis, a zoonosis of worldwide importance, is caused by Gram-negative bacteria of the genus *Brucella* (*B.*). Brucellosis in domestic water buffalo is generally caused by infection with *B. abortus*. Clinical signs in buffaloes include abortion, decreased fertility and milk production, and testicular degeneration in the bull, as a result of epididymitis - orchitis; because of this, it is considered one of the most damaging diseases to livestock and disturbing rates of productivity in buffalo herds, since prevention and control involves a high economic impact. Blood samples from 99 adult buffaloes were collected from Bagerhat and Mymensingh district of Bangladesh and then sera were and sent to OIE Reference Laboratory for Brucellosis (Germany). Rose Bengal test (RBT), serum agglutination test (SAT), complement fixation test (CFT) and the IDEXX Brucellosis Serum X2 Ab test were performed according to the procedures described by the manufacturers. The RBT-positive sera were re-tested with SAT, CFT, ELISA, and real-time PCR (qRT-PCR). For the qRT-PCR, DNA was isolated from 200 µL of seropositive serum using the High Pure PCR Template Preparation Kit according to the manufacturer's instructions. *Brucella* IS711 targeting genus specific qRT-PCR was done according to the established and routine protocol on a light cycler 2.0 instrument. Positive samples were then typed with the *Brucella* IS711 species specific qRT-PCRs for *B. abortus* and *B. melitensis.* 5 / 3 / 0 / 3 and 2 / 1 / 0 / 1 buffaloes from Bagerhat and Mymensingh district of Bangladesh reacted positive by means of RBT, SAT, CFT and ELISA, respectively. The genus specific screening by PCR detected *Brucella* DNA in seven sera, the species specific IS711 PCR revealed absence of *B. abortus* DNA. *Brucella* DNA was found in 7.1 % of the buffaloes investigated. It is the first time that we used real-time PCR for diagnosis of brucellosis in water buffalo (*Bubalus bubalis*) of Bangladesh.

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P69: Identification and differentiation of Greek *Brucella melitensis* and *B. abortus* isolates from solid tissues by a real-time PCR method

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As a part of an EU EMIDA ERA-NET project entitled '*Brucella melitensis*: biotyping and differential diagnostic- Brucmel' we established a procedure to isolate and identify directly *Brucella* (*B.) melitensis* and *B. abortus* strains from solid tissues. Identification procedures of *Brucella* sp. are based on microbiological or/and serological methods. The main disadvantage of the serological tests is the cross-reactivity with other related bacteria, e.g. *E. coli* and *Y. enterocolitica*, while the bacteriological tests are time-consuming and of high risk to people handling the infected tissues e.g. lymph nodes. To surmount these problems, nucleic acid amplification has been explored for the rapid detection and confirmation of *Brucella* but only from cultures and not from solid tissues.
Tissues samples (lymph nodes and spleen) for the laboratory examination were cut extensively and homogenized for 2 min with the addition of 1 ml distilled water or PBS. The homogenization was performed by a handle cordless grinder (NIPPON Genetics). 200 µl from the homogenized tissue were used for DNA extraction using the PureLink Genomic DNA Mini Kit (Life Sciences). In addition, the validity of the method was assessed using a ring test organized by IZS of Teramo. A panel of 30 spleen samples collected from cattle (n=10), sheep (n=10) and swine (n=10), were contaminated with a known number of *B. abortus, B. melitensis* and *B. suis* bacteria, respectively. The panel also included non-infected spleen tissue samples as negative controls.
In parallel, all homogenates were cultured in blood agar base (Oxoid) supplemented with 5 % equine serum and glucose (40 ml 25 % concentration per l). Moreover, the Farrell's medium was used, which is prepared by the addition of six antibiotics to a basal medium.
Real-time PCR was performed on genomic DNA. Target genes used for the identification of *Brucella* spp., *B melitensis* and *B abortus* were the multiple insertion element IS711, the BMEII0466 and BruAb2_0168 gene, respectively. Two duplex qPCR were performed, one for the identification of *Brucella spp.* and *B. melitensis* and a second one for the identification *Brucella* spp. and *B. abortus*. The procedure has proven to be highly specific giving better results than the microbiological tests. To test the specificity of the reaction, DNA extracts were tested from 30 tissues either from sheep or from cattle. No amplification was observed. To our knowledge for the first time, strains of *B. melitensis* and *B. abortus* were directly identified and differentiated from solid tissues by a real-time PCR method.

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P70: Novel identification of *B. abortus* strains using conventional and real-time PCR assays

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Brucella (*B.*) is a Gram-negative, intracellular and non-motile bacterium causing brucellosis, one of the most important zoonotic diseases and of animal and public health concern. Achieving fast and sensitive assays for detection of brucellosis play a key role in prevention, control and eradication programs. This project was aiming to present novel conventional and real-time PCR assays for identification of *B. abortus*. In this study, two primers were designed based on the unique locus encoding the autotransporter associated β-strand repeat containing protein ID:YP00113760. Twenty six *Brucella* strains (reference and vaccine strains, field isolates) and one *Yersinia enterocolitica* strain, representing a non-*Brucella* isolate, were used for evaluation of the conventional and real-time PCR systems. Results showed that 0.4 ng and 400 fg of genomic DNA of *B. abortus* strains can be detected by conventional and real-time PCR, respectively. The real-time PCR assay was 1,000-fold more sensitive than conventional PCR method. Furthermore, the data indicate that both methods were specific. The newly generated primers are suitable for both PCR methods. So, this study presents a novel technique for the identification of *B. abortus* strains.

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P71: MLVA-16 in *Brucella* spp.: Data interpretation considerations and limitations for epidemiological investigations

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Identifying genetic variants is important for the tracing of brucellosis outbreaks and is essential for surveillance programs in both human and animal health. The current optimal tool for *Brucella* (*B.*) strains epidemiological genotyping is the MLVA-16 assay (Multiple-Locus-Variable-number-tandem-repeat-Analysis), which involves 16 genetic markers, organized in panels, according to their evolution speed: panel#1 (eight mini-satellites), more stable than panel#2 (three micro-satellites for panel#2A and five micro-satellites for panel#2B).
For other pathogens it is known that closely related isolates could harbour some small VNTR pattern variations due to random genetic events. These events may impact MLVA data interpretation for epidemiological investigations. But, what is about *Brucella*?
This study aimed to investigate the genetic stability of MLVA-16 markers at species and biovar levels, and its consequences for *Brucella* MLVA-data interpretation. The Hunter-Gaston diversity index (HGDI) was analyzed from extensive MLVA data for the most relevant species/biovars (bv), i.e. > 500 in-house and published *B. melitensis* bv 3 (Bmel3) isolates. In parallel, the locus stability was investigated by comparing 76 animal and human Bmel3 patterns, clustered in 25 epidemiologically-related groups (2-9 members, 1-4 host species) (same outbreak, same contamination source, laboratory-acquired infection...).
HGDI values for each MLVA-16 genetic marker confirmed a polymorphism degree which is not only species-, but also biovar-dependent. Furthermore, 32 % of epidemiological groups showed at least one Single-Locus Variation (SLV), including 8 % with Double-Loci Variations and 12 % with multiple-loci variations (four loci), without apparent correlation with the host-range.
Most differences between closely related strains were attributable to variations within hypervariable panel#2B (28 %). Around 20% groups, including laboratory technician/patient clusters, harboured variations in panel#2A (1-2 U), but only one (6.7 %) harboured a SLV (1 U) in panel#1 (Bruce42), which is consistent with HGDI values. In addition, two distinct alleles (Bruce16) were reported in one same patient, reflecting a host adaptation.
Although MLVA-16 is adequate for epidemiological trace-back investigations, some pattern variations do exist between closely related *Brucella* strains which may alter data interpretation. Our results highlight the need of *Brucella*-specific guidelines for MLVA data interpretation.

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P72: Human brucellosis in Maghreb: Existence of a pre-independence lineage related to socio-historical connections between Algeria and France

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Despite control and eradication programs, brucellosis, a major worldwide zoonosis due to the *Brucella* genus, is endemic in Northern African countries and remains a major public health problem in the Maghreb region (Algeria, Morocco and Tunisia). *Brucella* (*B.*) *melitensis* biovar (bv) 3 is mostly involved in human infections. Although human brucellosis is a notifiable disease in these countries, its occurrence in the Maghreb seems still underestimated and its epidemiological situation remains hazy.
In France, no case has been reported in ruminants since 2003, apart from one recent isolated bovine outbreak resulting from wildlife contamination (*B. melitensis* bv 3 reservoir in Haute-Savoie).
In such non-endemic countries, where the disease has been effectively controlled for many years, human cases are mainly imported from endemic areas with high disease prevalence in animals.
Algeria and France are closely linked for political and historical reasons as Algeria was a French territory until 1962. These historical connections raise the question of their possible impact on the genetic variability of *Brucella* strains circulating in the Maghreb.
The purpose of this study was to assess the genetic diversity among Maghrebi *B. melitensis* bv 3 strains, and to investigate their possible epidemiological relationship with European strains, especially with French strains.
A total of 95 *B. melitensis* bv 3 Maghrebi (Algeria, Morocco and Tunisia) strains isolated over a 25 year-period (1989-2014), mainly from humans, were analysed by MLVA-16. The obtained results were compared with genotypes of French *B. melitensis* bv 3 strains (n=36), isolated from autochthonous human cases and animal outbreaks, from 1989 to 2001, and with genotypes of European *B. melitensis* bv 3 strains (n=130) (Spain, Italy, France, Portugal, Switzerland, etc.).
All Maghrebi strains belonged to the 'West Mediterranean' group. Molecular assays showed that Algerian strains were distributed into two distinct clusters, while Morocco and Tunisia strains constituted distinct and independent groups. Interestingly, one Algerian cluster was related to the Tunisian group, while the other Algerian cluster was closer to the Moroccan cluster, suggesting geographical patterns, probably related to socioeconomic links between Tunisia/Algeria and Morocco/Algeria (e.g. bilateral trade, breeding systems…). In addition, the Algerian/Tunisian cluster was related to the French sub-cluster. These results led to assume the existence of a lineage resulting from politico-historical connections between Algeria and France that evolved distinctly from the Algerian autochthonous group.
This study provides appropriate insights regarding the epidemiological situation of brucellosis in the Maghreb and is the first molecular investigation regarding *B. melitensis* bv 3 strains circulating in the Maghreb.

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P73: Multilocus variable number of tandem repeats analysis (MLVA) as an epidemiological tool to trace-back the re-emergence of *Brucella melitensis* biovar 1 in Italy

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Brucellosis is an important zoonosis caused by *Brucella* spp. Although the disease has been eradicated from Northern Europe, Australia, USA and Canada, it remains still endemic in most areas of the world. Control of brucellosis in animals is the key to protect humans, and the knowledge of the prevailing genotypes of *Brucella* spp. in a country represents an important epidemiological tool to formulate policies and strategies for the control of the disease in animal populations, and to identify the introduction of new strains previously considered as exotic. In recent years, multiple-locus variable number tandem repeat analysis (MLVA) has been proposed as complement of the classical biotyping methods, and it has been applied for genotyping large collections of *Brucella* spp. worldwide. MLVA may add important information to the classical epidemiological investigation and it has shown promising results in tracing-back sources of infection in brucellosis outbreaks in Italy. Sardinia is an Italian region officially-free from sheep and goats brucellosis since 1998, having a regional surveillance plan implemented to prevent the reintroduction of the disease. In 2011, serologically positive animals were found in two flocks that had recently introduced animals from abroad (Spain). In one flock, *B. melitensis* biovar 1, a biotype never more reported in Italy after 1995, was isolated. The genotyping MLVA-16 showed that isolates were belonging to a rare American lineage, confirming that it was introduced from other countries. The strain was considered as probably originating from Spain, where this lineage is endemic. This finding confirmed the results obtained by classical epidemiological investigation. *Brucella* MLVA-16 has been useful to analyze the epidemiological correlation of strains allowing to trace back its geographic origin by comparing their previously reported genetic patterns.

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P74: Multiple-Locus-Variable-number tandem repeat Analysis (MLVA) of *Brucella melitensis* strains isolated in Italy, Greece and Israel

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Since brucellosis was first discovered on Malta in the middle of the Mediterranean Sea in 1884, this zoonosis has still a huge burden in the area after two centuries. The high density of livestock farms with extensive farming system, paired, at least in the past, with an intensive trade of animals and animal products have permitted a free circulation of the *Brucella* over the borders. This study in the framework of EMIDA-ERANET project '*Brucella melitensis*: biotyping and differential diagnostic' aimed to study *Brucella* (*B.*) *melitensis* to discover common and discordant genetic lineages among Israel, Greece and Italy. We analyzed *Brucella* genetic diversity using MLVA using the 16 panel as previously published. For the study, *B. melitensis* field strains isolated in Italy (n=30), Greece (n=19), and Israel (n=2) were analysed. The MLVA-16 data were compared to genotypes from MLVA data available in the database at the website (http://mlva.u-psud.fr/brucella/), and Neighbor Joining cluster analysis was performed.

The MLVA-16 phylogenetic cluster analysis revealed that in Greece and Israel the East Mediterranean lineage is widely distributed. This clade seems well established because seven different MLVA-16 genotypes for 20 isolates were observed. These genetic clones are micro variants because the mutations occurred for the hyper variable minisatellite loci. On the other hand, the Italian strains showed the presence of several different MLVA-16 genotypes, belonging to the West Mediterranean lineage, suggesting that this lineage is dominant and that it may have been historically present in Italy. Furthermore, we revealed specific clades and their associated genotypes were often regionally localized. For example, Sicily had a multitude of genetic clones with specific clusters dissimilar from the other Italian strains.

Molecular characterization of *B. melitensis* is a useful tool to explore the genotypic diversity in a region. These fine-scale data will permit investigations into the spread of the disease and how the pathogen is dispersed among the countries. The final picture is showing that in two geographically distinct areas two different genetic clades of *B. melitensis* are present. All these isolates are part of two strain complex, which have succeeded in becoming ecologically established within the livestock. Geographically, the East Mediterranean lineage is spread over the eastern regions while the West Mediterranean lineage is present in the central area and northern Africa. It should be noted that Italy and Greece with their central position in the Mediterranean region do not have a mixtures of lineages, even though phenomena of introduction and reintroduction of the disease might have occurred in the past. The additional analyses of samples from a broader range of countries are needed to confirm this finding. Our results provide data for the European surveillance and increase information about brucellosis in Europe. This genetic characterization permits to draw the proper risk maps for brucellosis in the Mediterranean area.

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P75: Molecular characterization of *Brucella melitensis* isolates from brucellosis outbreaks in Israel

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Brucellosis, caused by *Brucella melitensis*, is a major zoonotic disease endemic in Israel. Over the past years, this pathogen has been isolated from sheep, goats, cattle, camels and humans. Recent molecular techniques, such as Multiple-Locus Variable-number tandem repeat Analysis (MLVA-16) have been indicated as valuable methods in deciphering the source of infection. We aimed to implement this technique to resolve epidemiological linkages between strains isolated from humans and the suspected animal source, as well as between different outbreaks in the country. We analyzed isolates from seven brucellosis outbreaks involving various animal and human sources. Thirteen out of the 16 analyzed loci were identical in all isolates. The remaining three loci (bruce 7, 16, 30) were variable, even within the same outbreak. Furthermore, no differences in the variable-number tandem repeat profile were found between different hosts. Our results indicate that the MLVA-16 method is not a sufficiently strong tool in resolving the source of brucellosis outbreaks in multiple infection areas such as Israel.

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P76: Use of genotyping to discern subtypes of *Brucella* species circulating in humans and livestock in the South Caucasus and Central Asia

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Brucellosis may be the most important zoonosis worldwide, a widespread bacterial disease of livestock and wildlife with high infectivity in humans with significant disease burden in many countries with large rural populations and limited control programs. Collaborative efforts were undertaken in three countries of the Former Soviet Union where brucellosis is a significant endemic problem, Kazakhstan, Georgia and Azerbaijan, to initiate parallel active surveillance in livestock and human patients using improved diagnostic tools, genetic subtyping for molecular epidemiology and Geographic Information Systems (GIS). In Kazakhstan and Georgia genetic subtyping by Multilocus Variable Number Tandem Repeat Analysis (MLVA) was used to characterize *Brucella* isolates with origin geolocated and analyzed using GIS. A streamlined MLVA method using eight markers from a 15-marker system allowed for the subtyping and comparison of a larger number of strains in Kazakhstan. This 8-marker approach was compared with the full 15-marker system. The combination of MLVA and GIS in a southern region of Kazakhstan indicated broader genetic diversity in strains isolated from livestock, with a limited genetic diversity apparently transmitted to humans. A genetic cluster was only observed in human isolates, indicating a potential need for improved animal brucellosis surveillance. MLVA-15 indicated that distinct genetic groups of *Brucella melitensis* are circulating in Georgia, Azerbaijan and Kazakhstan. MLVA results combined with GIS provide insights into brucellosis transmission patterns in Kazakhstan and Georgia and point to potential areas of focus for the improvement of national surveillance programs.

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P77: Investigation of *Brucella* spp. isolates of humans and livestock in Mongolia with Multi Locus Variable number tandem repeat Analysis (MLVA-16)

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Brucellosis is one of the most common zoonotic diseases worldwide with around half a million human cases reported annually. In Mongolia, human brucellosis has occurred in the 1960s. Since 1975, a mass vaccination strategy of small ruminants and cattle successfully reduced annual incidence in humans to less than one case per 10,000. Human brucellosis re-emerged since 1990 due to the break-down of medical and veterinary services and the lack of resources during the post-communist transition period. The country was ranked the second highest in terms of human brucellosis cases worldwide in 2003. It is therefore surprising that despite the high prevalence of brucellosis, there has been almost no genetic characterization of brucellosis in the country. In this study, we characterized 58 isolates of *Brucella* (*B.*) *melitensis* and *B. abortus* from humans and livestock from five provinces from Mongolia using MLVA-16. The 58 Mongolian strains are genetically diverse when compared with bordering Chinese and Kyrgyz strains. Human strains are most closely associated to *B. melitensis* strains from sheep and goat. To the best of our knowledge, this is the first report on MLVA-16 characterizing *Brucella* strains from Mongolia. MLVA-16 has a high potential to improve brucellosis surveillance and traces back outbreaks during the national control program.

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P78: Identification of specific sequences for *Brucella abortus* and *B. melitensis* based on in silico analysis of chromosome 2

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Brucella (*B.*) is a Gram-negative and intracellular bacterium causing brucellosis. Six classical species have been reported in genus *Brucella*. Recently, several new species have been allocated to the genus. Two of them were isolated from marine mammals. In previous studies, several PCR based methods for *Brucella*-typing were developed. The multiplex PCR-based method 'Bruce-ladder' is a comprehensive assay to differentiate *Brucella* species. In this study, we identified new primers based on the newly deposited *Brucella* genomes in GenBank (National Center for Biotechnology Information, NCBI). For this purpose we performed an in silico comparative analysis of 21 full genomes and 279 contigs in draft genomes of *Brucella*. We found four and ten new specific sequences for the detection of *B. abortus* and *B. melitensis* on chromosome 2, respectively. We suggest new primers for high efficient detection and differentiation of *Brucella* species.

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P79: Phage-host interaction peculiarities in *Brucella* bacteriophages

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Phage typing as an additional diagnostic tool to detect and identify *Brucella* (*B.*) spp. is widely used in many countries. Several phages are available for typing purposes, although detailed information on biology of these bacterial viruses is limited. Ten bacteriophages, including the internationally approved Tb phage from the Eliava Institute collection, have been selected for typing purposes. All the phages have similar morphology; they are temperate and reveal a slow growth cycle on the host cells. Phages are grown on two distinct hosts (*B. abortus* S19 and *B. abortus* 141) and show different lytic activity against various strains of *Brucella*, particularly *B. abortus* and *B. neotomae*. Efficiency of plating of these phages on corresponding hosts differs as well, and, in some cases, lysis without phage infection occurs. Genome sequence analyses of ten phages grown on two different host bacteria show several mutations in similar genes (at identical sites) across the phages. Genes containing the greatest number of shared and unique polymorphisms included putative genes encoding the HNH endonuclease, phage portal protein, and the tail collar protein, among others. Few phages show high variation across propagation on two distinct hosts, while most of them exhibit strong genetic stability. Genomic substitutes, including single nucleotide polymorphisms (SNPs), occur mainly in the tail collar protein gene. Based on the results, the polymorphisms in the relatively homogenous genomes of *Brucella* phages suggest that the phage collar protein genes may represent specific sites for host adaptive selection.

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P80: 1st bovine brucellosis interlaboratory proficiency test in Asia-Pacific in 2013

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FAO-RAP, the Animal Production and Health Commission for Asia and the Pacific (APHCA) and OIE-RRAP jointly organized the 'FAO-APHCA/OIE Regional Workshops on Brucellosis Diagnosis and Control – with an emphasis on *Brucella melitensis*' in the Asia and Pacific Region in collaboration with the Department of Livestock Development (DLD) of Thailand from 2008 to 2010 (three workshops). It was agreed upon that there was a need to establish a regional laboratory network. This important issue has been well considered through the framework of the OIE Twinning Programme on Brucellosis between the EU/OIE/FAO Reference Laboratory for Brucellosis in France and the Department of Livestock Development – National Institute of Animal Health (DLD-NIAH) of Thailand.
The first '2013 Asia-Pacific Bovine Brucellosis Interlaboratory Proficiency Test' was prepared (discussion and preparation of panels of 17 sera after selection of the sera, homogeneity and stability testing) from January to July 2013 under the ANSES/NIAH OIE Twinning Programme, together with FAO-APHCA and OIE-RRAP.
Announcement and information were delivered during the 37th Session of APHCA in September 2013. Expected/accepted results were defined according to ANSES-NIAH preliminary testing and taking into account the results of participants as well.
Qualitative, semi-quantitative and quantitative results, sensitivity, specificity and repeatability, as well as coherence between dilutions of the same serum were analysed for the assessment of the performance of each participating laboratory.
Thirty-three laboratories participated in this ILPT, including laboratories from 17 Asia-Pacific countries, eight DLD's Regional Veterinary Research and Development Centres, one government enterprise, four universities and two private companies in Thailand and ANSES. The shipment of sera to laboratories was made via several means and routes, but had apparently no effect on sample quality. Most of the results were returned in due time (84 %). Error of sample identification occurred for 0.36 % samples. Thirty-two participants performed the Rose Bengal test (RBT), 15 the complement fixation test (CFT) and nine I-ELISA. The same antigens were used in RBT whereas five different antigens/kits were used in CFT and I-ELISA. Overall results were very good for a first proficiency test.
All the results were presented and discussed during 'The 4th FAO-APHCA/OIE/DLD Regional Workshop on Brucellosis Diagnosis and Control in Asia-Pacific Region – Proficiency Test and Ways Forward', organized in Chiang Mai, Thailand, on 19-21 March 2014.
Despite the overall good results obtained, there is a real need of follow-up and/or refreshing training for several Asia-Pacific laboratories and the improvement expected in the test performance should be evaluated in a next ILPT.

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P81: First *Brucella* bacteriology and molecular biology interlaboratory-proficiency trial organized in the EU

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One of the main tasks of the brucellosis EURL specified in its 2012 work programme was to assess the identification and typing of *Brucella* (*B.*) strains in the EU, according to the corresponding EURL SOP. An interlaboratory proficiency ring-trial (ILPT) was organized between November 2012 and January 2013 in order to assess the reliability of methods used to identify and to type *Brucella* strains throughout the EU. This was the first ring-trial organised in the whole EU as regards direct diagnosis of brucellosis and it covered conventional bacteriology as well as molecular identification and typing methods. This trial first aimed at evaluating the capacity of EU NRLs in identifying *Brucella* strains, but it was also the opportunity to have an overview of all the methods used and to discuss their respective interest. Twenty-two EU NRLs participated to this ring trial. A panel of eight suspected *Brucella* strains was sent to each participant. The strains were selected in order to allow the evaluation of the NRLs capacity in identifying strains at *Brucella* genus level, at species level (different species of *Brucella* that are regulated within the EU for surveillance or for animal trade purpose: *B. melitensis*, *B. abortus*, *B. suis*, *B. ovis*) and at biovar level (different biovars that could be found usually in Europe). Most of the chosen *Brucella* (5/8) had typical characteristics, two strains were atypical and one strain was not a *Brucella*. NRLs were asked to confirm the *Brucella* genus or not, and to type them as precisely as possible with the techniques usually performed in the lab in their usual working conditions. At least one of following methods could be used:
-> Routine typing (bacteriology) according to EU-SOP, including cultural, morphological, biochemical, serological and phage-typing characteristics;
-> Nucleic acid methods such as PCR, real-time PCR, PCR-RFLP (PCR-Restriction Fragment Length Polymorphism), MLVA (Multiple-Locus Variable-number tandem repeat Analysis), etc.
Various methodologies and protocols were used by the participants during this ILPT. Most participating NRLs gave a very satisfactory identification, generally combining several methods. Phenotypic typing was mostly partially performed whereas molecular methods, especially genus-PCR and species-PCR were very well implemented. The EURL outlined that the combination of several methods should be chosen carefully to ensure the reliability of identification and to be consistent with routine lab work. Moreover, officially recommended methods - or at least sufficiently documented and validated methods - should be preferred. The typical strains sent were correctly identified, but various levels of identification were reached among the participants. Few laboratories faced growth problems and almost all laboratories except one succeeded in identifying the genus. Some laboratories stopped at genus- (n=3) or species- (n=3) level. Species and biovar were generally correctly identified through molecular methods. In phenotypic typing as well as in molecular typing, the investigation of problems faced oriented towards the need to check the correct implementation if the tests, since the use of relevant controls, the standardization of reagents and the respect of the protocol were the major critical points highlighted. The identification of atypical strains could be improved by completing phenotypic typing and by improving molecular data interpretation. Their identification requires a good knowledge of *Brucella* identification. Each laboratory received a general and an individual report with detailed results and EURL recommendations concerning the reliability of the strain identification (genus-species) and the relevance of the methodology used for *Brucella* identification and typing.

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P82: Brucellosis OIE laboratory twinning programme France/Thailand (2010-2013)

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The OIE laboratory twinning projects aim at improving global capacity for disease prevention, detection, and control through better veterinary governance. The discussion and preparation of the twinning project on brucellosis started during the 1st and 2nd FAO-APHCA / OIE Regional Workshops on Brucellosis Diagnosis and Control in 2008 and 2009. The objectives of the NIAH-ANSES twinning project were endorsed by the directors of both laboratories and respective national credentials verification organizations (CVOs). Main objectives were to strengthen the level of expertise in brucellosis (serological diagnosis/screening and bacteriological isolation of *Brucella* by culture) and implementing reference activities (*i*) control and standardisation of diagnostic reagents and vaccines, (*ii*) organization of regional serology proficiency ring-trials and (*iii*) *Brucella*-typing and molecular detection and identification. The work programme included three annual training sessions of NIAH staff at ANSES and three visits of ANSES experts to NIAH. The project was approved in June 2010 for a 3-year duration (2010-2013). The programme was fully successful in strengthening brucellosis diagnosis and implementing reference activities including three training sessions and three visits of ANSES experts. The main results were:

- a regional secondary standard bovine serum established in NIAH;
- the implementation of quality control of locally-produced RBT antigens by NIAH;
- the joint organisation (ANSES and DLD-NIAH) of the first '2013 Asia-Pacific Bovine Brucellosis Proficiency Test', the results of which were presented and discussed in 'The 4th FAO-APHCA/OIE/DLD Regional Workshop on Brucellosis Diagnosis and Control in Asia-Pacific Region – Proficiency Test and Ways Forward', Chiang-Mai, Thailand, March 2014;
- the participation of NIAH in the EU/OIE interlaboratory proficiency tests 2012 and 2014;
- the provision of NIAH with all the *Brucella* reference strains, brucellaphages and monospecific sera needed for a rapid implementation of *Brucella* biotyping in standard conditions;
- the validation by ANSES of the identification of *Brucella* strains stored in the NIAH *Brucella* collection;
- ANSES assistance in completion of the quality assurance system (ISO/IEC 17025:2005) in place in NIAH;
- ANSES support in designing research activities and a project of PhD thesis.

All activities in the programme were achieved and completed on December 31, 2013. The achievement of all project objectives is expected to strengthen the Asia Pacific regional impact of the NIAH brucellosis laboratory and to ensure the capability for the already ongoing implementation of those regional activities that are expected from an OIE Reference Laboratory. The benefit of this programme will be a guide for stimulating and enhancing an active network of regional diagnostic expertise for the surveillance and control of brucellosis for the benefit of the Regional Animal and Public Health.

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P83: Interlaboratory proficiency for quantitative and qualitative bovine brucellosis serological tests in Latin America in 2013

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All laboratories should participate in external proficiency tests according to their competence profile. Valid and confident laboratory results are essential for diagnosis. Proficiency testing provides an independent assessment of the testing methods used and the level of staff competence. Participation in proficiency testing enables the laboratory to assess and demonstrate the reliability of results by comparison with the expected results from other participating laboratories. Participation in such proficiency testing is a requirement for accredited laboratories.
The OIE/FAO Reference Laboratory Brucellosis of National Health Service and Agri-Food Quality (SENASA) of Argentine, organized for the third consecutive year an interlaboratory ring trial to ensure the validity of results of the laboratories conducting brucellosis diagnosis in Latin America. Nineteen Veterinary Services Laboratories of ten countries participated in this trial.
A set of 20 blinded field bovine serum samples (strong positive, weak positive and negative by replicate) was sent to each participant together with a set of instructions and necessary background information. The following tests were carried out by the laboratories: buffered plate antigen test (BPAT) by 5/19 laboratories, four different antigen were used, Rose Bengal test (RBT) by 16/19, seven different antigen were used, complement fixation test (CFT) by 3/19, with two different antigens, serum agglutination test (SAT) by 4/19, with 3 different antigens, Competitive ELISA (C-ELISA) by 9/19, all laboratories used the same commercial kit, Indirect I-ELISA (I-ELISA) by 5/19, three different kits were used and, fluorescence polarization assay (FPA) by 7/19 laboratories, two different commercial kits were used.
The results demonstrate very good performance in all laboratories participating in this trial. By participating in proficiency testing with the aim of continuous improvement, laboratory staff will gain satisfaction in the knowledge that they have improved or maintained their level of competence which is comparable to that of their colleagues. They can detect any difficulties they may have with analyses, can identify training needs and have a tool to meet their needs.
It is very important for those countries which want to develop campaigns for control and eradication of brucellosis to have laboratories which can guarantee reliable diagnosis.

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P84: Development of brucellosis laboratory diagnostics at the Laboratory of Ministry of Agriculture of Georgia (LMA) 2009-2014

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Brucellosis is a highly contagious zoonotic disease, caused by different species of *Brucella* (*B.*). It affects domestic and wild animals, as well as humans and is a widespread disease in Georgia, especially in regions with large animal populations. Therefore, usage of appropriate diagnostic methods is very important for the improvement of animal health as well as for the timely intervention and disease prevention in humans. Since 2005, the Laboratory of the Ministry of Agriculture (LMA) has started a program for the validation and implementation of new methods to detect and identify *Brucella*, including ELISA and PCR. These methods make it now possible to perform confirmatory testing in a timely manner in compliance with OIE requirements. Among others, *Brucella* culture isolation methods have been studied at the LMA to optimize the testing algorithm to identify and specify suspect Georgian strains. By means of AMOS PCR, only four common *Brucella* species/biovars can be identified, but *B. abortus* biovars 3, 5, 6, 7, and 9 which are very common and circulating in cattle population in Georgia cannot be detected with this method. In 2012, a new testing method, the fluorescent polarization assay (FPA), was validated and implemented, which allows confirmation from a single positive sample. FPA can replace ELISA and makes confirmatory testing more cost-effective when the laboratories have to deal with a relatively small number of positive samples. Validation studies for the FPA assay included 980 samples, 575 (33 confirmed positive) samples from cattle and 405 (13 confirmed positive) samples from small ruminants. In addition, the laboratory is currently implementing and validating the Bruce-ladder multiplex PCR which allows the identification of *Brucella* species and can distinguish between field and vaccination strains.

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Session 7: Human and Pet Brucellosis

Keynote Lecture: Assimoula Economopoulou
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Human Brucellosis

Brucellosis is a zoonosis existing worldwide. The disease is caused by bacterial species of the genus *Brucella*.
There are four terrestrial *Brucella* (*B.*) species known to cause human disease, and each of these has a specific animal reservoir: *B. melitensis* in goats, sheep and camels; *B. abortus* in cattle; *B. suis* in pigs; *B. canis* in dogs. *B. delphini, B. pinnipedialis and B. cetaceae,* are associated with marine animals, and have been reported to cause disease in humans. Of the seven species known to cause disease in humans, *B. melitensis* is the most virulent and having the largest public health impact.
Brucellae are Gram-negative coccobacilli, unencapsulated, nonsporulating. They grow aerobically, although they may require supplemental CO_2, especially for primo isolation. The species involved in human pathology are oxidase positive, catalase positive, and urease positive. To reach a diagnosis, a wide range of non-specific routine haematological and biochemical tests in addition to *Brucella* specific assays must be used. The latter are microbiological (culture), serological such as slide or tube agglutination, Coombs test, immunocapture agglutination, *Brucella*capt, immunochromatographic lateral flow, enzyme-linked immunosorbent assays, the indirect fluorescent antibody test, molecular polymerase chain reaction (PCR) and real-time PCR.
The incubation period of the disease varies from several days to six months, but is generally one to four weeks. The disease onset can be latent or acute. The symptoms of brucellosis are not specific; therefore, the clinician should conduct a thorough history looking for animal or food-borne exposures when attempting to explain non-specific symptoms suggesting brucellosis. Back pain, fever, fatigue, sweats, anorexia and headache are the most common presenting symptoms, while the most frequent clinical findings are splenomegaly, hepatomegaly, lymphadenopathy, and osteoarticular manifestations.
An undulant fever pattern is apparent in patients who are untreated for long periods of time, leading to the name undulant fever for brucellosis. Some patients report malodorous sweat. Depression is common and often out of proportion to the severity of other symptoms.
The treatment of brucellosis, recommended by World Health Organization (WHO), consists of a combination of oral doxycycline plus rifampin for the duration of six weeks. Alternatively, six weeks of doxycycline plus 21 days of streptomycin or gentamicin are recommended by WHO. Many studies have evaluated the use of fluoroquinolones or macrolides without demonstrating an *in vivo* superiority of these newer antibiotics.
The recurrence of symptoms after therapy may or may not be associated with relapse of the disease. Bacteriologic relapse generally occurs within three to six months after discontinuing therapy and is usually not caused by antibiotic resistance.
Brucellosis is especially prevalent in the Mediterranean basin, in the Arabian Peninsula, the Indian subcontinent, and in parts of Central Asia, Africa, Mexico, and Central and South America.
Humans can be infected by direct contact with infected animals or their secretions, through existing cuts or abrasions in the skin or conjunctival sac; by inhalation of contaminated aerosols; by ingestion of unpasteurized dairy products. Consequently, brucellosis is an occupational risk for farmers, veterinarians, abattoir workers, and laboratory personnel. Meat products are rarely the source of infection because they are not usually consumed raw and the numbers of organisms in muscle tissue are low. In areas, in which animal blood traditionally is drunk or raw liver is eaten, a foodborne infection unrelated to consumption of dairy products is possible.

The person-to-person transmission of brucellosis is unusual; however, rare cases in which sexual transmission was suspected have been reported and also of vertical transmission through breast feeding. Although persons with HIV are at risk of being infected with a number of zoonotic agents, very few cases of brucellosis are reported. It is not uncommon to find more than one case of brucellosis in a household; therefore, screening of contacts of index cases is warranted.

The surveillance of brucellosis is mandatory in the European Union (EU) and European Economic Area (EEA) countries.

In 2012, 26 of the 30 EU/EEA countries provided information on brucellosis in humans. Fifteen EU/EEA countries, (Austria, Belgium, Bulgaria, Finland, France, Germany, Greece, Ireland, Italy, the Netherlands, Portugal, Slovakia, Spain, Sweden, and the United Kingdom) reported human cases. In total, 359 cases of human brucellosis, of which 328 confirmed, were reported. The overall notification rate for confirmed cases was 0.07 cases per 100 000 population representing a 2.4 % decrease in confirmed cases compared with 2011 (Table 1). Since 2006, the reported human cases of brucellosis present a decreasing trend in EU/EEA countries (17).

Six of the 26 EU/EEA countries provided data on hospitalisation; on average, 78.0 % of the confirmed brucellosis cases were hospitalised, but hospitalisation status was provided for only 51.2 % of the confirmed cases in the EU.

Among the 26 EU/EEA countries reporting on brucellosis, seven provided information regarding the outcome; one death was reported in Portugal in 2012.

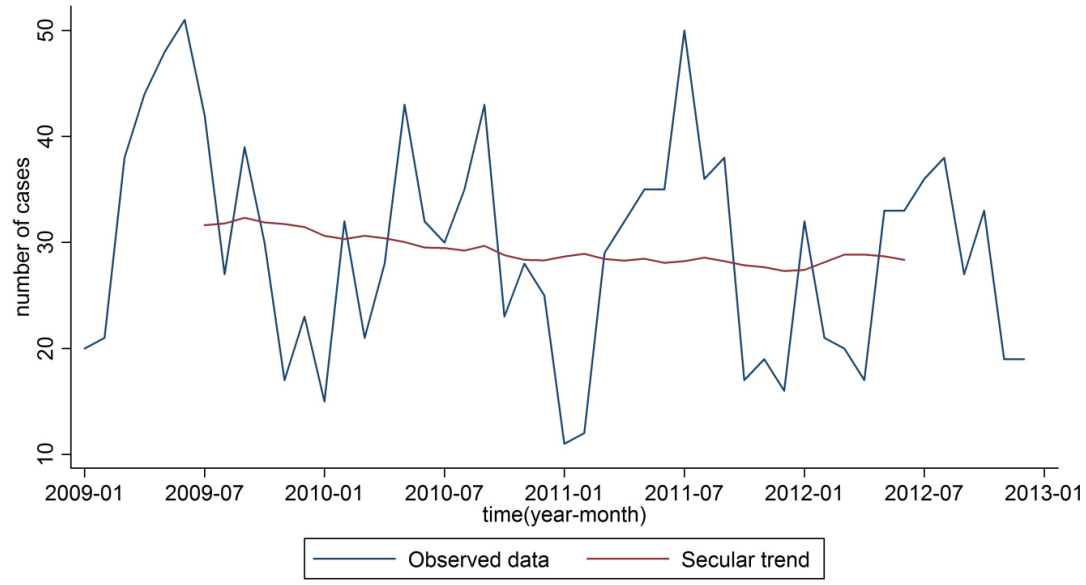
Information regarding *Brucella* species was provided for 99 of the 328 confirmed cases, reported in the EU and Norway. Of these, *B. melitensis* represented 83.8 % of the reported cases, *B. abortus* 10.1 % and *B. suis* 3.0 %.

The decreasing trend in reported cases of human brucellosis in Europe between 2006 and 2012 could be explained by a substantial decrease of infection in domestic small ruminants, which are the main reservoir for *B. melitensis* and *B. abortus*.

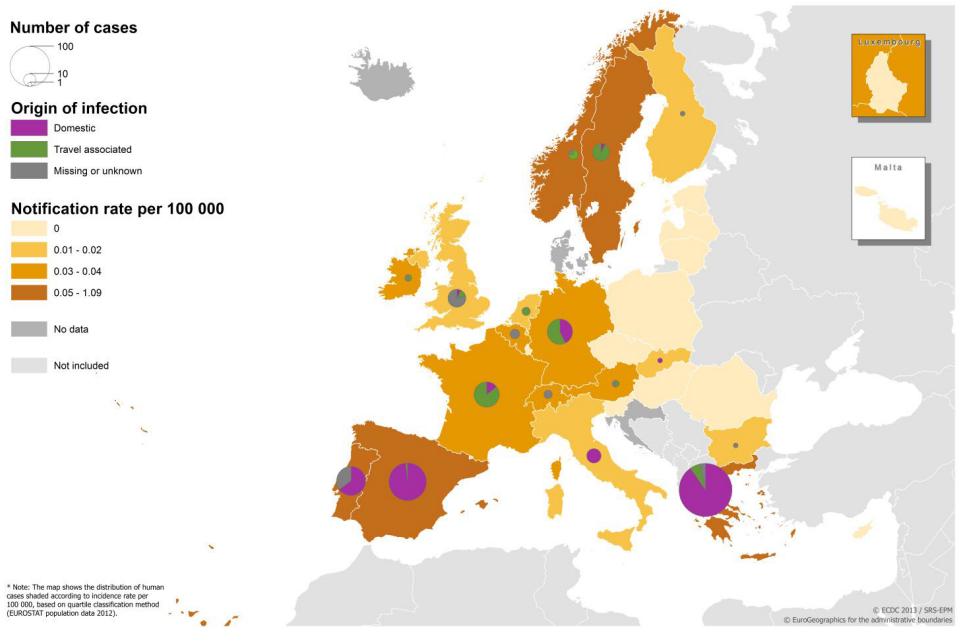
Despite the measures of eradication undertaken, a large outbreak of *B. melitensis* occurred in Greece in 2008. In France, which has been officially free of bovine brucellosis since 2005, a case of human brucellosis was diagnosed in January 2012. The investigation demonstrated that the case had been infected by raw milk cheese from a neighbouring dairy farm.

The occurrence of brucellosis in humans is directly linked to the epizootic of animal brucellosis. The eradication of brucellosis has been achieved in countries where a close collaboration between public health and animal health programs has been implemented by means of adoption of common eradication projects. This strategy has to be encouraged in order to prevent the reintroduction of the disease in areas which have been officially free of brucellosis and to continue the eradication or the disease.

Trend in reported confirmed cases of human brucellosis in the EU, 2009-2012



Numbers of reported cases, notification rates and origin of infection in human brucellosis in the EU/EFTA, 2012



Note: The map shows the incidence rate of human brucellosis cases per 100,000 based on quartile classification method distribution as shaded colors (EUROSTAT population data 2012).

Oral Presentations (O26-O29)

O26: Human *Brucella canis* infection transmitted by contact with an asymptomatic infected dog

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Brucella (*B.*) *canis,* the main etiologic agent of canine brucellosis, can be transmitted to humans by either laboratory accidents or close contact with infected dogs, principally after an abortion. Human *B. canis* infection is frequently mild or asymptomatic. In symptomatic cases, clinical signs are variable and typically the disease presents as an acute febrile illness with flu-like symptoms. Complications are infrequent and include arthritis, spondylitis and epididymo-orchitis. Neurologic signs (including meningitis, uveitis and optic neuritis), anemia, internal abscesses, nephritis, endocarditis and dermatitis can also occur and other organs and tissues can be affected, resulting in a wide variety of syndromes. Even though, antibiotic treatment is usually effective, relapses can occur. In untreated individuals, mortality rates vary from less than 2 % to 5 % and endocarditis and meningitis are the most frequent causes of death.

Case report: A 37-year-old previously healthy male sought medical care in a general hospital due to fever, myalgia and headache that had begun 3 days earlier. No pathological signs were found by physical examination, and laboratory studies showed moderate elevation of transaminases and alkaline phosphatase. At that moment, two samples for blood culture were taken and symptomatic treatment was administered. The patient attended to control again 48 hours later with symptoms worsening, hepatomegaly was demonstrated by ultrasonography, and increased levels of transaminases were found. Since the blood culture was positive for Gram-negative bacilli, hospitalization was decided and antibiotic treatment (ciprofloxacin 400 mg IV every 12 hours and ampicillin/sulbactam 1.5 g IV every 6 hours) was given. Few days later, the isolated bacteria were identified as *Brucella* spp. and the treatment was switched to streptomycin 1 g/d IM and doxycycline 100 mg b.i.d. orally. After treatment, the patient experienced clinical remission with normal transaminase values and negative results in subsequent blood cultures. After discharge, the patient was referred to the Hospital of Infectious Diseases FJ Muñiz for follow-up. Laboratory typing tests identified the isolated bacterium as *B. canis.* In addition, serum samples obtained during hospitalization were tested for antibodies against smooth *Brucella* strains (RBT, STA, STA-2ME, CFT and cELISA) and rough strains (RSAT and iELISA for *B. canis*). Positive results were obtained with RSAT and iELISA for *B. canis,* whereas the remaining tests were negative. The clinical examination and a questionnaire indicated that the patient was asymptomatic except for a mild intermittent diarrhea. Additionally, he reported to live with a healthy male dog which had presented positive blood cultures for *B. canis*. For this reason, we decided to carry out serological tests on the patient's family including his wife and their three children. His wife was asymptomatic and exhibited positive results by RSAT and iELISA*,* whereas the children were negative.

This case demonstrates the transmission of canine brucellosis to pet owners from an apparently healthy dog which had a *B. canis*-positive blood culture. This type of transmission is not commonly seen in immunocompetent individuals, but it highlights the need for intensified controls of pet dogs to exclude a latent canine *Brucella* infection.

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O27: Linked human and livestock study on seroprevalence and risk factors for brucellosis in Kenya, 2012

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Brucellosis is an endemic zoonotic disease in Kenya with dual burden i.e. significant economic losses among livestock and illness and disability in humans. Brucellosis studies in Kenya have previously focused on either animals or humans. We conducted a cross-sectional survey that investigated humans and livestock (cattle, sheep, goats and camels) in the same households. We assessed the seroprevalence and risk factors for human brucellosis, animal brucellosis and their association. The study was conducted in three counties with different livestock production systems; households were selected randomly using a two-stage cluster sampling method by sub-location and then by household. Three persons were enrolled and livestock species proportional to the herd size were sampled per household. Questionnaires were answered by household heads and the persons sampled. Human sera were tested for *Brucella* IgG antibodies using competitive ELISA and animal sera were tested using an indirect ELISA. Risk factors were assessed using multivariate logistic regression. A total of 1,099 households, 2,811 persons and 11,039 production animals were enrolled. Overall, 14 % (95%CI 12-16) of households had at least one *Brucella* seropositive person and 15 % (95%CI 12-18) of the herds had at least one seropositive animal. Among humans sampled, 6.7 % (95%CI 5.6-7.8) were seropositive. Risk factors for human seropositivity included consuming unboiled milk (aOR=3.9, 95%CI 2.0-7.6), exposure to goats [herding, milking, feeding] (aOR=2.5, 95%CI 1.6-4.0) and handling hides (aOR=3.9, 95%CI 2.5-6.2). Animal seropositivity was linked to intermingling with wildlife (aOR=4.3, 95%CI 2.3-8.1) and keeping goats (aOR=2.7, 95%CI 1.4-5.2). The odds of human seropositivity given a seropositive animal in the same household was 5.3 (95%CI 3.2-8.8).This linked survey shows that human and animal brucellosis seropositivity is associated with factors that increase exposure to seropositive animals. Our survey contributes to a better understanding of the burden of brucellosis in Kenya and helps to target health education programs.

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O28: Risk factors for human brucellosis in the Kween District, Eastern Uganda, 2011 – a case-control study

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The global burden of brucellosis remains enormous, accounting for over 500,000 new infections per year. The disease is endemic in most countries of Africa, Asia, South and Central America with incidence rates of up to 70 cases per 100,000 people. In Uganda, brucellosis is reported as an increasing public health hazard with a prevalence of 18-24 % in endemic areas. This study was conducted to determine the risk factors for transmission of brucellosis to humans in the Kween District, Eastern Uganda and to recommend prevention and control methods.

A facility based case-control study was carried out in a rural community in March 2011. A total of 56 cases and 141 controls were included. Cases and controls were residents of the Kween District at any age who were willing to participate in the study. Participants were identified and enrolled when they attended outpatient services at selected facilities. A brucellosis case was defined to be a patient with a positive competitive ELISA result and presenting with at least two clinical symptoms that are suggestive of brucellosis. A control was an individual from the same village as the case having a negative serological test result. Blood samples were drawn and aliquoted before shipping to the Central Public Health Laboratory for laboratory examination. Those who tested positive on rapid screening tests were further subjected to ELISA which was considered as the gold standard. Risk factor data were collected using individual structured questionnaire interviews. Data were entered into SPSS and exported to Stata and Epinfo statistical software programs for analysis. Cross tabulations, logistic regression models and Fishers exact tests were used to establish associations at univariate and multivariate levels. P-values and odds ratios (OR) were used to determine statistical significance of independent variables.

After adjusting for gender and age being in the age group of 21-50 years (OR 11, P=0.03), handling cow dung (OR 0.1, P=0.01), and being a male herding animals (OR 2.3, P=0.04) were found to be risk factors for brucellosis.

In summary, there is a need to strengthen and prioritize the veterinary public health services at the community level in order to control brucellosis in humans. Communication programs aiming at behavior change are essential for empowering livestock keeping communities to prevent brucellosis transmission to humans.

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O29: Epidemiological status of brucellosis in the Russian Federation

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Brucellosis has caused about 300-500 reported new human cases per annum in the Russian Federation during the last decade. The official number of human brucellosis cases decreased from 465 to 341 cases in the period 2012 to 2013. The morbidity rate was 0.33-0.24 per 100,000 people and in children below 17 years of age 0.17-0.08. Most new cases of human brucellosis were detected in three federal districts of Russia: 298 cases (3.16 per 100,000 people) to 211 (2.23) in the North Caucasian Federal District, 75 (0.54) to 64 (0.46) in the Southern Federal District, 69 (0.36) to 44 (0.23) cases in the Siberian Federal District. These three districts covered 93.55 % of all human cases reported in the country. In the North Caucasian Federal District brucellosis was reported in almost all of the administrative territories except for the Republic of Ingushetia. The highest percentage of human cases were reported to be 45.81 % and 41.64 % (7.32 and 4.86 per 100,000 people) in the Republic of Dagestan, 16.34 % and 18.48 % cases (2.73 and 2.26) in the Stavropol region in 2012 and 2013, respectively. Human brucellosis was also reported in all of the administrative territories of the Southern Federal District with the highest percentage of cases in the Republic of Kalmykia i.e. 10.97 % and 12.9 % (17.62 and 15.29) in 2012 to 2013, respectively. In the Siberian Federal District, morbidity was 4.52 % and 3.52 % (6.82 and 3.89) in the Republic of Tyva, and 4.09 % and 4.12 % (1.72 and 1.27) in Trans-Baikal Territory in the respective years.

In 2013, there were about 20.3 million cattle and over 22 million sheep in the country. The most affected regions for animal brucellosis were again the three federal districts already mentioned. In 2013, bovine brucellosis was reported in 22 regions of Russia in which 209 holdings (state and private farms) were affected by bovine brucellosis with 13.800 cattle infected. The highest number of bovine brucellosis cases was reported from the North Caucasus Federal District with 9,022 infected cattle or 65.2 % of all cases in Russia, the Southern Federal District with 3,389 cattle (24.5 %), and the Siberian Federal District with 1,134 cattle (8.2 %). In 2013, caprine brucellosis was reported in 30 holdings with 2,000 goats and sheep involved. About 80 % of sheep and goats and more than 50 % of cattle were located in private farms. Livestock owners were not capable to carry out expensive anti-brucellosis measures due to restricted financial resources. Most animals were infected on farms where goats and cattle were kept together and *B. melitensis* biovar 3 was the most prominent species originating from the Republic of Dagestan and Kalmykia. Summing up it can be pointed out that reported cases of human brucellosis have been found in 29 regions (34.94 %) of Russia, in 8 regions (9.64 %) brucellosis was only found in cattle (maybe accompanied by under-reporting of human brucellosis), in 6 regions (7.2 %) 'free' of brucellosis (travel-associated cases may still occur), in 42 regions (50.6 %) brucellosis was not detected in humans and animals in 2013.

Thus, in Russia the epidemiological situation is complicated due to missing controls of private animal farms, not ideal veterinary service, limited use of laboratory methods for active brucellosis surveillance in animals and humans. This lack of public veterinary health contributes to the spread of animal brucellosis among cattle and sheep, a delayed culling of sick animals, the refusal of private owners to obey veterinary control measures, under-registration of new livestock. The situation is also worsened by uncontrolled introduction of animals suffering from brucellosis (mainly sheep) into territories previously free from brucellosis, the uncontrolled movement of animals inside Russia and uncontrolled importation of animals from other CIS states bordering the Russian Federation (Kazakhstan, Azerbaijan, Mongolia).

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Poster Presentations (P85-P99)

P85: Clinical and epidemiological parallels in human brucellosis cases

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The aim of our study was to assess the significance of peripheral blood and urine parameters in brucellosis patients and their correlation to clinical and epidemiological data.
We investigated patients suffering from brucellosis as well as healthy volunteers from endemic regions (Oblasts). The phenotype of acetylation (PA) and the levels of antigen-binding antibodies directed to tissue-specific antigens (ABL to TA ratio) were measured in all patients and some volunteers to determine the extent of pathogenic effects on joints, heart, brain and liver. The fast acetylation phenotype was determined in urine samples using 10 mg/kg of sulfathiazole as a test drug. The capacity of acetylation was considered slow unless it reached 50 % of average, and fast when it exceeded 50 % of average. Interviews were conducted according to an established protocol to identify possible cases of brucellosis in various social segments of the population. In addition, bacteriological and serological tests were performed to diagnose a Brucella infection.
Brucellosis patients in various stages of disease were examined. As a result of the questionnaires over 80 % of the residents in endemic regions turned out to be cattle owners and 57 % owned sheep and goats. At the same time, only 3 % of them belonged to the occupational risk group of livestock owners. A total of 17.4 % of the participants were involved in cattle slaughtering and processing of livestock products. 31 % of the healthy volunteers were involved in lambing campaigns. 9.4 % revealed positive serological test results for brucellosis. Peripheral blood parameters examined in a limited number of healthy volunteers were similar to that of patients with brucellosis.
Acute brucellosis was characterized by a more foudroyant clinical course and a fast acetylation phenotype. Chronic brucellosis was characterized by a subclinical and dull course and slow acetylation phenotype, which could be documented in nearly 60 % of the examined interviewees. More significant effects on joints, brain and the myocardium were found in patients with a slow acetylation phenotypeas compared to fast acetylation. According to comparative analysis of clinical and epidemiological data 9.4 % of the patients had experienced clinically non-manifesting brucellosis.

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P86: Brucellosis laboratory diagnostics at Armenia's National Center for Disease Control and Prevention, 2009-2013

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Brucellosis is one of the most common global zoonotic infections with over 500,000 new human cases annually reported. Brucellosis is endemic in Armenia, with an average of 300 human cases recorded each year. As part of a continuous improvement process, we evaluated the laboratory surveillance system for brucellosis at the national level.
A retrospective survey of brucellosis laboratory data originating from the National Center for Disease Control and Prevention (NCDCP) was conducted. Case files of all samples suspecting brucellosis and submitted to NCDCP in the years 2009-2013 were analysed for diagnostic tools used and results obtained.
From 2009 to 2013, a total of 1,365 samples of any suspect human brucellosis case on the basis of standard case definition were sent to NCDCP for confirmatory laboratory diagnosis. Brucellosis was confirmed by Serum Agglutination Test (SAT) in combination with the Wright-Huddleston test. The samples were also tested by PCR. All 1,365 samples were positive in SAT and PCR. The confirmed cases were recorded at the national level and reported to the World Health Organization (WHO). The highest incidence was recorded in 2009, with a rate of 10.7 % per 100,000 population, and the lowest incidence was recorded in 2013, with a rate of 7.2 % per 100,000 population.
In all suspect samples which reached NCDCP brucellosis was laboratory confirmed. These results indicate that the standard case definition for brucellosis is accurate and is used appropriately by the health care providers in Armenia. The results also indicate that PCR is an alternative diagnostic tool for brucellosis; however, it is not recommended to be used as standalone test because SAT titres provide critical information for the diagnosis of brucellosis. In order to improve the surveillance system in Armenia, genotyping of *Brucella* isolates should also be performed.

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P87: Role of laboratory methods in active surveillance of human brucellosis outbreaks on a part-time farm of the Moscow Zoo

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Brucellosis is a dangerous zoonotic infection with chronic courses and disability of patients and of serious public health concern in the Russian Federation. Human cases are caused by the transmission of brucellae from their natural hosts (goats, sheep, pigs, and cattle).
The aim of our study was to assess the role of laboratory methods in active surveillance of human brucellosis outbreaks on a part-time farm of the Moscow Zoo located about 100 km from Moscow. Epidemiological surveys were performed from 2003 to 2013 (twice a year in spring and autumn) involving more than 200 part-time farm employees who were working on the farm during outbreaks of sheep or cattle brucellosis. Blood sera were analysed using tube agglutination (Wright test), agglutination on glass slides (Huddelson), Coombs test, iELISA (indirect enzyme-linked immunosorbent assay) with the immunodominant s-LPS, real-time PCR targeting *bcsp31*.
After the elimination of epizootic caprine brucellosis in 2003, the percentage of positively tested employees decreased from 42.7 % to 17.2/15.9 % in 2005/2006. Real-time PCR detected *Brucella* DNA in infected humans more efficiently than iELISA detected anti-*Brucella* antibodies. In 2007 and 2009, 5 and 11 workers were found infected, respectively. They had negative results from 2003 to 2006. The infection of these workers might be associated with outbreaks in 2007 and 2009 which were traced back to an infected 2-year-old yak. In 2012 and 2013, no acute brucellosis case was found, and a decrease of iELISA antibody titres in previously infected employees was noted. During the study period of 11 years, 42.2 % of the employees were diagnosed with brucellosis. These employees had contact to *Brucella* positive animals, but only 14 % of these presented clinical signs and symptoms of disease.
Active brucellosis surveillance by various laboratory techniques has been conducted during brucellosis outbreaks on a part-time farm of the Moscow Zoo. The laboratory proven human cases could be traced back to brucellosis outbreaks in sheep and in a yak in 2003, and 2007 and 2009, respectively. Real-time PCR was an effective tool to identify *Brucella* infections. The iELISA proved to be more sensitive in chronic patients than real-time PCR. Traditional serological methods were less sensitive and informative, but less time consuming compared to real-time PCR and iELISA.

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P88: Combined diagnostics in a case study on human brucellosis

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Brucellosis is an infectious disease which affects small ruminants, bovine animals and pigs, and is dangerous for people. Currently, brucellosis is not officially reported in Ukraine. However, because of the threat of import of the pathogen from abroad, timely and correct diagnosis are extremely important.
The aim of our study was to identify efficient approaches to diagnose human brucellosis. Epidemiological, clinical, and laboratory methods were used. Laboratory tests comprised tube agglutination (Huddelson), the Rait agglutination, and an indirect hemagglutination test. An Ukrainian citizen who worked at a construction side of stable at a livestock enterprise in Russia since October 2011 asked for medical help at the city hospital on 15th May 2013. He suffered from fever up to 40 °C, weakness, fatigue, drowsiness, joint pain in the upper limb, and a productive cough. An acute respiratory disease was initially diagnosed. However, the patient reported that two human brucellosis cases have been diagnosed on the cattle farm he was working at. Subsequently, the patient was tested positive for brucellosis and negative for tularemia. The Huddelson test was positive, the Rait test and the indirect hemagglutination revealed anti-*Brucella* antibody titres, 1:200 and 1:640, respectively. Hence, the final diagnosis was 'acute brucellosis, moderately severe'.
A combination of laboratory tests and epidemiological investigation led to the timely detection of brucellosis in our patient. Whether the disease was acquired abroad cannot be clarified. In Ukraine, the implementation of effective countermeasures to prevent the introduction of brucellosis from endemic areas is of high relevance to control the spreading of the disease.

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P89: Human brucellosis in Georgia: Clinical and laboratory manifestations

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Human brucellosis is one of the most prevalent zoonosis in Georgia. According to national statistics, approximately 150 confirmed cases of brucellosis were recorded in the country annually. The disease in humans is mainly associated with animal contact and/or consuming animal products.

We analyzed medical records of 543 patients at the Virsaladze Research Institute of Medical Parasitology and Tropical Medicine from 2009-2013 of whom brucellosis was diagnosed and treated. We assessed age, gender, clinical signs, laboratory investigations including complete blood count (CBC) and serological tests of the patients.

During 2009-2013, a total of 2,423 patients were tested for brucellosis at the Institute. Among them, 543 patients (22.4 %) were positive tested for the disease. Confirmation of diagnosis was performed by Wright Agglutination test at a titer ≥1/200 and/or by enzyme-linked immunosorbent assay (ELISA) to detect *Brucella*-specific IgM antibodies. Among patients, males predominated significantly (88.7 %). Brucellosis-confirmed patients were allocated to three different age groups. 67 % of the patients (363/543) belong to the largest group with ages ranging from 14-60 years. The second group consists of 24.4 % (133/543) of the patients within the age group of >60 years, and only 8.6 % (47/543) of patients were in the third age group ranging between 0-14 years. The youngest patient of the study was 18 months. Most of the children with brucellosis reported a family history of the disease.

158 (29 %) out of 543 patients were hospitalized and treated at the clinic. The average duration from symptoms onset to the diagnosis was 18 days. Delayed diagnosis for ≥1 month was observed in about 20 % of cases. Fever was recorded in 94.3 % (510/543), sweating in 75.8 % (412/543), chills in 77.7 % (422/543), fatigue in 77.3 % (420/543), osteoarticular and neuromuscular pain in 88 % (482/543), hepatomegaly in 34 %, (189/543) and splenomegaly in 16.3 % (89/543) of the investigated patients. Polylymphadenopathy was rare and observed only in 8.8 % (48/543) of cases. Among 543 brucellosis patients hematologic tests were performed for 454 patients. Anaemia was detected in 45.8 % (208/454) of cases. Leukopenia (WBC <4,300/mm^3) was present in 7.54 % (34/454), thrombocytopenia (platelet count <150,000/mm^3) in 26 % (118/454) and pancytopenia in 5.2 % (24/454) of the patients. Additionally, a slight increase of liver enzyme levels was observed in 35.4 % (56/158) of cases. The most frequent complication of brucellosis was sacroileitis, which was observed in 13 % of the patients while epididymo-orchitis was detected only in 9 % of the cases. Both of these complications were associated with delayed diagnosis for more than 1 month. All adult patients were treated with an antibiotic combination of streptomycin or gentamicin and doxycycline. Vast majority of patients 88.9 % (483/543) were cured after the full course of the treatment, 6.6 % (36/543) of patients interrupted the therapy and 4.5 % (24/543) of patients experienced relapse of the disease.

Brucellosis in humans remains one of the main health problems in Georgia. Nonspecific clinical signs cause delayed diagnosis of the disease, which can be associated with serious complications. Brucellosis should be considered for all patients who show signs of systemic inflammation and arthritis in endemic areas.

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P90: Using molecular tools to identify the geographical origin of a case of human brucellosis

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Brucellosis is a zoonosis of great socio-economic importance that causes reproductive problems in ruminants and swine as well as a number of ailments in humans including general malaise. Although this disease has a global presence, a number of countries have invested a great deal of time and money on brucellosis eradication schemes and attain officially brucellosis-free status. One of these countries is the Mediterranean nation of Malta.
A strain of *Brucella* (*B.*) *melitensis* that had been isolated from a human in Malta was submitted in late 2013 to the WHO Collaborating Centre for Reference and Research on Brucellosis at AHVLA Weybridge. Maltese colleagues wanted to establish whether the causative agent had been imported or was an example of a local strain that had been present on the island prior to eradication. To this end, molecular typing tools were used to try and provide an answer to this question.
Multi Locus Sequence Analysis (MLSA) and Variable Number Tandem Repeat (VNTR) testing are well-established tools widely used for typing and molecular epidemiology. As very little molecular data existed on *Brucella* strains from Malta, 15 *B. melitensis* isolates taken from both humans and livestock in Malta between 1950 and 1960 and present in the AHVLA strain archive were processed by these two methods alongside this current isolation.
Using MLSA, we found that whilst the historical isolations all shared a common sequence type (ST7) which is found in other isolations in the Mediterranean/Southern Europe, the recently submitted case belonged to a ST associated with countries in the Horn of Africa. This observation was backed up by subsequently provided field epidemiological data which identified the case as a recent arrival to Malta from the Horn of Africa. As expected, VNTR data showed more diversity within the 16 isolates tested but confirmed that the recent human isolate was unrelated to historical local strains. In addition, whilst the MLVA-16 profiles of the historical local isolates were similar to each other, the profile generated from the recent human isolation was very different to these.
The example in this study illustrates the usefulness of molecular techniques in the unbiased geographical placement of a strain to add value to field epidemiological observations. Given advances in whole genome sequencing technology and its potential applications in phylogeography and microbial forensics, the strains are currently undergoing whole genome sequencing to assess the added value of this approach.

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P91: Human brucellosis in Belgium: serological trends, isolates and molecular typing, 1998-2014

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Belgium is a country free of brucellosis in livestock, with the latest domestic animal case occurring in 2000. In wildlife *Brucella* spp. can still be isolated from wild boars (*B. suis* bv 2 in *Sus scrofa*) and marine mammals. In late 2010 and spring 2012, the country was affected by two cluster cases of bovine brucellosis which, properly controlled, did not hamper the officially brucellosis-free (OBF) status of Belgium. To increase knowledge of human brucellosis in Belgium, we analyzed historical data of serological samples and clinical isolates collected in a reference setting during a 16-year period. Clinical samples were those submitted to the National Reference Centre outside of the labor medical testing. Confirmed laboratory case definition was that recommended by the World Health Organization (isolation of the bacteria or sero-convertion assessed with the IgG ELISA-(iELISA)). A total of 201 samples were serologically analyzed by Rose Bengal test (RBT) and iELISA. Thirty-three samples (15.7 %) were positive to RBT of which nine (27.3 %) were confirmed by the Wright agglutination test and fourteen (42.4 %) by the iELISA (with one associated with the direct isolation of *Brucella* spp.). Although not quantifiable due to inconsistent information on clinical data of the patients, Rose Bengal test yielded false-positive results in various cases.
A total of 32 clinical cases were identified by bacterial isolation. Biotyping of the isolated strains identified 26 as *B. melitensis* bv 3, three as *B. melitensis* bv 2, two as *B. melitensis* bv 1 and one as *B. abortus* bv 1. Multiple Loci VNTR Analysis confirmed and highlighted the diversity of those strains. Because *B. melitensis* is not present in small ruminants in Belgium, and because only *B. abortus* bv 3 has been isolated in cattle since the early 1990s, it is highly likely that all human cases cited here were imported or associated to a contamination having occurred abroad. Human brucellosis is yet a rare disease in Belgium, mainly allochthonous (or associated to professional work). Early detection of imported brucellosis in domestic animals is a prerequisite to prevent any human contamination associated to animals to occur in Belgium.

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P92: Investigation of the risk factors to human brucellosis in Mubende district, Uganda

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Brucellosis is a globally distributed zoonotic disease associated with more than 500,000 cases annually. Prevalence of 16 and 18-24 % has been reported in Africa and in Uganda, respectively. Human brucellosis infections are mainly associated with the consumption of unpasteurized milk and its products or the direct contact with infected animals. In 2011, an increasing number of brucellosis cases from Mubende district were reported to the Ministry of Health Uganda. These cases prompted us to establish the prevalence and the main risk factors for both human and animal brucellosis for this district. An unmatched one to one case control study involved extraction of brucellosis cases biodata from laboratory records. Active search for patients was done in communities. 52 cases of the target number of 100 cases were enrolled. Fifty-two controls accessed from immediate neighborhood considering age and gender were interviewed using structured questionnaires. Children's questionnaires were answered by their parents. Cattle older than two years and goats older than one year originating from cases' and controls' homesteads were randomly selected and bled. Seroprevalence in animals was determined using cELISA, risk factors were analysed using Chi square test that generated odds ratios.
For humans, 52 cases and 52 controls were recruited for the study. The brucellosis seroprevalence determined from hospital records was 31 %. The seroprevalence of brucellosis in cattle was 11 % at animal level and 38 % at herd level. In goats, prevalence was 36 % and 58 % at animal and herd level, respectively. Significant risk factors associated with contracting brucellosis in humans were: consumption of undercooked meat (the risk was eight times higher); not using protective wear while handling animals' aborted fetuses and faeces (four times higher). In animals main risk factors were: history of animal abortions on farm and use of rented breeding bulls (eight times and five times higher, respectively).
The increase in number of cases in Mubende district was found to be associated with the consumption of undercooked meat by farmers and failure to use protective wear while handling animals. History of abortions on farms and use of rented breeding bulls were associated with high prevalence of brucellosis in animals. The government should strengthen projects that will rise the awareness of rural farmers for the risk factors and the control measures for brucellosis. Regular testing of animals for brucellosis is advised.

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P93: Changing incidence of human brucellosis, Azerbaijan 1983-2009

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We assessed spatial and temporal trends in the epidemiology of human brucellosis in Azerbaijan from 1983 to 2009. Up to the mid-1990's there was a substantial increase in brucellosis incidence (25 % annually) followed by an average decline in reporting of 5 % annually after the initiation of a livestock test-slaughter campaign. Our findings confirmed that brucellosis was widespread in both humans and livestock. However, recent changes in the case distribution indicated an annual increase in human incidence among districts clustered in southeast compared to a decrease elsewhere. Males were disproportionately represented among cases (71 % male vs. 29 % female). The age specific average incidence was highest in the 15-19 years age group with 18.1 cases per 100,000 population, suggesting a primarily occupation route of exposure. Livestock control campaigns have likely improved the current brucellosis situation in Azerbaijan, although the disease appears to be re-emerging in the southeast. Our findings highlight the need for dynamic surveillance that takes into account the changing patterns of disease while focusing on high risk populations.

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P94: Time series analysis of human brucellosis in Azerbaijan 2002-2010

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In Azerbaijan, the zoontic disease brucellosis is considered an endemic public and veterinary health threat. To assess the temporal dynamics of human brucellosis and target areas for control, we analysed monthly reporting from 2002 to 2010 using a time series analysis. Due to strong seasonality in the data, seasonal and non-seasonal Autoregressive Integrated Moving Average (ARIMA) models were used to forecast future brucellosis case reporting 24 months ahead of time by fitting a series of models to the data and selecting the model with the lowest Akaike Information Criterion (AIC) score. Spatial differences in the seasonality of cases were assessed using geographic information systems (GIS). A total of 3,898 cases of human brucellosis were reported during 2002-2010 with significant seasonality in the summer months. Model diagnostics and the AIC score indicated the ARIMA model with a (2,1,3) was the best fit. The trend in the data suggested a gradual decrease in the reporting of human cases; however, we identified district local level heterogeneity in reporting trends and spatial differences in the seasonality of case reporting. Control of brucellosis requires cooperation among multiple health agencies in order to properly formulate management strategies. Recent national reductions in the occurrence of human brucellosis in Azerbaijan highlight the impact that veterinary control measurements such as test-slaughter campaigns can have for human health. Further reduction of the number of human cases can only be reached if areas of high human and animal transmission are targeted.

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P95: Brucellosis epidemic situation dynamics in the Armenian population, 2011-2013

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Brucellosis is a dangerous disease for both humans and animals. It is endemic in several countries of the Caucasus region, including Armenia, and thus considered as a priority disease for the region. In 2013, 219 new cases of brucellosis were recorded in Armenia (incidence rate of 7.2 per 100,000); in 2012, 227 cases (incidence rate of 6.9 per 100,000); in 2011, 260 cases (incidence rate of 8.0 per 100,000); in 2010, 295 cases (incidence rate of 9.2 per 100,000); in 2009, 346 cases (incidence rate of 10.7 per 100,000); and in 2008, 289 cases (incidence rate of 8.95 per 100,000). In this study, we used data from the Armenian epidemiological surveillance system to study the demographic distribution of human brucellosis cases and identify risk factors of infection.
Data were deduced from medical records of confirmed brucellosis patients registered in the country from 2011-2013. In total, 706 records were analysed with regard to demographic data (age, gender, residence [urban/rural]) and history of possible exposure.
The epidemiology of human brucellosis in Armenia can be characterised as follows: 12 % of patients lived in urban and 88 % in rural communities; 92 % were adults (aged >18 years) and 8 % children (aged <18 years); and 70 % were women and 30 % men. Following risk factors were identified: exposure to animals was noted in 51 % (cows, sheep, horses and pigs), the purchase of milk and dairy products purchased from local farmer's markets (i.e. non-state regulated markets) was recorded for 28 %, and no risk factor was identified in 21 % cases.
Most brucellosis patients (88 %) were from rural communities. The prevalence of brucellosis was much higher in women (70 %). This is likely due to the fact that in Armenia women are responsible for milking animals and preparing food from animal products that may contain *Brucella* species. Disease cases occurred primarily in adults (92 %). A large majority of patients had contact with animals before symptoms developed (79 %). This study provides some initial information on brucellosis cases concerning demographic structures and risk factors in Armenia. Our results are consistent with results of similar studies from endemic countries around the world.

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P96: *Brucella melitensis* in humans in Venezuela: Mistaken identity of *Brucella* infection

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Brucellosis is a zoonotic disease transmitted by direct or indirect contact with infected animals or their products. Nowadays, *Brucella* (*B.*) *melitensis* is the species most frequently reported as the cause of human brucellosis in the world. Human brucellosis is a multisystemic disease with a wide range of clinical signs and symptoms, and despite being a centenary problem, still presents many challenges, particularly in diagnosis. In Venezuela, brucellosis is a forgotten disease among physicians. Laboratory diagnosis is based on serological tests, because applying direct methods is only possible in a few laboratories. Serological results can be questionable and advanced molecular techniques are now always available. Considering these facts it is not surprising that *B. melitensis* was frequently misdiagnosed in the past.

This report describes a case of a 9-year-old girl with a prolonged febrile illness whose serology screening tests for brucellosis were positive (Rose Bengal test, Buffered Plate Antigen test, *Fluorescent Polarization* Assay). The medical interview revealed that the patient had often consumed fresh goat and cow milk cheese. For bacteriological diagnosis, two blood cultures were processed by Bact/Alert SA, being positive after 60 hours. Bacterial colonies were identified by the Vitek-2 system with a 99 % match for *B. melitensis*. The result was automatically reported by the software. Molecular diagnosis was performed at the National Polytechnic Institute of Mexico using the Bru-Multiplex PCR. The isolate was unmistakably diagnosed as *B. abortus*.

The database of the Vitek-2 system considers all *Brucella* species as biovars of *B. melitensis* and can consequently not discriminate among species of the genus. Misidentification of *Brucella* species by commercial bacterial identification systems including the Vitek-2 has been previously reported. Diagnostic microbiology laboratories should be aware of potential misidentifications of brucellae using automated identification systems.

This report confirms the presence of human brucellosis in Venezuela. However, the presence of *B. melitensis* could not be confirmed as the causative agent. *B. melitensis* is widely spread in sheep and goats, where it is endemic, whereas *B. abortus* is usually found in cattle.

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P97: *Brucella* seroprevalence study among stray and pet dogs in Georgia

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Brucellosis is one of the most important zoonotic diseases worldwide. Brucellosis continues to cause significant medical, veterinary and socioeconomic problems and is endemic in Georgia. Although exposure to *Brucella* (*B.*) has been studied in cattle and small ruminants, no information is available of that in pets or wild animals in the country of Georgia. This study is the first attempt to record the detection of antibodies against *Brucella* spp. in serum samples from stray and pet dogs collected in Tbilisi, Georgia in 2011.
The Rose Bengal test (RBT) was performed to analyze serum samples collected from dogs for the presence of anti-*Brucella* spp. antibodies. RBT-positive samples were further investigated by fluorescence polarization assay (FPA) and ELISA to attempt confirmation. In addition, positive samples were also tested by the slide agglutination test against five different pathogens: *Yersinia* (*Y.*) *enterocolitica, Salmonella* (*S.*) *enteritidis, S. typhi, S. typhimurium,* and *E. coli* O:157 to exclude false-positive results due to antigen cross-reactivity.
Sera of 141 stray and 145 pet dogs were screened by RBT. The overall seroprevalence varied slightly between stray and pet dogs; a lower prevalence was detected in stray than that in pet dogs, 43.26 % and 51.7 %, respectively. Confirmation by FPA was successful in 5 of 11 RBT-positive samples that were negative for other infections. ELISA results were positive for all RBT-positive samples, regardless of infection status with other species. Out of the RBT-positive samples the samples were also positive for the following: *Y. enterocolitica* (69.73 %), *S. enteritidis* (25.65 %), *S. typhi* (37.5 %). *S. typhimurium* (21.71 %), *E. coli* (26.97 %).While the RBT assay does not allow the discrimination of antibodies against *B. abortus* and *B. melitensis*, the demonstrated high seroprevalence rate in both stray and pet dogs in Georgia suggests frequent exposure to one of the smooth lipopolysaccharide (LPS) *Brucella* spp. among these animals. Confirmation of RBT results was inconclusive as commercial FPA and ELISA assays are not validated for canine testing. Although the extent is unclear to which dogs transmit *Brucella* to human, the results obtained indicate a necessity to consider dogs as potential sources for human infection. To the best of our knowledge these are the first data reported for seroprevalence of *Brucella* spp. in dogs in Georgia. Future work will also include investigation in areas where the RBT test cross-reacts with non-*Brucella* microorganisms. This project will provide unique results to be incorporated into the public health approach in Georgia.

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P98: Evaluation of human brucellosis surveillance system in Georgia, 2010-2012

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Brucellosis is a highly contagious zoonosis affecting livestock and humans worldwide. It is recognized as a significant public health challenge with major economic and financial burdens. According to the WHO, about 500,000 cases of this disease are registered worldwide every year. The disease is transmitted to humans by contact with infected animals or the consumption of infected food products. The economic impact of the disease occurs through livestock disease and loss. According to national statistics, approximately 150 human brucellosis cases were reported per year in Georgia in 2010-2012, although the actual numbers may be higher. The objectives of our evaluation were to identify strengths and weaknesses of the human brucellosis surveillance system in Georgia and to recommend improvements to the current surveillance system. For this purpose, current existing Georgian Guidelines for Brucellosis, policies and data from NCDC and EIDSS reports were reviewed. Data were also obtained from The Parasitology Center, Infectious Pathology Center, and Sepsis Center Laboratories. Brucellosis case definition was based on NCDC/DTRA definition, updated CDC 2001 evaluation guidelines, and other references. According to quantitative and qualitative assessments of the system attributes, the system is not simple and not flexible. Data quality, acceptability, representativeness of the system and timeliness are low. On average, 52.7 % of the monthly notification forms were filled completely in 2010-2012. A total of 190 samples were tested by ELISA and 34 (17.89 %) were positive. 2,279 Wright-Huddleston tests were performed, 440 (19.3 %) were positive. During the same period, 287 samples were cultured and 29 (10.1 %) showed *Brucella* growth. In 2010-2012, complete surveillance was conducted for 472 probable cases and 19 *Brucella* outbreaks (two or more patients) were revealed. We could not calculate system sensitivity; however, the positive predictive value (PPV) for all tests in the surveillance system was approximately 18 %. In 2010-2012, the brucellosis incidence rate in Georgia decreased from 4.49 to 2.96 per 100,000 residents. This finding may be attributed to undiagnosed cases or a decreasing number of ruminants (for the last two years large numbers of sheep have been exported) or to a lack of patients seeking medical care. Culture is still considered the 'gold standard'. However, it may not be sensitive. Not all cases are confirmed by culture and the system does not provide follow-up for all patients. Data quality and acceptability is low due to missing fields in EIDSS and slow reporting (interval between onset of symptoms and date of diagnosis is on average from 8 days until 5 months and up to one month from diagnosis until case registration). Cases entered into the system, however, have high validity. Representativeness is low, as this is a passive surveillance system and national screening has not been conducted for 30 years. Georgia does not have a national algorithm for brucellosis used by all labs. Brucellosis surveillance should be ongoing for better control of the disease in the country. The surveillance system should be evaluated periodically and recommendations should include quality, efficiency and usefulness variables. Finally, a national testing algorithm should be standardized.

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P99: First outbreak of *Brucella canis* in a Finnish breeding kennel

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Canine brucellosis is caused by *Brucella* (*B.*) *canis*. In female dogs, the most common symptom is abortion, but an infected bitch can give birth to healthy, weak or dead puppies as well. In male dogs, the infection can cause infertility. Canine brucellosis is a zoonotic disease that is globally distributed. In Finland, *B. canis* was detected from a biopsy sample from lumbosacral vertebra of a stray dog imported from Russia for the first time in 2008. According to the Finnish legislation, *B. canis* infections belong to the category of immediately notifiable diseases. In 2011, a four-year-old Cane Corso bitch gave birth to 16 puppies. Six of them were dead and the rest required human assistance after birth. One and a half years after the delivery, the bitch had vaginitis which did not respond to treatment and the bitch was spayed. At the age of two and a half years, one of the female puppies was brought to a clinic for neutering as she did not show signs of estrus. Because the dog had foreign background, the clinic advised to test her for *B. canis* infection. The dog was tested positive for *B. canis* antibodies by rapid slid agglutination test (RSAT) without and with 2-mercaptoethanol. Consequently, other offspring and the parents were tested. Five out of nine puppies (four females and one male) and the bitch were positive for *B. canis* antibodies, and the parent male had a suspicious result. In addition, 21 dogs connected to the outbreak were tested, but all with negative results. Samples from both euthanized parents and one female puppy were sent for bacteriological investigation. The first seropositive dog was euthanized, but the owner refused to send the dog for autopsy. Bacteria out of the samples were investigated by Stamp staining and culturing on Farrel agar. Cultures were incubated at 37 °C under normal and 5 % CO_2 atmosphere. All samples were negative for *Brucella* by culture and staining. This is the first report on brucellosis in a breeding kennel in Finland. Brucellosis was not suspected when the infected litter was born. It is known that infected dogs can be asymptomatic, or the symptoms can be mild or unspecific. The infection can persist in pet dogs for a long time without knowing of the owner. Maybe the chronic stage of the disease or antibiotic treatment was the reason for the failure to recover *Brucella* from the seropositive dogs. Usually dogs suffering from *Brucella* infection do not respond to normal antibiotic treatment, but antibiotics may still hamper the isolation of *B. canis*. In our opinion, the risk of canine brucellosis is underestimated in Finland. The risk of contracting canine brucellosis in the country is certainly low, but imported dogs and the use breeding services abroad are clear risk factors. The veterinarians and owners should be aware that Finnish dogs can carry *B. canis*. Thus each case of abortion, abnormal delivery or weak offspring should be investigated for *B. canis*.

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Session 8: Livestock Brucellosis and Food Hygiene

Keynote Lecture: Jakob Zinsstag-Klopfenstein
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Towards a science of brucellosis elimination

Brucellosis control by livestock mass vaccination and subsequent test-and-slaughter is effective and in this way brucellosis has been eliminated in several countries. Yet, its control is stagnating or even re-emerging in many parts of the world, especially in Africa and Asia. Depending on the context, the cross-sector nature of brucellosis involving wildlife, livestock and humans require a systemic integrated approach when aiming towards elimination. However, as this conference also shows, the public health and animal health sectors work too much in separation. While research on better vaccines is urgent and on-going, control and elimination of brucellosis is not merely an operational problem. Control and elimination of brucellosis is a science challenge by the complex involved processes and by its contribution to fundamental properties of the biology of brucellosis. From a matrix mathematical point of view, a brucellosis intervention aiming at elimination can be considered as a major perturbation towards a disease free equilibrium. Here we outline the different components of a science of brucellosis elimination involving all actors in public and animal health.

Prior to embark on brucellosis control and elimination activities the main reservoirs and their population dynamic must be known. The population dynamics of reservoir hosts directly determines the effective reproductive number (R_e) of brucellosis transmission and the persistence of its transmission. Because of its long incubation period, the role of density dependence and contact networks in brucellosis transmission is not well understood and needs more research. Effective reproductive numbers were estimated at 1.2 in small ruminants and 1.7 in cattle in Mongolia and a vaccination coverage of 80% was recommended for the forthcoming mass vaccination campaign from 2000-2010. A recent study in Mongolia showed that since the end of the socialist period in 1990 the livestock population of goat, cattle and sheep changed in different ways. Especially the goat population rose sharply because of the high price of cashmere. However, all livestock species suffered heavy losses from recurrent snow storm catastrophes in 2001 and 2009. The near doubling of the Mongolian livestock population very likely contributed to the largely ineffective mass vaccination campaign (2000-2010) because the number of vaccinations was not sufficiently adjusted to the growing number of animals. The high human brucellosis seroprevalence in 2012 indicates that brucellosis transmission in Mongolia is ongoing and a new mass vaccination started, whereby the achieved vaccination coverage is now systematically assessed.

Effective interventions against brucellosis rely on important enabling public conditions. Not only public or private veterinary services need be sufficiently staffed and being able to cover the area of intervention. Sufficient human and veterinary laboratory capacity is needed with the ability to characterized bacterial strains. Implementing test-and-slaughter control schemes require sufficient public funds to compensate farmers for culled stock and a relatively corruption-free environment. Above all, a societal consensus is needed by including all actors in so called ‘transdisciplinary’ stakeholder processes. Thereby, all involved stakeholders contribute to the identification of priority actions and may create trust among themselves.

Understanding the disease ecology allows to identify the interventions with the highest effect. For this purpose, joint animal-human cross-sectional studies provide a snapshot of disease frequencies in the most important reservoir hosts and may indicate the main source of human infection. In a representative study in Kyrgyzstan human seroprevalence was significantly related to sheep seroprevalence but not to goat and cattle. Such studies should be

complemented by molecular epidemiological studies ascertaining transmission pathways and reservoir hosts. In the Kyrgyz, example *Brucella* (*B.*) *melitensis* strains were isolated mostly from sheep and a few from cattle but none from goats. Unfortunately, the Kyrgyz health authorities refused to share human strains which would have allowed to ascertain the sources of human strains. In addition, diagnostic tests are most often not validated properly. Recent Bayesian analytical work allowed estimating true seroprevalences for several diagnostic tests without a Gold Standard. In addition to the validation of diagnostic test, the shear lack of diagnostic tests in district public health centres leads to a huge underreporting of the disease. The Mongolian authorities have recently adopted a modified Rose Bengal test for human diagnosis in district health centres.

Mathematical modelling of the transmission dynamics of brucellosis assists follow up the effectiveness of interventions. Such models are the backbone to intervention economic analysis and can be used to assess the animal-human interface. In this way it can be shown that *B. melitensis* seems to be more readily transmitted to humans than *B. abortus*. In Mongolia, the small ruminant to human transmission constant was 13 times lower than that between small ruminants, i.e. one infected small ruminant infected 13 other small ruminants before one person was also infected. Assuming the cattle were mostly infected with *B. abortus*, the cattle to human transmission constant was 165 times lower than that between cattle transmission. Such findings are still very rare and need to be further assessed. A mathematical analysis of an elimination intervention provides the tool to validate fundamental properties of transmission dynamics like the threshold population below which brucellosis transmission is interrupted. For the pastoral system in Mongolia we estimated that the threshold density to interrupt transmission for cattle was 1.2 (min. 0.6; max. 8) cows/km2 and for small ruminants at about 6.8 (min. 4.5; max. 21) small ruminants/km2. Mathematical models of brucellosis should be combined with molecular strain characterization using full sequencing to address the risk of re-introduction into a previously brucellae free zone.

The effectiveness of any intervention is the product of the vaccine efficacy times the achieved coverage. The coverage, the proportion of animals effectively reached by mass vaccination is determined by the vaccine availability, accessibility, affordability, acceptability and adequacy. Further it depends on the service provider compliance and the adherence to the mass vaccination by the animal holder. Understanding the determinants of the effectiveness of an intervention requires a close interdisciplinary collaboration among others between vaccine biology, health systems research, health economics, social and cultural sciences and animal health. Even if all interventions factors have a relatively high performance, they are all related to each other in a multiplicative way. This may lead to very low community effectiveness below the threshold to interrupt the transmission of brucellosis. Yet, the detailed understanding of the intervention factors is critical for achieving a successful intervention. Understanding the effectiveness of an intervention is a core element of the science of brucellosis elimination and is the focus of this talk. Novel quantitative models of intervention effectiveness should allow identifying the most sensitive effectiveness factors, which need to be harnessed to make an intervention successful.
From our research on dog rabies elimination in African cities we have learned that above all, the monitoring and evaluation of vaccination coverage is a critical element for the follow up of an effective intervention. Often, the basic principles of random sampling proportional to size are not understood and it does not help if vaccination coverage assessments are only done in vaccinated herds. Vaccination coverage data, i.e. the proportion of effectively vaccinated animals, combined with field epidemiological seroprevalence and sequence data of isolated strains can be used to validate the effectiveness of the intervention as predicted by models like the ones cited above.

Intervention methods depend on the prevalence of the disease and the available funds. It is well established that mass vaccination of livestock is recommended as long as brucellosis seroprevalence is above 1 %. Below this, a test-and-slaughter method is recommended,

whereby seropositive animals would be culled after systematic sampling. However, we should bear in mind that most of the developing countries would not be able to compensate farmers for culled stock and the level of corruption would probably not allow a successful implementation of such schemes. Intervention methods should thus be carefully analysed within a given political and socio-economic context. Cross-sector economic analyses provide a societal perspective which could lead to the sharing of intervention cost between the livestock and public health sectors (Roth *et al.* 2003). To achieve elimination, novel financing instruments could be examined like development impact bonds (DIB) which are currently examined for the elimination of sleeping sickness in Uganda. Thereby risks are shared between national governments, institutional donors and private investors. Another precedent is the successful elimination of Rinderpest which could be re-invigorated for the elimination of livestock brucellosis at least in areas with negligible wildlife reservoirs.

Brucellosis elimination is achievable also in developing countries. Above all, it requires a societal consensus addressing issues like compensation or intervention types which should not be decided over the head of livestock owners. Successful examples of disease elimination shows that all actors need to be involved from the start, as all of them play an important role. Specifically the public health and animal health sectors should work as closely together as possible. Unfortunately, the structure of this conference reflects the poor communication between human and animal health and I hope that future brucellosis conferences will be integrated, aiming at a joint problem solving of doctors and veterinarians. Regional approaches, for example involving Mongolia, China and Russia will be needed to address issues of cross border transmission. In addition, in this way brucellosis control would probably makes a significant practical contribution to the creation of trust and peace building. The proposed science of brucellosis elimination is not alone, it can learn from other initiatives, like the science of Malaria eradication or the science of rabies elimination. Further orientations could aim at combining for example brucellosis and echinococcosis mass vaccination. We could also think about a locally adapted extended farm package including dog rabies, echinococcosis, brucellosis, anthrax, and FMD. Brucellosis can be eliminated but we need to work all together in an evidence based systemic way.

Oral Presentations (O30-O35)

O30: Seroprevalence survey of animal brucellosis in Afghanistan

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The approach to control, prevention or eradication of brucellosis in a country or region will depend on many factors, such as the level of infection in the herds or flocks, type of husbandry, economic resources, public health impacts, and potential international trade implications. Decision making by those charged with policy making is likely to be intuitive unless accurate and current epidemiological information is available. Several Middle Eastern and Central Asian countries have recently reported an increase in the incidence of human brucellosis and the appearance of new foci. In recent years, the disease has re-emerged as being of major importance in Central Asia and has been found to be common in several localities of Afghanistan. Evidence based surveys carried out by officers from MoPH, MAIL (national and provincial level), AHDP and Massey University EpiCentre for New Zealand Agriculture Support Program (ASP) have demonstrated that the disease is especially common in certain areas of Bamyan, Kabul, Kapisa, Kunduz, Baghlan and Badakhshan, where human clinical cases have been detected and subsequently, traced back to infection in cattle, sheep and goats in the same villages. During the winter and spring of 2010-2011, the US State Department funded the FAO to conduct a passive-surveillance survey of 'Causes of Abortion' on behalf of the Directorate of Animal Health. This included cases of abortions in sheep, goats and cattle, with the collection and testing of samples from aborted fetuses and placentas. In the study, Q fever and brucellosis were found to be two common causes of abortion.
Before implementation of a brucellosis control program, accurate epidemiological information was required for decision makers and funding agencies. To provide this, a National Brucellosis Sero-Survey (NBSS) was designed and conducted to determine the prevalence of brucellosis in Afghanistan. Collection of data involved carrying out a national two-stage cross-sectional brucellosis serological survey of ruminants in all 34 provinces in Afghanistan. From November 2012 to April 2013, sera from 14,489 sheep, goats and cattle from 24 Kuchi flocks and 166 sedentary herds in 190 epidemiological units were collected (through the Sanitary Mandate: public-private partnership) and screened for antibodies to *Brucella* using the Rose Bengal test as the primary test and cELISA as the confirmatory test. The baseline individual brucellosis serological prevalence was 3.5 % (95%CI=3.1-3.9) for sheep, 2.3 % (95%CI=1.9-2.8) for goats and 2.6 % (95%CI=1.3-3.0 %) for cattle. The baseline herd & Kuchi seroprevalence was 24 % (95%CI=18.7-30.8), which indicated that there are about 593,100 seropositive animals throughout the country. According to the analyzed data and test results, one or more sero-positive animals were found in 46 of 190 (166 Villages, 24 Kuchi flocks) randomly selected villages/Kuchi flocks, and about 7,524 villages/Kuchi flocks in Afghanistan have seropositive animals. The total number of villages in Afghanistan is 31,353 and about 10,000 Kuchi flocks. The herds with highest prevalence were found to be located in the western part of Afghanistan, while the medium and low herd prevalence areas were situated in the middle and the eastern part of Afghanistan (high herd prevalence >40 %, 40 %> medium >20 %, low <20 %).
The information derived from this survey will serve as a baseline from which to measure the success (or otherwise) of the National Brucellosis Control Program (NBCP), which is the subject of a separate program running from 2013-2018.

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O31: Descriptive epidemiology of bovine brucellosis in Gauteng Province, South Africa, 2009-2013

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Brucellosis is a worldwide, zoonotic disease of economic importance. The prevalence of bovine brucellosis has increased in South Africa in recent years despite a national control programme utilising serological surveillance, test-and-slaughter and preventative vaccination. There is a paucity of published data on risk factors for both cattle and human health, and programme optimisation. This study is the first in a series to evaluate and mitigate the economic and zoonotic impact of brucellosis in Gauteng province, the smallest but most populous province in the country.

We analysed data from all serological samples (n=150,000; 2,746 herds) tested by the reference laboratory (Onderstepoort Veterinary Institute) between 2009 and 2013. A complement fixation test (CFT) >60I U/ml was used to define a positive animal or herd to exclude S19 vaccine reactors. Data were captured using Access (Microsoft 2010) and analysed in Excel (Microsoft 2010) and RStudio (version 0.98.597). 2013 accounted for 36 % of all cattle tested. The median tested herd size was 24 (range 1–1,205). Herd prevalence increased from 17 % (2009) to 21 % (2013) in the province while animal prevalence decreased from 2.1 % in 2009 to 1.0 % in 2013. Herd size was a significant risk factor (Chi-square for trend =113.0, $p<0.05$; OR for herd size >150=3.85 that of smaller herds), which differs from anecdotal reports of higher prevalence in smallholder herds compared to larger, commercial units.

This study emphasised the need to determine the zoonotic impact of brucellosis in Gauteng and to evaluate the current control strategy. We discuss the implications of this study and the further analyses that are underway to evaluate the representativeness of the herds tested, the effect of test thresholds and the relative sensitivity of the tests employed.

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O32: A nationwide cross-sectional study of ruminant brucellosis in Jordan

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Brucellosis is a neglected zoonosis that remains endemic in many of the Middle Eastern countries including Jordan. Control of brucellosis in ruminants may the keystone for the prevention of human infection. Reliable frequency estimates of ruminant brucellosis are needed to inform control but they are notably lacking in Jordan.
Our objectives in this study are i) to estimate the true seroprevalence of brucellosis in cattle and small ruminants ii) to identify risk factors associated with *Brucella* spp. seropositivity at herd/flock level and iii) to describe the spatial distribution of ruminant brucellosis in Jordan.
We conducted a cross-sectional study from May to October 2013 including all the twelve Jordanian governorates. A total of 204 cattle herds and 393 small ruminant flocks (289 sheep flocks, 52 goat flocks and 52 mixed flocks containing both sheep and goats) including 1,810 cows, 2,123 sheep and 541 goats were tested for *Brucella* spp. antibodies using ELISA. The true seroprevalence of herds/flocks with at least one seropositive animal was estimated at 22 % (95%CI: 16, 29), 45 % (95%CI: 30, 62), 70 % (95%CI: 55, 80), 34 % (95%CI: 28, 40) and 19 % (95%CI: 11, 25) for sheep, goat, mixed, all small ruminant flocks and cattle herds, respectively. The seropositive herds and flocks were heterogeneously distributed all over the country with the Northern governorates having the highest seroprevalence compared with Southern and middle governorates.
Out of twelve studied variables at cattle herd level; adding new animals during the last year (OR=11.7; 95%CI: 2.8, 49.4) enhanced herd seropositivity, while disinfecting herds (OR=0.04; 95%CI: 0.01, 0.15), separate newly added animals (OR=0.09; 95%CI: 0.03, 0.29) and having calving pens (OR=0.14; 95%CI: 0.05, 0.43) reduced the risk of herd seropositivity. In small ruminant flocks; lending/borrowing ram (OR=8.9; 95%CI: 3, 26.1), feeding aborted material to dog (OR=8; 95%CI: 3.5, 18.1), flocks with goats (OR=6.9; 95%CI: 3.1, 15.4), adding new animals to the flock in the last year (OR=5.8; 95%CI: 2.5, 13.6) and large flock size (OR=2.2; 95%CI: 1, 4.6) enhanced flock seropositivity, while separating newly added animals (OR=0.16; 95%CI: 0.05, 0.47), isolating aborted animals (OR= 0.19; 95%CI: 0.08, 0.46) and pen disinfection (OR=0.37; 95%CI: 0.16, 0.83) reduced the risk of flock seropositivity .
To our knowledge, this is the first nationwide study with probabilistic sampling aiming to estimate the prevalence of ruminant brucellosis in Jordan. The results of this study confirm that ruminant brucellosis is widely spread in Jordan with high seroprevalence. The decision on how to better allocate resources to brucellosis control within a certain country or region should be based, among other considerations, on the existing frequency of infection. Furthermore, an understanding of spatial and demographic heterogeneities could help targeting control efforts towards specific subpopulations. Accordingly, the current study provides baseline information on the frequency and distribution of ruminant brucellosis in Jordan to inform the national control program.

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O33: The identification of *Brucella* strains isolated during mass vaccination campaign with *B. melitensis* Rev1 and *B. abortus* S19 vaccines in Turkey

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Brucellosis still continues to be one of the major zoonotic agents in many parts of the world. The only logical way to control the disease in endemic regions or countries is vaccination of all animals in a flock or herd. In Turkey, a mass vaccination campaign was launched in the beginning of year 2012. According to this control program, all abortion cases were to be transmitted to relevant authorities. A total of 3,542 *Brucella* isolates received by the National Brucellosis Reference Laboratory were identified by conventional and molecular typing procedures from 2012 to the end of 2013. 1,671 were isolated from cattle, 681 from sheep and 1,190 from goats. Of the cattle isolates, 1,273 were identifed as *Brucella* (*B.*) *abortus* biotypes, 394 *B. melitensis* biotypes and four *B. abortus* S19 vaccine strain. From the goat isolates, 1,076 were *B. melitensis* Rev1 vaccine strain, 104 *B. melitensis* biotypes and ten *B. abortus* biotypes. Isolates from sheep showed similar identification profile to those obtained from goats and 290 of the isolates were identified as *B. melitensis* Rev1 vaccine strain. The results showed high numbers of vaccines induced abortions in especially goats followed by sheep, while *B. abortus* S19 induced abortions were very few and considered as negligible. Such dramatic abortions due to vaccination could be happen if pregnancy status of the animals is ignored, especially when mass vaccinations are practised. Cattle brucellosis caused by *B. melitensis* strains were found as dramatically high whilst *B. abortus* induced abortions were relatively much lower in sheep and goats. Regarding to atypical characteristics, penicilline and thionine sensitivities were encountered mostly among others. According to the results, it was recommended that animals should only be vaccinated when they are not pregnant. Husbandry practices that include animals of different species being herded together increase the likelihood of animals being exposed to the disease. This factor should be taken into consideration in the planning and execution of control and eradication programs.

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O34: Temporal analysis and costs of ruminant brucellosis eradication program in Egypt between 1999 and 2011

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Brucellosis is a highly contagious disease affecting almost all domestic species, leading to severe economic losses due to abortion, infertility and reduced milk production. Data for the prevalence of brucellosis in ruminants in Egypt are scarce. Recent studies suggest the disease is endemic in all ruminant species, with a high prevalence. Since 1981, the national control program involves testing all female ruminants older than six months and slaughtering serological positives, with voluntary vaccination of calves using *Brucella abortus* S19 vaccine and lambs and kids using Rev1 vaccine. The aim of this study was to assess the financial costs and the impact of the current control program on the temporal pattern of brucellosis among cattle, buffalo, sheep and goats using data from the active surveillance program between 1999 and 2011. Statistical analyses were conducted to allow comparison between different years and ruminant species using IBM SPSS (IBM Corp.). To compare between seropositive proportions for different years for each species, a univariate binary logistic regression model, with seropositive as the response variable and year 1999 as a reference was used. The results showed that, the frequency of testing cattle was higher than other species with a maximum of 4.34 % in 2006. The proportion of tested buffalo and sheep was below 2 % except in 2001 (2.3 % in buffalo). The proportion of tested goat was <1 % apart from 2008 and 2009 in which 1.04 % and 1.06 % were tested, respectively. The highest proportion of seropositive was in sheep, followed by goat, cattle and buffalo with an average of 1.68 %, 1.18 %, 0.73 % and 0.48 %, respectively. The highest proportions of seropositive cattle (1.27 %), buffalo (1.08 %), sheep (3.65 %) and goat (3.22 %) were observed in 2002, 2008, 2009 and 2008, respectively. The proportion of seropositive cattle was significantly increased from 2000 to 2004 then significantly decreased from 2005 to 2011. The proportion of seropositive buffalo fluctuated year to year, however there was a significant increase in 2008 (OR 3.13, 95%CI 2.69-3.66, $p<0.001$). There was a decrease in the proportion of tested sheep that were seropositive during the study period except in 2001 and 2009 in which there was a significant increase. The proportion of seropositive goats increased in 2000 and 2001, and then decreased from 2002 to 2007. In 2008 there was a significant increase in the seropositive proportion of goats (OR 2.53, 95%CI 2.21-2.90, $p<0.001$). The average annual cost for sampling and testing all ruminant species was US $1,658,048. The total costs for sampling and testing ruminants in the study period was US $21,554,630; the cost for cattle accounted for more than 50 %. The average annual compensation cost was US $1,427,546. The total compensation in the study period was more than US$18.5 million; about 65 % was spent on cattle. The average annual total cost for the eradication program including testing and compensation was more than US $3 million. The highest costs were for cattle particularly in year 2002 and 2003. The total cost for the eradication program including testing and compensation in the study period was more than US $40 million, from which more than 56 % for cattle. According to our assumption for voluntary vaccination, the annual cost was US $703,436. Despite significant government expenditure on brucellosis eradication, these results indicate that brucellosis is still endemic in ruminants and a serious threat to humans in Egypt. Further studies are required for the effectiveness of the current control strategies and alternative strategies should be considered. The socio-economic impact of brucellosis and its control measures should be investigated.

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O35: Investigating strategies to reduce the risk of brucellosis: Opinions of Albanian sheep farmers

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Brucellosis is considered an important zoonotic disease in Albania, with over 300 human cases reported annually. A national brucellosis control programme was initiated in 2012, involving the annual vaccination of all small ruminants for two consecutive years, followed by vaccination of replacement animals in subsequent years. The aim of the programme is to reduce brucellosis prevalence in small ruminants, thereby reducing transmission of the disease to humans and cattle. The programme also aims to support private farm-level measures to reduce the risk of brucellosis entering into or spreading within a flock. The success of the programme is dependent on economic, social and political factors that influence its implementation and sustainability. Therefore an understanding of farmers' knowledge of brucellosis and its control will provide core information to enhance success.

However, very little is known about the level of knowledge regarding brucellosis transmission and the perception of risk amongst livestock owners in Albania. An understanding of farmers' knowledge of the ways how brucellosis is introduced and maintained in flocks, and identifying the levers and barriers of risk mitigation strategies are needed. Important information includes what measures they take to mitigate risk to themselves and to their livestock, and why they choose certain biosecurity measures and not others. The aim of the study was to investigate the determinants of brucellosis prevention and control practices used by farmers in Albania to aid the design of feasible and sustainable disease control and public health policies.

Focus group discussions with sheep farmers were conducted to assess the importance of brucellosis and to explore the range of social, political and economic factors that influence the transmission of brucellosis or its control. The focus groups were conducted in Albanian, transcribed and translated into English, then analysed using the Framework Method technique for qualitative data.

Mapping transhumance, purchases and sales of live ruminants demonstrated that all regions of Albania were epidemiologically linked and transboundary movement with neighbouring countries occurs, including Kosovo, Macedonia and Greece. Brucellosis was considered a constraint to livestock production, although other causes of abortion were also mentioned. Participants frequently raised concerns that a definitive diagnosis was rarely reached. Knowledge on the foodborne risk of brucellosis was universal, and all participants reported heating milk before consumption or processing at home, although few farmers treated milk before selling it. In general, farmers were aware that direct contact with animals was a risk; however, many felt that 'it wouldn't happen to them' thus rarely protected themselves when handling ruminants during parturition.

The study identified a range of factors affecting the decision-making process by Albanian farmers with respect to brucellosis risk mitigation. It highlighted the need to shift from a national brucellosis control programme to a more comprehensive strategy for zoonotic diseases in the Balkans. It also suggested that advising farmers on the broader benefits of biosecurity may be more successful than a disease focussed education policy.

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Poster Presentations (P100-P124)

P100: *Brucella suis* biovar 2 in cattle in Europe: Results of an experimental infection

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Last year, three member states of the EU (Belgium, Poland and France), that are 'officially free of bovine brucellosis (OBF)', reported the isolation of *Brucella* (*B.*) *suis* biovar 2 (BS2) in cattle showing brucellosis positive serological reactions. In the EU, infection associated with BS2 in cattle is the result of a spill over from a wildlife reservoir: wild boars (*Sus scrofa*) and hares (*Lepus europaeus*). In order to study the virulence of BS2 in cattle and to define accurate testing strategies, the following experimental infection was set up: three groups of heifers were infected; three animals with BS2; three with *B. abortus* biovar 3 (BA3) and two with *Yersinia enterocolitica* O:9 (YO:9). *Brucella* spp. were inoculated subcutaneously ($1.5\ 10^7$ BA3 and $1.08\ 10^7$ BS2). YO:9 ($1.5\ 10^{12}$) were administrated orally (per os).
Animals were followed for 1 month post infection. Blood samples were taken every three days for brucellosis serology (indirect ELISA, SAW-EDTA, RBPT and CFT). For animals infected with BS2, positive reactions were observed with SAW-EDTA, RBPT and CFT. Serological reactions appeared later (5 to 10 day after positive serology of *B. abortus* bv 3) and were of lesser amplitude compared to those observed for animals infected with BS3. In addition, mild and transient serological reactions were observed in animals infected with YO:9: results in indirect ELISA test were rapidly positive (when and for how long) but negative at the end of the experiment for the two cattle. Combined SAT-EDTA, CFT and RBPT positive results were observed only for one animal. Lastly, a skin test with Brucellergen (Synbiotics®, Lyon, France) was performed at day 21 post infection. Animals infected with YO:9 tested negative. Conversely animals infected by *Brucella* spp. tested positive with slight reactions in animals infected with BS2 (average 1.36 mm for BS2 infected animal and 4.8 mm for the *B. abortus* infected group). After slaughter, bacteriological tests were performed in all the animals on 11 pairs of organs. For animals infected with BS2, two were positive (BS2 was isolated from three organs for one animal and only from one organ for the other animal). For the animal infected by BA3, bacteria were isolated in all animals (from five, seven and two organs respectively). Our results suggest that BS2 is less virulent than BS3 in cattle and that BS2 is unlikely to persist in cattle herds ($R01<1$).
The results of the experimental infection with *B. suis* bv 2 reproduce field observations in OBF member states, and thus BS2 is likely to be the cause of positive serological reactions in cattle.
In conclusion, the only diagnosis of certainty is the isolation of *Brucella* spp. YO:9 does not induce positivity in the brucellosis skin test, whereas BS2 does. In OBF-member states, transient serology of limited amplitude, associated with positive skin test in a small number of animals in cattle herds, is suggestive of a BS2 spill over from the wildlife reservoir.

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P101: *Brucella abortus* isolated for the first time from the cattle livestock in central region of Algeria

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In Algeria, brucellosis was described for the first time at the end of 19th century. It has proved to be an endemic and widespread disease in the bovine population and several cases were detected in human. This disease causes significant economic losses. The available previous data were based only on serological surveys. Up today, no bacteriological investigations were carried out. Hence, the prevailing species and biotypes of *Brucella* in Algerian livestock are not yet known. In order to elucidate the epidemiology of this disease, isolation of *Brucella* is required. That is why our study aims to isolate and to identify the different biotypes of *Brucella* prevailing in the central region of Algeria from infected cattle in order to establish their distribution in this region. Indeed, the central region of Algeria includes 25 % of Algerian cattle population. A survey conducted between 2004 and 2005 showed that brucellosis was an important prevailing disease in this area. We found that the estimated herd and individual seroprevalence rates of the bovine brucellosis were about 3 % [CI 95 %: 2.31-3.69 %] and 0.8 % [CI 95 %: 0.68-0.94 %], respectively. In the same time, numerous human cases were detected (1,973 which corresponds to 19 per 100,000 habitants).
Between October 2011 and May 2014, 160 samples, originating from infected animals (detected during screening and slaughtered under the control program) including 81 milks and 79 lymph tissues (41 retropharyngeal, 36 supramammary and two inguinal lymph nodes) were collected from 91 cows coming from seven departments of the central region.
Brucella strain identifications were performed according to the technique described by the French standard AFNOR NF U47-105. The sensitivity of the isolated strains to several antibiotics (streptomycin, rifampicin, gentamicin, tetracycline, doxycycline and trimethoprim-sulfamethoxazole) was tested by E-test method.
A total of 50 strains of *Brucella* (*B.*) were isolated, 24 from milks and 26 from lymph nodes (seven from retropharyngeal, 17 from supramammary and two from inguinal lymph nodes). Forty-six strains belong to *B. abortus* bv 3, one to *B. abortus* bv 2, two to *B. melitensis* bv 3 and one to *B. melitensis* bv 2. It is the first time that *B. abortus* bv 2 and *B. melitensis* bv 2 were isolated from a region of the Maghreb. It is noteworthy that two of the isolated *Brucella* strains were found to be resistant to streptomycin, the main used antibiotic for the treatment of human brucellosis in Algeria.
Far as we know, the study reported herein represents the first investigation in Algeria on the characterisation of *Brucella* strains from cattle. The obtained results may contribute (i) to establish the epidemiological map of the distribution of different *Brucella* strains prevailing in the central region, (ii) to provide information which may be helpful in planning control program (iii) and to implement a better fight against this zoonosis.

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P102: Isolation and characterisation of *Brucella abortus* biovar 3 from cattle in a dairy herd in the southern highlands of Tanzania

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Brucellosis is a disease of veterinary public health and economic importance worldwide. Almost all studies on livestock brucellosis carried out in Tanzania have reported brucellosis based on serology results. The aim of the present study was to isolate, identify and characterize *Brucella* spp. and to estimate it's within-herd prevalence in dairy cattle and to investigate spillover effect to small ruminants and dogs from a dairy herd in Tanzania. This herd had about 350 cattle that are crossbred of Friesian/Ayshire with zebu or boran mingling together with 130 goats, 90 sheep and six dogs. Animals are taken into unfenced pasture area during the day and kept indoors during the night with minimum contact with pastoral and other dairy cattle around during grazing. Anti-brucellosis vaccination has never been performed in this farm. Serum from cattle, goats, sheep and dogs; milk and aborted materials from cows were used. Rose Bengal plate test, indirect ELISA and MRT were used to detect *Brucella* antibodies. Bacteriology was done using selective media whereas identification and characterization was done using PCR, gene sequencing and classical biotyping procedures. A high within-herdprevalence of 48 % (95%CI 0.4-0.5) for cattle was observed in this study by testing with a commercial indirect ELISA. In sheep and goats 2/35 and 1/50 positive individuals respectively were observed while all dogs were negative. *Brucella* spp. was isolated from aborted fetal organs and membranes from one cow. *Brucella* spp. specific PCR targeting IS711 indicated that the isolate belongs to the genus *Brucella*. Multiplex PCR (Bruce ladder) identified the isolate as *B. abortus* wild type, and classical biotyping classified as *B. abortus* bv 3. This is the first report of isolation and characterization of *B. abortus* bv 3 from dairy cattle in Tanzania. Importantly, dairy replacement heifers are sourced from this herd by smallholder communal farmers in the area posing risk of infection transmission. Serology has indicated spillover of *Brucella* infection from cattle to small ruminants but not to dogs. Since *B. abortus* is a potential zoonotic agent, further studies on human infection are necessary.

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P103: Isolation of a *Brucella suis* strain from raw milk from a *Bos indicus* x *Bos taurus* cow, in Barinas State, Venezuela

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Brucellosis is a zoonosis present in the countries of the American continent. It has been known in Venezuela since 1930. The most important biotype in our country is *Brucella* (*B.*) *abortus* causing disease in cattle, followed by *B. suis* in pigs and wild fauna. Brucellosis is considered endemic in Barinas which is a livestock zone, from where confirmed cases in personnel at risk in slaughter and milking rooms have been reported. In general, isolation and classification are not done, diagnosis relies on serology.
Fifteen individual samples from cow milk, collected from dual purpose dairy farms, with crossbreed cattle *Bos taurus* x *Bos indicus*, located in Sucre Municipality, Parish of Ticoporo, were studied in the months of May and June. The samples were aseptically taken, refrigerated and sent within the first 24 hours to the processing laboratory.
The milk samples were examined for antibodies to *Brucella* using the milk ring test (MRT) as screening test. Both suspected and positive samples were inoculated on *Brucella* agar and classic identification for morphology, biochemistry and special tests were carried out. The strains suspected to be *Brucella* were identified using API 20NE and those strains identified as *Brucella* spp. were subjected to DNA extraction and multiplex PCR for *Brucella* spp. to type them.
The MRT detected four suspicious (26.66 %) and nine positive (60 %) cows. Five suspicious colonies were gained of which one was identified as *Brucella* spp. Finally, it was transferred to the *National Polytechnic Institute* of the NSBS (Mexico) and typied as *B. suis*. The last isolation of *B. suis* in Venezuela was done in 1991 from a Capybara (*Hydrochoerus hydrochaeris*) in the Venezuelan llanos.

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P104: Study of *Brucella* spp. circulating in milk-producing animals in Goygol region of Azerbaijan

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Brucellosis was first diagnosed in the Republic of Azerbaijan (AJ) in 1922 and spreads quickly to more than two thirds of the country's districts (or rayons) in <30 years. Changes in governmental system brought on by the collapse of the Soviet Union in 1991 have likely contributed to the persistence of the disease due to decreased funding for surveillance and eradication programs. AJ currently ranks 13th among countries with the highest incidence of disease, with an estimated annual incidence in the year 2,000 of >50 cases per million people. Six species of *Brucella* have been identified in AJ by previous research: *Brucella (B.) suis, B. abortus, B. melitensis, B. neotome, B. ovis,* and *B. canis*.

Based on the information mentioned above, a project will be created that will characterize human and animal brucellosis in the Goygol region of AJ. The long term aim of this project is to enhance the quality of the surveillance system of AJ using appropriate sample collection and analytical methods. Thus, appropriate response measures can be taken, to reduce the overall incidence of brucellosis in man and animal. The Goygol region is located in a high risk area category according to a study of historic data that examined records from 1995 to 2009 and analyzed the spatial and temporal distribution of human brucellosis in AJ. It is also important to emphasize that Goygol region is located in a seasonal movement area of farm animals (especially sheep).

The project will be led by the Goygol Zonal Veterinary Laboratory and Rayonal Veterinary Office and sets out to accomplish the following objectives: 1) improve sample collection and transportation methods for *Brucella* surveillance; 2) determine the species and biovars of *Brucella* circulating in milk-producing animals in the selected region; 3) compare results of diagnostic assays testing blood and milk by bacteriology and serology (Rose Bengal/Milk Ring test, complement fixation test); 4) determine any *Brucella* isolate identified in milk as a potential source of infection for humans in the area; and 5) enhance skills of laboratory personnel on *Brucella* diagnostic techniques. The hypothesis of this study is that monitoring of brucellosis in lactating livestock by using bacteriological detection methods on milk samples is more effective than on blood samples.

In addition, expected outcomes of the project are the education of animal owners on brucellosis; enhancement of laboratory and field sampling skills of veterinarians; decrease of economic losses and risk of transmission to humans; and implementation of anti-epidemic activities at the proper level in Azerbaijan. Overall, this project will serve as a baseline for further research on the characterization of *Brucella* in other areas of Azerbaijan and will enhance overall surveillance and epidemiological analytical capabilities for brucellosis in the country.

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P105: Update about typing of *Brucella* field strains isolated from livestock and wildlife populations in Italy, 2007-2012

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Brucellosis is a disease of public health concern in many countries around the world, where the disease is endemic and surveillance plans as well as animal vaccination programs are in place. Also territories free from the disease may be at risk given the fact that the geographical distribution of brucellosis is constantly changing and new foci may emerge or re-emerge. Ten species are currently recognized within the genus *Brucella* (*B.*), and each species has distinctive host preferences, pathogenicity and epidemiology. The knowledge of the prevailing species and biovars of *Brucella* field strains isolated in outbreaks is an important epidemiological tool to better understand the epidemiology of the disease and to give support to the tracing-back of its origin, giving important information for the formulation of policies and strategies for the control of brucellosis in animal populations. The present study considers *Brucella* strains submitted for typing by the Italian Istituti Zooprofilattici Sperimentali to the National Reference Laboratory for Brucellosis from January 2007 to December 2012. *Brucella* field strains were identified using both classical and molecular techniques. Species and biovar identification were performed by evaluating agglutination with anti-A, anti-M and anti-R monospecific sera, the production of H_2S, CO_2-dependence and the growth in the presence of basic fuchsin and thionin at final concentrations of 20 µg/ml. Results were confirmed with AMOS-PCR (Abortus Melitensis Ovis Suis-PCR) and PCR-fragment length polymorphism (PCR-RFLP) techniques. For biovar identification, amplification of *omp2a*, *omp2b* and *omp31* genes was performed, followed by restriction analysis of the amplicons using endonucleases (PstI, HinfI, TaqI, AvaII, NeoI). The total number of strains typed during the period considered has been 4,390: 2,458 strains of *B. abortus* (56.0 %), 1,786 strains of *B. melitensis* (40.7 %), 143 strains of *B. suis* (3.3 %) and three strains of *B. ceti* (0.1 %). These figures include also the isolation of vaccine strains (58 strains of *B. melitensis* Rev1 and four strains of *B. abortus* RB51). 2,337 strains (53.2 %) isolated from cattle were typed as *B. abortus* bv 1, 3, 6, *B. abortus* strain RB51 and *B. melitensis* bv 3, 275 strains (6.3 %) isolated from water buffaloes were typed as *B. abortus* bv 1 and 3, *B. abortus* strain RB51 and *B. melitensis* bv 3. 1,618 strains (36.9 %) isolated from sheep and goats were typed as *B. melitensis* bv 1, 2 and 3; *B. abortus* bv 1, 3, 6, and *B. melitensis* strain Rev1. 136 strains (3.1 %) isolated from wild boar were typed as *B. suis* bv 2 and *B. melitensis* bv 3. Nine strains (0.21 %) isolated from pigs were typed as *B. suis* bv 2. Ten strains (0.23 %) isolated from man were typed as *B. melitensis* biovar 3 and *B. abortus* bv 3. Two strains (0.05 %) isolated from horses were typed as *B. abortus* bv 1 and three strains (0.07 %) isolated from dolphins were typed as *B. ceti*. The animal species affected, the biovars typed, the geographical origin and the spatial distribution of isolates are analysed and discussed.

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P106: *Brucella* species circulating in animals in Georgia, 2013-2014 (Interim report)

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Brucellosis is an endemic zoonotic disease in the country of Georgia. Prevalence among humans is 3.7 per 100,000 population (Georgia, NCDC). The prevalence of brucellosis among the cattle and sheep-goat population has not been precisely estimated yet. In 2012, a seroprevalence study found that 17 % of Georgian villages had a brucellosis prevalence of at least 10 % in cattle; and 10 % of villages had at least 10 % brucellosis prevalence in small ruminants. This investigation sought to characterize the prevalent *Brucella* species.
Clinical samples (blood, serum, lymph node, milk and vaginal swab) from both cattle and small ruminants were collected from the animals (including the placenta and aborted fetal tissue (stomach, spleen lung). We cultured samples on Farrell's medium. Serum was tested by Rose Bengal test and confirmed by fluorescent polarization assay (FPA). Isolated cultures were retested by the *Brucella abortus/melitensis/ovis/suis (*AMOS) PCR assay to differentiate specific *Brucella* species. Further identification of *Brucella* species was based on morphology, staining, and the metabolic profile (catalase, oxidase, and urease) and was compared to the AMOS test results.
140 samples from 35 cows and their aborted fetuses were submitted to the Georgia Laboratory of the Ministry of Agriculture in 2013 (including four blood, 28 serum, 31 milk, 27 vaginal swabs, one lymph node, nine placentas, and 40 samples of aborted fetuses). The number of samples per animal varied from 2 to 7; twelve animals (34 %) had four samples. In total, 16 out of 35 cattle tested positive: 15 by both FPA and culture and one by culture only. Out of the 35 animals tested in this study, 15 tested positive using the Rose Bengal test and the FPA. A total of 14 positive cultures were isolated. All positive cultures were also tested by AMOS assay and classical microbiology identification methods; all 14 cultures were identified as *B. abortus*, however, several of these strains could not be typed using the AMOS PCR assay.
This study shows that *B. abortus* is the common species in cattle; however, several of these strains exist as a *B. abortus* variant because they could not be properly identified with the *Brucella* AMOS assay.

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P107: *Brucella* seropositivity among cattle in and around Kolkata, India

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Brucellosis is one of the important emerging zoonosis in developing countries and surprisingly a neglected disease in India. The disease has manifold pathological consequences without specific symptoms among animals and human beings. Sporadic occurrence of brucellosis is mainly reported from the Southern and Northern part of India. No generalized data are available regarding the disease in and around Kolkata, a metropolitan city of Eastern India. The present study was aimed to evaluate the burden of bovine brucellosis in this part of the country. A total of 988 blood samples from apparently healthy cattle were collected at a centrally located government registered slaughterhouse of Kolkata catering all varieties of cattle in this area mainly from four adjoining districts of Kolkata. Brucellosis seropositivity was tested by Rose Bengal plate test (RBPT) and ELISA. The result of the study revealed the *Brucella* seroreactivity of 4.85 % and 5.46 % by RBT and ELISA respectively. A possible cross reactivity was checked among the positive serum against salmonellosis (14.5 %) which is highly prevalent in this area. Other cross reactive agents like *Yersinia enterocolitica* O:9, *E. coli* O:157 H:7, *Pseudomonas maltophilia* 555, *Vibrio cholerae* were not tested as they are not prevalent in this area. This study reflects the significant burden of bovine brucellosis in this area. Interestingly, all of the seropositive samples were from apparently healthy animals. This cross sectional study may contribute in developing strategies in diagnosis and control of this ignored disease in this part of the country.

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P108: Assessment of brucellosis epidemiology in the Republic of Georgia and proposal of sustainable prevention and control

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Animal brucellosis is present in cattle, sheep and goats in Georgia. Regarding disease occurrence, the most recent available information has been provided by a National-Survey conducted by the National Food Agency (NFA) of the Ministry of Agriculture of Georgia, with the assistance of international stakeholders in 2011. The purpose of the survey was to estimate prevalence and geographical distribution of brucellosis at village level by regions. The survey was based on a random probability-based sampling scheme aiming to detect 10 % or greater prevalence with a 95 % confidence. At national level, the proportion of positive village-level herds/flocks was superior in cattle (16.3 %; 95%CI 4.2, 28.4) than in sheep and goats (8.1 %; 95%CI 0.1, 16.1), but with pronounced differences by regions. Territories where brucellosis was detected at a higher level (East and South Georgia) are epidemiologically linked by intense seasonal movements of flocks and herds for the use of natural pastures. Available data of human cases point to a non-homogeneous distribution along the country as well, and disease cases (166 in 2011) are higher again in the East and the South. The greatest risk factors for human beings are animal-related work, dairy production, and sheep ownership. In relation to species of *Brucella (B.)* involved, both *B. abortus* and *B. melitensis* have been isolated.

Under the framework defined by the Georgia National Animal Health Program 2013-18, and the Animal Health Action Plans 2013-14, the NFA sought the assistance of the Food and Agriculture Organization of the United Nations (FAO), and other international stakeholders, to support the national veterinary authorities in their efforts to contain the disease in animals and to assist them in developing scientific-sound strategies for a sustainable and cost-effective national brucellosis control program. Under the approach of *One Health Concept* and the horizon of OIE and EU standards, FAO is currently assisting the NFA[*] in building veterinary capacities for an effective brucellosis risk assessment, risk management and risk communication in the country, through the development of working teams receiving intensive on-the-job training and permanent advice. The basis of the control will be the vaccination of animals at risk and the development of an active surveillance system. The vaccination of cattle and sheep and goats will be first implemented in high prevalence regions and extended after to other parts of country, if necessary. The surveillance system, based on serology and bacteriology, will be designed to monitor the effectiveness of the vaccination campaign and also to give answers to the main existing information gaps regarding disease distribution and most prevalent *Brucella* species. Public awareness and collaboration between the public health and veterinary sectors will be part of the proposed program.

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P109: Prevalence of antibodies against *Brucella* in cattle production systems around Lake Mburo National Park

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The problem of brucellosis among animal owners and handlers is escalating day after day given its economic importance and the fact that it is transmissible to humans especially in developing countries where exposure risks and transmission avenues are not fully understood. The threat is worse among farmers along the wildlife-livestock interface where animals mingle during critical periods of marked shortage of pasture and water. A cross sectional study was conducted around Lake Mburo National Park in South Western Uganda, aiming at finding out and comparing the prevalence of antibodies against *Brucella* in the varying cattle production systems. The study was based on laboratory examination of bovine sera taken from varying production systems and breeds and animals aged older than one year. *Brucella* agglutination plate test was used as a screening test and confirmed by indirect ELISA. Furthermore, information was obtained through administration of a structured questionnaire targeting various stake holders in the communities. Results revealed the lowest individual animal prevalence in the Traditional Dual Purpose Production system, followed by Commercial Dairy Production system, and the highest prevalence was recorded in the Transitional Dairy Production system. The Chi Square test showed significant variation in prevalence among production systems. The study revealed that introduction of new stock is an important way of transmission of brucellosis between herds. Re-stocking is also an important risk factor for transmission in the Transitional Dairy production system. Congested and restricted confinements in the commercial dairy production also provide better opportunities for intra herd transmission of brucellae. In conclusion, cattle that are less confined under Traditional Dual Purpose Production even when mixed with wild animals at the interface are at a lower risk of contracting the disease compared to those animals confined in the other two production systems. The production system affects the risk of exposure. The study findings imply that control measures directed to more vulnerable groups of animals and screening animals before they are allowed to move should be implemented to control inter herd transmission.

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P110: Retrospective analysis of serological screening of ovine epididymitis in Ukraine

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Brucella (*B.*) *ovis* was first identified in Australia and New Zealand in 1953. The disease is found in many countries. In Ukraine it was detected for the first time in 1971. Economic losses are caused by culling of animals, disruption of stock breeding, conducting veterinary-sanitary and management measures aiming at the elimination of the disease.
Epizootic analysis of the results of serological screening was based on the official reports from the regional state laboratories of veterinary medicine and the State R&D Institute of Laboratory Diagnostics and Veterinary-Sanitary Expertise for 2008-2012.
In Ukraine scheduled serological screening and clinical examination of sheep for brucellosis is conducted once a year prior to mating campaign, formation of herds before and after the pasturing season, and during the preventive quarantine if breeding animals are on sale. Diagnosis is considered positive in case of the isolation of *B. ovis* or positive prolonged complement fixation test or immunodiffusion test.

Table: Positive results of CFT in different oblasts (2008-2012)

Year	2008	2009	2010	2011	2012
Samples tested by CFT	43,512	33,169	39,133	38,840	43,629
Altogether positive	542	217	260	1092	276
Crimea	40		73		33
Dnipropetrovsk	40	5	71	170	28
Kherson	62	51	1		4
Cherkasy	400	48		7	63
Kharkiv		83	106	832	59
Chernivtsi		30		7	
Donetsk			9	71	64
Chernihiv				2	
Rivne				3	4
Sumy					21

Results of the serological screening for 2008-2012 showed the increased number of oblasts where sheep were seropositive for infectious epididymitis.

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P111: Estimation of the prevalence of brucellosis and identification of *Brucella* species among cattle and small ruminants in Georgia

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Brucellosis is endemic in Georgia with several of thousands of positive animal cases and hundreds of human cases every year. Currently, Georgia uses a passive surveillance system and the endemic disease is still considered to be severely underestimated. This study was developed to identify which regions face the greatest risk so that cost-effective control programs can be used in these areas. The study was planned in two phases. Phase one included the serology of animals in all regions of the country to identify 'hot spots' for *Brucella* infected animals. Phase two included isolation, identification, and typing of *Brucella* isolates that are circulating among cattle and small ruminants. Phase one: in total, 17,372 samples were randomly collected from cattle and small ruminants and tested by both the Rose Bengal test and the fluorescence polarization assay (FPA) at the Laboratory of the Ministry of Agriculture. The results of this work demonstrated that brucellosis is not equally distributed between different regions of Georgia. Some villages are hyper-endemic with a prevalence greater than 10 % in susceptible animals. Phase two: a total of 110 tissue samples from 35 cows and their aborted fetuses (lymph nodes, vaginal swabs, aborted fetus, placenta, and milk) were collected throughout the country. Samples were cultured on selective media to isolate brucellae, and then suspected isolates were analyzed by AMOS-PCR. In total, 14 samples from seven different animals were positive by culture. None of them was confirmed by AMOS PCR, demonstrating the limitation of the test in determining suspected isolates e.g. of *B. abortus* which may circulate in Georgia. The study results are being used to design new cost-effective brucellosis control programs to be managed at the regional level and the efforts will focus on the regions with the highest levels of infection.

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P112: Seroprevalence of brucellosis and risk factors assessment in caprines of Ramechhap District

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A study was conducted to assess the seroprevalence of brucellosis and its associated risk factors in goats of Ramechhap District. Two-stage sampling procedure was adopted; in the first step, VDCs were selected from the district and in the second step individual animal was selected randomly from the respected VDCs. In all, three VDCs (Bhaluwajor, Okhreni and Chuchure) were selected and then 502 animals were sampled. Serum was screened for *Brucella melitensis* employing Rose Bengal plate test (RBPT) and indirect ELISA (i-ELISA). The overall prevalence of brucellosis was found to be 5.8 % by i-ELISA and 6.3 % by RBPT. The prevalence varied from 3.4 % to 7.1 % in various VDCs. Correlation and regression analysis was made to check the relationship between the prevalence of disease in various VDCs in relation to the goat population. The results indicated strong and positive correlation ($r=0.99$) and regression statistics indicated significant association (F-statistic: 928.6; $p=0.02$). Seropositivity significantly increases when herd size increases ($p<0.05$). Higher seroprevalence was found in the animals at lower altitude than at higher altitude ($p<0.05$). The disease prevalence was found to be significantly increasing with age ($p<0.05$). No statistically difference was observed ($p>0.05$) in prevalence between males and females. However, disease was significantly associated with abortion ($p<0.01$). The highest prevalence was also found in goats kept in close association with other species in comparison to those kept separately.

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P113: The status of bovine brucellosis in some parts of northern Nigeria

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The prevalence of bovine brucellosis in northern Nigeria appears to be on the increase. This study considered recent reports of the status of brucellosis in northern Nigeria. Multistage cluster sampling strategy, random sampling techniques and multiple logistic regression models were used. The various serological tests carried out included Rose Bengal plate Test, milk ring test and ELISA. The states studied were Kaduna, Kano, Adamawa, Taraba, Jigawa and Bauchi. The individual animal-level prevalence ranged from 4.0 % to 26.3 %, while a herd-level prevalence of up to 77.5 % was reported. Several factors have been attributed to the increase of brucellosis such as management system, attitude of the pastoral Fulanis towards brucellosis, nomadism, crossing national borders, mixed herds/flocks, poor surveillance and control programs etc. Several other risk factors were significantly associated with brucellosis such as age, sex, pregnancy status, pastoral systems, herd size, handling facility, and presence of small ruminants in the same farm, introducing new animals from different sources and borrowing or sharing of bulls. The seemingly increase in brucellosis in cattle is of immense concern especially the fact that cattle and their products, particularly contaminated milk, transmit the disease to humans. Consuming unpasteurized milk by Fulani herdsmen in northern Nigeria is a very common practice, making them susceptible to brucellosis. This study highlights the epidemiological picture of brucellosis in parts of northern Nigeria. The control of brucellosis can be achieved by enlightening the farmers on brucellosis and its public health significance and instituting an annual vaccination program of cattle at national level.

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P114: Serological survey on brucellosis of domestic animals in Srem and South Backa (Autonomous Province of Vojvodina)

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Brucellosis is a very important zoonosis in Mediterranean countries, in terms of public health and economy. Brucellosis in Serbia has been monitored and controlled during the past few years within the Ministry of Agriculture, which is financing this control program. Brucellosis in pigs occurs sporadically as individual case. However, considering the presence of brucellosis in sheep and goats in the southern part of Serbia and the events that followed, brucellosis in sheep and goats has spread to the north of the country. The first case of brucellosis in sheep and goats in Vojvodina region was detected in 1999, and since then the disease has been spreading. Contribution to the spreading of the disease is made by uncontrolled animal trade, a nomadic way of keeping animals. Because of that, the decision was made that brucellosis should be monitored in sheep and goats and cattle. The categories of animals which are screened every year are the following: sheep and goats older than 6 months and cattle older than 12 months. The examination is done once a year for each animal. Serologically positive animals are safely disposed and the expenses for the diagnostic procedure and disposal are taken from the governmental budget.
Within the control program of brucellosis in the region of Vojvodina during the period from 2001 to 2013, in total, 1,371,478 samples from domestic animals were analysed. Positive reactions were found in 1,344 sera samples, which is 0.097 %. The examined samples were from different animal species: 787,494 sera samples were from sheep and goats, 486,495 sera samples were from cattle, 95,807 from pigs, 1,151 from horses and 163 samples were from dogs. Following diagnostic methods were used: RBT, CFT and iELISA, and cELISA as conformation test. Positive findings were detected in 1,316 (0.17 %) sheep and goats, during the period from 2003 to 2007. During the same period, brucellosis was detected in eight human patients as well. After this period of time, no new positive animal cases were found. Positive findings were detected in twelve (0.0024 %) sera from cattle which were from the same farms where brucellosis in sheep was found. Fifteen (0.015 %) serum samples from pigs and only one from horses were diagnosed as positive for the same period.

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P115: Risk factors for bovine brucellosis in emerging or indigent farms Nkangala District, Mpumalanga, South Africa – a case control study.

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Brucellosis is a controlled animal disease in South Africa in accordance with the Animal diseases Act 35 since 1984. It is a disease of zoonotic importance, because it causes significant reproductive losses in animals, annually.
A case control study is being conducted with at least 40 farms known to be infected and 40 control farms. A case is defined as a bovine herd with at least one culture positive animal or at least two animals positive to the CFT between 2014 and 2015. Controls are bovine herds with negative test results in the last 3 years and no recorded outbreak within the last 5 years and no untested abortions in the last 6 months. A questionnaire is given to emerging or indigent farmers with a range of questions encompassing farm size and farming practices, location relative to game reserves specifically those with buffalos, milking practices, incidences of abortions in past 3 years and how they were managed, new animals introduced into herds and their source and history, details of animals culled or removed from herd, vaccination program against brucellosis in the herd, whether farmers share equipment/facilities/grazing with other herds (and the status of these herds), artificial insemination program (methods used and source of semen) and incidences of flu like symptoms in farm personnel.
There is little known documented information about this group of farmers in Mpumalanga and their practices and attitudes. The study will help provide data on risk factors for brucellosis in these farms. The study is currently still underway.
Farming practices, grazing practices and vaccination programs employed are expected to be major risk factors for brucellosis for emerging farmers. Vaccination of pregnant animals should be encouraged despite the risk of abortions because they are the most likely source of infection in a herd and thereby assist in eradication of the disease in communal farms.

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P116: Brucellosis outbreak in a rural endemic region of Mexico

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Brucellosis is a worldwide zoonotic disease. Humans can be infected mainly by either the consumption of raw milk and unpasteurised fresh cheeses or by contact with infected animals, mainly in endemic regions. Here, we investigated a brucellosis outbreak in an endemic region of Mexico. Microbiological culture of human blood, raw milk from cows and goats, and fresh cheeses was performed to isolate *Brucella*. Identification of the bacteria was done by bacteriological procedures and by multiplex Bruce-ladder polymerase chain reaction. *Brucella melitensis* was isolated from patients, infected goats, and fresh goat cheeses, while *B. abortus* was isolated from cows. All patients had eaten fresh cheese but no occupational exposure to animals was reported. Results of molecular typing did not show any *Brucella* vaccine strains. The isolation, identification, and molecular characterisation of *Brucella* spp. in human brucellosis cases and infected animals are very important to identify the source of infection and to take control measures in endemic regions.

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P117: Role of agricultural animals in brucellosis transmission in southern Kazakhstan

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Here we report on field surveillance made jointly by veterinary specialists and medical specialists at settlements in the Almaty and Zhambyl oblasts of southern Kazakhstan to examine backyard animals belonging to people who had confirmed brucellosis. 406 whole blood samples were collected from cattle, sheep and goats kept by different owners. 86 whole blood samples were from cattle and sheep owned by people of Alakol rayon of Almaty oblast; Rose Bengal test, CFT and FPA detected 17 seropositive blood samples. *Brucella* (*B.*) strains were isolated from the blood of two cows (*B. abortus*) and one sheep (*B. melitensis*) by culturing on solid nutrient media. Sick animals belonged to three persons from different settlements - from Aktubek village, Zhaupak village and Terekty village. It is worth noting that all these owners were seropositive for brucellosis as determined by the rayon Sanitary and Epidemiology Service (SES). In settlements of the Yenbekshikazakh rayon, in the same oblast, 24 cattle were tested for brucellosis-specific antibodies using *Brucella* antigen. Six cattle were seropositive and only two cows were bacteremic. Further testing identified these *Brucella* isolates as *B. abortus*. Both cows belonged to a private owner living in Karaturuk village, who was already registered to have brucellosis. In Sarkan rayon of Almaty oblast, there was another examination of animals from those private farms where owners were sick. However, no brucellosis-seropositive animals were detected among 36 cattle and 25 sheep. A bacteriological survey of all whole blood samples also showed negative results. This may be explained by the removal and slaughter of all *Brucella*-positive animals in these settlements earlier that year (in spring 2013) under the national program. A similar situation was observed in two rayons of Zhambyl oblast where 235 animal units were tested for brucellosis. Testing detected five seropositive cows and 22 seropositive small ruminants (sheep and goats). *Brucella* cultures were isolated from whole blood samples collected from three sheep kept in farms in Kilisbai village, Moinkum rayon, and four sheep from Shu rayon. It should be noted that sheep were kept in different farms and backyards, and there were also cases of brucellosis among members of the owners' families. Analysis of the data above indicated that people in these southern Kazakhstan areas are infected through contact with diseased animals they own. The isolation rate of *B. abortus* is rare when compared to previous studies of our group in 2007-08 and 2012-13, when *B. melitensis* was commonly isolated. In order to shed light on animal-to-human transmission dynamics in this region we will conduct further studies to identify the genotypic characteristics of these isolates.

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P118: Epidemiological study of brucellosis in camels (*Camelus dromedarius*) in Central Sudan

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Camels in Sudan are farmed in forested grasslands together with sheep, goats and occasionally cattle; therefore, they are very susceptible to brucellosis caused by *Brucella* (*B.*) *melitensis* and *B. abortus*. Under these conditions, brucellosis has spread between these species and has appeared as a major constraint to camel breeding.
Diagnosis of camel brucellosis is complicated by many difficulties, mainly because this disease barely causes any apparent clinical signs in comparison to its clinical course in sheep and cattle.
Until now, none of the commonly used serological tests can be considered as a recommended test for brucellosis diagnosis in camel and most serological tests used have been driven from cattle without enough validation.
This work has been formulated to contribute to the enhancement of diagnostic capabilities by studying bacteriological and different serological diagnostic instrumental features for detection of brucellosis in camels in a distinct chosen district, Tamboul area, ElGazira State, Sudan.
A cross-sectional study was carried out during the year 2012 to estimate the seroprevalence of camel brucellosis. The sample size of the studied animals was estimated using the formula given for simple random sampling method, following the relevant formula for 95 % confidence and 5 % precision as by Thrusfield, 2005.
A total of 500 serum samples were collected randomly from different herds, and 30 tissue samples were also collected from brucellosis seropositive camels in Tampoul slaughter house. Serum samples were subjected to serological investigation using Rose Bengal plate test (RBPT) as screening test, the results were then confirmed by competitive ELISA. The prevalence of the disease was found to be 11.6 % and 7.7 % by RBPT and cELISA, respectively. Further serological tests are still under investigation including the modified RBT and the complement fixation test (CFT).
All 30 tissue samples (lymph nodes, placentas and spleens) were bacteriologically cultured, purified, identified and then characterized; four *Brucella* isolates were then recovered from the tissues. The isolated *Brucella* spp. were characterized as *B. abortus* bv 1; this finding document the isolation of biovar 1 from camels for the first time in Sudan.
The study recommended continuous investigation of the disease in camels to reveal the various impacts of the disease: epidemiologically, economically and its public health threat. All these collectively will help in planning for applied operational control approaches in different parts of Sudan.

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P119: Case study in brucellosis re-emergence: the current status of brucellosis in humans, domestic livestock and the Saiga antelope in Kazakhstan

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The collapse of the Soviet Union in 1991 was followed by an increase in the reported incidence of brucellosis (and other diseases) in several Central Asian countries, including Kazakhstan. In Kazakhstan there was also a major shift in the way that livestock were kept, i.e. from large state-owned farms towards household-based subsistence farms. This has caused a major challenge for the veterinary services which were previously organized around the state-farm system. This also meant that more people are exposed to direct contact with livestock now, and are therefore at risk to contract livestock zoonoses. In addition, the changing livestock systems may mean that livestock has increased contact with wildlife, such as the Saiga, a critically endangered migratory antelope that is probably susceptible to brucellosis. In 2007, a nationwide test-and-slaughter program was introduced in Kazakhstan, and there have been more than 100 million blood samples been tested. However, the official incidence of brucellosis in humans remains as high as 60 cases per 100,000 in some areas of the country, annually.

There is a lack of published data on the burden of brucellosis in Kazakhstan in the peer-reviewed literature. A study in 1997-1998 found a seroprevalence of 2.4 % amongst domestic livestock; however, there was no random sampling done.

The aim of this project was to analyze official data from the brucellosis test-and-slaughter program, official data on brucellosis in humans and available information on brucellosis in Saiga and to discuss the implications for brucellosis control in Kazakhstan.

Official human incidence data were obtained from the Kazakh Ministry of Agriculture for the years 2007-2012, analysed for every oblast (province), age-group and rural versus urban area if available. Official livestock seroprevalence data were also obtained from the Kazakh Ministry of Agriculture for the years 2008-2012, and analysed for every oblast (province). Serosurvey results from Saiga conducted in 2012 and 2013 were obtained from the Research Institute for Biological Safety Problems. Descriptive statistics and mapping were done.

The official statistics suggest that the incidence in humans may be decreasing, although caution must be exercised in interpreting official data, particularly as there are ongoing changes within the Kazakh health system. There is a strong spatial pattern in human incidence, with a much higher incidence in the south of the country. Potential reasons for this include the higher density of livestock in the south. The official seroprevalence in livestock is consistently very low, given the high incidence in people. This could be explained by some flexibility in interpretation of the tests. Although the numbers of Saiga tested were limited, there were no positive test results to *Brucella* antibodies. Current low numbers of Saiga and a likely high-prevalence in livestock suggests that the role of Saiga is currently not critical to brucellosis control; however, this fact could change in the future. Further work is needed to obtain more reliable seroprevalence data for domestic livestock and to evaluate the current control program.

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P120: System of the prevention of brucellosis in horses in Ukraine

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Brucella abortus is a causative agent of brucellosis in horses, bovine animals, yaks, buffalos, camels and possibly dogs. Most often horses become infected during joint pasturing and via contacts with infected animals, which had aborted. Horses may be a source of infection for people depending on the spread of the disease. Transmission of the *Brucella* species is of epizootic importance. Brucellosis in horses has no clinical manifestation. Documented are infiltrations in withers and nape, rarely in other parts of the body, which then turn into abscesses, lameness because of arthritis of the forelegs and only sometimes of the hindlegs, and tendovaginitis.
Results of the serological survey of brucellosis in horses in various regions were analyzed. Measures for the prevention of import and spread of the infections were developed for Ukraine. Over the last years, brucellosis in horses was registered in China, Kyrgyzstan, Russia, and in some regions of Ukraine. The disease is connected to horse breeding. Brucellosis in horses has not been registered in Ukraine for over 40 years. Brucellosis in sheep and in bovine cattle was not registered since 1967 and 1980, respectively. According to veterinary instructions, horses are tested with Rose Bengal test and CFT when symptoms of brucellosis are noted or if there was contact to other animals of an outbreak or when horses are imported from abroad. In addition, seed bulls, cows, heifers, buffalos, rams, ewes without offspring, breeding boars and main breeding pigs are tested twice a year in an area where brucellosis was possibly imported.
Horses positive to brucellosis were not registered during 5 years of serological survey. Due to well-organized work and adherence to the veterinary instructions Ukraine remains free from brucellosis in horses despite close relations with countries endemic for brucellosis.

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P121: The brucellin skin test: A step towards more effective control of bovine brucellosis in South Africa?

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Bovine brucellosis is a bacterial disease of public health and veterinary significance. It is characterised by variable and sometimes latent incubation periods which make it difficult to control. No single diagnostic test can identify all stages of the disease with high sensitivity. While only a handful of countries have successfully eradicated brucellosis, South Africa is currently not only far from eradication but has seen an increase in outbreaks in recent years. The brucellin skin test (BST), which has been proved to identify some acute and chronic latent stages of brucellosis, was studied for the first time in South Africa by conducting a comparative evaluation with serological tests. The objective of this study was to evaluate the BST in brucellosis free, as well as confirmed infected herds, particularly prior to routine brucellosis vaccination, under South African farming conditions. The results indicated that the BST, with a limited sensitivity of 42.86 % and a high specificity of 99.18 %, was unsuitable for use as a sole diagnostic test. However, the BST detected more brucellosis cases among unvaccinated four to nine months old heifers in infected herds compared to the routinely used Rose Bengal test and complement fixation test. It was concluded that the BST, used in parallel with routine serology, particularly iELISA, could play a complementary role to enhance diagnostic sensitivity in the identification of infected heifer calves prior to vaccination and breeding; and thus expedite the elimination of brucellosis in chronically infected herds where eradication has proven problematic.

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P122: A retrospective analysis of animal brucellosis research in Egypt in the last 25 years

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Brucellosis is a highly contagious zoonotic disease of significance for public health and economic performance in endemic and non-endemic countries. In developing countries, brucellosis is often a very common disease. A comprehensive, evidence-based assessment of literature and officially available data on animal brucellosis is missing for Egypt. National and international scientific publications on serological investigations (n=53), isolation and biotyping (n=34) of brucellae at the last 25 years were reviewed to verify the current status of brucellosis in animals in Egypt. Serological assays for brucellosis are well-established in Egypt and the results give indirect proof for the presence of brucellosis in cattle, buffaloes, sheep, goats and camels.
Brucella (*B.*) *melitensis* bv 3*, B. abortus* bv 1 and *B. suis* bv 1 are the predominant isolates in Egypt and have been isolated from farm animals and Nile catfish. There seems to be a discrepancy between official seroprevalence data and data of scientific publications. Thus, it can only be assumed that Egypt is endemic with brucellosis and the disease is prevalent in all farm animal species, in the environment and in carrier hosts e.g. rats. The epidemiological situation of brucellosis in Egypt is complicated and needs clarification by a nationwide survey including genotyping of circulating brucellae.

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P123: Development of a forecasting model for brucellosis spreading using the Italian cattle trade network

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Brucellosis caused by *Brucella abortus* is an important zoonosis that constitutes a serious hazard to public health. Prevention of human brucellosis depends on the control of the disease in animals. Livestock movement data represent a valuable source of information to understand the pattern of contacts between premises, which may determine the spread of the disease. The present study shows the development of a probabilistic brucellosis spreading simulation model, based on the Italian cattle trade network. The model takes into account both time and sequence of the events used for the simulation (cattle movements), and considers two main components: the dynamics of the disease at herd level (intra-herd spreading) and the dynamics of the disease following cattle movements (inter-herd spreading). The parameters for brucellosis spread used in the simulation model (rate of infection, rate of recovery, etc.) were calculated on the basis of data derived from international peer-reviewed literature. Results of the simulation model have been compared to those generated through a deterministic approach. The model has been applied to the network of cattle movements recorded in 2009 and validated using real data on cases of cattle brucellosis recorded in the same year in Sicily, Italy. In particular, the following data have been extracted from the Animal Health Information System (SANAN): brucellosis infected herds during the year and number of heads present. The reference network was generated from a brucellosis infected herd from Sicily by extracting from the National Database for Animal Identification and Registration (BDN) all movements starting from that herd since the last brucellosis negative control until the date in which the herd was found as infected (i.e. a one-year period). In particular, the data considered from BDN about the network of movements were the following: node of origin, type of node, node of destination, number of animals moved, date(s) of movement(s), number of animals present in the herd on the date of movement, number of females aged equal or more than 18 months and present in the herd on the date of movement.
The nodes of the network were those commercially connected directly or indirectly with the infected herd, and for which the number of animals entering and the timing of introduction were consistent to allow the transmission and the development of the infection. The numbers of nodes involved by the simulation, the numbers of movements concerning the network as well as the numbers of outbreaks identified by the model are discussed. The simulation model involves complex computational analysis; future developments may include the identification of additional tools for analysis (to optimize the design of the model) as well as of a fine tuning of the proposed algorithms (to maximize its performance).

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P124: OIE Laboratory Twinning Sudan-UK: An empowering initiative for local laboratories which had a remarkable outcome for the control of brucellosis in Sudan

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OIE Laboratory Twinning was aimed to strengthen the candidate *Brucella* laboratory in Sudan by identifying its needs for laboratory capacity building, overcoming barriers and obstacles, networking and joint scientific activities. The candidate laboratory's visibility is raised at national, regional, international conferences and meetings.
The candidate laboratory at VRI sucessefully implemented the project activities.These activities included the production and standardization of biologicals (diagnostics and vaccines). Standard Operating Procedures (SOPs) for each product were implemented. The quality control of these products is done according to the OIE Manual and SOPs received from AHVLA. *Brucella* (*B.*) antigens (RBT, MRT, CFT and SAT), *Brucella* vaccines (S19 and Rev1), mono specific antisera (A and M) and The National Standard Serum are readily produced with high quality.
The biological products also serve strategic aims: The amount of produced antigens is sufficient to test to 2 to 3 million animals of livestock to be exported (sheep, goats and camels) and the produced antigens can also be used for large scale diagnosis of thousands of samples annually.
Several research activities were done this year: cultural, serological and molecular diagnosis of camel brucellosis was investigated; *B. melitensis* Rev1 vaccine and Rose Bengal antigen of *B. melitensis* were produced for the control of small ruminants' brucellosis in Sudan; a study of acquired immunity against brucellosis using *B. abortus* S19 and *B. melitensis* strain Rev1 vaccines was done in Sudanese camels (*Camelus dromedaries*); an epizotological study of brucellosis in animals in Darfur State, Sudan, was conducted. Several activities towards the '*One Health*' idea wereundertaken: the isolation of *Brucella* from humans with suspected brucellosis was initiated by a collaboration of the Ministry of Health, the Faculty of Medicine, University of Khartoum and the Nidiag (European Research Network). An external quality testing procedure (EQT) was set into force: The parent laboratory in UK sent various samples to be tested with the different technologies implemented in the Sudanese laboratory. These tests were successfully accomplished. In Khartoum State an epidemiological survey was done and the spatial distribution of brucellosis was sucsessfully investigated. Additionally, training courses in serological diagnosis of brucellosis using ovine, caprine, bovine and camel sera, and different serological tests (RPT, SAT, MRT and cELISA) were done at thedepartment of *Brucella*. The trainees were veterinarians, technical staff, veterinary paraprofessionals and vet students. Various national, regional and international activities were done. The parent laboratory experts will continue to give guidance to the Sudanese colleaguese.

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Session 9: Wildlife Brucellosis

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Wildlife brucellosis: Spillover or sustainable infection?

Interaction of humans and livestock with wildlife exposes them to the risk of pathogen spillover. Livestock and wildlife may become intermediate or amplifier hosts from which pathogens can spill over into humans or humans can be infected via vectors. It has been estimated that 58 % of human pathogens are zoonotic, and 13 % are emerging, of which 73 % are zoonotic. Additionally, 26 % of human pathogens infect both domestic and wild animals. From a zoonotic perspective, brucellosis in humans is strongly linked to the management of infected animals and ingestion of unpasteurized dairy products. In this regard, there is a clear connection of brucellosis with the domestication of even-toed ungulates, milking practices, and fabrication of cheese and other dairy products. Domestication of sheep, cattle and goat, which occurred some 11,000-8,000 BCE, and clustering of wildlife reservoirs with narrower genetic backgrounds have provided a steady supply of emergent pathogens for humans. Domestication, anthropogenic modification of the environment and selection of animals by humans are not neutral phenomena. Consequently, it is expected that the prevalent extant *Brucella* strains have been selected through 'narrow funnels' connected to these processes.

Nonetheless, reservoirs of *Brucella* spp. existed in indigenous African artiodactyla species (which include a significant number of potential *Brucella* hosts) long before the introduction of domesticated animals. A paleopathological study has suggested the presence of *Brucella* infections in australopithecines, already 2.5–2.3-million years ago.
The first *Brucella* genome sequences were released in the early 2000s, and their analysis confirmed the previously held ideas concerning just how closely related they were. One of the key aspects of the biology of *Brucella* spp. that genomic sequencing has not yet been able to fully explain is the genetic basis of host specificity of the different *Brucella* spp. It is however clear that the preference of different *Brucella* spp. for a host is not based on a specific gene or pathogenicity island, but may rather be related to small genomic changes that affect expression or function of shared genomic content. Likewise, minor differences in the physiology of the different host species of *Brucella* spp. will also play a role in establishing the host specificity and preference of the different species.

Most wildlife brucellosis studies have consisted of serological surveys, and pathogenesis studies are lacking. Differences in the disease course exist and are likely to be attributable to immunological and behavioral differences among species. Additionally, anthropogenic drivers may result in breaking down the species barrier by multiplying exposure, allowing 'species jump' for *Brucella* spp. which may favor the persistence of a particular *Brucella* (*B.*) spp. clone in a different 'preferred' host.

Natural infection of *B. abortus* has been confirmed by culture in many wild artiodactyls, including bison (*Bison bison*), elk (*Cervus elaphus*), and African buffalo (*Syncerus caffer*). These species are known maintenance hosts of *B. abortus* under certain circumstances and may serve as reservoirs of the infection with the potential for transmission mainly to cattle and humans. Moose (*Alces alces*) appear unable to mount a protective immune response against the organism, usually resulting in mortality from *B. abortus* infection and the species is considered likely to be a dead-end host. Retained placentas, commonly seen following abortion or parturition in *B. abortus* infected cattle and bison are not observed in elk. *Brucella abortus* infection in female elk often results in abortion of the first calf. Mammary gland involvement is less frequent in elk than in cattle. Hygromata, carpal bursitis, synovitis and ten-

donitis are commonly seen. Based on natural observations and experimental work, of *B. abortus* infected wild canids are considered spillover hosts with little role, if any, in maintenance of the infection in other populations.
Nowadays, in countries where bovine brucellosis eradication programmes are close to their end, there are no known sustainable reservoirs of *B. abortus* in wild species, other than bison and elk in and adjacent to the American National Parks of the Greater Yellowstone Area (GYA) and bison in the Canadian Wood Buffalo National Park. However, recent studies in feral pigs on the Atlantic coast of South Carolina, USA, may challenge these facts. Indeed, *B. abortus* wild-type and the *B. abortus* S19 and RB51 vaccine strains have been isolated from feral pigs that had been spatially separated from domestic livestock for decades. This is the first report of a wildlife reservoir of *B. abortus* outside the GYA in the USA. The epidemiological consequences of these findings are of the utmost importance. Indeed, if experimentally confirmed, this would mean that *B. abortus* was able to perform a 'host species jump' and feral pigs may have become a true reservoir of *B. abortus*. In Europe, natural infection of *B. abortus* in wild boar has, so far, not been reported.
A recent survey in the Iberian Peninsula highlighted that wild ruminants were not a brucellosis reservoir for livestock. *Brucella abortus* was only isolated from a single red deer (*Cervus elaphus*), likely as a spillover from infected cattle. Thus, these results suggest that, in Europe, wild ruminants are occasional victims of brucellosis transmitted from infected livestock, rather than true reservoirs of the infection for livestock, to the contrary of what occurs in the GYA.

Some years ago, spillover of *B. melitensis* from infected small ruminants has been documented in a few wildlife species, such as chamois (*Rupicapra rupicapra*) and ibex (*Capra ibex*) in the French and Italian Alps, and the Iberian wild goat (*Capra pyrenaica*) in Spain. These reports highlight the fact that *B. melitensis* infection in European wildlife was always linked to the small ruminant reservoir suggesting that wildlife species are unable to sustain the infection and cannot act as reservoirs for domestic animals and human beings. Given the progress in national eradication brucellosis programs in small ruminants in Europe, it was speculated that only anecdotal *B. melitensis* spillovers in wildlife would be reported. Surprisingly, the wildlife 2012 surveillance campaign in France, revealed a very high prevalence of brucellosis within the Alpine ibex population (>45 %) of the Bargy Massif, despite the absence of any new outbreak in domestic animals and while wild ungulates had been considered, up to now, as an epidemiological dead-end host in Europe.

Brucella suis biovar 2 infection remains restricted to wild boar (*Sus scrofa*) and European brown hares (*Lepus europaeus*) in Europe, where *B. suis* has been eradicated in the pig industry for decades. Several outbreaks of *B. suis* biovar 2 have occurred in outdoor rearing systems of domestic pigs, with the source of infection being traced back to contacts with wild boar. Transmission from wild boar to pigs is believed to be through the venereal route, as crossed (striped) piglets have been reported, at least in France and Portugal; however, other routes might also be possible. The role of wild boar hunting (migration pressure, offal remaining in the field, hunters working on the premises, etc.) has not been fully investigated. *Brucella suis* biovar 2 strains isolated from hares and wild boars from Hungary showed substantial genetic divergence, suggesting separate lineages in each host and no instances of cross species infections, while across Europe, isolates from domestic pigs were closely related to isolates originating from both hares and wild boars.
Feral pigs are rapidly expanding their ranges across the United States, with 44 of 50 states reporting populations. The rapid expansion into new areas has primarily been caused by unregulated human transportation of feral pigs to establish new populations for hunting. In at least 14 states, brucellosis has been documented to be present in feral pigs, with some populations showing high seroprevalence. Feral pigs have been implicated as the source of *B. suis* biovar 1 infections in humans in a number of cases in the United States. As behaviour and topography make elimination of established populations of feral pigs unachievable, effective separation from domestic livestock, and feral pig population control by hunting or

targeted depopulation efforts has been implemented in order to avoid transmission of *B. suis* biovar 1 to livestock.Nevertheless, *B. suis* biovar 1 is regularly isolated from cattle in the United States and is becoming an emerging problem in cattle in South America. *Brucella suis* biovars 1, 2 and 3 are not considered to be pathogens in cattle. Indeed, cattle naturally infected with *B. suis* biovar 1 from feral pigs, and closely monitored for two years, did not experience abortion, did not transmit the agent to co-mingled negative cattle, and did not shed the organism except in the milk, which remained positive for two years. In Belgium, *Brucella suis* biovar 2 has recently been isolated from cattle without any induced pathology. After *B. suis* biovar 2 experimental infection in cattle, no gross pathology was seen and *B. suis* biovar 2 could not be isolated from carcasses 8 weeks post infection. Importantly, *B. suis* biovars 1 and 3 are true pathogens for humans, whereas *B. suis* biovar 2 is not.

Recently, two independent studies suggest that hooded seals are not the definitive host of *B. pinnipedialis*. Indeed, gross pathology associated with *B. pinnipedialis* infection has not been described in seals and vertical transmission is not likely to occur, since *B. pinnipedialis* has never been isolated from females of reproductive age. These studies point toward the existence of a reservoir of *Brucella* spp. in the aquatic environment, likely in the food web. Fish constitute a significant part of the diet of seals and further studies are needed to determine if fish, or other aquatic organisms that are part of the diet of seals, can sustain a *B. pinnipedialis* infection in seal.
There have been three reported cases of natural human infection associated with *Brucella* spp. from marine mammals. They were all associated with *Brucella* sequence type (ST) 27, although no contact with marine mammals was reported. *Brucella* ST27 has also been isolated in a dolphin and in a fur seal along the Californian coast. These findings suggest the hypothesis of a reservoir of *Brucella* spp. in the aquatic environment, possibly in fish or aquatic organisms, and that these marine *Brucella* spp. may spill over to marine mammals and humans.

The basic reproductive number (R_0) of an infection is the number of cases one case generates on average over the course of its infectious period, in an otherwise uninfected population. That is, for brucellosis to spread and for an effective animal to animal transmission of *Brucella* spp., it is required that R_0 of *Brucella* spp. > 1. In contrast, if $R_0 < 1$, then *Brucella* spp. will die out. Higher the R_0 value, higher would be the chances for *Brucella* spp. to achieve a steady and successful adaptation in the host.
The prevalence of brucellosis in some wildlife species is very low and thus, besides the classical factors considered to be important in transmission (host susceptibility, shedding, survival in the environment, etc.), the behaviour of individuals and interactions between wildlife and livestock may actually be the most important drivers for transmission. For instance, the reclusive calving behaviour of elk, which keeps the elk cow and calf away from the herd for several days after calving, minimises the risks of *B. abortus* transmission to other animals. On the other hand, in situations with important anthropic effects (i.e. intentional winter feeding, or co-mingling of wildlife on livestock feed grounds), the risk of infection increases dramatically, due to possible exposure to an infectious abortion near the feeding grounds. In the GYA, winter feeding changed the behaviour and density of free-ranging elk, the exposure rate to *B. abortus*, probably converting what would otherwise be a dead-end host in nature to a maintenance host of particular clones of *B. abortus*.

A study of the *B. melitensis* infected Alpine ibex population of the Bargy Massif concluded on the persistence and circulation of *B. melitensis* local clone in the area, over a two decade-period, in this protected population. The high brucellosis prevalence reported in wildlife, and especially in ungulates, reflects the direct effects of artificial management on wildlife and brucellosis transmission. The important anthropogenic effects (e.g. habitat modifications, increased wildlife density, sedentary behavior, contacts with domestic animals, artificial feeding facilitating contacts, etc…) could account for the high brucellosis prevalence found in ibex in the Bargy Massif. Moreover, the genetic integrity of Alpine ibex in the Alpine massifs,

strongly modified by anthropic effects, might have also played a role. Indeed, the founding of new Ibex population in the Bargy Massif in the Alps came from a pool of few (15) animals, narrowing their genetic diversity. All these factors may have contributed to make the Bargy Massif ibex population a semi-domestic population able to self-sustain the *B. melitensis* infection, with high prevalence, which has never been reported before in wild ungulates.

Brucella pinnipedialis has limited ability to survive within primary cells from hooded seals, thought to be the preferential host species, as well as human/murine cell lines. A lack of, or low, capacity to replicate and survive for prolonged periods within host cells, particularly macrophages, abolishes the ability to produce chronic infections. Seals may thus not be the primary host for *B. pinnipedialis*, but rather a 'dead-end' or spillover host susceptible to infection derived from other sources in the marine environment. Future research should aim at identifying the primary reservoir of *B. pinnipedialis* along with investigating potential virulent traits in marine mammal *Brucella* spp., important in the establishment of disease, either acute or chronic, in marine mammals and humans.

The fact that *B. abortus*, *B. melitensis* and *B. suis* are isolated from non-preferential hosts more and more frequently indicates shortcomings in livestock control programmes and/or changes in husbandry practices or wildlife management. Anthropogenic drivers are thus some of the most important determinants for transmission of *Brucella* spp. to non-preferred species.
Intracellular survival and replication is the hallmark of *Brucella* spp infection. Chronicity is linked to multiplication in macrophages, a feature facilitated by overriding the intracellular response following bacterial infection. A stealthy entry into host cells is essential to avoid detection by the immune system. Unfortunately, there are only very few studies of the pathobiology and the mechanisms of bacterial intracellular invasion and multiplication involving *Brucella* spp. in wildlife and this warrants further investigations.
Lastly, the determinants of host specificity of *Brucella* spp. at the molecular level have not yet been identified, which remains one of the most important research issues.

Oral Presentations (O36-O40)

O36: *Brucella melitensis* at the wildlife-livestock-human interface in the Emirate of Abu Dhabi

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Brucellosis has been reported in almost all livestock species in the Middle East, especially in goats and camels. In the emirate of Abu Dhabi (UAE), 55.1 % of the 247 domestic farms sampled in 2010 were *Brucella* seropositive. *B. melitensis* has been isolated once in a Nubian Ibex (*Capra ibex nubiana*) in Dubai. Nevertheless there are no recent data accessible on the species and biovars circulating in the UAE. Both *B. melitensis* biovar 1 and 3 were reported on human cases from Tawam Hospital, Al Ain (Abu Dhabi), hence were co-circulating in the UAE in 1996. From 2000 to 2003, 6.5 % of the 998 patients admitted in the same hospital were *Brucella*-seropositive. The livestock management system in the UAE plays a role in *Brucella* spreading with the coexistence of several livestock species and because of misdiagnosis and/or under reporting of animal and human cases. *B. melitensis* is rarely reported in wildlife but direct contact between free roaming livestock and wild ungulates is likely in the region. In this study we investigated the brucellosis seroprevalence within a large captive herd of scimitar horned oryx (SHO / *Oryx dammah)* to trace the source of infection. SHO are extinct in the wild since 2000 and conservation programmes rely heavily on captive stocks for possible future re-introduction. Among the 480 juvenile/subadult SHOs and the 400 adult SHOs tested, 75 % and 95 % were *Brucella*-seropositive, respectively, based on a Single Rose Bengal test. 15 sand gazelles housed within the same collection but 2 km away from the oryx herd were also tested seropositive. Clinically most of the male oryx suffer from orchitis while sand gazelles present mainly hygroma at the tibio-tarsal and metacarpo/tarsophalangeal joints. *B. melitensis* biovar 1 was isolated from both oryx foetal stomach content and metacarpal fluid from a gazelle and genotyped using multiple-locus variable-number tandem-repeat (MLVA) at CODA-CERVA (Belgium). Both strains have a similar genetic profile to the BfR25 strain previously isolated from a goat in the UAE. Supposedly, oryx and/or gazelles initially acquired brucellosis infection from livestock, and later it started circulating among wildlife. Moreover, *Brucella* spp. that has not been genotyped has clinically infected three ungulate keepers handling contaminated materials in the same collection. All were cured following the Word Health Organisation treatment recommendations.
A holistic ecosystem based approach should be used to tackle *Brucella* transmission and maintenance in heavily contaminated environment. Efforts in identifying species and subtyping of *Brucella* isolates are paramount for any preventive (awareness campaign) and epidemiologic surveillance-control programme in endemic areas. Active surveillance of susceptible animals and occupational health screening of the workers will give a more accurate picture of *Brucella* distribution in the country and will help defining an appropriate control strategy. In heavily affected countries, mass vaccination programmes seem inevitable but require proficient veterinary services. Implementation of an eradication strategy to fight brucellosis in scimitar horned oryx will require preliminary studies on diagnosis tests, host/pathogen/environment interactions, vaccine assessment and appropriate veterinary protocols.

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O37: *Brucella* infection cases among stranded marine mammals recovered from the North Sea coasts of Belgium, the Netherlands and France (1999-2013)

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A total of 203 stranded animals have been recovered from the North Sea coast during a fifteen years surveillance program. The aim of this study was to determine the prevalence of *Brucella* infections among the stranded sea mammals population. A total of 15 *Brucella* strains were isolated from porpoises (n=6), grey seals (n=5), harbor seals (n=3) and a short-beaked common dolphin. In most cases, the infection was localized in the respiratory system. Additionally, two porpoises carried lung parasites harboring *Brucella*. Isolation of bacteria was confirmed by real-time PCR and strains were typed by Multi-Locus Variable Number Tandem Repeats Analysis (MLVA). Different genetic MLVA profiles were identified, demonstrating high strain variability among *Brucella* spp. circulating in marine mammals. As a potential zoonotic disease, the presence of *Brucella* spp. in marine mammals constitutes a biohazard for human health.

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O38: First isolations of *Brucella ceti* from long-finned pilot whales (*Globicephela melas*) and a sowerby's beaked whale (*Mesoploden bidens*)

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The isolation of *Brucella* from cetaceans was first reported in 1994 from two harbour porpoises (*Phocoena phocoena*) and a short-beaked common dolphin (*Delphinus delphis*) in Scotland and from the aborted foetus of a captive bottlenose dolphin (*Tursiops truncatus*) in the USA. In the years since, *B. ceti* has been reported from several further species of cetacean including Atlantic white-sided dolphin (*Lagenorhyncus acutus*), striped dolphin (*Stenella coeruleoalba*), white-beaked dolphin (*Lagenorhyncus albirostris*), and minke whale (*Balaenoptera acutorostrata*). While further evidence of *Brucella* infection in further cetacean hosts have been provided by serological tests and detection of *Brucella* DNA in tissues from Bryde's whales (*Balaenoptera edenii*), isolation of *B. ceti* following bacterial culture remains the gold standard for detection of *Brucella* in cetaceans. We report the first isolations of *B. ceti* from long-finned pilot whale (*Globicephala melas*) (LFPW) and Sowerby's beaked whale (*Mesoploden bidens*) (SBW).
Animals were reported through the Scottish Marine Animal Strandings Scheme (SMASS) and were subject to post mortem examinations which included selection of tissues for bacteriology and histopathology.

Case 1: *Brucella ceti* was isolated from spleen, kidney and colorectal lymph node of a SBW. Although there was no correlation between isolation of *B. ceti* and histopathological lesions in individual tissues, a non-suppurative, necrotising encephalitis was detected, which was reminiscent of that described in other cetacean species infected with *B. ceti*, some of which have only small numbers of colonies recovered from brain. It remains a possibility therefore that culture of brain in this case was not sufficiently sensitive to detect *Brucella*.

Case 2: *B. ceti* was isolated from testis and pus collected from the shoulder of the scapulohumeral joint of a LFPW. The animal was one of ten LFPW for which bacteriology was carried out following a mass stranding event (MSE) of 70 LFPW, in which 19 animals died

Case 3: *B. ceti* was isolated from the CSF and brain of a single LFPW. Histopathology supported a diagnosis of non-suppurative meningo-encephalitis as reported previously for Atlantic white-sided, striped and common dolphins.
Multi-locus sequence typing identified the three isolates as belonging to ST26, a strain which is associated with pelagic delphinids in the north-east Atlantic. The recovery of *B. ceti* from a LFPW involved in a mass stranding event and from the brain of another LFPW, provide some evidence towards a potential role for *B. ceti* involvement in LFPW mass stranding events under the sick lead animal hypothesis. The recovery of *B. ceti* from the testis of a LFPW is of interest with regard to the impact of reproductive disease on cetaceans, which remains unknown and will likely require input from researchers in other fields.

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O39: Atlantic cod (*Gadus morhua*): A potential transmission host for *Brucella pinnipedialis* hooded seal (*Cystophora cristata*) strain?

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Investigations of hooded seals (*Cystophora cristata*) have revealed high prevalences of *Brucella*-positive seals in the dramatically reduced Northeast Atlantic stock. However, pathological changes due to infection with *B. pinnipedialis* have not been identified in the hooded seal (HS). *B. pinnipedialis* HS strain is not able to multiply or establish a chronic infection in HS macrophages or epithelial cells *in vitro*. Age-dependent serological and bacteriological patterns for *B. pinnipedialis* have been identified in HS with a low probability of being positive for pups, a high probability for yearlings, followed by a decreasing probability with age, suggesting an environmental exposure post weaning during the first year of life followed by clearance of infection. This raises the question of a reservoir of *B. pinnipedialis* in the HS food web. For adult HS, the diet consists of Atlantic (*Gadus morhua*) and polar cod (*Boreogadus saida*) along with other species like e.g. deep-sea squid (*Gonatus fabricii*), redfish (*Sebastes* sp.), and Greenland halibut (*Reinhardtius hippoglossoides*).
Primary leukocytes were isolated from head kidney of Atlantic cod and challenged with *B. pinnipedialis* sp. nov. (NCTC 12890) and a HS isolate (B17). Both bacterial strains entered the leukocytes and survived intracellularly without any major reduction in retrievable numbers for at least 48 hours. This is in contrast to the rapid elimination of intracellular bacteria from HS alveolar macrophages.
Atlantic cod was experimentally infected with *B. pinnipedialis* HS strain. The fish was challenged with either 10^5 (low dose) or 10^8 (high dose) *B. pinnipedialis* intraperitonally. At day 24 post infection (pi) no mortality in any of the infected groups has been recorded. Samples of blood, liver, spleen, muscle, heart, head kidney, female gonads and feces have been collected from 5 fish in each group at day 1, 7, 14 and 28 pi to determine the bacterial load. Our results show that *B. pinnipedialis* induce an extended bacteremia in Atlantic cod receiving the high dose. Bacteria were found in blood samples from all fish at day 1, 7, and 14.
So far, preliminary results indicate that *B. pinnipedialis* HS strain may have a prolonged course of infection in the Atlantic cod, at least according to *in vitro* results generated from the use of primary cell cultures. A sustained bacteremia, compared to what has been observed in mammalian hosts, points in the same direction. This could imply that Atlantic cod may act as a transmission host for *B. pinnipedialis* HS strain in the marine environment.

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O40: Characterization of *Brucella* species from stranded cetaceans in the United States since 2010

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The first reported isolation of a *Brucella* species from a marine mammal was in 1994 from an aborted fetus of a captive bottlenose dolphin in the United States (US). Since then, dozens of studies have reported culture and serologically-positive animals in captive and wild (stranded or hunted) cetacean populations. This includes both animals exhibiting obvious clinical signs of disease and animals appearing disease free. There have also been reports of human brucellosis cases, although few, caused by certain marine *Brucella s*train types emphasizing the zoonotic potential of these species. In 2007, the *Brucella* marine species, *B. ceti* and *B. pinnipedialis,* were formally described and taxonomically named after the marine mammals they preferentially infect. *B. ceti* is most commonly associated with dolphin and porpoise populations and depending on the strain types, there have been varying degrees of virulence reported within this species. Genetic analysis of *B. ceti* isolates using a multi-locus sequence analysis (MLSA) scheme of nine housekeeping genes has shown three sequence types (STs) identified as STs 23, 26 and 27 associated with this species. Although brucellosis in cetaceans is globally distributed, much of the strain typing data have been gathered from isolates predominantly originating from Europe, South and Central America. In essence, there is little known about the diversity of *Brucella* strains circulating in the cetacean populations in US coastal waters. Due to an increase in cetacean 'strandings' and Unusual Mortality Events in the United States, a multi-agency investigation began in 2010 to better understand the disease burden of brucellosis in cetaceans and describe the strain diversity of *Brucella* species in cetaceans. Since 2010 tissue samples for molecular testing, culture, and histopathology have been obtained from 50 cetaceans stranding from 17 states throughout the US. Preliminary molecular analysis indicates the prevalence of previously described STs 23, 26 and 27. The identification of ST 27 in the US *B. ceti* isolates is notable and is the first report of this sequence type in the wild cetacean population. ST 27 has only been reported previously from a single cetacean and three human cases. A unique sequence type, ST 28, has also been discovered in this group of North America *B. ceti* isolates. Genetic analysis of marine mammal *Brucella* species in the US is ongoing. Future work will continue to reveal the true diversity of *Brucella* among marine mammals and their global distribution.

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Poster Presentations (P125-P135)

P125: Genetic relatedness of *Brucella suis* biovar 2 isolates from wild boars, hares and pigs in Hungary and Europe

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Porcine brucellosis generally manifests as disorders in reproductive organs potentially leading to serious losses in the swine industry. *Brucella suis* biovar 2 is endemic in European wild boar and hare populations, thus these species may play a significant role in the spread of the disease and serve as potential sources of infection for domestic pigs. The aim of this study was an epidemiologic analysis of porcine brucellosis in Hungary and a comparative analysis of *B. suis* bv 2 strains from Europe using multiple-locus variable-number tandem repeat analysis (MLVA). MLVA-16 and its MLVA-11 subset were used to determine the genotypes of 68 *B. suis* bv 2 isolates from Hungary. The results werefurther compared to the MLVA genotypes of European isolates. The analyses indicated a high genetic diversity of *B. suis* bv 2 in Hungary. Strains isolated from hares and wild boars showed substantial genetic divergence, suggesting separate lineages in each host and no instances of cross species infections. The assessment of the European MLVA genotypes of wild boar isolates generally showed clustering based on geographic origin. In contrast, the hare isolates were more closely related to one another and did not cluster based on geographic origin. The limited relationship between geographic origin and genotype of the isolates from hares might be the result of cross-border live animal exchange. The results could also suggest that certain *B. suis* strains are more adapted to hares. Across Europe, isolates from domestic pigs were often closely related to isolates originating from both hares and wild boars, supporting the idea that wild animals are a source of brucellosis in domestic pigs.

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P126: Anti-*Brucella* antibodies in wild boars in Poland

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Porcine brucellosis is an infectious disease caused by *Brucella suis* biovar 1, 2 or 3. The main clinical feature of *B. suis* infection is reproductive failure characterized by abortion, stillbirth and infertility in sows, testicular lesions, asymmetry of testicles and infertility in boars. In Europe the most common agent of brucellosis in pigs is *B. suis* biovar 2, and wildlife (wild boars and hares) constitutes a source of infection for domestic pigs. Several outbreaks of *B. suis* biovar 2 have been confirmed in porcine outdoor rearing systems, even in fenced premises. The source of infection has been traced to contacts with wild boars. Transmission from wild boars to pigs is thought to be through the venereal route, but other routes are also possible. Systematic brucellosis monitoring in wildlife does not exist. In contrast veterinary surveillance of the animal health status is strictly regulated for domestic animals. In this study we determined the prevalence of anti-*Brucella* antibodies in wild boars from Poland by indirect ELISA. The ELISA kit developed at the National Veterinary Research Institute in Pulawy and utilized for examination of swine sera for anti-*Brucella* antibodies, was used for testing. The lipopolysaccharide (LPS) obtained from the strain *B. abortus* S19 was used as antigen, anti-swine immunoglobulins, conjugated with horseradish peroxidase, were used as the conjugate, and ABTS with H_2O_2 as substrate. Assay controls consisted of strong positive (S++) and weak positive (S+) sera prepared on the basis of samples obtained from naturally *Brucella* infected pigs. A negative control (N-) was prepared from sera of healthy animals. Same criteria for the assessment of sera from wild boars and porcine sera were used. A total of 4,407 serum samples of wild boars (Sus scrofa) shot by hunters in Poland in 2012 were tested. Samples originated from 11 out of 15 voivodeships and 1,077 (24.44 %) seroreagents were detected. The highest prevalence of anti-*Brucella* antibodies in wild boars was found in Opolskie (39.9 %) and Wielkopolskie (37.29 %) voivodeships. On the other hand, the lowest percentage of positive sera was observed in Kujawsko-Pomorskie (13.74 %) and łódzkie (15.47 %). Results revealed vital differences in the level of prevalence of anti-*Brucella* antibodies in different districts, varying between 0-100 %. Results of the surveys show that wild boars constitute a huge reservoir of *B. suis* biovar 2 in Poland.

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P127: *Brucella suis* in wild boar (*Sus scrofa*) in the Netherlands

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So far, *Brucella suis* has been only detected in wild boars (*Sus scrofa*) originating from in countries bordering the Netherlands, but not in populations within the Netherlands.
A monitoring program in wild boars in the Netherlands is currently in place to test for Food and Mouth Disease and Classical Swine Fever at the Central Veterinary Institute (CVI). Additionally, these wild boars were tested for the presence of anti-*Brucella* antibodies to investigate the status of *B. suis* among wild boars in the Netherlands. A few wild boars were found serologically positive. Therefore, tonsils were collected and studied for the presence of *Brucella* spp. using classical culture techniques and PCR.
A total of 54 animals were tested, mostly originating from the southern part of the Netherlands. All tonsils were handled using methods described for the isolation of *Brucella* species by the OIE, subsequently tested by PCR targeting the IS711 element and if needed typed by MLVA.
Two animals that originated from the southern part of the Netherlands close to the German and Belgium border showed viable brucellae in their tonsils. These samples were also positive for the genetic marker IS711. Four tonsils of different animals were only positive tested by PCR. *Brucella*-typing of the isolates from enrichment cultures indicates that both strains belong to the species *B. suis* biovar 2.
In conclusion, this is the first study that shows that *B. suis* is present in wild boars at least in the southern part of the Netherlands.

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P128: *Brucella suis* biovar 2 infection in atypical hosts in France

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Brucella suis, mainly responsible for porcine brucellosis, is a zoonotic disease of public health and economic concern worldwide. *B. suis* biovar 2 has only been described in Europe and infects preferentially suids and hares, causing reproductive troubles. In France, *B. suis* bv 2 has been eradicated in domestic pigs with farming intensification and industrialisation (indoor commercial pig holdings using controlled artificial insemination), but an important wildlife reservoir remains (wild-boar population with a prevalence exceeding 30 % in certain areas, hares). At national level, no dedicated surveillance plan is implemented in production animals as well as in wildlife. Since 1993, 0-10 outdoor pig holdings have got infected with *B. suis* bv 2 due to contact with wild boars (generally through mating). Surprisingly, a *B. suis* bv 2 contamination was discovered in a cow in 2012 in Belgium. Retrospectively, similar cases in cattle in Poland were reported. in 2014, a *B. suis* bv 2 infection was confirmed in a cow in France. Two other cases were previously identified in France: a cow (2000) and a ewe (2009). All cases were discovered by accident during serological testing and no clinical signs were observed in infected animals. Infected animals remained isolated cases within the herd (negative serology on the entire herd) suggesting that *B. suis* bv 2 was not likely to be transmitted between ruminants. However, the isolation of *B. suis* bv 2 from different organs raises the question of *B. suis* bv 2 a sustainable infection and excretion. Epidemiological investigations evidenced a close exposure of these ruminants to the wild reservoir (wild-boar field dressing in the cowshed, sheep/wild-boar cohabitation in pastures). To date, the consequences of ruminant *B. suis* bv 2 infections are unknown. Nevertheless, based on the few cases described, the infection seems asymptomatic and little/not contagious, suggesting a less pathogenicity in these species. The low pathogenicity of *B. suis* bv 2 in ruminants is also observed in humans. Unlike *B. suis* bv 1 and 3, *B. suis* bv 2 is considered as an opportunistic pathogen for humans. Indeed, human infection with *B. suis* bv 2 is rare, even in endemic areas and in exposed population (e.g. hunters). However, three *B. suis* bv 2 human cases, with clinical signs classically observed in human brucellosis, have been confirmed in France since 2004 in patients suffering from co-morbidity. Epidemiological investigations on these *B. suis* bv 2 atypical infections in humans and ruminants evidenced contamination risk factors. Indeed, in almost all cases described, these atypical hosts were exposed to the *B. suis* bv 2 wild reservoir and were suffering from co-morbidity. Together with *Brucella* biochemical typing, recommended molecular methods allowed a rapid and specific identification of *B. suis* bv 2 (Bruce-Ladder suis) which is necessary to implement the adapted control strategy in ruminants (stamping out not necessary). Molecular typing approaches are under study, in order to investigate possible epidemiological relationship of these *B. suis* bv 2 'atypical' strains with strains isolated in wildlife. *B. suis* bv 2 contamination of atypical hosts may be underestimated, especially in ruminants due to the absence of clinical signs. For now, *B. suis* bv 2 appears as a limited public health issue, but, as wild boar density is steadily increasing, *B. suis* bv 2 contamination could become an increasing threat to exposed immunocompromised people. To reduce this risk, preventive measures against *B. suis* bv 2 should be implemented (biosecurity awareness, responsible hunting practices).

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P129: Unexpected *Brucella suis* biovar 2 infection in a dairy cow in Belgium

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A spill over infection with *Brucella suis* biovar 2 in a dairy cow was detected during the Belgian national brucellosis screening program in spring 2012 (positive bulk tank milk indirect ELISA). All animals of this 'reactor' farm were further serologically tested. One animal classified positive by SAT-EDTA and two independent indirect ELISAs, was slaughtered on April 23, 2012. This was a non-pregnant dairy cow (last calving in March 2011) older than four years, born on a farm that showed no obvious brucellosis cases. Bacteriological examination was conducted on the following samples collected at the slaughterhouse: spleen, uterus, lymph nodes (supramammary, retropharyngeal, iliac and submandibular) and the udder. *Brucella* spp. were only cultured from the spleen and the uterus and were further identified as *B. suis* bv 2. This case was epidemiologically linked to discarded offal in a pasture during the 2011 wikd boar hunting season. In livestock, the different *Brucella* species have preferred (but not exclusive) animal hosts. In bovines, in addition to *B. bortus*, infections caused by *B. melitensis* and *B. suis* have been previously reported. These cases are always epidemiologically linked to the presence of a reservoir of *Brucella* spp. in their preferential hosts. Infection associated with *B. suis* in cattle is considered to be a 'spill over' from a wildlife reservoir. In Europe, wild boars (*Sus scrofa*) and hares (*Lepus europaeus*) constitutes the reservoir of *B. suis* bv 2. Only two cases of infection in cattle have been described previously in the literature in Denmark and France, presumably related to *B. suis* bv 2 infected hares and wild boars, respectively. Since then, two other cases were reported in Europe, one in Poland (result of bacteriological investigations in cattle slaughtered due to positive serological reactions for brucellosis) and one in France (under investigation). The results of the presented study indicate that *B. suis* bv 2 can infect cattle, and plays a role in the epidemiology and need to be considered in the control of bovine brucellosis. Lessons to be learned: Firstly, preventive measures must be implemented by hunters (awareness campaign, education on biosecurity and responsibility) and farmers (double fences for 'at risk' pastures). Secondly, typing of *Brucella* spp. is necessary to trace back the infection. Thirdly, a sound epidemiological inquiry must always be done in case of positive laboratory results in order to identify or exclude *B. abortus* infections as well as to investigate possible *B. suis* bv 2 infections. Fourthly, our bacteriological results (i.e., the absence of the bacterium from the samples collected at the abattoir from 111 additional bovines) suggest that stamping out is not necessary because *B. suis* bv 2 is not likely to be transmitted between cattle given that cattle are spill over hosts, not preferential hosts for *B. suis* bv 2 (i.e. the basic reproduction number, $R_0<1$). Lastly, from a veterinary public health perspective, according to the literature *B. suis* bv 2 has a very low residual pathogenicity in humans.

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P130: Impact of small mammal populations as a potential reservoir or vector for *Brucella* spp. dissemination

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Brucellosis is a widespread zoonotic disease transmitted from an animal reservoir to humans. In Germany, bovine and ovine/caprine brucellosis were eradicated more than a decade ago and nowadays livestock is well-controlled. In contrast, the surveillance of wildlife is still challenging, and reliable data on the prevalence of brucellae in small mammal populations do not exist. In order to assess the role of small mammals as a potential reservoir or vector for *Brucella* spp. dissemination a molecular survey was carried out. In this study, we used large scale rodent monitoring data to identify (a) the occurrence of *Brucella* in small mammal populations and their potential host species, (b) the spatial distribution of mammal populations with high prevalence as well as (c) intrinsic factors associated with the transmission of brucellae.
A total of 537 small mammals (rodents and shrews) which were trapped in four federal states (Mecklenburg-Western Pomerania, North Rhine-Westphalia, Thuringia and Baden-Wuerttemberg) located throughout Germany were investigated. Our survey provides evidence that *Brucella* is more commonly disseminated in Germany than previously expected. Using a two-step molecular real-time assay based on the detection of the *Brucella*-specific *bcsp31* and IS711 sequences, 14.2 % (n=76) of the investigated animals were positive and originated mainly from Western and Southwestern Germany, where preliminary analyses suggest density dependent *Brucella* prevalence in bank voles (*Myodes glareolus*) and *Apodemus* spp. The molecular detection of brucellae in various rodent taxa and for the first time in shrew species shows that these animals may be naturally infected or at least have a history of exposure to the pathogen. Although the isolation of the bacteria from liver samples failed, results from molecular studies suggest that rodents and other small mammals might have an impact on the transmission of *Brucella* in the sylvatic and domestic cycle.

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P131: Unexpected *Brucella melitensis* outbreak in the Alpine Ibex (*Capra ibex*) population in the French Alps

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France has been officially free of bovine brucellosis since 2005, with no case in domestic/wild ruminants since 2003. In 2012, a human case and a related bovine outbreak due to *B. melitensis* biovar 3 occurred in the Bargy massif (Haute-Savoie, France). Epidemiological investigations did not identify any other case in domestic ruminants. An extensive survey was therefore implemented in wildlife: hunted chamois (*Rupicapra rupicapra*) (n=114), red deer (*Cervus elaphus*) (n=56) and roe deer (*Capreolus capreolus*) (n=65), as well as randomly captured Alpine ibex (*Capra ibex*) (n=77 – a protected species in France) were serologically tested in 2012-2013. Positive animals as well as Alpine ibex with clinical signs (arthritis and orchitis) were further submitted to bacteriological analyses. Only two chamois were found infected, while, surprisingly, the apparent seroprevalence reached 38 % in investigated Alpine ibex animals (72 % in >5 year-old females). *B. melitensis* biovar 3 was isolated from lesions/organs and from urogenital secretions of 16 out of 37 seropositive Alpine ibex. However, complementary surveys did not evidence the presence of infection in Alpine ibex in the two neighbouring massifs (Aravis, n=60; Sous-Dine, n=30).
The isolates originating from the 2012 domestic/human outbreak harboured an identical genotype, confirming a recent and direct contamination from cattle to human. Interestingly, they clustered not only with isolates from wildlife in 2012, but also with local historical domestic isolates, in particular with the 1999 last bovine case in the same massif. Altogether, our results suggest that the recent bovine outbreak could have originated from the Alpine ibex population.
Twenty captured Alpine ibex were equipped with GPS collars and 53 with VHF collars in order to monitor their movements, spatial behaviour and habitat selection.In order to minimize the risk of transmission to domestic ruminants, in October 2013 it was decided to cull all Alpine ibex aged over 5 years. The follow-up of the younger Alpine ibex population could provide the necessary epidemiological information to adapt the control measures.
This is the first report of a *B. melitensis* spill-over from wildlife to domestic ruminants and of the sustainability of the infection in Alpine ibex.

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P132: A rapid test for identifying *B. melitensis* infection in an Alpine ibex (*Capra ibex*) reservoir in the French Alps

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France has been officially free of bovine brucellosis since 2005 and though a regular exhaustive surveillance in the whole French territory. Since 2003 no infection has been reported in all domestic ruminants. In the French Alps, a bovine outbreak due to *B. melitensis* biovar 3 was confirmed in April 2012 and identified as the source of two human cases. Epidemiological investigations implemented in the concerned Bargy massif did not identify any other case in domestic ruminants, but a huge prevalence was observed in wild Alpine ibex (*Capra ibex*) which isa protected species in France. Thus, unlike the hunted species, animals may be killed only for sanitary reasons (i.e. seropositive or diseased animals). In order to control the disease in the Alpine ibex local population, a test-and-slaughter programme has been implemented since fall 2012. Animals are first captured (chemical immobilization) for blood sampling (laboratory tests required) and identified (Ear-tags and GPS/VHF collar). Animals with positive Rose Bengal test (RBT)/ complement fixiation test (CFT) results are then tracked (collars), and killed. Laminar flow immuno-chromatographic assays (LFIA) are rapid tests applicable on many samples, including whole-blood, and efficient for rapid diagnosis, in human brucellosis particularly. In order to reduce time and cost of the eradication strategy (expensive GPS/VHF equipment, two field interventions), our study aimed at assessing, in Alpine ibex, a commercial LFIA designed for sheep and goat brucellosis diagnosis, in comparison with the OIE prescribed tests for small ruminant brucellosis, i.e. RBT and CFT.
This study included 172 ibex sera sampled in 2012-2013 in the Bargy massif, the International Standard anti-*Brucella melitensis* Serum (ISaBmS) and the EU Goat *Brucella* Standard Serum (EUGBSS). Standard sera were tested at the lower limit of detection defined for the indirect ELISA (ISaBmS 1/64; EUGBSS 1/8). The standards were diluted in whole sheep blood in order to evaluate the sensitivity of LFIA in field *versus* laboratory conditions (dilution in negative serum). LFIA (Bionote, South Korea) was then compared on field serum samples to RBT and CFT (Idexx, France), and to two ELISA assays: a sheep and goat specific indirect ELISA (iELISA; Idexx, France) and a blocking ELISA (Ingenasa, Spain).
Our results showed a very good correlation between the LFIA and the other tests. On the 172 sera, the consistency of LFIA with other tests exceeded 97.6 % no matter which test was applied. Moreover, the ISaBmS and EUGBSS standards, directly diluted in whole blood, were positive in LFIA.
The reliability of LFIA in Alpine ibex seems very good in terms of sensitivity and specificity in comparison with OIE approved laboratory tests for ovine/caprine brucellosis. Furthermore, this test can be performed by unskilled people, in the field, with fast results (<20 min) and easy interpretation. A first trial in field conditions on Alpine ibex whole-blood confirmed the reliability obtained in laboratory conditions. LFIA could therefore be used for the immediate serological diagnosis of brucellosis the Alpine ibex in order to make a rapid sanitary decision.

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P133: First report of the isolation of *Brucella pinnipedialis* from a bearded seal (*Erignathus barbatus*)

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The isolation of *Brucella* from seals was first reported in 1994 from common seals (*Phoca vitulina*) in Scotland. In the two decades since, *Brucella* has been recovered from several other species of seal, including Pacific harbour seal (*Phoca vitulina richardsii*), grey seal (*Halichoerus grypus*), hooded seal (*Cystophara cristata*), ringed seal (*Phoca hispida*) and harp seal (*Phoca groenlandica*) and *Brucella pinnipedialis* has been described for isolates with seals as their preferred host. Serological studies have provided further presumptive evidence that infections are widespread amongst other pinniped hosts and populations, however, isolation following bacterial culture remains the gold standard for detection of *B. pinnipedialis* in seals.

Bearded seals (*Erignathus barbatus*) are members of the phocid family of 'true seals' and represent the only species within the genus *Erignathus.* They have a patchy circumpolar distribution throughout the Arctic between 45 and 85 N and are rarely observed in Scottish waters.

In February 2012, a stranded bearded seal was reported through the Scottish Marine Animal Strandings Scheme (SMASS). The animal had stranded on the Aberdeenshire coast of the north-eastern Scottish mainland and represented the first report of a stranded bearded seal in Scotland since records began in 1992.

The carcase was transported to SAC Consulting Veterinary Services, Inverness, for a post mortem examination and tissues selected for bacterial culture and histopathology. Samples of lung, liver, spleen, kidney, brain, mesenteric lymph node, bladder and small intestine were cultured on Columbia sheep blood agar and Farrell's medium and incubated at 37° C in air with 5 % added CO_2. Colonies typical of *Brucella* were recovered from the mesenteric lymph node after four days and further characterisation identified the organism as *B. pinnipedialis*. Multi locus sequence analysis demonstrated that the isolate belonged to ST24, which is associated typically with seals.

Histopathological examination of a range of tissues suggested that the likely cause of death of this animal was verminous granulomatous encephalitis. Mesenteric lymph node was not amongst the tissues examined for histopathology.

In contrast to cetaceans, for which a broad range of pathologies associated with *Brucella* infection has been reported, there has been a paucity of such reports from pinnipeds, with isolation of *B. pinnipedialis* made in several cases from animals that were apparently healthy, including seals that had been shot by fishermen. *Brucella* infection was suggested as a possible cause of abortions in California sea lions (*Zalophus californianus*) and the possibility that *Brucella* may be causing hitherto unrecognised reproductive problems in different species and populations of free-ranging pinnipeds, including bearded seals, is a concern and represents a major challenge for researchers studying these animals.

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P134: No evidence of *Brucella* spp. infection in free living Amazon River Dolphins (*Inia geoffrensis*) in Amazon region, Brazil

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The objective of this study was to investigate the occurrence of *Brucella* spp. infection in free living Amazon River Dolphin (*Inia geoffrensis*), from the Mamirauá Sustainable Development Reserve, in Tefé municipality, Amazonas, Brazil. Samples were collected from 161 free living animals of both sexes and different ages during two expeditions, in 2010 and 2011. Samples of serum (n=67), milk (n= 29) and genital, anal, nasal, oral and cutaneous lesions swabs (n=340) were collected. Detection of antibodies against smooth *Brucella* was performed using the Rose Bengal test (RBT) and the Fluorescent Polarization Assay (FPA). Milk and swab samples were cultured on Tryptose Agar with 5 % of fetal bovine serum and the following antibiotic mixture: vancomycin (3 mg/L), colistin methanesulphonate (7.5 mg/L), nystatin (12.500 UI/L), amphotericin B (2.5 mg/L) and nitrofurantoin (10 mg/L). DNA from Gram-negative bacteria was extracted by boiling preparation and were tested by PCR using a pair of primers directed to the 16S-23S interspace region (ITS-PCR). Those with positive results were characterized by sequencing the 16S ribosomal DNA and the *recA* gene. All 67 serum samples were negative in RBT and FPA. No *Brucella*-like bacteria were isolated from the 340 samples tested by microbiological culture; however, an isolate showed a positive result in ITS-PCR and was characterized as *Ochrobactrum intermedium* according to the sequence of the 16S ribosomal DNA and *recA* gene. The genus *Ochrobactrum* has been considered an expanding genus, with several species being identified in the last decades and *O. intermedium* is an emerging human opportunistic pathogen. It was not possible to evaluate the sanitary relevance of this detection since the isolate was obtained from a free living animal that appeared clinically normal during sampling.

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P135: *Brucella pinnipedialis* in harbor seals (*Phoca vitulina*) in the Netherlands

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Brucella spp. were detected in marine mammals in the '90s of the last century. *Brucella pinnipedialis* was identified in pinnipeds in countries around the North Sea, but not along the Dutch coast.
Stranded harbor seals (*Phoca vitulina*) were investigated at the Faculty of Veterinary Medicine (Utrecht University). Selected organs and nematodes from lungs were sent to the Central Veterinary Institute (CVI) to test for the presence of *Brucella* spp. In total, 132 samples from 39 animals were analysed using classical bacteriology and PCR.
All samples were cultured using the standard protocol for the isolation of *Brucella* as previously described by the OIE. Suspected culture samples were screened by PCR, targeting the *IS*711 element. One third of the harbor seals (12 of the 39: 31 %) were found to be positive for *Brucella* spp. Brucellae were detected in lungs, lungworms, pulmonary lymphnodes, spleen and liver. All *Brucella* isolates were typed as *B. pinnipedialis*.
In conclusion, the *B. pinnipedialis* prevalence in harbor seals stranded at the Dutch coast was estimated to be around one third within the population. This is in accordance with previous reports on the prevalence along the coasts of Scotland, Northern Ireland and Germany and thereby extends the geographical distribution of *B. pinnipedialis* to the Dutch coastal areas.

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