European Mycoplasma Meeting:

Mycoplasmas – A Practical Approach

Organized by:
Faculty of Veterinary Medicine
University of Zagreb

Sponsored by:
Boehringer Ingelheim
Mycoplasma Experience
Croatian Veterinary Institute

BOOK OF ABSTRACTS

Dubrovnik, Croatia
June 6th and 7th, 2013

Publisher
Medicinska naklada
Zagreb

Editors:
Krešimir Matanović
Roger Ayling
Franjo Martinković
Branka Šeol Martinec

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Welcome

We would like to welcome you all to this European Mycoplasma Group meeting held in Dubrovnik, Croatia organized by Veterinary Faculty University of Zagreb. This meeting continues the tradition of *ad hoc* mycoplasma meetings held in the years between the biennial conferences of the International Organization of Mycoplasmology. The last European meeting, held at the Weybridge site of the newly merged Animal Health and Veterinary Laboratories Agency (AHVLA) organized by AHVLA and the Royal Veterinary College in 2011 entitled “What’s new in mycoplasmology” attempted to look forward at new and emerging diseases, technologies and control strategies. The present conference will try to give us insight in human mycoplasmosis and some new-old stories about bovine and sheep infections as well as practical approach especially in new achievement in vaccine area.

We hope you will enjoy and fully participate in the meeting and this magnificent old city of Dubrovnik.

Finally we would like to acknowledge the generosity of our sponsors: Boehringer Ingelheim and Mycoplasma Experience.

Branka Šeol Martinec, Krešimir Matanović and Selma Pintarić (Department of Microbiology and Infectious Diseases)

June 2013

General Information

Meeting venue
The meeting is held at the Conference center (ELAFITI 1, ground floor) of the hotel Valamar Lacroma Dubrovnik.

Registration
Registration desk will be situated in front of the ELAFITI 1 conference room.

Language
The official language of the meeting is English.

INFORMATION FOR PRESENTERS

ORAL PRESENTATIONS
Please bring your Power Point presentation on a CD, a DVD or on a USB Memory stick at the time of registration or **at least 1 hour before the start of the session**.

POSTER PRESENTATIONS
Posters will be displayed at all times during the meeting and presenters are kindly requested to dismount their posters at the end of the meeting.
Tacks and technical equipment will be available for the mounting of posters.
Please refer to the Final Programme for the poster number assigned to you and use the board with the same number.

BREAKS
Coffee breaks will take place in the lobby in front of the conference room or, in case of good weather, on a main hotel terrace.

INTERNET CONNECTION
WiFi internet connection is available in rooms and in the conference room.
Contact person: Vlaho Sabljić, tel. +385 (99) 7312-600

All participants are kindly asked to wear their conference badges during scientific sessions and social events during the meeting.

PRELIMINARY PROGRAMME

THURSDAY, June 6, 2013

08.30 - 09.00	**Registration**	
09.00 - 09.20	**Welcome**	
Session 1	**Chaired by Branka Šeol Martinec and Michael Szostak**	
09.20-09.50	Laboratory science and how that relates to veterinary mycoplasma diseases	Roger Ayling
09.50-10.20	Role of mycoplasmas in respiratory disease in small ruminants	Robin Nicholas
10.20-10.50	**Break**	
Session 2	**Chaired by Inna Lysnyansky and Roger Ayling**	
10.50-11.10	Mycoplasmal pneumonia as the most common respiratory disease complex of pig illnesses (PRDC)	Biljana Radojičić
11.20-11.40	Antimicrobial susceptibility of *Mycoplasma bovis* strains isolated from dairy cattle herds in Croatia	Jana Pospichalova
11.40-12.00	Isolation and analysis of tetracyclne-resistant *Mycoplasma agalactiae* strains from an infected goat herd in Cyprus	George Filioussis
12.00-12.30	*Mycoplasma bovis* ribosomal operon loci are hot spots for insertion like sequences	Inna Lysnyansky
12.30-13.40	**Lunch**	
Session 3	**Chaired by Victoria Chalker and Salvatore Catania**	
13.40-14.10	Innate immune recognition and evasion of *Ureaplasma*; the role of multiple banded antigen	O Brad Spiller
14.10-14.40	*Mycoplasma salivarium* found as a dominant colonizer of an oral cancer in a patient with Fanconi anemia	Birgit Henrich
14.40-15.00	*Mycoplasma agalactiae* infection of the natural host : identification of a key virulence fator	Eric Baranowski
15.00-15.30	**Break**	
Session 4	**Chaired by George Filioussis and Krešimir Matanović**	
15.30-16.00	Contagious agalactia: how to manage an outbreak	Guido Loria
16.00-16.20	New insights into *Mycoplasma bovis* regulation of host immunity and potential pathways towards a protective vaccine	Musa Mulongo
16.20-16.40	The application of denaturing gradient gel electrophoresis (DGGE) in avian mycoplasma diagnosis	Salvatore Catania
16.40-17.00	*Mycoplasma bovigentalium* infection in a cow herd: a practical approach using antibiotic susceptibility testing of isolates	Salvatore Catania
17.00-18.30	**Poster Session**	
18.30 -	**Reception sponsored by Mycoplasma Experience**	

FRIDAY, June 7, 2013

Session 5	**Chaired by Anna Ramirez and Joachim Frey**	
08.40-09.10	*Mycopasma bovis* in Ireland	Hywel Ball
09.10-09.40	Extrachromosomal forms and strain variable integration sites define Tra Islands as novel mobile genetic elements of *Mycoplasma leachii* and *Mycoplasma capricolum* subsp. *capricolum*	Michael Calcutt
09.40-10.00	Characterization of free exopolysaccharides secreted by *Mycoplasma mycoides* subsp. *mycoides*	Clothilde Bertin
10.00-10.20	Dramatic decrease of antibiotic susceptibility of *Mycoplasma bovis* strains isolated from bovine respiratory diseases during the last 30 years in France	Anne Gautier-Bouchardon
10.20-10.50	**Break**	
Session 6	**Chaired by Hywel Ball and Michael Calcutt**	
10.50-11.20	Contagious bovine pleuropnemonia CBPP: Molecular and patho-evolution of *Mycoplasma mycoides* subsp. *mycoides*	Joachim Frey
11.20-11.40	Identificatiion of resistance mechanisms to quinolones in Ureaplasma urealyticum and Ureaplasma parvum in Croatian patients	Blaženka Hunjak
11.40-12.00	Role of porcine mycoplasmas in the porcine respiratory disease complex: a story of association	Ruben Rosales
12.00-12.30	A chimeric protein vaccine containing *Mycoplasma hyopneumoniae* antigens: protective efficacy against enzootic pneumonia in pigs	Annelies Michiels
12.30-12.50	Identification of haemagglutinin and neuraminidase of *Mycoplasma cynos*	Saša Kastelic
12.50-14.00	**Lunch**	
Session 7	**Chaired by Robin Nicholas and Umit Ozdemir**	
14.00-14.30	Motility of *Mycoplasma gallisepticum*	Michael Szostak
14.30-14.50	Putative pathogenic factors of *Mycoplasma synoviae*	Dušan Benčina
14.50-15.10	Herd level diagnosis of *Mycoplasma bovis* - lessons learnt from a pilot study in Denmark 2012	Krogh Kaspar
15.10-15.30	Rapid diagnosis of *Mycoplasma pneumoniae* respiratory infection in children by nested polymerase chain reaction.	Surinder Kumar
15.30-16.00	**Break**	
Session 8	**Chaired by Brad Spiller and Ruben Rosales**	
16.00-16.30	Mollicutes of non-mammal marine animals	Anna Ramirez
16.30-17:00	Understanding cyclic epidemics of *Mycoplasma pneumoniae* infections in England and Wales (1975-2009) using the time series analysis and mathematical modelling	Victoria Chalker
17.00-17.15	Formation of ESCMID Study Group for Mycoplasma infections (ESGMI)	Victoria Chalker
17.15- 17.30	**Close**	

Oral Presentations

Oral Presentation Abstract 1.

Laboratory science and how that relates to veterinary mycoplasma diseases

Roger D. Ayling

Animal Health and Veterinary Laboratories Agency, (Weybridge), Woodham Lane, Addlestone, Surrey, KT15 3NB, UK.
E mail: roger.ayling@ahvla.gsi.gov.uk

Mycoplasma species are the smallest organisms capable of self-replication, but can cause some of the most economically important diseases in farmed animal species. Understanding how these small organisms work has interested scientists for many years, as they have tried to understand life, and the minimum requirements for life. As a consequence *Mycoplasma* species have had a lot of interest from scientists, which includes the first genome to be sequenced, and the expression of one organism's genome by another organism. Whilst this and other scientific achievements may not appear to have had a direct benefit to veterinary practice or for animal health, some major achievements are highlighted. These include the development and application of improved laboratory and pen-side diagnostic tests; the application of molecular epidemiology, which can help understand disease; the organisms' variability; survivability, possibly in biofilms; and the role of antimicrobials and vaccines in controlling disease. However, science still has a lot to do, as nature counteracts these advances with the development of antimicrobial resistance and the hosts' variable responses to novel vaccines.

Oral Presentation Abstract 2.

Role of mycoplasmas in respiratory diseases in small ruminants

Robin Nicholas[1], Guido R. Loria[2], Ruben Rosales[1], Roger D. Ayling[1]

[1]Animal Health and Veterinary Laboratories Agency, Woodham Lane, Addlestone, Surrey KT15 3NB. UK
[2]Istituto Zooprofilattico Sperimentale della Sicilia "A. Mirri" – Palermo, Italy
E mail: robin.nicholas@ahvla.gsi.gov.uk

Respiratory disease in small ruminants is responsible for enormous financial losses worldwide. It may result in sudden death or in protracted illness causing much suffering to affected animals. In addition to death and sickness, reduced feed efficiency, slaughter condemnations, prevention and treatment measures contribute substantially to losses. It is clear with few exceptions that the aetiology of respiratory disease is multifactorial and complex. However, mycoplasmas have been increasingly linked to respiratory disease particularly in goats. The OIE listed contagious caprine pleuropneumonia, caused by *M capricolum* subsp. *capripneumoniae,* has been shown to have a much wider distribution worldwide thanks to improved laboratory diagnosis and is not so host specific as previously believed. Closely related mycoplasmas like *M. mycoides* subsp. *capri* are also responsible for clinically similar though less serious disease. The ubiquitous and highly variable *Mycoplasma ovipneumoniae* has been linked to multifactorial disease in both young and adult sheep and goats and has recently been recognised as a possible aetiological agent of pneumonia in endangered big horn sheep. The talk will cover comparative gross pathology of the different diseases, improved diagnostic tools available and control methods.

Oral Presentation Abstract 3.

Identification of resistance mechanisms to quinolones in *Ureaplasma urealyticum* and *Ureaplasma parvum* in Croatian patients

Blaženka Hunjak[1], Ivan Sabol[2],Gordana Vojnović[3], Andrea Babić Erceg[3], Zdenka Peršić[1], Magdalena Grce[2]

[1]Department of Microbiology and Bacteriology, Croatian National Institute of Public Health, Rockefellerova 2, 10000 Zagreb, Croatia
[2]Rudjer Boskovic Institute, Bijenicka 54, 10000 Zagreb, Croatia
[3]Department of Molecular Diagnostics, Croatian National Institute of Public Health, Rockefellerova 2, 10000 Zagreb, Croatia
E mail: blazenka.hunjak@hzjz.hr

Ureaplasma urealyticum (*U. urealyticum*) and *Ureaplasma parvum* (*U. parvum*), in women of childbearing age are associated with pregnancy complications, premature birth, infertility, pelvic inflammatory disease and urethritis. Those bacteria are members of the class *Mollicutes*. The number of resistant ureaplasma strains to quinolones, tetracyclines and macrolides antibiotics is increasing every year following the wide spread use of antibiotics. The objectives of this study were to genotype *U. parvum* and *U. urealyticum* and determine the molecular mechanism of resistance by analyzing changes in the Quinolone Resistance-Determining Regions (QRDRs) of *gyrA*, *gyrB*, *parC*, and *parE* genes in quinolone resistant ureaplasma isolates.

The study was carried out at gynecological practices of the health centers in the Zagreb region, Croatia, during 2010. Cervicovaginal and urethral swab specimens were obtained from each patient for microbiological analysis. In order to distinguish *U. urealyticum* and *U. parvum*, identification was performed by real-time PCR. The high resolution melting (HRM) method was used to determine changes in the DNA molecule based on changes in thermodynamic stability of double-stranded structure of DNA. After the HRM analysis, strains were selected for further nucleotide sequencing.

The genotyping was successful in analyzing 244 isolates, and there were much more *U. parvum* (92.6%) than *U. urealyticum* (7.3%). The molecular analysis of QRDRs, which is considered responsible for the resistance to quinolone, showed indeed several mutations, mostly of them were silent mutations (8/10) found in the *parE* gene. The isolates with mutations within the *parE* gene were more resistant and moderately resistant (7/8) to quinolones. Although there were most silent mutations in the QRDRs it seems that they still contribute to the occurrence of resistance to quinolones.

Oral Presentation Abstract 4.

Antimicrobial susceptibility of *Mycoplasma bovis* strains isolated from dairy cattle herds in Croatia

Jana Pospichalova[1], Branka Šeol Martinec[2], Krešimir Matanović[2], Hywell J. Ball[3], Dagmar Zendulkova[1], Roger D. Ayling[4]

[1]Faculty of Veterinary Medicine, University of Veterinary and Pharmaceutical Sciences Brno, Palackého tř. 1/3, Brno 612 42, Czech Republic
[2]Department of Microbiology and Infectious Diseases with Clinic, Faculty of Veterinary Medicine University of Zagreb, Heinzelova 55, Zagreb 10000, Croatia.
[3]Agri-Food and Biosciences Institute, Veterinary Sciences Division, Stoney Road, Stormont, Belfast BT4 3SD, UK
[4]Animal Health and Veterinary Laboratories Agency, Woodham Lane, Addlestone, Surrey KT15 3NB, UK
E mail: mvdr.pospichalova@seznam.cz

Mycoplasma bovis is the causative agent of severe clinical mastitis in several allied cattle herds in Croatia and appeared resistant to conventional antimicrobial therapy. Other clinical conditions including pneumonia, arthritis, otitis and reproductive problems are associated with this pathogen and are reported worldwide.

In 2002 and between 2007 and 2009 eighty-seven *Mycoplasma bovis* isolates, recovered from milk, lungs, nasal swabs and joint fluid of Croatian dairy cows or calves were confirmed by PCR and antigen capture ELISA. Subsequently minimum inhibitory concentrations (MIC's) for seven antimicrobials were determined using a microbroth dilution method. Antimicrobials were supplied freeze-dried at specified concentrations in 'Sensititre' plates. The plates were designed to provide doubling dilutions of the antimicrobials from 0.12 µg/ml to 32 µg/ml (or from 0.25 µg/ml to 128 µg/ml for tulathromycin). The inoculum was standardised at approx 5×10^5 CFU per ml and the tests read after 48 hours incubation at 37°C.

The MIC_{50} for enrofloxacin, marbofloxacin, lincomycin, tulathromycin, florfenicol, oxytetracycline and tylosin were 0.25, 0.5, 1, 2, 8, 8 and >32 µg/ml, respectively. Lincomycin, tulathromycin, florfenicol, oxytetracycline and tylosin had a wide range of MIC values indicating the development of antimicrobial resistance and the involvement of different strains in outbreaks. *In vitro* susceptibility should give an indication of the antimicrobials effectiveness for treating animals *in vivo*, although many factors may affect their efficacy and the successful treatment of infected cattle. Our results provide an indication of the antimicrobials that are most likely to be effective. With the range of MIC's obtained from the isolates tested we recommend testing *M. bovis* isolates to help select the best therapeutic treatment.

Oral Presentation Abstract 5.

Isolation and analysis of tetracycline-resistant *Mycoplasma agalactiae* strains from an infected goat herd in Cyprus

George Filioussis[1], Ioannis Ioannou[2], Evanthia Petridou[1], Maria Avraam[2], Nektarios D. Giadinis[3], Spyridon K. Kritas[1]

[1]Department of Microbiology and Infectious Diseases, Faculty of Veterinary Medicine, Aristotle University of Thessaloniki, Thessaloniki 54124, Greece;
[2]Veterinary Services, Nicosia, Cyprus;
[3]Clinic of Farm Animals, Faculty of Veterinary Medicine, Aristotle University of Thessaloniki, Thessaloniki, Greece
E mail: georgefilious@vet.auth.gr

A major concern with the use of tetracycline against mycoplasmas is the development of resistance. Infections in small ruminants due to tetracycline resistant *Mycoplasma agalactiae* strains are becoming a frequent problem worldwide.

In the present paper the detection and analysis of three tetracycline-resistant *M. agalactiae* strains, isolated from infected goats in Cyprus, are reported. The three field isolates were identified as *M. agalactiae* by polymerase chain reaction (PCR) showing 98% identity to the *M. agalactiae* PG2 reference strain. Furthermore, they were found sensitive to tylosin, enrofloxacin, spiramycin and lincomycin. In contrast, they were resistant to tetracycline. None of the putative genes [*tet*(M), *tet*(O) and *tet*(S)] that commonly contribute to high-level resistance to tetracycline could be amplified from their genome. Contrarily, the field isolates were found to carry IS*Mag1,* an insertion sequence related to the IS30 family of mobile elements. Although IS*Mag1* is widely believed to induce high-frequency chromosomal rearrangements resulting in phenotypic changes of microorganisms, its potential role in tetracycline resistance of mycoplasmas requires further studies.

Oral Presentation Abstract 6.

Mycoplasma bovis ribosomal operon loci are hot spots for insertion like sequences

E. Amram[1, 3], R. D. Ayling[2], S. Harrus[3], I. Lysnyansky[1]

[1]Mycoplasma Unit, Division of Avian and Fish Diseases, Kimron Veterinary Institute, Israel
[2]Animal Health and Veterinary Laboratories Agency, Weybridge, UK
[3]Koret School of Veterinary Medicine, Faculty of Agriculture, Hebrew University of Jerusalem, Israel
E-mail: innal@moag.gov.il

Field isolates of the bovine pathogen, *Mycoplasma bovis,* that had acquired antimicrobial resistance to macrolides were analysed. Southern blot analysis was performed to quantify the number of *rrs* (16S rRNA) and *rrl* (23S rRNA) alleles, as well as the size of the *EcoR*I-fragments that contained the *rrs* and *rrl* genes. We identified the presence of four different configurations (C-F) of the ribosomal RNA (rRNA) operons, in addition to the configurations found in the published genomes of *M. bovis* PG45 (A) and *M. bovis* HB0801 (B). Sequence analysis of the A-F configurations revealed the presence of the following features: (i) two tandem 16S-23S rRNAs alleles in configuration A; (ii) an insertion like sequence (IS) IS*Mbov4* in the intergenic region 3 (IGR), which was located between *rrl3* and *rrs4* genes in configuration B; (iii) an insertion like sequence IS*Mbov1* in the IGR-3 in configuration C; (iv) the insertion of IS*Mbov1* and IS*Mbov4* elements in the IGR-3 and insertion of IS*Mbov1* in ISR-5, which was located between *rrl4* and MBOV_0287 genes in configuration D; (v) the insertion of IS*Mbov4* and IS*Mbov1* elements in IGR-3 in configuration E; (vi) one allele of 16S and 23S rRNAs in configuration F. These sequence data suggest that *M. bovis rrn* loci might serve as hot spots for IS elements.

Oral Presentation Abstract 7.

Innate immune recognition and evasion by *Ureaplasma*; the role of the multiple banded antigen

O. Brad Spiller

Cardiff University School of Medicine, Cardiff, U.K.
E mail: SpillerB@cardiff.ac.uk

While *Ureaplasma* spp. are often found as commensals in the genito-urinary tract, there is mounting anecdotal evidence to show that it can be invasive: frequent detection in amniotic fluid and cord blood, as well as bacteria-mediated arthritis in agammaglobulinemic patients. Therefore, *Ureaplasma* spp. are subject to detection by, clearance through and evasion from the innate immune system. The innate immune system is composed of cells armed with pattern recognition molecules as well as the complement system (a soluble amplification cascade composed of 20+ serum proteins), both of which probe the surface of microbes for "non-self" signals. The multiple banded antigen (MBA) separates Ureaplasma into 14 serovars, is thought to be the most abundant and important Ureaplasma surface protein, and has been reported to undergo phase variation as a mechanism of immune evasion.

We found whole *U. parvum* and *U. urealyticum* were detected by and activated toll-like receptors (TLR) 2, 6 and 9 using human transfected reporter cell lines and human amniotic epithelial cells. TLR2 and TLR6 also directly recognised recombinant soluble MBA, while TLR9 detected intracellular Ureaplasma. An adaptive immune response was required for optimal complement activation and clearance of *Ureaplasma* spp. via high levels of anti-Ureaplasma antibodies, which included antibodies against MBA. Serial attack of Ureaplasma with seropositive serum led to evolution of strains *in vitro* that were completely resistant to serum killing; however, no phase variation loss of MBA expression was observed for any of the resistant strains. Investigation of *in vivo* isolates recovered from serial endotracheal samples from intubated neonates also showed no loss of MBA expression for isolates recovered from 2-4 weeks apart from infected patients. While loss of MBA as a surface target for antibodies was not found to mediate immune evasion, we have identified a Ureaplasma protein that is consistently induced with serum resistance following human immune system pressure.

Mycoplasma salivarium found as a dominant colonizer of an oral cancer in a patient with Fanconi anemia

Birgit Henrich[1], Eunike Velleuer[2], Ralf Dietrich[3], Wolfgang Gerlach[4], Alexander Sczyrba[4], Madis Rumming[2, 4], Jens Stoye[4], Arndt Borkhardt[2], and Ute Fischer[2]

[1]Institute of Medical Microbiology and Hospital Hygiene, Medical Faculty, Heinrich Heine University, Moorenstr. 5, 40225 Düsseldorf, Germany
[2]Department of Pediatric Oncology, Hematology and Clinical Immunology, Center for Child and Adolescent Health, Medical Faculty, Heinrich Heine University, Moorenstr. 5, 40225 Düsseldorf, Germany.
[3]German Fanconi-Anemia-Help e.V., Böckenweg 4, 59427 Unna, Germany
[4]Genome Informatics, Faculty of Technology, Center for Biotechnology (CeBiTec), University Bielefeld, Universitystr. 25, 33615 Bielefeld, Germany
E mail: Birgit.Henrich@uni-duesseldorf.de

Mycoplasma salivarium belongs to the class of the smallest self-replicating *Mollicutes* and is predominantly found in the oral cavity of humans. In general it is considered as a non-pathogenic commensal. However, some reports point to an association with human diseases. For example *M. salivarium* was found as the causative agent of a submasseteric abscess, in material of pulpal necrosis, in brain abscess and clogged biliary stent.

Here we describe the detection of *M. salivarium* on the surface of a squamous cell carcinoma of the tongue of a 41 year old patient with Fanconi anemia. Fanconi anemia is an inherited bone marrow failure syndrome based on a defect in DNA-repair. Clinical characteristics are congenital malformations, progressive bone marrow failure and an increased risk of malignoma, especially squamous cell carcinoma. Employing high coverage, massive parallel Roche/454-next-generation-sequencing of 16S rDNA amplicons we analysed the microbiome at four distinct regions of the oral cavity: one swab was taken from the tumour of the tongue, one from the neighbouring gingival tissue and two swabs from both corresponding mirror-inverted sites.

The microbiome at the mirror-inverted, healthy sites was dominated by more than 40% reads for *Pseudomonas* spp. which changed to a *Mollicutes*-predominance of 96% at the tumour region. The neighbouring gingival tissues showed intermediate prevalence for both species. Homology search using the reads of the *Mollicutes* branch in BLAST analysis revealed that the exclusive species found was *M. salivarium*. Quantification of the *M. salivarium* load of each sample by TaqMan-PCR confirmed the prevalence of *M. salivarium* at the tumour site and led to the suggestion that this mycoplasma species with its reduced coding capacity found ideal breeding grounds at the tumour site. It remains to be elucidated in future whether *M. salivarium* can function as a predictive bio-marker for tumour development.

Mycoplasma agalactiae infection of the natural host: Identification of a key virulence factor

Eric Baranowski[1], Dominique Bergonier[2], Eveline Sagné[2], Marie-Claude Hygonenq[1], Patricia Ronsin[2], Xavier Berthelot[2], Christine Citti[1]

[1]INRA, UMR 1225, IHAP, F-31076 Toulouse, France
[2]Université de Toulouse, INP-ENVT, UMR 1225, IHAP, F-31076 Toulouse, France
E mail: e.baranowski@envt.fr

Mechanisms underlying pathogenic processes in mycoplasma infections are poorly understood, mainly because of limited sequence similarities with classical, bacterial virulence factors. Recently, large-scale transposon mutagenesis in the ruminant pathogen *Mycoplasma agalactiae* (MA) identified the NIF locus, including *nifS* and *nifU*, as essential for mycoplasma growth in cell culture, while dispensable in axenic media. To evaluate the role of this locus *in vivo*, the infectivity of two knock-out mutants was tested upon experimental infection in the natural host. In this model, the parental PG2 strain was able to establish a systemic infection in lactating ewes, colonizing various body sites such as lymph nodes and the mammary gland, even when inoculated at low doses. In these PG2-infected ewes, together with a specific antibody response, dynamic changes in the mycoplasma surface composition were observed over the course of infection, with rapid in-host oscillation of variable proteins (Vpma) expression and multiple Vpma profiles co-existing in the same animal. In contrast and despite a sensitive model, none of the knock-out mutants were able to survive and colonize the host. The extreme avirulent phenotype of the two mutants was further supported by the absence of an IgG response in inoculated animals. The exact role of the NIF locus remains to be elucidated but these data demonstrate that it plays a key role in the infectious process of MA and most likely of other pathogenic mycoplasma species as many carry closely related homologs.

Contagious agalactia: how to manage an outbreak

Guido R. Loria[1], Roberto Puleio[1], Anna Tamburello[1], Francesca Messina[1], Giusi Macaluso[1], Robin A. J. Nicholas[2]

[1]Istituto Zooprofilattico Sperimentale della Sicilia "A. Mirri" – Via G. Marinuzzi 3, 90129, Palermo, Italy
[2]Animal Health Veterinary Laboratories Agency, Woodham Lane, Addlestone, Surrey KT15 3NB, UK
E mail: guidoruggero.loria@izssicilia.it

Contagious agalactia (CA) mainly affects the small ruminant dairy industry in Mediterranean areas, where it has a high economic impact. CA is a priority in this sector because of costly production losses such as low milk yields, weak lambs, poor fertility as well as the indirect costs of treatment, veterinary time and additional labour required to look after sick animals. In several countries including Italy, in spite of improved antibiotics and commercial vaccines against mycoplasmas, control of CA still, more often than not, results in failure, with a rapid development of agalactia to the majority of animals. Moreover the strict regulations concerning CA in the EU and Italy in particular seems to compound the situation resulting in further depression to the economy of the affected farm over a prolonged time

What should be done in the case of an outbreak and how can the damage be limited?

A realistic chance of veterinary control requires rapid clinical and laboratory confirmation of a CA outbreak: the fewer the ewes and lambs are involved the easier we can limit the general impact/losses on farm production. Improvements in hygiene and basic farm husbandry (such as disinfection procedures, milking machine and availability of a separated quarantine paddock) could help in the management of an aggressive outbreak especially when observed during middle period of lactation. Control is mainly based on repeated booster doses of inactivated vaccines while antibiotics such as the fluoroquinolones, macrolides or, even the cheaper, tetracyclines, should be recommended only for those few ewes showing clear clinical signs and these should be kept separated in a quarantine paddock until they become dry to avoid the risk for human consumers and massive contamination of the farm. The authors describe the experience and the veterinary approach against CA performed in one of the historically endemic areas of Sicily.

New insights into *Mycoplasma bovis* regulation of host immunity and potential pathways towards a protective vaccine

Musa Mulongo, Tracy Prysliak, Erin Scruten, Scott Napper, Andy Potter, Jose Perez-Casal

Vaccine and Infectious Disease Organization; International Vaccine Centre. University of Saskatchewan, 120 Veterinary Rd. Saskatoon, SK. Canada S7N 5E3
E mail: musa.mulongo@usask.ca

Mycoplasma bovis (*M. bovis*) is one of the major causative pathogens of the bovine respiratory disease (BRD) complex that is characterized by enzootic pneumonia, mastitis, pleuritis and polyarthritis. The nature of the interaction between *M. bovis* and both adaptive and innate immunity is poorly understood. Our laboratory is currently undertaking experimentation to characterize *M. bovis* - host interaction and also identification of *M. bovis* candidate vaccines for subsequent use in experimental vaccination and challenge trials. We have used a combination of bovine peptide kinome arrays, traditional immunoassays and lymphocyte stimulation assays to develop a tentative picture of how *M. bovis* regulates host immunity. Our findings indicate that *M. bovis* induces inflammatory responses at respiratory surfaces but stimulates a more regulatory anti-inflammatory peripheral immune response. Further, *M. bovis* delays the apoptosis of PBMC, alveolar macrophages and purified blood monocytes. In two vaccine trials involving the use of *M. bovis* Gapdh and total membrane extracts and fractions, we observed induction of high IgG1 and IgG2 titres that were not protective. We also observed non-responsive PBMC suggesting suppression of T cell activity *in vitro* as stimulation indices in treated PBMC were lower than untreated cells. We are currently undertaking studies to identify novel *M. bovis* proteins responsible for this immunosuppression as well as investigating any potential role of Th17 $CD4^+$ T cells in mucosal immunity against *M. bovis*.

The application of denaturing gradient gel electrophoresis (DGGE) in avian mycoplasma diagnosis

Salvatore Catania[1], Federica Gobbo[1], Stefania Rodio[1], Elisa Baldasso[1], Alice Fincato[1], Marialuisa Moronato[1], Robin A.J. Nicholas[2]

[1]Istituto Zooprofilattico delle Venezie, Mycoplasma Unit, Viale Università 10, Legnaro (PD), 35020, Italy.
[2]Animal Health and Veterinary Laboratories Agency, Woodham Lane, Addlestone, Surrey KT15 3NB. UK
E mail: scatania@izsvenezie.it

The pathogenic role of some mycoplasma species has been widely reported in the poultry industry which represents a very important sector in animal production. Usually in this sector the diagnosis of mycoplasma is focused on the detection by molecular methods of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* based on the suspicion of the practitioners in the field. However, focusing on only two species can lead to inadequate diagnosis as many other species, whose role in disease has never been fully explored, have been isolated from diseased birds. The real situation as regards mycoplasma flora in an important sector such as the poultry industry could represent an important issue. In addition genetic recombination in mycoplasmas may also have serious consequences.

This PCR/DGGE has been used for several years in some European laboratories for the detection and identification of over 80 *Mycoplasma* species. It is rapid, sensitive and can uniquely detect new and mixed mycoplasma species in clinical samples. At the IZS Venezie we have applied the PCR/ DGGE identification test for 3 years. During this period we have seen a significant improvement in our diagnostic activities because the DGGE method combines the merits of biomolecular and traditional isolation methods. It allows us to rapidly identify mycoplasma species involved while enabling us to perform other and deeper analysis on the isolates. Finally by this approach we have the opportunity to identify any species of live and cultivable mycoplasma present in the samples, typical of microbiological procedure, but with a considerable saving of time.

The goal of this work is to share our considerations on this powerful technique in the avian mycoplasma sector.

Mycoplasma bovigenitalium infection in a cow herd: a practical approach using antibiotic susceptibility testing of isolates

Salvatore Catania[1], Alex Tavella[2], Michael Puff[3], Anna Sturaro[1], Barbara Flaminio[1], Luciano Iob[1], Robin A. J. Nicholas[4]

[1]Istituto Zooprofilattico delle Venezie, Mycoplasma Unit, Viale Università 10, Legnaro (PD), 35020, Italy.
[2]Istituto Zooprofilattico delle Venezie, Diagnostic Service Bolzano, Via Laura Conti 4, Bolzano, 39100, Italy
[3]Pratictioner, Bolzano, Italy
[4]Animal Health and Veterinary Laboratories Agency, Woodham Lane, Addlestone, Surrey KT15 3NB. UK
E mail: scatania@izsvenezie.it

Mycoplasma bovigenitalium, a pathogenic mycoplasma of cattle, can cause vulvovaginitis, epididymitis, infertility, and has been associated with mastitis, pneumonia, conjunctivitis and arthritis. The transmission routes are via sexual contact and/or aerosol. During our diagnostic activities we were contacted by a practitioner who reported an infertility syndrome in a dairy cattle herd where artificial insemination was the sole means of reproduction. Cows and heifers required at least 4 or more service to conceive. We carried out diagnostic tests for the most important infectious agents that could affect the reproductive system. The results of the all routine diagnostic tests were negative with the exception of mycoplasma isolation on vaginal swabs that showed mycoplasma colonies which were identified as *M. bovigenitalium* by PCR/DGGE. In order to provide the practitioner with some advice to manage the problem, we decided to perform antibiotic susceptibility testing on the isolated strains. This showed that the isolates had low *in vitro* MIC values for tylosin. Based on this guidance the practitioner treated 10 heifers with an injection of tylosin following the manufacturer's dose regime 1 day before the animals were "on heat" and 3 days after the insemination. The treatment was focused only on the heifers in order to avoid any problem of drug residues in the milk. All 10 treated heifers became pregnant with a maximum of 2 services, corresponding to a significant fertility improvement in young stock. Further investigations on the source and way of infection are required.

Mycoplasma bovis in Ireland

Hywel J. Ball

Agri-Food and Biosciences Institute, Veterinary Sciences Division, Stoney Road, Stormont, BELFAST BT4 3SD
E mail: Hywel.Ball@afbini.gov.uk

Ireland was the last part of the Britain to record *Mycoplasma bovis* involvement in calf pneumonia following the relaxation of the EU border regulations during 1993 and the subsequent large increase in calf importation. Although mastitis and arthritis have been recorded, *M. bovis* has been more significant as a contribution to calf pneumonia in Northern Ireland being demonstrated in up to 27% of terminal cases submitted per annum to the institute for post mortem examination, as determined by a culture enrichment sELISA. Recent studies using PCR for detection has demonstrated that this is an underestimate of the prevalence. Experimental studies involving the role of *M. bovis* in calf pneumonia has included the demonstration of the involvement of the host immune response in the development of multi-focal pulmonary coagulative necrosis lesions that are characteristic of the chronic stage of disease. This work and additional finding from experimental infection studies will be reviewed and used as the basis of some speculative suggestions concerning the involvement of *M. bovis* in calf pneumonia and in cases of primary arthritis that have recently re-occurred in Ireland.

Extrachromosomal forms and strain variable integration sites define Tra Islands as novel mobile genetic elements of *Mycoplasma leachii* and *Mycoplasma capricolum* subsp. *capricolum*

Michael J. Calcutt, Mark F. Foecking, Kim S. Wise

University of Missouri, Columbia Missouri 65211 USA
E mail: calcuttm@missouri.edu

Comparative analysis of whole genome sequence sets has greatly facilitated the identification of mobile genetic elements (MGEs) in bacteria. Analysis of the genome sequence of *Mycoplasma capricolum* subsp. *capricolum* Kid led to the detection of two putative MGEs integrated into the chromosome, in addition to the previously identified unit Integrative Conjugative Element (ICE). As each MGE encoded a gene with homology to TraE, a protein associated with conjugative DNA molecules, the units were designated Tra Island I (43 kb in length) and Tra Island II (24 kb). The genome sequences for two strains of *Mycoplasma leachii* also encode a Tra Island I-like sequence (in strain PG50) and two possible Tra Island II-related units in strain 99/014/6, each of the latter being associated with a break in overall gene synteny with strain PG50. Analysis of the identified Tra Islands disclosed the following features: (i) presence of multiple mobility-related genes including a terminal ORF with significant homology to IS*3*-family transposases, (ii) multiple ORFs containing tandem copies of the previously reported PARCEL domain and (iii) extrachromosomal forms detectable by PCR. Consistent with the designation as MGEs, Tra Islands were found to be strain variable in genomic location and copy number. Regions of Tra Island II exhibit homology to both available *Mycoplasma mycoides (Mmy)* subsp. *mycoides* SC genome sequences; however Tra Islands are not ubiquitous in the "*Mmy* cluster" as such units are not present in the publically available genomes of *Mmy* subsp. *capri*, *M. capricolum* subsp. *capripneumoniae* or *Mycoplasma feriruminatoris*. These data indicate that Tra Islands represent a novel family of integrative elements that insert via an IS*3*-like transposition mechanism with potential for promoting intraspecies chromosomal re-arrangements and lateral gene transfer between co-resident mycoplasmas.

Characterization of free exopolysaccharides secreted by *Mycoplasma mycoides* subsp. *mycoides*

Clothilde Bertin[1,2,3,4], Corinne Pau-Roblot[5], Josiane Courtois[5], Lucía Manso-Silván [3,4], François Thiaucourt[3,4], Florence Tardy[1,2], Dominique Le Grand[2,1], François Poumarat[1,2], Patrice Gaurivaud[1,2]

[1]Anses, Laboratoire de Lyon, UMR Mycoplasmoses des ruminants, 31 avenue Tony Garnier, 69364 Lyon, France
[2]UMR Mycoplasmoses des ruminants, Université Lyon VetAgro Sup-Campus Vétérinaire de Lyon, 69280 Marcy-l'Etoile, France
[3]CIRAD, UMR Contrôle des maladies animales exotiques et émergentes, 34398 Montpellier, France
[4]INRA, UMR1309 Contrôle des maladies animales exotiques et émergentes, 34398 Montpellier, France
[5]Unité de Biologie des Plantes et Innovations, Université de Picardie Jules Verne, 80000 Amiens, France
E mail: patrice.gaurivaud@anses.fr

Contagious bovine pleuropneumonia is a severe respiratory disease of cattle that is caused by *Mycoplasma mycoides* subsp. *mycoides* (*Mmm*). In the absence of toxins or other classical virulence determinants, the pathogenicity of *Mmm* is thought to rely on intrinsic metabolic functions and on specific components of the outer cell surface. Among them, the capsular polysaccharide (galactan) has been notably demonstrated to play a role in *Mmm* persistence and dissemination. The free exopolysaccharides (EPS), also produced by *Mmm* and shown to circulate in the blood stream of infected cattle, have received little attention so far. Indeed, their characterization had been compromised by polysaccharide contaminations present in the complex mycoplasma culture medium.

In this study we developed a suitable methodology to produce and purify *Mmm* EPS, to measure their carbohydrate content and to compare them biochemically to the capsular polysaccharide. By NMR analyses we showed that purified, free EPS had a β(1->6)-galactofuranosyl structure identical to that of capsular galactan. We analyzed intraclonal *Mmm* variants that produce opaque / translucent colonies on agar. First, we demonstrated that colony opacity was related to the production of a capsule, as observed by electron microscopy. We compared the EPS extracts and showed that non-capsulated, translucent colony variants produced higher amounts of free EPS than capsulated, opaque colony variants. This phenotypic variation was associated with an antigenic variation of a specific glucose phosphotransferase permease. Finally, we conducted *in silico* analyses of candidate polysaccharide biosynthetic pathways in order to decipher the potential link between glucose phosphotransferase permease activity and attachment / release of galactan. The co-existence of variants producing alternative galactan forms (capsular versus free extracellular galactan) and associated with an antigenic switch constitutes a finely tuned mechanism that may be involved in virulence.

Dramatic decrease of antibiotics susceptibility of *Mycoplasma bovis* strains isolated from bovine respiratory diseases during the last 30 years in France

Anne V. Gautier-Bouchardon[1], Séverine Ferré[1], Dominique Le Grand[2], François Poumarat[3].

[1]Anses, Ploufragan/Plouzané Laboratory, Mycoplasmology-Bacteriology Unit, BP 53, 22440 Ploufragan, France
[2]Université de Lyon, VetAgro Sup, UMR Mycoplasmoses des ruminants, 69280 Marcy-l'Etoile, France
[3]Anses, Lyon Laboratory, UMR Mycoplasmoses des ruminants, 31 avenue Tony Garnier, 69364 Lyon Cedex 07, France
E mail: anne.bouchardon@anses.fr

Over the past decade, it has become clear that *Mycoplasma bovis,* as primary or contributory agent, is a major cause of bovine respiratory disease (BRD) worldwide, and must thus be taken into account in its treatment. Evolution of antimicrobial susceptibility of *M. bovis* in France was studied by comparing two batches of strains: 27 and 46 strains isolated in 1978-1979 and 2010-2012, respectively. These strains came from 73 unrelated outbreaks of BRD scattered all over France and were all isolated from lung lesions of young cattle, half of them in co-infection with other bacteria (mostly *Pasteurellaceae*). Six antimicrobials representative of the main families usually recommended for mycoplasma treatment were tested: tylosin and tilmicosin (macrolides), spectinomycin (aminoglycosides), oxytetracycline (tetracyclines), enrofloxacin (fluoroquinolones) and florfenicol (amphenicols). Minimum inhibitory concentrations (MIC) were determined by a standardised agar dilution technique. Highly significant differences in susceptibility level were evidenced between old and recent strains for most of antimicrobials. MIC_{50} values increased from 4 to >64 µg/mL for spectinomycin, and from 2 to >64 µg/mL for tylosin and tilmicosin. Significant differences were also evidenced for enrofloxacin and oxytetracycline but to a lesser extent. No evolution was observed for florfenicol. As no breakpoints were available for veterinary mycoplasmas, MIC values were compared to CLSI breakpoints given for *Pasteurellaceae* in BRD. With these values, all the recent strains of *M. bovis* were considered resistant to tylosin, tilmicosin, spectinomycin and oxytetracycline and most of them to florfenicol. Enrofloxacin was the most effective antimicrobial *in vitro* with susceptible or intermediate strains. This study shows that in France, despite the diversity of geographical origin, 100 % of recent strains tested are resistant to most of the antimicrobials formerly active on mycoplasmas. We hypothesize that the general antimicrobial therapy pressure has probably been a major selection factor for *M. bovis* strains.

Contagious bovine pleuropneumonia (CBPP): Molecular and patho-evolution of *Mycoplasma mycoides* subsp. *mycoides*

Joachim Frey[1], Jörg Jores[2], Jan Naessens[2], Musa Mulongo[2], Anne Fischer[3], Declan McKeever[4]

[1]Institute of Veterinary Bacteriology, University of Bern, Laenggasstrasse 122, 3012 Bern, Switzerland
[2]International Livestock Research Institute, ILRI, P.O. Box 30709 - 00100 Nairobi, Kenya
[3]International Centre for Insect Physiology and Ecology, Nairobi, Kenya
4Royal Veterinary College, University of London
E-mail: joachim.frey@vbi.unibe.ch

Mycoplasma mycoides subsp. *mycoides*, the ethological agent of contagious bovine pleuropneumonia (CBPP) is a member of the '*M. mycoides* cluster' of Mycoplasmas together with *Mycoplasma capricolum* subsp. *capripneumoniae* the agent of contagious caprine pleuropneumonia (CCPP), *Mycoplasma capricolum* subsp. *capricolum*, *Mycoplasma leachii* and *Mycoplasma mycoides* subsp. *capri*. CBPP and CCPP are major livestock diseases and impact the agricultural sector especially in developing countries through reduced food-supply and international trade restrictions. Analysis of strains of the '*M. mycoides* cluster' using multilocus sequence typing (MLST) revealed the origin of the '*M. mycoides* cluster' dates to about 10,000 years ago, suggesting that the establishment and spread of the cluster coincided with livestock domestication while *M. mycoides* subsp. *mycoides* itself seems to have evolved about 300 years ago as recently revealed by researchers from France. One of the central mechanisms of virulence of *M. mycoides* subsp. *mycoides* and also *M. leachii* is its particular metabolism of physiological glycerol that includes a highly efficient ABC glycerol transporter system and the glycerol phosphate oxidase GlpO that is membrane located and translocates the toxic metabolite H_2O_2 from the bacterial surface into the host cell. While rabbit and mouse antibodies directed against GlpO of *M. mycoides* subsp. *mycoides* neutralize the enzymatic activity of GlpO and thus prevent production of H_2O_2, bovine serum obtained from cattle that were immunized with recombinant GlpO or serum from cattle that suffered from CBPP has no neutralizing activity. *In silico* analysis of the enzymatically important epitopes of GlpO and comparison with epitopes of related enzymes in mammals indicates host adaptation of this central enzyme for pathogenicity to the bovine host. This patho-evolution of *M. mycoides* subsp. *mycoides* represents a particular challenge in development of efficient long lasting vaccines against CBPP.

Mycoplasmal pneumonia as the most common respiratory disease complex of pigs' illnesses (PRDC)

Biljana Radojičić[1], Maja Bukvić[1], Marko Stepanov[2]

[1]University of Belgrade, Faculty of Veterinary Medicine, Bulevar Oslobodjenja 18, 11000Belgrade, Serbia
[2]Farm of pigs "Lacarak", Sremska Mitrovica, Serbia
E mail: biljanar@vet.bg.ac.rs

In this study we investigated the etiology and incidence of porcine respiratory disease complex (PRDC) in two pig farms in the Sremska Mitrovica region of Serbia. One farm was a large closed farm (farm 1); the other a small farm (farm 2) was open for fattening pigs in two seasons (Spring and Autumn). Diagnosis was made by clinical presentation, laboratory diagnosis using PCR and ELISA; and pathological diagnosis determined by morphological examination for PRDC. In the Institute of Veterinary Medicine in Novi Sad (NIV-NS) the submitted material from dead pigs included lung tissue and tonsils. In Spring and Autumn the presence of *Mycoplasma hyopneumoniae* and *Actinobacillus pleuropneuominae* and the viruses PRRSV and PCV2 were reported on both farms. Bacterial diagnosis was confirmed by PCR and the presence of viruses by serological diagnosis. Farm one had a slightly higher number of reported cases of PRDC, but the difference was not statistically significant.

In all categories of pigs, about 56 percent had mycoplasma pneumonia in the PRDC. Non-specific factors contributing to higher frequencies of the disease were recorded on a farm 1 (dust and poor ventilation). The season did not have a significant impact on the occurrence of disease. The PRDC affected pigs from farm 2 were not vaccinated against mycoplasmas. Different antimicrobials to treat porkers were evaluated which included: lincomycin-spectinomicin; florfenicol, and tulathromycin. Tulathromycin gave the best therapeutic efficacy against mycoplasmas, especially in unvaccinated animals where the clinical manifestations were more pronounced.

Role of porcine mycoplasmas in the porcine respiratory disease complex: a story of association

Ruben S. Rosales, Angel Ortiz-Pelaez, Roger D. Ayling, Robin A. Nicholas

Animal Health and Veterinary Laboratories Agency, Woodham Lane, Addlestone, Surrey KT15 3NB. UK
E mail: ruben.rosales@ahvla.gsi.gov.uk

The porcine respiratory disease complex (PRDC) represents one of the most economically important diseases in intensive porcine production systems. This complex is characterised by a multifactorial association of various porcine respiratory pathogens, including viruses, bacteria, and different environmental stressors. Porcine mycoplasmas play an essential role in this complex, particularly *Mycoplasma hyopneumoniae*, the etiological agent of Enzootic pneumonia (EP) and *Mycoplasma hyorhinis*, a common bacterial isolate associated to cases of polyserositis, arthritis and EP-like disease.

To further elucidate the role of porcine mycoplasmas in PRDC, a retrospective analysis of more than 900 porcine submissions received at the Animal Health and Veterinary Laboratories Agency (Weybridge, UK) between 2005 to 2011, which had suspicion of respiratory disease, were analysed and the prevalence of mycoplasmas in these cases determined. In addition the association between these mycoplasmas and other pathogens involved in porcine respiratory disease was evaluated. For this purpose, the final diagnostic result for each submission was obtained and the frequency of association with other microorganisms involved in porcine respiratory disease determined.

Our results demonstrate the central role of both *Mycoplasma* species in PRDC, either in their own right, or in association with other porcine respiratory pathogens, further confirming the status of *Mycoplasma hyorhinis* as a key pathogen in respiratory disease of pigs.

A chimeric protein vaccine containing *Mycoplasma hyopneumoniae* antigens: protective efficacy against enzootic pneumonia in pigs

S. B. Marchioro[1,2], A. T. M. G. Michiels[1], R. Del Pozo Sacristán[1], F. Conceição[2], O. Dellagostin[2], F. Haesebrouck[1], D. Maes[1]

[1]Ghent University, Faculty of Veterinary Medicine. Salisburylaan 133, B-9820 Merelbeke, Belgium.
[2] Laboratório de Biologia Molecular, Núcleo de Biotecnologia, Centro de Desenvolvimento Tecnológico, Universidade Federal de Pelotas, RS, Brazil
E mail: silmarchioro@yahoo.com.br

A recombinant chimeric protein containing three *Mycoplasma hyopneumoniae* antigens; (R1, the C-terminal portion of the adhesin P97, the heat shock protein P42 and NrdF) fused to an adjuvant, the subunit B of heat-labile enterotoxin of *Escherichia coli* (LTB), was used to immunize pigs intramuscularly and intranasally against enzootic pneumonia. The humoral and local immune responses and the efficacy against experimental *M. hyopneumoniae* infection were evaluated. In total, 60 male piglets of 4 weeks of age were randomly allocated into six different experimental groups of 10 animals each: 1) recombinant chimeric protein by intramuscular (IM) application; 2) recombinant chimeric protein by intranasal (IN) application; 3) commercial bacterin by IM; 4) positive control group a (vaccinated with the adjuvant LTB by IM); 5) positive control group b (vaccinated with the adjuvant LTB by IN). All groups were vaccinated at 24 and 38 days of age and challenged at 52 days of age. One group was kept as negative control (vaccinated with the adjuvant LTB by IN (5 animals) or IM (5 animals) and not challenged.

At necropsy, the chimeric protein elicited a significant response ($p<0.05$) of IgG in serum and IgA locally in brochoalveolar lavage against all the antigens present in the protein, compared to the negative control group. The highest mean values for macroscopic lesions were observed in group that received just the adjuvant LTB via IM (1.28), followed by the LTB via IN (0.67), the lowest in the groups vaccinated with the chimeric protein IM (0.41) and IN (0.38). Significant differences were found between the group vaccinated with the commercial bacterin (0.00) and the group that received just adjuvant (LTB) via IM (1.28) ($p<0.05$). Macroscopic lung lesions were not observed in the negative control group and in the group vaccinated with the commercial bacterin.

The chimeric protein induced an immune response and conferred protection, but the bacterin-based commercial vaccine induced better protection than the chimeric protein. These results suggest that immunodominant antigens other than the ones used in the chimeric protein and possibly also the adjuvants are also important in the induction of a protective immune response. This should be taken into account in the future development of *M. hyopneumoniae* subunit vaccines.

Identification of haemagglutinin and neuraminidase of *Mycoplasma cynos*

Saša Kastelic[1], Mojca Narat[1], Nataša Tozon[2], Dušan Benčina[1]

[1]Department of Animal Science, Biotechnical Faculty, University of Ljubljana, Groblje 3, SI-1230 Domžale, Slovenia.
[2]Veterinary Faculty, University of Ljubljana, Gerbičeva 60, SI-1000 Ljubljana, Slovenia.
E mail: sasa.kastelic@bf.uni-lj.si

Species from the genus *Mycoplasma* have small genomes and no cell wall. Many of them cause diseases in animals and humans. Pathogenic canine mycoplasma, *Mycoplasma cynos*, is commonly associated with pneumonia in dogs. However, its factors that enable colonization of host organs are largely unknown. Haemagglutinins and neuraminidases are possible factors that help *Mycoplasma cynos* colonization and survival in immunocompetent hosts.

Neuraminidases (sialidases) are enzymes, capable of hydrolysing the glycosidic bond between sialic acid (commonly found as a terminal residue of host glycoproteins) and neighbouring sugar. They are putative virulence factors of several mycoplasmas, including *M. synoviae*, *M. gallisepticum*, *M. neurolyticum*, *M. alligatoris* and *M. canis*. We have shown that *M. cynos* has considerable levels of neuraminidase activity. Zymogram showed that the putative neuraminidase of *M. cynos* is approximately 105 kDa protein. *M. cynos* has a gene for neuraminidase of such size. Our studies indicate that neuraminidase is capable of desialylating glycoproteins in mucus of dogs and that it preferentially cleaves sia(α2-3)gal moiety.

Haemagglutinins are highly immunogenic proteins that are capable of haemagglutination and haemadsorption of host erythrocytes. Many of them bind to receptors containing sialic acid, which are also present on glycophorin in erythrocyte membranes. We have shown that cells as well as “cell-free” supernatants of *M. cynos* can haemagglutinate chicken and dog erythrocytes. *M. cynos* synthesizes ~65 kDa protein, which binds to erythrocytes and is most probably its haemagglutinin. Furthermore, sera of several infected dogs contained antibodies, which prevented haemagglutination of erythrocytes in the haemagglutination-inhibition tests. Thus, haemagglutinin (termed HAP) represents a major immunogenic protein of *M. cynos*.

In conclusion, HAP and neuraminidase of *M. cynos* seem to play important roles in interactions with dog cells and sialylated glycoproteins.

Motility of *Mycoplasma gallisepticum*

Michael P. Szostak, Ivana Indikova, Martin Vronka, Renate Rosengarten

Institute of Bacteriology, Mycology and Hygiene, University of Veterinary Medicine Vienna, Austria
E mail: michael.szostak@vu-wien.ac.at

Motility enables a bacterium to gain access to new niches, better food supplies, and may help escape from hostile environments. Mobility seems advantageous for host-dependent microbes like Mycoplasma however, only a few have developed the ability to move in a directed manner. Mycoplasmas have developed a motility mechanism distinct from most other bacteria lacking any noticeable flagella, filaments or protrusions they are able to glide over smooth surfaces.

Motility of *M. pulmonis* was described in 1965 but the motility of the fish pathogen *M. mobile* became the most studied species. Being the fastest moving mycoplasma, even at room temperature, this was beneficial for research on motility. Thus, details about the gliding machinery of *M. mobile* accumulated over the last decade, has been translated into a "centipede" model of locomotion.

Members of the *M. pneumoniae* phylogenetic group seem to represent the biggest group of motile mycoplasmas, with *M. pneumoniae, M. genitalium, M. amphoriforme* and *M. gallisepticum* being able to glide. It seems as mycoplasmas have evolved different solutions for the same task: to glide on surfaces.

To identify proteins involved in the gliding motility of *M. gallisepticum,* we created transposon mutants and screened them for satellite microcolony formation in medium with low agar concentration. The majority of mutants with gliding deficiencies had also lost the ability to hemadsorb and further investigations confirmed the disruption of the major cytadherence genes *gapA* or *crmA* in those mutants. It is difficult to link either GapA or CrmA with the effects on hemadsorption or motility seen in natural mutants. Therefore, artificial mutants were constructed expressing engineered CrmA molecules, and the effects on morphology, hemadsorption and motility were analyzed.

A group of non-motile mutants, however, carried the transposon in *mgc2*, which is encoded directly upstream of *gapA*/*crmA*. Lack of Mgc2 did not result in a loss of hemadsorption activity, suggesting that this protein is not involved in cytadherence. The essential role of this gene in the gliding mechanism of *M. gallisepticum* could be confirmed by complementation of the non-motile transposon mutant with an *mgc2-His* gene hybrid delivered by an integration/expression vector. Similarities to the participation of P30 in the motility of *M. pneumoniae* are evident, with subtle distinctions that will be addressed.

Putative pathogenic factors of *Mycoplasma synoviae*

Dušan Benčina, Ivanka Cizelj, Rebeka Lucijana Berčič, Mojca Narat

Department of Animal Science, Biotechnical Faculty, University of Ljubljana, 1230 Domžale, Slovenia
E mail: Dusan.Bencina@bf.uni-lj.si

Mycoplasma synoviae is a major poultry pathogen causing respiratory disease and infectious synovitis (IS). *M. synoviae* synthesizes hemagglutinin VlhA which enables attachment to chicken cells containing sialic acid receptors e.g. to glycophorin of erythrocytes. The N-terminal part of VlhA, the lipoprotein MSPB, stimulates chicken macrophages and other cells to produce NO, proinflammatory interleukins IL-1β, IL-6 and other cytokines. They were also found in synovial fluids (SF) in chickens with acute forms of IS. *M. synoviae* invades chicken cells, including chondrocytes and causes apoptosis and/or necrosis. Its hemolysin HlyC seems to be involved in haemolytic anemia, whereas its nuclease (also extracellular) efficiently cleaved DNA of chickens' cells. *M. synoviae* synthesizes a cysteine protease, termed CysP. CysP cleaves chicken proteins, including IgG, which is cleaved into Fab and Fc. Such a cleavage of IgG was found also in SF of chickens during the acute stage of IS and presumably contributes to the appearance of rheumatoid factor (RF) i.e. to autoantibodies reacting with Fc of IgG. Neuraminidase NanH is another potential virulence factor of *M. synoviae*. NanH desialylates a variety of chicken glycoproteins, including those on chicken chondrocytes and Fc (CH2 domain) of IgG. Whereas almost all chickens with IS raised antibodies to VlhA, about 70% of them, had also antibodies to CysP and NanH. In addition, many samples of SF from chickens with IS contained autoantibodies that reacted with Fc, enolase, collagen (type II), several proteins of chondrocytes, fibronectin and autoantibodies which agglutinated erythrocytes. In conclusion, *M. synoviae* has proteins causing direct damage to host cells and proteins, as well as factors inducing severe inflammation and autoimmune processes.

Herd level diagnosis of *Mycoplasma bovis* – lessons learned from a pilot study in Denmark 2012

Kaspar Krogh[1], Erling Kristensen[1], Liza Rosenbaum Nielsen[2]

[1]Knowledge Centre for Agriculture, Cattle, Agro Food Park 15, DK-8200 Aarhus N, Denmark.
[2]University of Copenhagen, Faculty of Health and Medical Sciences, Groennegaardsvej 8, DK-1870 Frederiksberg C, Denmark
E mail: ksk@vfl.dk

Six large dairy herds with more than 250 cows (3 case herds and 3 control herds) were selected based on previous monthly PCR-results for *Mycoplasma bovis* (MB) (PathoProof Mastitis Complete 16, Thermo Fisher Scientific Inc. Finland) on bulk tank milk (BTM). Case-herds were identified as herds with concomitant clinical signs (e.g. arthritis, mastitis or pneumonia) and Ct-values for MB in BTM below 37. Control herds had no clinical signs and Ct-values above 37. Antibody levels in BTM samples collected every 4th week were obtained using a MB specific ELISA (Bio-X Diagnostics, Belgium) with a cut-off-value at 37 ODC%. Case herds were positive in BTM in 11 of the 12 test rounds. Control herds were negative in all 12 test rounds. Generally, the antibody results from the BTM samples were stable during the study period. However, 6 of the 12 samples from control herds were above 30 OCD% (and below 37 OCD%). This indicates that we need more information about the herd infection status and other herd characteristics to understand the responses of the ELISA test at herd level.

Each farm was visited 3 times with 4-7 weeks intervals in the second half of 2012. Blood samples were collected from 7 distinct age groups (including new calves born since last visit) and, if possible, repeated samples were collected from the same animal (in total 1,532 blood samples from 691 animals were analyzed). Blood samples were tested for MB antibodies. Antibody levels and seroprevalence from the individual animals were generally higher in case herds than in the control herds. However, we also identified unexpectedly high antibody levels among individual animals in all of the control herds. Most likely, the best age group to identify the true MB status at herd level is calves below 6 months old, as we found the largest differences in seroprevalence between case and control herds at this age. Luckily, this age group is also relatively easy to handle during the blood sampling procedure.

Oral Presentation Abstract 26.

Rapid diagnosis of *Mycoplasma pneumonia*e respiratory infection in children by nested polymerase chain reaction.

Surinder Kumar[1], Sanjeev R. Saigal[1], Gulshan Rai Sethi[2]

[1]Department of Microbiology, Maulana Azad Medical College, New Delhi-110 002, INDIA
[2]Department of Paediatrics, Maulana Azad Medical College, New Delhi-110 002, INDIA
Email: kumarsurinderdr@yahoo.com

Detection of *Mycoplasma pneumoniae* (*M. pneumoniae*), a causative agent of pediatric respiratory infections, is challenging because of the lack of standardized rapid tests. Serological tests and polymerase chain reaction (PCR) based methods are used with different diagnostic criteria. We applied nested polymerase chain reaction (PCR) and serology for rapid detection of *M. pneumoniae* in community-acquired lower respiratory tract infections (LRTIs) in Indian children. Two hundred children aged 2 months to 12 years, with community acquired LRTIs were investigated clinically, radiologically and sera processed for IgM and IgG antibodies to *M*. *pneumoniae* by enzyme linked immunosorbent assay (ELISA). Nasopharyngeal aspirates were obtained on admission for analysis of *M*. *pneumoniae* by nested PCR. Fifty (25%) children documented serological evidence of *M. pneumoniae* infection. Nested PCR was positive in 20 (10%) children for *M. pneumoniae*; 15 (75%) with serologically proven and 5 (25%) serologically unproven *M. pneumoniae* infection. Thirty (30%) children were diagnosed with *M. pneumoniae* infection by serology/PCR. The clinical and radiological features in *M. pneumoniae* positive and negative groups were comparable. In conclusion, nested PCR together with ELISA is effective in the detection of *M. pneumoniae* in pediatric LRTIs. Nested PCR bypassing culture could be employed for rapidly detecting and characterizing *M. pneumoniae* directly from clinical specimens in pediatric LRTIs.

Mollicutes of non-mammal marine animals

Orestes M. Vega-Orellana[1], José B. Poveda[1], Lidia E. Mederos-Iriarte[1], Carlos G. Poveda[1], M. José Caballero[2], Mónica B. Betancor[2], Ramón Roselló-Móra[3], Ana S. Ramírez[1]

[1]Unidad de Epidemiología y Medicina Preventiva,
[2]Grupo de Acuicultura y Genética Marina, Instituto Universitario de Sanidad Animal y Seguridad Alimentaria (IUSA), Universidad de Las Palmas de Gran Canaria, C/Trasmontaña s/n, Arucas, 35413, Canary Islands, Spain
[3]Institut Mediterrani d'Estudis Avançats (CSIC-UIB), Esporles, Islas Baleares, Spain
E mail: aramirez@dpat.ulpgc.es

Mollicutes have been found in nearly all living beings on the planet. *Mycoplasma* species have been reported in marine mammals (cetaceans and pinnipeds) and fish. Among marine invertebrates, Mollicutes have been identified in bryozoans, American oysters, corals and a variety of marine crustacean species like crayfish and shrimps. This work describes the methodology and the attempts to isolate mollicutes from non-mammal marine animals. One hundred and seven fish of twenty one different species, seven octopus (*Octopus vulgaris*), two European flying squids (*Todarodes sagittatus*) and four batches of jellyfish (*Pelagia nocticula*) were tested for the presence of mollicutes. Samples were cultured using a variation of the SP4-II medium. Originally the SP4-II medium was supplemented with 1.5% of NaCl and incubated at 18ºC anaerobically. However, after the first isolation was obtained, the growth conditions were adjusted to 3% of NaCl and aerobically incubation at 25ºC. DNA was extracted after one month of incubation and a Class Mollicutes real time PCR has been done. Mollicutes isolations were obtained from squid in three samples; two from octopus and four from jellyfish. It was not possible to get isolations from fish, although two samples gave positive to the class PCR. Isolates were analyzed biochemically and genetically (16S-23S Intergenic Spacer Region (ISR) and 16S rDNA). PCR products were sent for sequencing. Nucleotide sequences were compared using the GeneBank software. A partial 16S rDNA sequence from a jellyfish isolation was classified as *Spiroplasma* spp. 16S rDNA and ISR from squids and octopus isolations differs significantly from all known *Mycoplasma* species, but have 98-99% of similarity with a partial 16S rDNA sequence (738 bp; AY226323) detected in environmental samples (brine water, Mediterranean Sea).

Oral Presentation Abstract 28.

Understanding cyclic epidemics of *Mycoplasma pneumoniae* infections in England and Wales (1975 -2009) using time-series analysis and mathematical modelling

P. Nguipdop-Djomo[2], E. Vynnycky[2], K. Halsby[1], H. Durnall[3], PEM Fine[2], V. Chalker[1]

[1]Public Health England, London, NW9 5EQ, UK.
[2]London School of Hygiene and Tropical Medicine, London, WC1E 7HT, UK
[3]RCGP Research and Surveillance Centre, Birmingham, UK
E mail: Vicki.chalker@hpa.org.uk

Mycoplasma pneumoniae infections (MPI) cause pneumonia and recurrent epidemics have been observed in the UK. Regular multiannual epidemic cycles have been reported across the world with a periodicity of 3-7 y. This investigation focuses on factors driving recurrent epidemics of MPI in England and Wales. We used time-series analysis to describe laboratory reports of MPI to the UK Health Protection Agency (HPA) from 1975 to 2009. A reversible catalytic model was fitted to seroprevalence survey data to estimate transmission parameters for MPI; then we built and quantified a mathematical model to simulate their transmission dynamics and investigate factors that sustain the epidemic cycles.

Epidemics with lasting 18 months on average, recurring at ~4y intervals were observed. Epidemic peaks were synchronous across the regions of England and Wales and always occurred during winter. A strong seasonal pattern with higher reported incidence during winter and lower in summer was observed. Average incidence during epidemics (3.7 per 100,000 person-y) was 3 times higher than that in inter-epidemic periods (1.2 per 100,000 person-y), similar to recent observed cumulative rates for England and Wales in 2012-2013 (3.1 per 100,000). The basic reproduction number (R0 – average number of secondary infectious individuals resulting from an infectious person in a totally susceptible population) was estimated at 1.7 (95%CI 1.6-1.9). The catalytic model predicted that by age 40 y, ~90% of the population has been infected at least once. The mathematical model was able to qualitatively reproduce MPI epidemic cycles observed in England and Wales. Simulations suggested that epidemic cycles are intrinsic to MPI natural transmission dynamics in the population, and they are sustained by the interaction between seasonality in the transmission and random variations in other environmental factors that affects its transmissibility.

Poster Presentations

Poster Presentation Abstract P1.

The bactericidal activity of normal human serum against 21 clinical isolates of *Mycoplasma pneumoniae*

Rebecca J. Brown[1], O. Brad Spiller[1], Victoria J. Chalker[2]

[1]Department of Child Health, Institute of Molecular and Experimental Medicine, School of Medicine, Cardiff University, CF14 4XN
[2]Public Health England, 61 Colindale Avenue, London, NW9 5EQ
E mail: BrownRJ6@cardiff.ac.uk

Mycoplasma pneumoniae is a common cause of upper and lower respiratory tract infections and may be responsible for up to 15-20% of community-acquired pneumonias. However, as a consequence of *Mycoplasma pneumoniae* infection, extrapulmonary complications may occur (e.g. encephalitis), related to immunosuppression or direct spread of infection. Understanding of the host immune response to *Mycoplasma pneumoniae* infection has advanced over the recent years although little is known about the role of serum killing and serum resistance to *Mycoplasma pneumoniae* infection. The ability of normal human serum (NHS) to kill bacteria, via the complement system, plays an important role in the host immune defence against infection. The complement system is activated via three pathways and is directly able to kill many microorganisms.

We have screened for complement killing of 21 *Mycoplasma pneumoniae* clinical isolates by normal human serum. Two seropositive and two seronegative normal human sera for *Mycoplasma pneumoniae* were identified and the presence of specific antibodies was confirmed by Western blot and Lineblot analysis. The serum killing assay was performed by incubating clinical isolates in 50% NHS (or heat-inactivated control serum) at 37°C for one hour. Complement specific killing was measured as the fold decrease in survival relative to the matched heat-inactivated control serum.

Our initial findings indicate varying susceptibility of the 21 *Mycoplasma pneumoniae* clinical isolates to seropositive serum killing. Five isolates were particularly susceptible, with less than 0.1% survival, including one with survival of approximately 0.001%. One resistant strain has been identified with greater than 10% survival. Further investigation is on-going to determine the pathway of complement activation responsible for killing the highly susceptible isolates. A comparison of the inherent killing ability of seropositive and seronegative normal human serum against these *Mycoplasma pneumoniae* clinical isolates is underway.

Poster Presentation Abstract P2.

Protein and antigenic profile among *Mycoplasma ovipneumoniae* field strains isolated from goats

Zinka Maksimović, Maid Rifatbegović

Veterinary faculty, Department of Microbiology and infectious diseases, University of Sarajevo, Zmaja od Bosne 90, Sarajevo 71000, Bosnia and Herzegovina
E mail: maid.rifatbegovic@vfs.unsa.ba

Respiratory disease in small ruminants is responsible for enormous financial losses worldwide. Goats are highly susceptible to a number of mycoplasmas, particularly *Mycoplasma capricolum* subsp. *capripneumoniae* and other mycoplasmas of *Mycoplasma mycoides* cluster. *M. ovipneumoniae* is considered to be one of the most important mycoplasmas involved in respiratory disease of sheep. In contrast to the case in sheep, the role of this mycoplasma in the etiology of goat pneumonia is not well understood. The objective of this study was to characterize the protein and antigenic profiles among *M. ovipneumoniae* strains isolated from goats by sodium dodecyl sulphate polyacrylamide gel electrophoresis and immunoblotting. Tested strains were isolated from goats during two outbreaks of severe respiratory disease (n=10) in Bosnia and Herzegovina and Kosovo, and from asymptomatic goats (n=2). Protein and antigenic heterogeneity was observed in the field isolates, which were compared with the reference strain Y 98. These data confirm the importance of this mycoplasma in respiratory disease of goats and indicate strain variation.

Detection of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* infections in poultry

A. Feberwee, J. J. de Wit

GD-Animal Health Service, Arnsbergstraat 7, 7418 EZ Deventer, the Netherlands
E-mail: a.feberwee@gddeventer.com

Mycoplasma gallisepticum (Mg) and *Mycoplasma synoviae* (Ms) are important vertically and horizontally transmitted pathogens for poultry, which exhibit a wide variation in virulence resulting in different clinical manifestations and causing considerable economic losses. Control programmes for Mg and Ms are based on the identification of infected and non-infected poultry flocks by means of serological tests. For the detection of an infection in a poultry flock, both the number of samples and the characteristics of the used tests are important. With regard to the available serological tests, new tests have replaced the old ones, with an increased need to evaluate the new serological tests for its use in Ms and Mg monitoring programmes. For this purpose GD-Animal Health evaluated 300.000 samples from Ms and Mg infected and non-infected poultry flocks in a Ms and Mg RPA test and three ELISA tests. The test specificity and sensitivity results (individual samples) and the flock specificity and flock sensitivity (10-60 samples per house) results showed that it is not advisable to rely on one single test to determine a flock to be infected or free of infection from Mg or Ms. Moreover the results also showed that a low number of false positives can occur in all tests which can lead to an unjust identification of an uninfected flock as being infected.

Stability of tandem repeats using for typing of *Mycoplasma pneumoniae* strains

Roger Dumke, Enno Jacobs

TU Dresden, Institute of Medical Microbiology and Hygiene, Fetscherstrasse 74, 01307 Dresden, Germany
E mail: roger.dumke@tu-dresden.de

Mycoplasma pneumoniae is a common agent causing a broad spectrum of respiratory tract infections in humans. Despite remarkable genetic homogeneity between clinical isolates, circulating strains can be typed by sequence differences in the repetitive elements of the main P1 adhesin and with higher discriminatory power by multi locus variable number of tandem repeat analysis (MLVA). The primary panel of tandem repeats (TR's) comprises five loci (Mpn1, Mpn13-16) located in most cases between open reading frames or in hypothetical orf's. To further discriminate strains with identical MLVA result, the number of TR's in the inter-repetitive part of the gene (MPN142) encoding for the P1 protein (refered as MpnP1) was introduced as additional criterion. However, the overall discriminatory power of the method is based on the stability of the number of TR's during the growth of bacteria in the host as well in artificial media used for cultivation. Here, we analyzed the number of TR's in the mentioned six loci after culture passage to contribute to the overall usefulness of MLVA for typing *M. pneumoniae*. In addition, the number of TR's of *M. pneumoniae* strains in patients tested at different days of infection and of samples obtained from infected guinea pigs representing the influence of host passage of the bacteria on the number of TR's were determined. At present, the results confirmed the stability of the TR's investigated suggesting the usefulness of the typing scheme.

Does *Mycoplasma bovoculi* play a role in the development of infectious bovine keratoconjunctivitis (pinkeye)?

Martin Heller, Christiane Schnee, Evelyn Schubert, Konrad Sachse

Friedrich-Loeffler-Institut, Federal Institute for Animal Health, Institute of Molecular Pathogenesis, Naumburger Str. 96a, D-07743 Jena, Germany
E mail: martin.heller@fli.bund.de

Infectious bovine keratoconjunctivitis (IBK) is the most common and important ocular disease in cattle. Both adult cattle and calves are susceptible. *Moraxella* (*Mor*.) spp. and several *Mycoplasma* (*M*.) spp. have been associated with IBK. To study the role of mycoplasmas, we have investigated the presence of *M. bovoculi* and *M. bovis,* as well as *Mor. bovis, Mor. ovis* and *Mor. bovoculi* in two herds with clinical IBK in comparison to six groups of healthy calves.

Herd 1 consisted of approximately 250 animals, of which 90 have been randomly selected and tested. Heifers were particularly affected by the disease in this herd. In Herd 2 comprising 231 animals, of which all were tested, clinical symptoms occurred predominantly in young calves. Clinical signs ranged from monolateral and bilateral conjunctivitis, tearing, uveitis, corneal opacity and keratitis to ulcerations on the corneal epithelium. In both herds, animals with clinical symptoms were treated with antibiotics resulting in disease mitigation in Herd 2, whereas treatment was less effective in Herd 1. The six control groups comprised a total of 61 healthy calves from the animal facilities of Friedrich-Loeffler-Institut.

In herds with IBK, *M. bovoculi* and all three *Moraxella* spp. were detected by PCR. In Herd 1, 88% of conjunctival swabs tested positive for *M. bovoculi* and 90% for *Moraxella* spp., while in Herd 2, 35% of conjunctival swabs were positive for *M. bovoculi* and 12 % for *Moraxella* species. *M. bovis* was not detected. Using an in-house ELISA, specific antibodies against *M. bovoculi* were detected in sera of 42% of animals of Herd 1 and in 27% of animals of Herd 2. However, we also found positive PCR results for *M. bovoculi* and *Moraxella* spp. in healthy calves (26% and 41%, respectively). Interestingly, *Mor. bovoculi* was exclusively detected in the two IBK-affected herds, thus possibly indicating a closer association of this agent with the disease compared to the other *Moraxella* species. Although our results suggest that additional pathogens as well as environmental factors play a role in the pathogenesis of clinical IBK, it seems that *M. bovoculi* could be a predisposing factor for IBK.

Evaluation of an antigen cocktail ELISA to diagnose Contagious Bovine Pleuropneumonia (CBPP)

Malin Wennlund[1], Christina Ferreira[2], Ana Botelho[2], Anja Persson[1]

[1]Division of Proteomics and Nanobiotechnology, School of Biotechnology, AlbaNova University Center, Royal Institute of Technology (KTH), Stockholm, Sweden
[2]Bacteriology Laboratory, Production & Animal Health Unit, Instituto Nacional de Investigação Agrária e Veterinária. (INIAV, IP), Lisboa, Portugal
E mail: wennlund@kth.se

In the present investigation, two variants of an ELISA that targets multiple surface antigens of *Mycoplasma mycoides* subsp. *mycoides* SC, the etiological agent of CBPP, were validated for their diagnostic capacity of this disease. The validation was performed on 150 bovine sera from the most recent European CBPP outbreaks. The antigens were previously selected amongst 65 recombinant proteins by screening African sera in a bead based microarray setting (Neiman *et al.*, 2009).

ELISA wells were coated with antigen cocktails consisting of either eight surface proteins or a subset of five proteins. The assays were performed on 76 positive sera from naturally infected bovines and 74 CBPP negative sera, in two independent laboratories; the Royal Institute of Technology (KTH) and Instituto Nacional de Investigação Agrária e Veterinária (the OIE reference laboratory for CBPP in Portugal. The complement fixation test (CFT) and immunoblotting assay (IBT), performed by experienced staff in a routine base, were used to evaluate each serum.

The eight protein cocktail preformed slightly better than the five protein ELISA. The areas under the curve (AUCs) in receiver operator characteristics (ROC) plots were 0.97 and 0.95, respectively, meaning that both assays allowed the definition of a cut off that gives a low false positive ratio, in addition to a low false negative ratio. This cut off of two standard deviations resulted in 92% and 89% agreement with previous classifications for each assay.

In this investigation it was demonstrated that both antigen cocktail ELISAs had good sensitivity and specificity for detecting CBPP among European cattle and also that the assay is stable and reproducible in different laboratories. The data supported previous studies with African sera, suggesting that this method can be a useful diagnostic tool.

Local and systemic immune responses in pigs intramuscularly injected with an inactivated *Mycoplasma hyopneumoniae* vaccine

S. B. Marchioro[1, 4], D. Maes[2], B. Flahou[1], F. Pasmans[1], R. Del Pozo Sacristán[2], K. Vranckx[1], V. Melkebeek[1], E. Cox[3], N. Wuyts[5], F. Haesebrouck[1]

[1]Department of Pathology, Bacteriology and Avian Diseases
[2]Department of Reproduction, Obstetrics and Herd Health, Unit Porcine Health Management
[3]Department of Virology, Parasitology and Immunology
Ghent University, Faculty of Veterinary Medicine. Salisburylaan 133, B-9820 Merelbeke, Belgium.
[4]Laboratório de Biologia Molecular, Núcleo de Biotecnologia, Centro de Desenvolvimento Tecnológico, Universidade Federal de Pelotas, RS, Brazil
[5]Pfizer Animal Health. Pleinlaan 17, 1050 Elsene, Brussels
E mail: silmarchioro@yahoo.com.br

The immune response induced by intramuscular administration of a commercial inactivated *Mycoplasma hyopneumonie* whole-cell vaccine (Suvaxyn®MH One) was investigated in conventional *M. hyopneumoniae*-free pigs. The animals were assigned randomly to two groups: non-vaccinated and vaccinated. Pigs in the vaccinated group were injected intramuscularly with the vaccine at 7 days of age, whereas non-vaccinated pigs received physiological saline solution (PBS). Pigs were euthanized and necropsied at 30, 36 and 58 days of age. Blood, bronchoalveolar lavage (BAL) fluid, spleen, lung and bronchial lymph nodes (BLN) were collected. Serum and BAL fluid were tested for the presence of antibodies by ELISA. Monomorphonuclear cells from the peripheral blood and tissues were isolated to quantify the T cell subsets by flow cytometry, and cytokine production by ELIspot and ELISA. Antibodies against *M. hyopneumoniae* were detected in serum of most vaccinated pigs at 30 days of age. *M. hyopneumoniae* specific IgG, IgM and IgA were detected in BAL fluid from vaccinated animals, but not from control animals. Significantly higher numbers of IL-12 secreting cells were observed in the lung at day 58 in the vaccinated than in the non-vaccinated group ($P<0.05$). The number of IL-10 secreting cells from BLN was also higher in the vaccinated group at day 58 ($P<0.05$). After re-stimulation *in vitro*, lymphocytes from BLN and lungs secreted significantly higher levels of IL-12 in the vaccinated group at day 58. These results show that the vaccine induced both systemic and mucosal cellular and humoral immune responses.

Molecular detection of macrolide-resistant *Mycoplasma pneumoniae* isolates from Slovenian pediatric and adult patients

Rok Kogoj[1], Malena Aldeco[2], Darja Keše[1]

[1]University of Ljubljana, Medical Faculty, Institute of Microbiology and Immunology, Zaloška 4, 1000 Ljubljana, Slovenia
[2]University Medical Centre Ljubljana, Division of Paediatrics - University Children's Hospital, Bohoričeva ul. 20, 1000 Ljubljana, Slovenia
E mail: rok.kogoj@mf.uni-lj.si

Mycoplasma pneumoniae is a fastidious, slowly growing, cell wall-less bacterium that can be the causative agent of up to 40% of cases of community-acquired respiratory tract infections in school-aged children and young adults. Infections with macrolide-resistant *M. pneumoniae* strains are being increasingly discovered all over the world and consequently methods able to detect mutations in the domain V of the *M. pneumoniae* 23S rRNA are being developed with greater interest. In this study we modified a previously published pyrosequencing assay that targets two regions in the domain V of the *M. pneumoniae* 23S rRNA gene (2063 to 2141 and 2606 to 2621; *M. pneumoniae* M129 numbering) in order to detect possible macrolide-resistant *M. pneumoniae* strains isolated between 2007 and 2011 from Slovenian pediatric and adult patients. Our modified assay was able to detect all so far known macrolide resistance conferring mutations: A2063G, A2063C, A2063T, A2064G, A2067G and C2617G or C2617A. Moreover the 79 base long pyrosequencing product of the first region also made it able to distinguish *M. pneumoniae* from other mycoplasmas. From a total of 174 analyzed *M. pneumoniae* isolates obtained from respiratory samples of 135 pediatric and 39 adult patients respectively, three (1.8%) were found to harbor macrolide resistance conferring mutations. One macrolide-resistant isolate was obtained from an adult (35 years old) and two from children (9 and 15 years old). All three isolates had an A to G transition at position 2063 which is the most commonly detected mutation in macrolide-resistant *M. pneumoniae* strains. No other mutations were detected in the sequenced areas of the domain V in this study. Our results show that macrolide resistant *M. pneumoniae* is already present in Slovenia. Fortunately its incidence seems to be low for now, which is in concordance with the data from other European countries.

Comparison of antigen capture ELISA and PCR for detection of *Mycoplasma bovis* in milk samples

Krešimir Matanović[1], Selma Pintarić[1], Hywel Ball[2], Franjo Martinković[3], Miljenko Martinec[4], Branka Šeol Martinec[1]

[1]Department of Microbiology and Infectious Diseases with Clinic, Faculty of Veterinary Medicine University of Zagreb, Heinzelova 55, Zagreb 10000, Croatia
[2]Agri-Food and Biosciences Institute, Veterinary Sciences Division, Stoney Road, Stormont, Belfast BT4 3SD, UK
[3]Department of Parasitology and Parasitic Diseases with Clinic, Faculty of Veterinary Medicine University of Zagreb, Heinzelova 55, Zagreb 10000, Croatia
[4] Veterinary station Remetinec, Zagreb 10000, Croatia
E mail: kmatanov@vef.hr

Mycoplasma bovis is a significant cause of bovine mastitis at large dairy farms in Croatia. In our laboratory, we routinely use antigen capture ELISA combined with culture on modified Hayflick agar for detection of *M. bovis* in milk, nasal swabs or lung tissue. It takes at least three days to detect *M. bovis* using this technique, and the presence of antimicrobials in samples may have a negative effect on the growth and sensitivity of the method. PCR based techniques have great sensitivity, are faster, and are not influenced by antimicrobial therapy. In this work we compared antigen capture ELISA and PCR combined with two different methods of DNA extraction to detect *M. bovis* in bulk tank or individual milk samples. The aim was to develop a cheap and quick method for DNA extraction from milk without using commercial kits or expensive chemicals for purification of DNA.

Approximately 1000 milk samples were examined for the presence of *M. bovis* using antigen capture ELISA. A smaller subset consisting of ELISA-positive and ELISA-negative milk samples was further investigated using PCR. Extraction of DNA from milk samples was performed using Chelex-100 resin (Bio-Rad, USA) or proteinase K digestion. PCR was performed using primers specific for *uvr*C or 16S rRNA gene of *M. bovis*.

Although the PCRs performed after Chelex or proteinase K based DNA extraction showed similar efficiency, antigen capture ELISA outperformed both methods when testing either bulk tank or individual milk samples. In addition, the sensitivity of PCR performed after the investigated DNA extraction methods was below 80%, rendering them unsuitable for detection of *M. bovis*, possibly because of the presence of PCR inhibitors due to the absence of purification step, or too low DNA content.

Poster Presentation Abstract P10.

Comparison of PCR assays with culture for the diagnosis of bacterial pathogens in bovine respiratory disease

C. J. Bell, P. Blackburn, H. J. Ball

Agri-Food and Biosciences Institute, Veterinary Sciences Division, Stoney Road, Stormont, Belfast, BT4 3SD, UK

Bovine respiratory disease (BRD) is a global problem causing severe economic losses to the farming industry through mortality, loss of production and treatment costs. BRD is the single biggest cause of mortality in calves in Northern Ireland accounting for 34% of deaths in juvenile calves (1 – 5 months), 41% of deaths in weanlings (6 – 12 months) and 14% of deaths in adult cattle in 2011 based on post-mortem submissions (All-island Animal Disease Surveillance Report 2011). The major bacterial organisms associated with the BRD complex are *Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni*, *Trueperella pyogenes* and *Mycoplasma bovis*. All of these organisms are ubiquitous in the cattle population and can infiltrate into the lungs following stress or viral infection. Currently the post mortem detection of these organisms in pneumonic lung tissue at this Institute is by standard culture techniques and, in the case of *M. bovis*, by a capture / enrichment sandwich ELISA (sELISA) combined with culture. Isolation of these organisms can be influenced by the presence of antibiotics in the tissue, administered during the terminal stages of pneumonia or, in the case of *M. bovis*, by overgrowth of less fastidious bacteria present during culture. End point and real-time PCR were used to assess the prevalence of these organisms in grossly pneumonic lung samples from 60 animals submitted for post-mortem examination. The PCR assays detected a significantly higher prevalence of these organisms in cattle pneumonia in Northern Ireland than can be detected by current standard procedures.

Association of *Mycoplasma pneumoniae* with respiratory tract infections in children

Osama M. S. Abdul-Wahab[1], Ahmed Mossa Al-Hakami[1], Ayed A. M. Shati[2], Ali M. A. Alsuheel[2], Ashish Kumar[1], Fateha Benahmed[3]

[1]Department of Microbiology, Faculty of Medicine, King Khalid University, Saudi Arabia.
[2]Department of Child Health, Faculty of Medicine, King Khalid University, Saudi Arabia.
[3]Serology Section, Laboratory Department, Asser Central Hospital, Ministry of Health, Saudi Arabia.
E mail: osamaabdulwahab@hotmail.com

Mycoplasma pneumoniae is one of four most common species of organisms that are responsible for most clinically significant infections in humans. It is a frequent cause of acute respiratory infections in both children and adults. The organism can cause pharyngitis, otitis, tracheobronchitis, or community-acquired pneumonia, but patients may also remain totally asymptomatic. The aim of this retrospective study on children was to investigate the association of *M. pneumoniae* with respiratory tract infections in a Saudi population. This work was designed as a case-control study in which 90 patients (mean age of the patients in case group was 5.94±2.73 and in control group was 6.51±2.26) of either sexes were included. These patients were classified into two groups: first group (case group), included 45 patients who had been admitted in hospital with diagnosis of respiratory tract infections and the second group (control group), included 45 healthy patients who had no history of respiratory tract infections. Both groups were age and sex matched. Presence of IgM antibodies to *Mycoplasma pneumoniae* was assessed by ELISA technique in both groups. In the case group, 4 (9%) cases out of 45 children were positive for anti-mycoplasma antibody whereas in the control group, all children were negative. All positive case group patients had symptoms of acute bronchopneumonia. 18 (40%) of the patients were diagnosed with bronchial asthma (40%) inclusive of all the four cases diagnosed with *Mycoplasma pneumoniae* infection. The relative risk for the occurrence of mycoplasma infection was estimated to be 9 (95% CI=0.49-162.43). However, on comparing the case and control groups, the result was not found to be statistically significant. (Fischer Exact Test *p=0.0583*). Children in Saudi Arabia are at a relatively higher risk of developing *Mycoplasma pneumoniae* infection especially those predisposed with underlying chronic respiratory illnesses such as asthma. This is a first study of its kind from the region reporting such a disease in children using a serological assay as ELISA. Further studies are required to evaluate the risk of co infection by *Mycoplasma pneumoniae*, *Streptococcus pneumoniae* and *Chlamydia pneumoniae.* Evaluating and establishing a correlation between *Mycoplasma pneumoniae* and the onset of asthma among infected children can be a retrospective field of study.

Respiratory and extra-pulmonary *Mycoplasma hominis* cases submitted to the Mycoplasma Reference Laboratory, England & Wales, 2003-2013

Teresa Stocki and Victoria Chalker

Public Health England, London, NW9 5EQ, UK.
E mail: Teresa.Stocki@phe.gov.uk

Mycoplasma hominis is an opportunistic pathogen that has been isolated from organ transplantation, septic arthritis, respiratory infection (especially in neonates), haematomas, post-surgical and wound infections. Laboratories in England and Wales refer isolates for identification from neonatal respiratory samples, unusual and invasive infections to the PHE Mycoplasma Reference Laboratory wherein identification of referred isolates and isolation from PCR positive extra-genital clinical samples is undertaken. A review of all cases in which *M. hominis* was confirmed by PCR/16S sequencing from 2003-2013 is presented.

120 isolates were identified as *M. hominis* from 2003-2013 from 115 patients. Isolates were grouped according to age (neonates/infants <1y, children 1-15y and adults >15y). Of 42 neonates 27/115 (23.5% 16.6-32.1 95%CI) yielded isolates from respiratory samples with respiratory distress (sex 13F/13M/1U) and 12/115 (10.4% 5.9-1.8.1 95%CI) from extra-pulmonary sites (CSF, skin swabs, gastric aspirates, sex 5F/6M/1U). Other than a single isolate from a neck swab (age unknown), isolates from three children (2F/1M, median age 1y, range 1-4) and 73 (73/115, 63.5% 54.4-71.7 95%CI) adult patients (age 16 – 85y, median age 28) were received (sex 60F/12M/1U). Adult patients fell into three categories, pre/post-partum (post Caesarean-section, mid-stream urine/uterine swabs, abdominal pus, placental isolates), post-operative (non- gynaecological) and other. The largest proportion of isolates were from adult post-operative cases 27/73 adults, 27/115 total (23.5% 16.6-32.1 95%CI), post renal/lung transplantation, hysterectomy, laparotomy, brain/cardiac/spinal surgery, carcinoma). Other isolation sites 17/73 adults, 17/115 total (14.8% 9.3-2.3 95%CI) included abscess, respiratory, joint, tissue and peritoneum.

M. hominis was isolated from respiratory and invasive infections in neonates and from pre/post-partum, post-operative and a variety of other sites in adults from 2003-2013.

Cultural morphological characteristics of the isolate *Mycoplasma genitalium* obtained from the patients with reproductive disorders

Julia A. Shishporenok[1], Lyudmila V. Rubanik[1], Andrei N. Astashonok[1], Tatiana V. Rudenkova[2], Zinaida B. Kvacheva[1], Nikolai N. Poleshchuk[1]

[1]Republican Research & Practical Centre for Epidemiology and Microbiology, Filimonova 23, Minsk, Belarus
[2]Belorussian Medical Academy of Postgraduate Education, P. Brovki 3/3, Minsk, Belarus
220013
E mail: expert5@tut.by

Mycoplasma genitalium is an obligate intracellular pathogen that causes human inflammatory diseases of the urogenital tract. Currently, the selection of the nutrients and conditions for its cultivation still remains a problem which requires further investigation to facilitate studies on the biological properties of this pathogen.

Urethral and cervical swabs were obtained from 23 patients with reproductive disorders and examined for the presence of *Mycoplasma genitalium*. The use of a modified selective growth medium has enabled growth of two isolates of the pathogen (MG-20, MG-9) to titers of 10^3 and 10^4 cfu/ml. Comparative analysis of the developed culture medium with the standard medium ("Mycoplasma genitalium", Russia) revealed no differences in the growth parameters of the pathogen. The *M. genitalium* MG-9 isolate was passaged in Vero cell culture. Electron microscopy has been used to analyse the morphological characteristics of the agent and specific changes in the ultrastructure of the infected cells have been described and specified stages of morphogenesis of *M. genitalium* were clarified. The resulting model system is based on the initial accumulation of the pathogen in the growing medium and its subsequent introduction into the Vero cell culture which can be used not only for studying of morphological changes of *M. genitalium* but also for describing the mechanism of its pathogenesis on sensitive target cells.

Modified culture medium is promising for the isolation and examination the strains *M. genitalium*.

Measurement of immune response against inactivated *Mycoplasma capricolum* subsp. *capripneumoniae* vaccine by cELISA

Umit Ozdemir[1], M. Ali Türkyilmaz[1], François Thiaucourt[2]

1Pendik Veterinary Control Institute, Ankara cad. No.1 34890 Istanbul, Turkey
2CIRAD, UMR CMAEE, F-34398 Montpellier, France
E mail: uozdemir@penvet.gov.tr

This study aimed to evaluate the efficacy of a saponin-adjuvanted inactivated vaccine prepared with a Turkish field strain of *Mycoplasma capricolum* subsp. *capripneumoniae* (Mccp) in experimental and field conditions by cELISA. A field isolate of Mccp was cloned to obtain pure culture, followed by identification using growth inhibition test and PCR. Mccp was propagated in broth medium, the antigen was harvested by centrifugation, the pellet was purified by washing twice with PBS, protein concentration was measured by spectrophotometric absorbance at 280 nm and an inactivated vaccine containing 2 mg/ml protein and 2 mg/ml saponin was prepared. In the first experiment, 20 goats were inoculated with 1 ml of the vaccine by subcutaneous route. Ten goats were inoculated with 1 ml of PBS as control. All animals were bled before inoculation and also 21 days, 3 months and 6 months after inoculation. Antibody titers were measured by cELISA. Mean percent inhibitions of vaccinated group were 69.47, 64.07 and 62.63 for 0-day21-day, 3-month and 6-month sera respectively, exceeding cut-off value of 55 and 0-day mean of 35.25. The results indicated seroconversion and high antibody levels at least for 6 months. In the field experiment, a herd consisting of 450 goats was vaccinated with the vaccine by the same dose and route as the first experiment. The animals showed lameness lasting 1-2 days, inappetence for one day and minor swelling at the injection site. A representative group of 30 animals was drawn from the herd for serologic assessment by cELISA. Animals were bled before inoculation and also 21 days and 6 months after inoculation. Mean percent inhibitions of 0-day, 21-day and 6-month sera were 38.55, 62.7 and 58.46 respectively. The results showed that the Mccp vaccine was immunogenic under field conditions.

Poster Presentation Abstract P15.

Production of rapid serum plate (RSP) antigen from local isolates of *Mycoplasma gallisepticum* in Pakistan and its comparison with the imported commercial antigen

Mushtaq Ahmad Gondal[1], Masood Rabbani[1], Mazhar I. Khan[2]

[1]Internationally Accredited University Diagnostic Lab, University of Veterinary and Animal Sciences, Lahore-54000, Pakistan
[2]Department of Pathobiology and Veterinary Science, University of Connecticut, Storrs 06269-3089, CT, USA
E mail: mazhar.khan@uconn.edu

Throughout the world the poultry industry is facing a lot of microbial challenges including mycoplasmosis. Amongst other avian diseases, *Mycoplasma gallisepticum* (MG) infection is one of the major constraints in the rapidly growing poultry industry of Pakistan and is causing significant economic losses in the infected flocks. Considering the economic impact of MG and a requirement to improve current diagnostic test methods, this study was undertaken to produce the local rapid serum plate (RSP) antigen. This was then used to investigate the involvement of *Mycoplasma gallisepticum* in respiratory diseases of commercial poultry flocks. The local isolate of *Mycoplasma gallisepticum* grew well in Frey's medium at 37^0C in 10 % CO_2 for 48 hours and identification was confirmed by PCR. The antigen is usually prepared from the culture medium showing good growth of MG; the culture was harvested by centrifugation in Hopkin's tube at 20,000 *g* for 45 minutes. Cells were re-suspended in phosphate buffer saline (PBS pH 7.0) and then washed thrice. The cells were finally re-suspended in PBS pH 7.0 and stained with Rose Bengal dye at a concentration of 1:10000. Rapid serum plate agglutination (RSPA) performed with this local antigen showed that 64 (32%) samples contained anti MG agglutinating antibodies while 136 (68%) serum samples showed no agglutinating antibodies. RSPA with the imported (commercial) antigen showed that 60 (30%) samples contained anti MG agglutinating antibodies and 140 (70%) samples had no agglutinating antibodies. This means that the local antigen is comparable with the imported MG antigen for detecting anti MG agglutinating antibodies. Comparative efficacy of both antigens showed 60 (30%) of samples were true positive samples, 136 (68%) samples as true negative samples, 4 (2%) were false positive and 4 (2%) were false negative. Sensitivity of the local antigen was 93.7% while specificity of the test was 97.1%. We conclude that results of the RSPA performed with the local RSP antigen are comparable with the imported one.

Poster Presentation Abstract P16.

A comparison of methods used to measure the *in vitro* antimicrobial susceptibilities of *Mycoplasma* species of animal origin

Omer Kibeida, Moritz van Vuuren, Johan Gouws, Jackie Picard

Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, Private Bag X04, Onderstepoort 0110, South Africa.
E mail: omerkibeda@yahoo.com

Antibiotics are commonly used to treat mycoplasmosis in animals. In spite of this and the fact that antimicrobial resistance has been recorded for this group of bacteria there are no universally accepted *in vitro* means of testing for this resistance, nor is resistance testing for mycoplasmas routine in most veterinary laboratories. So prior to testing for resistance to a number of mycoplasmas isolated from animals in South Africa it was necessary to compare different tests, including broth and agar microdilution tests to find out which one would perform best and was the easiest to do.

Using reference strains *M*. *mycoides* Y-*goat* and *M*. *gallisepticum* 56USDA, vaccine strain *M*. *mycoides mycoides* T1/44, and field strains *M*. *bovis*, *M*. *crocodyli*, *M*. *felis* and *M*. *gallisepticum*, the following tests were compared: the microbroth dilution test using either Hayflicks or *Mycoplasma synoviae* broth and agar microdilution test. Sugar fermentation using phenol red and either glucose or pyruvate and oxidation using Alamar^{TM}Blue were compared as indicators of metabolism. It was also tested whether amoxicillin and clavulanic acid could be used to reduce bacterial contamination. Statistical analyses of the tests indicated that the broth microdilution test using phenol red and a sugar as an indicator was the most reproducible and accurate method. Amoxicillin with clavulanic acid was found to have a negligible effect on the MIC results.

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