Programme and Proceedings

4th Havemeyer Workshop on *Rhodococcus equi*

Edinburgh, 13-16 July 2008

THE UNIVERSITY of EDINBURGH
CID
CENTRE for INFECTIOUS DISEASES

Havemeyer Foundation

THE UNIVERSITY OF EDINBURGH

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John Prescott, University of Guelph, Canada
Wim Meijer, University College Dublin, Ireland
Desmond Leadon, Irish Equine Centre, Ireland

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Michal Letek
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Workshop Secretary

Emma Pineau, Tel +44 (0)131651 3624, E-mail jvbgroup@ed.ac.uk

Workshop Sponsor

The Dorothy Russell Havemeyer Foundation, Inc.
60 East 42nd Street - 40th Floor New York, NY 10165-0006, USA
http://www.havemeyerfoundation.org/

Acknowledgements

The organizers are grateful to the Dorothy Russell Havemeyer Foundation, and particularly its Chief Executive Officer and President, Mr. Gene Pranzo, for their continued support. This Workshop series has critically helped, and we hope will continue helping, scientists interact to improve the quality of science that we are doing.

Thanks are also due to Sarah-Jane Johnston, Louise Baird and Chris Barnes from Edinburgh First, for their help and advice in the organization of the workshop, Morag Laidlaw and Garry Robertson for assistance in the financial management, and Nadine Matthes for making available the poster boards.

Overview of the workshop

The workshop is intended as a forum to discuss current topical aspects of *R. equi* infection, summarise the key achievements of the pre-genomic era of *R. equi* research, and discuss future developments based on the genome sequence of the organism, made recently available to the international scientific community thanks to the efforts of the IREC platform. Having the first *R. equi* genome sequence will certainly make a difference to control of this infection in animals and in humans.

Brief history of the Havemeyer *R. equi* workshops

This is the 4th International Workshop on *Rhodococcus equi* supported by the generosity of the Dorothy Russell Havemeyer Foundation:

The first Workshop was held in Guelph, Ontario, Canada in 1986, organized by John Prescott and Julie Yager. Highlights of that Workshop included taxonomic presentation by Mike Goodfellow which convinced everyone to drop the name *Corynebacterium equi*, and presentations by Corinne Sweeney and by Chris Hillidge that confirmed the clinical efficacy of treatment of foals using erythromycin and rifampin combinations. This Workshop managed to attract most of the relatively few people working on *R. equi*.

The second Workshop was held ten years later also in Guelph and focused on the immunological aspects that predispose foals to develop *R. equi* infection. This 1996 Workshop was organized by John Prescott, Julie Yager, Mark Holmes and Shinji Takai. By this time the virulence plasmid had been discovered in Shinji Takai`s and in John Prescott`s laboratories, VapA had to some extent been characterized and shown to be important in immunity, and Steve Hines`s group had done its ground-breaking work on the importance of type 1 and type 2 immune responses in determining the outcome of a rhodococcal infection.

A mini-Workshop was organized at very short notice in Guelph in 2000 on the occasion of the sequencing of the virulence plasmids from two foal isolates, ATCC 33701 and strain 103. This Workshop was attended by delegates from Steve Hines, Wim Meijer, Jesus Navas, John Prescott, Shinji Takai and Jose Vazquez-Boland`s groups. This was the meeting at which the `*R. equi* community` decided to try to pursue obtaining funding to sequence the genome.

The last (3rd) Workshop was held in Washington State University in Pullman, Washington, in 2002, and was organized beautifully by Steve Hines, Mary Hondalus, Steeve Giguere, Wim Meijer, and Iain Sutcliffe.

Workshop venue at Pollock Halls

The conference venue is at Pollock Halls, University of Edinburgh, 18 Holyrood Park Road, Edinburgh, EH16 5AY.

Bus information, including the airport bus link from the city centre, can be obtained from www.lothianbuses.co.uk. Visit www.edinburghshuttlebus.com for the alternative door-to-door shuttle service to the airport (9 GBP per single journey). The approximate cost of a taxi Pollock Halls-airport is 20 GBP. If you are hiring a car, itineraries can be obtained from www.multimap.co.uk.

If you need help or assistance during your stay at Pollock Halls, please report to the Reception Centre which is open 24 hours a day.

Please see the accompanying Pollock Halls precinct map with the location of the Reception Centre and relevant buildings i.e. St. Leonard`s Hall (Sunday 13th reception and delegate registration), South Hall block A (conference and poster display rooms), John McIntyre Centre (canteen for lunches), and the accommodation sites (Holland House, Chancellor`s Court, Salisbury Green).

Workshop dinner at Playfair Library, Old College

The Workshop dinner venue is the Playfair Library, Old College, South Bridge, EH8 9YL. Please consult the attached maps for directions of how to get there from Pollock Halls.

Tourist information

There are plenty of cafes and restaurants in the following areas of the city: Clerk and Nicolson Street (the number 14, 30 or 33 buses), near the main University of Edinburgh campus (the number 2, 14, 30 or 33 buses), at the top of Leith Walk (the number 14 bus), in the Grassmarket area, along the Royal Mile (both with the number 2 bus), and in the New Town (around George Street).

When using the bus service in the city centre, please make sure that you have the correct money for the fare, as the bus drivers do not give change. A single journey costs 1.10 GBP and a day ticket costs 2.50 GBP.

If you do have some free time and would like to visit the city here are some ideas:

Holyrood Park. This park is adjacent to Holyrood Palace, the residence of Her Majesty the Queen when she visits Edinburgh. The park is within walking distance from Pollock Halls and there are a variety of footpaths with some panoramic views of the city.

The Royal Mile and Castle. The street that links Edinburgh Castle and Holyrood Palace is called the Royal Mile. If you are feeling energetic you can walk the full mile (up hill!) from the Palace to the Castle and visit the Old Town at the same time.

The Scottish Parliament. The new Scottish Parliament building is also near to Holyrood Palace and is open to the public.

The University of Edinburgh. The main campus for the University of Edinburgh is located at George Square, close to the old Faculty of Medicine, Faculty of Law and the McEwan Hall, which is used for all the graduation ceremonies at the University. Close to George Square is The Old College, with its distinctive dome. Most of the science-based disciplines are located at the King`s Buildings campus, near to the Royal Observatory. The new Medical School and Royal Infirmary relocated to a new campus at Little France at the southeast of Edinburgh. The Royal (Dick) School of Veterinary Studies is based at Summerhall near George Square and the Meadows, and at the Easter Bush site near the Roslin Institute, a few miles south to Edinburgh.

Visit http://websiterepository.ed.ac.uk/explore/ and http://websiterepository.ed.ac.uk/explore/places/buildings/ for information about the University of Edinburgh and its history, architectural heritage and buildings.

Princes Street and Gardens. This is the main shopping street in Edinburgh alongside Princes Street Gardens. The Castle and the Old Town overlook Princes Street and the Gardens.

Grassmarket. This is an historic market square located in the Old Town, with a variety of restaurants and pubs, also overlooked by the Castle and near to the famous Greyfriars Kirk and the Museum of Scotland.

The New Town. This is a masterpiece, a magnificent example of city planning of the neo-classical Georgian period, and is a UNESCO World Heritage Site. Although still referred to as the New Town, it was built in stages between 1765 and around 1850, and retains much of the original architecture. Walk along George Street and explore the area with its elegant shops, trendy cafes and restaurants.

Royal Botanic Garden. Edinburgh`s botanic garden is world renowned for its horticultural excellence. Established in 1670 and over 70 acres of beautifully landscaped grounds, it provides a tranquil haven just one mile from the city centre.

Water of Leith and Dean village. The Water of Leith is the main river of Edinburgh, Scotland. It flows to the port of Leith and into the sea via the Firth of Forth. Its course through the Dean village and Stockbridge down near the Royal Botanic Garden worth a visit. The Dean village was known as the "Water of Leith Village" and was a successful grain milling hamlet for more than 800 years. Today is a picturesque spot at the west end of Edinburgh with nicely restored workers' cottages, warehouses and mill buildings. One of its landmarks is the four-arched Dean bridge over the Dean Gorge, 106 feet above the water level.

Visit http://websiterepository.ed.ac.uk/explore/city/index.html for information about Edinburgh and http://www.visitscotland.com/guide/scotland-factfile/ for information about Scotland.

The University of Edinburgh

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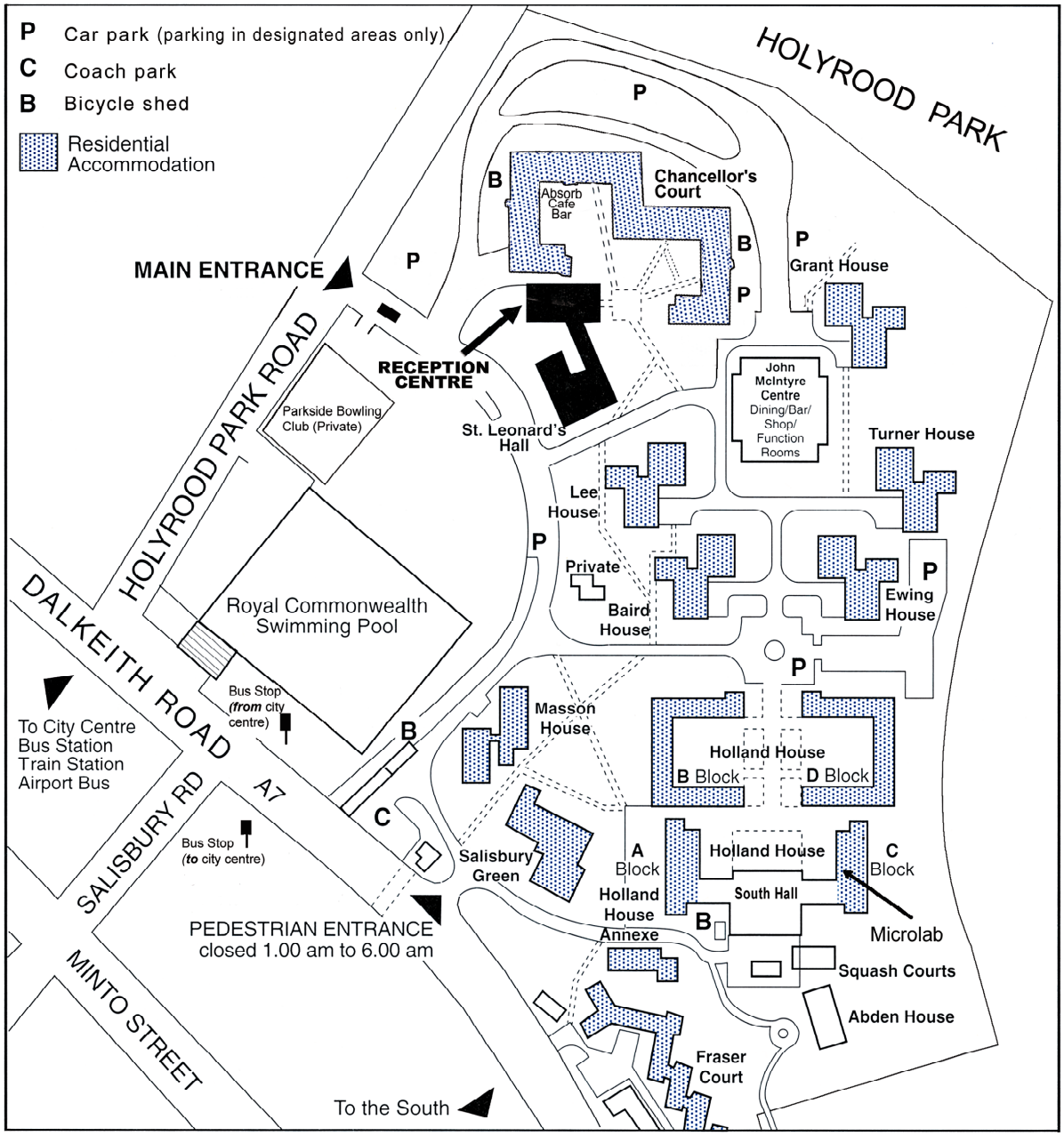
Pollock Halls of Residence



Useful phone numbers

Visit Scotland: 0131 332 2433
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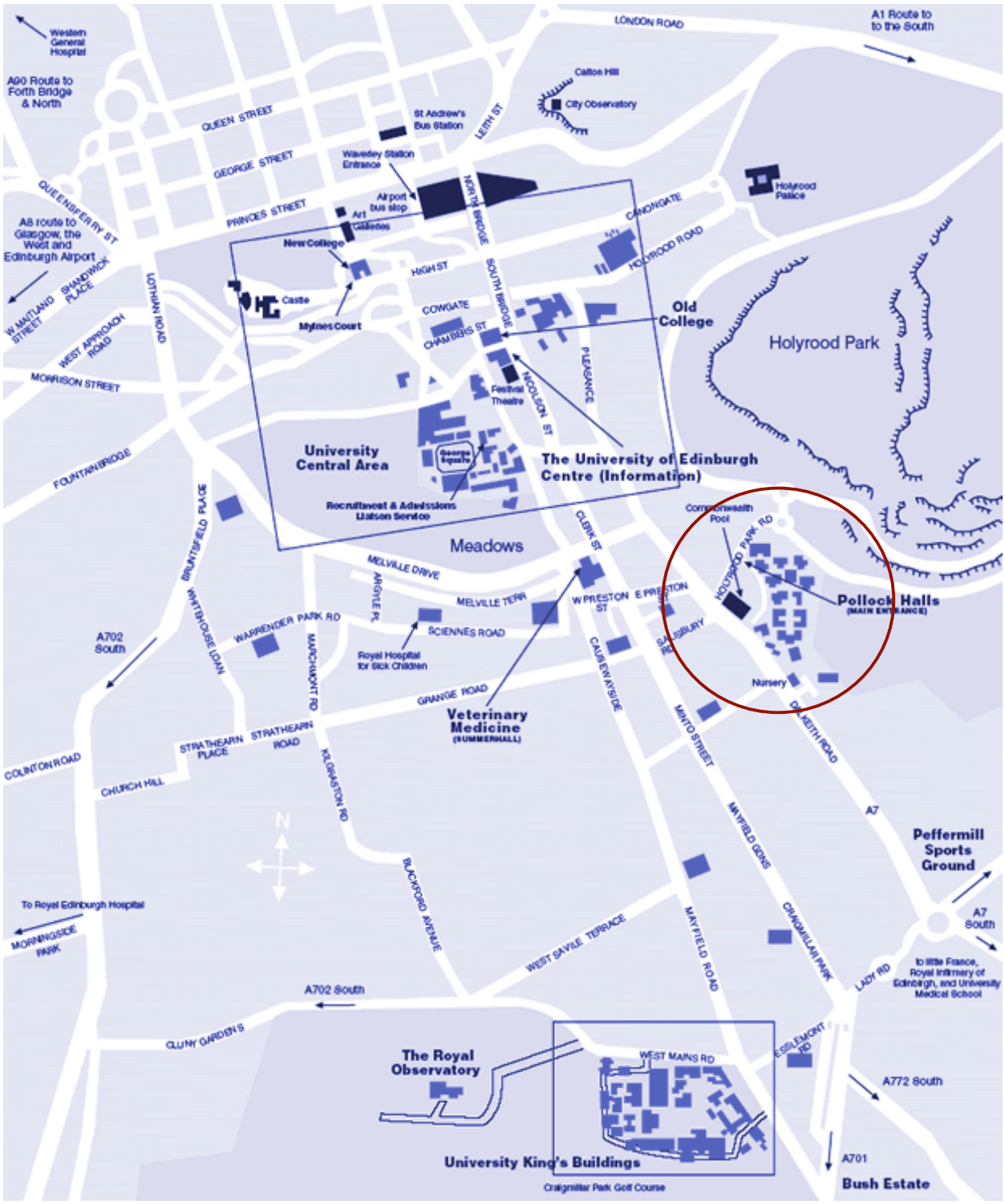
Lothian Buses info: 0131 555 6363
First Edinburgh bus info: 08708 72 72 71
Rail enquiries: 08457 48 49 50

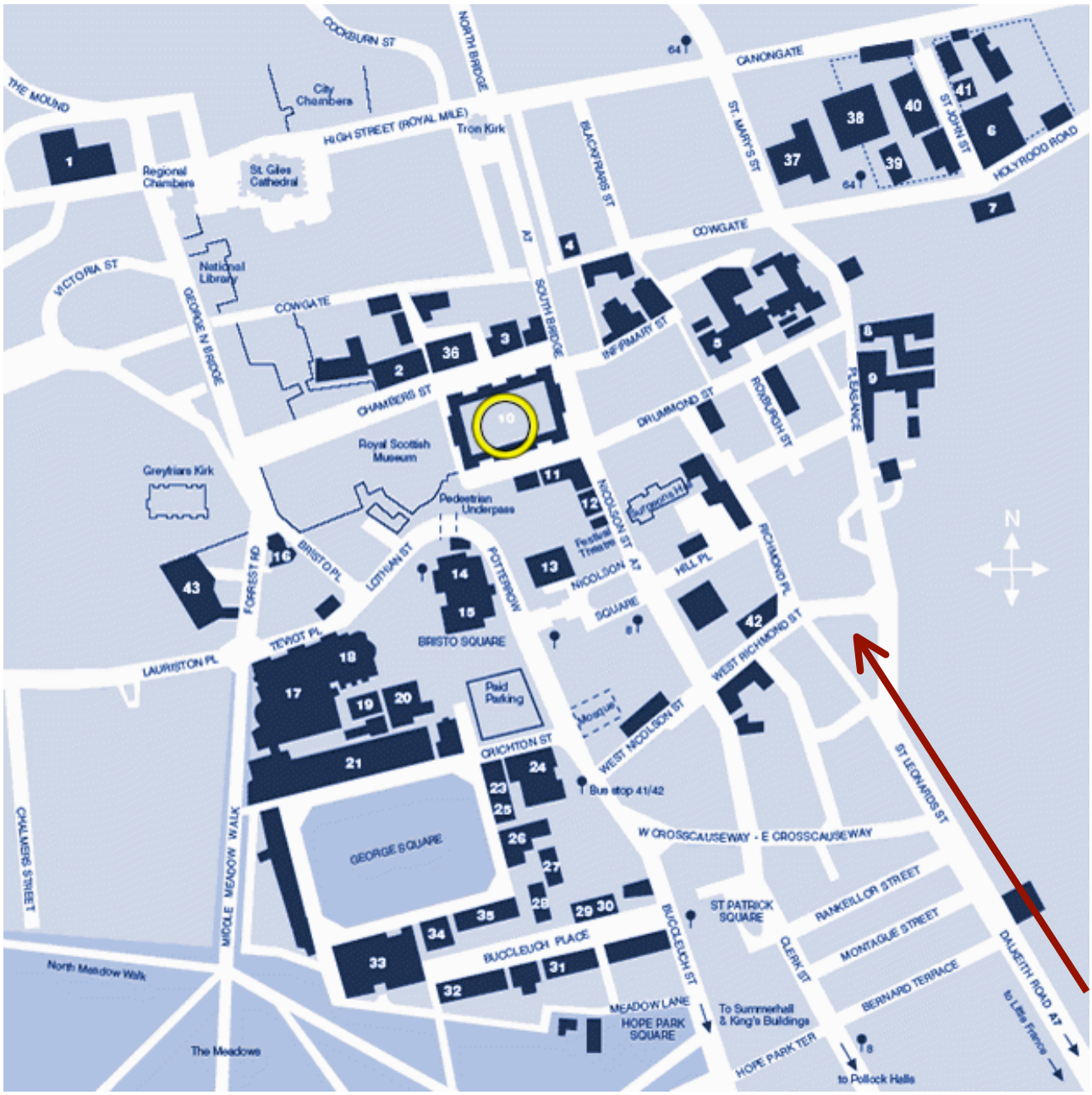
Edinburgh Airport: 0131 333 1000
British Airways (reservations): 08708 50 98 50
British Airways (flight info): 0870 55 111 55
British Midland Airways: 0870 60 70 555
KLM UK: 08705 074 074

Edinburgh city centre map with location of Pollock Halls (red circle) and University central area (rectangle)



Close up view of the University central area
(rectangle on map of previous page)

Location of Old College and Workshop dinner venue (Playfair Library) indicated by a yellow circle. Red arrow indicates Dalkeith Road and St. Leonards Street that you will take from Pollock Halls to reach the University central area (see map previous page).



PROGRAMME

Rhodococcus equi Havemeyer Workshop, Edinburgh 13-16 July 2008

TIMETABLE

Sunday 13

17.50	Registration opens (St. Leonard's Hall)
18.00 -19.00	Reception / get together at St. Leonard's hall, Pollock Halls

Monday 14

09.00-09.10	Introductory address
09.10-09.50	Plenary lecture: ***Rhodococcus equi*: the past, the present, and the future** *Shinji Takai, Kitasato University, Towada, Aomori, Japan*
SESSION 1	**ORGANISMAL BIOLOGY**
	(Chairs: Wim Meijer & José Vázquez-Boland)
09.50-10.20	Keynote lecture: **The *R. equi* genome** *Michal Letek, University of Edinburgh, UK & Irish Equine Centre, Ireland*
10.20-10.50	Coffee break – Poster viewing
10.50-11.20	Invited lecture: **Genetic engineering of steroid catabolism in *R. equi*** *Robert van der Geize, University of Groningen, The Netherlands*
11.20-11.50	Invited lecture: **Membrane anchored molecules of *R. equi*: insights from the genome**. *Iain Sutcliffe, Northumbria University, Newcastle upon Tyne, UK*
11.50-12.10	**Iron acquisition in *R. equi*** *Raúl Miranda-Casoluengo, University College Dublin, Ireland*
12.10-12.30	**Definition of *R. equi* secreted proteins by electrospray mass spectrometry and *in silico* sequence analysis**. *Corinne Barbey, AFSSA, Goustranville, France*
12.45-14.15	Lunch (John McIntyre Centre)
SESSION 2	**PATHOGENESIS**
	(Chairs: Mary Hondalus & John Prescott)
14.20-14.50	Keynote lecture: **Overview of *R. equi* pathogenesis and virulence** *Mary Hondalus, University of Georgia, Athens, Georgia, USA*
14.50-15.30	Invited lecture: **Microarray experiments: some lessons from mycobacteria** *Neil Stoker, Royal Veterinary College, London, UK*
15.30-16.00	Coffee break – Poster viewing

16.00-16.30 Invited lecture: **The virulence plasmid of *R. equi*: how does it work?**
Wim Meijer, University College Dublin, Ireland

16.30-17.00 Invited lecture: ***R. equi* phagocytosis: when macrophages suffer a digestive disorder**
Albert Haas, University of Bonn, Germany

17.00-17.15 **PhoP-PhoR and virulence in *R. equi***
Valeria Parreira, University of Guelph, Canada

17.15-17.30 **VapA is not all there is**
Garry Coulson, University of Georgia, Athens, Georgia, USA

Tuesday 15

SESSION 3 **IMMUNITY**

(Chairs: Steve Hines & David Horohov)

09.00-09.30 Keynote lecture: **Current understanding of immunity to *R. equi* pneumonia in foals**
Steve Hines, Washington State University, Pullman, Washington, USA

09.30-09.45 **Foal dendritic cells become activated upon *R. equi* infection *in vitro***
Maria Julia Flaminio, Cornell University, Ithaca, New York, USA

09.45-10.00 **Identification of immunologically relevant genes in equine dendritic cells infected with *R. equi* and a potential role for indoleamine 2,3 dioxygenase (INDO) in *R. equi* infection**
Meera Heller, University of California, Davis, California, USA

10.00-10.15 **TLR9-mediated cytokine production in neutrophils of new born foals and its therapeutic potential in preventing *R. equi* infection**
Mei Liu, Texas A&M University, Texas, USA

10.15-10.30 Poster slam

10.30-11.00 Coffee break – Poster viewing

11.00-11.15 **Immunization with *Salmonella enterica* expressing the VapA protein of *R. equi* induces a Th1 immune response, associated with a long-term protection**
Aline Oliveira, Universidade de São Paulo, Brazil

11:15-11:30 **Recognition of *R. equi* lipid antigens by cytotoxic T-lymphocytes**
Seth Harris, Washington State University, Pullman, Washington, USA

11.30-11.45 **Activation of cells of the monocyte lineage results in more robust production of IL-10 in neonatal foals compared to adult horses**
Brett Sponseller, Iowa State University, Ames, Iowa, USA

11.45-12.00 **Interferon-gamma expression in young foals when treated with an immunostimulant or plasma**
David Horohov, University of Kentucky, Lexington, Kentucky, USA

12.00-12.15	**Experimental infection of neonatal foals with *R. equi* triggers adult-like gamma interferon induction** *Steeve Giguère, University of Florida, Gainesville, Florida, USA*
12.15-12.30	Moderated general discussion on immunity and vaccination against *R. equi*
12.45-14.15	Lunch (John McIntyre Centre)
SESSION 4	**CLINICAL ASPECTS**
	(Chairs: Desmond Leadon & Steeve Giguère)
14.15-14:30	Keynote: **The priorities of the industry that we serve** *Des Leadon, Irish Equine Centre, Johnstwon, Naas, Ireland*
14.30-14.45	***R. equi* disease in foals in Ireland's temperate climate - clinical studies 2002 to 2007** *Mariann Klay, Irish Equine Centre, Johnstwon, Naas, Ireland*
14.45-15.00	**Diagnostic of pulmonary abscesses in foals: comparison of sonographic and radiographic examination.** *Monica Venner, University of Hannover, Germany*
15.00-15.15	**Effectiveness of azithromycin or tulathromycin in preventing pulmonary abscesses in foals of a *R. equi* endemic breeding farm** *Monica Venner, University of Veterinary Medicine, Hannover, Germany*
15.15-15.30	**Chemoprophylactic effects of azithromycin against *R. equi* pneumonia among foals at endemic equine breeding farms.** *Keith Chaffin, Texas A&M University, Texas, USA*
15.30-16.00	Coffee break – Poster viewing
16.00-16.15	**Safety of enteral gallium maltolate in neonatal foals** *Ron Martens, Texas A&M University, Texas, USA*
16.15-16.30	**Pharmacokinetics of a methylcellulose oral formulation of gallium maltolate in neonatal foals.** *Keith Chaffin, Texas A&M University, Texas, USA*
16.30-16.45	***R. equi in adult horses*** Ursula Fogarty, *Irish Equine Centre, Johnstwon, Naas, Ireland*
16.45-17.00	**Resistance studies of erythromycin and rifampin for *R. equi* over a 10-year period** *Thomas Buckley, Irish Equine Centre, Johnstwon, Naas, Ireland*
17.00-17.15	**Prevalence of *R. equi* isolates resistant to macrolides or rifampin and outcome of infected foals.** *Steeve Giguère, University of Florida, Gainesville, Florida, USA*
19.30-21.00	Dinner at Playfair Library Hall, Old College, University of Edinburgh

Wednesday 16

SESSION 5	**EPIDEMIOLOGY**
	(Chairs: Gary Muscatello & Noah Cohen)
08.30-09.00	Keynote lecture: **Epidemiology of *R. equi* disease: facts and thoughts** *Gary Muscatello, University of Sydney, Australia*
09.00-09.25	Invited lecture: **Foal- and farm-level risk factors for *R. equi* pneumonia** Noah Cohen, *Texas A&M University, Texas, USA*
09.25-09.50	Invited lecture: **Epidemiology and evolution of *R. equi*: new insights from molecular studies** *Jose Vazquez-Boland, University of Edinburgh, Scotland, UK*
09.50-10.20	Coffee break – Poster viewing
10.20-10.35	**Proliferation of virulent *R. equi* in the gastrointestinal tract of foals** *Catherine Chicken, University of Melbourne, Australia*
10.35-10.50	**Shedding of *R. equi* in fecal and tracheal secretions of foals with pulmonary abscesses** *Mark Lämmer, University of Hannover, Germany*
10.50-11.05	**Assessment of the genetic diversity of the virulence plasmid present in *R. equi*, the agent of equine rhodococcosis.** *Fabien Duquesne, AFSSA, Goustranville, France*
11.05-11.20	***Mycobacterium avium* subsp. *paratuberculosis*: another challenging pathogenic actinomycete** *Karen Stevenson, Moredun Research Institute, Penicuik, Scotland, UK*
11.20-11.35	**Comparison of *R. equi* strains isolated from swine, wild boars and humans in Hungary** *Laszlo Makrai, Szent István University, Budapest, Hungary*
SESSION 6	**WORKSHOP GENERAL DISCUSSION**
11.35-12.15	General commentary and discussion on key issues from the sessions
12:15-12:30	Travel Prize Awards to graduate student presenters (Oral/Poster)
12.30	End of meeting

POSTER COMMUNICATIONS

(only presenting author indicated; see abstracts for complete list of authors)

SESSION 1 – ORGANISMAL BIOLOGY

Bacteriophages infecting *Rhodococcus equi*: diversity and genome analysis
Sophie Foley, Napier University, Edinburgh, Scotland, UK

Identification of a sensor kinase and arylsulfatase interacting with the orphan response regulator VirS (Orf8) encoded by the virulence plasmid of *Rhodococcus equi* 103
Ciaran Finn, University College Dublin, Ireland

Enumeration of proliferating *Rhodococcus equi* inside macrophage cells
Aleksandra Gnojska, University College Dublin, Ireland

The cholesterol response of mycobacteria is controlled by two independent transcriptional regulators, KstR and KstR2
Neil Stoker, Royal Veterinary College, London, UK

SESSION 2 – PATHOGENESIS

Which two-component system sensor kinase phosphorylates the Orf8 response regulator of *Rhodococcus equi*?
Iain MacArthur, University of Guelph, Canada

A long way down – The intracellular pathogen *Rhodococcus equi* in activated macrophages
Kristine von Bargen, University of Bonn, Germany

Real time PCR analysis of *Rhodococcus equi* virulence plasmid genes under inducing and non-inducing conditions
Ruth Fahey, University College Dublin, Ireland

Study of C4 dicarboxylic acid transport in *Rhodococcus equi*
Vibhay Tripathi, University of Georgia, Athens, Georgia, USA

SESSION 3 – IMMUNITY

Differential gene expression of equine pulmonary alveolar macrophages and monocyte-derived dendritic cells in response to *Rhodococcus equi* infection *in vitro*
Johanna Watson, University of California, Davis, California, USA

Treatment of neonatal foals with immunostimulants enhances phagocytic cell activity against *ex vivo* infection with *Rhodococcus equi*
Clare Ryan, University of Florida, Gainesville, Florida, USA

CpG-induced stimulation of cytokine expression by peripheral blood mononuclear cells of foals and their dams
Angela Bordin, Texas A&M University, Texas, USA

Antibacterial properties of the equine a-defensin DEFA1 against *Rhodococcus equi*
Julien Cauchard, AFFSA, Goustranville, France

Susceptibility of *Rhodococcus equi* to equine antimicrobial peptides
Marianela Lopez, University of Saskatchewan, Saskatoon, Canada

Bronchoalveolar lavage component comparison between foals and adult horses
Marianela Lopez, University of Saskatchewan, Saskatoon, Canada

Analysis and genomic structure of the equine CD1 gene family
Robson Dossa, Washington State University, Pullman, USA

Antibody mediated regulation of immunity to *Rhodococcus equi*
Crystal Montoya, Washington State University, Pullman, USA

Development of live-attenuated metabolic drift mutant strains of a virulent *Rhodococcus equi* strain
Monika Krueger, University of Leipzig, Germany

Immune response anti-*Rhodococcus equi* after immunization of neonate foals with live attenuated *Salmonella* vaccine strain expressing the VapA protein
Ana Carolina R. Correa Porto, Universidade de São Paulo, Brazil

Mice inoculation by nasal route of a live attenuated *Salmonella* vaccine strain expressing the VapA protein induces a long-term protection against *Rhodococcus equi* infection
Silvia Cardoso, Universidade de São Paulo, Brazil

Development of a live attenuated VapA-producing *Rhodococcus equi* oral vaccine to protect neonatal foals against pneumonia.
Ashley Whitehead, Department of Clinical Studies, University of Guelph, Guelph, Ontario, Canada

SESSION 4 – CLINICAL ASPECTS

Studies on the persistence of transfused *Rhodococcus equi* antibodies in Thoroughbred foals
Lorraine Palmer, Beaufort Cottage Stables, Rossdales & Partners, Newmarket, UK

Immune-mediated arthritis and uveitis associated to *Rhodococcus equi* infection in foals.
Ignacio Corradini, Universitat Autònoma de Barcelona, Spain

Detection of *Rhodococcus equi* by microbiological culture and by polymerase chain reaction in samples of tracheobronchial secretions of foals
Monica Venner, University of Veterinary Medicine, Hannover, Germany

Evaluation of tulathromycin in the treatment of pulmonary abscesses in foals
Monica Venner, University of Veterinary Medicine, Hannover, Germany

A double blind study comparing the effect of hyperimmune plasma and standard equine plasma on reducing the incidence of *R. equi* infection and requirement for treatment on an endemic stud farm.
Tamsin Dawson, Veterinary Immunogenics, Penrith, Cumbria, UK

SESSION 5 – EPIDEMIOLOGY

Retrospective study of *R. equi* infection from a population of 1352 autopsied foals in France
Jacky Tapprest, AFSSA, Goustranville, France

***Rhodococcus equi* infection in foals in Poland**
Lucjan Witkowski, Warsaw University of Life Sciences, Warsaw, Poland

ABSTRACTS

PLENARY LECTURE

Rhodococcus equi as a pathogen: past, present and future

S. Takai, Department of Animal Hygiene, School of Veterinary Medicine, Kitasato University, Towada, Aomori 034-8628, Japan.

Since its discovery by Magnusson (1923) *Rhodococcus equi* has been recognized as a significant pulmonary pathogen of foals worldwide. However, there had been no workshop or meeting on *Rhodococcus equi* before the first workshop on *R. equi* pneumonia of foals held at the Ontario Veterinary College in July 1986 by Drs. Prescott and Yager. At the workshop, topics on the epidemiology, immunology and diagnosis of *R. equi* infection in foals were presented and discussed. The second workshop was held at the same place in July 1996. The discovery of the virulence plasmid in foal and pig isolates has improved our understanding of the pathgenesis of *R. equi* infection in foals. The third workshop was held at the Washington State Unversity in July 2002. Complete sequence of the virulence plasmid has revealed the Vap family and the pathogenicity island. Protective immune responses in horses has been discussed from the standpoint of the function of Vap genes. The fourth workshop will be held at Edinburgh and it will open the postgenomic era of *Rhodococcus equi* research. As an introduction of this workshop, I will review the virulence plasmids and molecular epidemiology in horses and other animals.

SESSION 1

The *Rhodococcus equi* genome

M. Letek [1,2,3], M. Blanco [2], A. Ocampo-Sosa [2,3], D.A. Lewis [2], N. Bason [4], I. Cherevach [4], K. Mungall [4], M.A. Quail [4], M. Sanders [4], I. McArthur [5], R. Fahey [6], P. Gonzalez [1,3], M. Scortti [1,3,7], J. Navas [8], V. Armendarez [9], I. Sutcliffe [10], J. Prescott [5], T. Buckley [3], D. Leadon [3], U. Fogarty [3], W. Meijer [6], S. Bentley [4], J. Parkhill [4], J.A. Vazquez-Boland[1,3,7]. [1]Microbial Pathogenesis Unit, Centre for Infectious Diseases, University of Edinburgh, UK; [2]Veterinary Molecular Microbiology Section, Faculty of Medical and Veterinary Sciences, University of Bristol, UK; [3]Irish Equine Centre, Johnstown, Naas, Ireland; [4]Pathogen Genomics, Wellcome Trust Sanger Institute, Genome Campus, Hinxton, UK; [5]Department of Pathobiology, University of Guelph, Canada; [6]School of Biomolecular and Biomedical Sciences, University College Dublin, Ireland; [7]Grupo de Patogenómica Bacteriana, Instituto de Biología Molecular y Genómica, Universidad de León, Spain; [8]Departamento de Biología Molecular, Universidad de Cantabria, Spain; [9]Department of Molecular Microbiology, John Innes Centre, Norwich, UK; [10]School of Applied Science, Northumbria University, Newcastle upon Tyne, UK;*

We determined the complete genome sequence of *Rhodococcus equi* 103S, a prototypic equine isolate from a case of foal pneumonia. The *R. equi* 103S genome is just above 5 millions of bp in size, with a circular chromosome of 5,043,170 bp and 4,521 predicted genes, and a virulence plasmid of 80,609 bp with 73 predicted genes. The GC content is 68.82% and the coding density 90.1 % or 0.894 genes per kb (1,118 bp per gene in average). The *R. equi* genome size is similar to that of other pathogenic nocardioform actiomycetes (*Mycobacterium tuberculosis*, ≈ 4,400 to 4,800 kb; *Nocardia farcinica*, 6,290 kb). Genome comparisons by reciprocal best-match BLASTP and orthologue plots showed that *R. equi* 103S is most similar to the xenobiotic-degrading *Rhodococcus* sp. RHA1, indicating a close phylogenetic relatedness and justifying their inclusion in the same genus. RHA1 has however a substantially larger genome, 7,804 kb, mainly accounted for by an extensive complement of genes involved in secondary metabolism which are absent in *R. equi*. Only 13 pseudogenes were identified in the *R. equi* genome, indicative of a low rate of gene decay. Thus, the genome differences between *Rhodococcus* RHA1 and *R. equi* 103S are likely due to massive secondary metabolism gene expansion in the former rather than reductive genome evolution in the latter. Next in (decreasing order of) similarity based on number of shared gene orthologues are *N. farcinica*, *Mycobacterium smegmatis* and *Streptomyces coelicolor*. *R. equi* encodes a large number of surface-associated proteins (1,057 genes i.e. 23.37% of total CDS), many of which are transporters (406 genes, 8.9% of total CDS). There is also a large secretome (610 genes, 13.5% of total CDS) and many regulatory genes (464 i.e. 10.26% of total CDS), consistent with the multiplicity of niches potentially colonized by *R. equi* in his dual lifestyle as soil saprophyte and animal pathogen. No genes related to sugar transport were identified and there is a large set of genes involved in lipid catabolism, suggesting that, as in mycobacteria, lipids are the major carbon source for *R. equi*. We also identified a number of putative virulence genes, some of which were horizontally acquired DNA. Interestingly, members of the mycobacterial PE/PPE multigene family were present in *R. equi*. We found many putative antibiotic resistance genes (7 encoding aminoglycoside phosphotransferases, 22 beta-lactamases, 3 small multidrug resistance proteins, and one a multi-antimicrobial extrusion family protein), revealing a new facet of *R. equi* as multiresistant pathogen. Our findings highlight the phenomenal niche-adaptive plasticity of the mycolata genomes.

Acknowledgements:

The *R. equi* genome project was supported by grants from the Horserace Betting Levy Board (UK), the Irish NDP fund, and the Grayson-Jockey Club Research Foundation (USA).

SESSION 1

Genetic engineering of steroid catabolism in *Rhodococcus equi*

Robert van der Geize and Lubbert Dijkhuizen. Department of Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, P.O. Box 14, 9750 AA Haren, The Netherlands.*

Steroids constitute a large class of organic compounds with different bioactive properties and biological functions. Members of the genus *Rhodococcus* are of environmental and biotechnological importance and degrade steroids. Rhodocci therefore are interesting candidates for the industrial production of bioactive steroids. This requires construction of strains blocked at crucial catabolic steps. Using transcriptome analysis we have been able to identify a cholesterol catabolic gene cluster in the saprophyte *Rhodococcus jostii* RHA1 [1]. Up-regulated genes are putatively involved in cholesterol uptake, sterol side chain degradation and steroid ring opening. Rhodococcal genes specifically expressed during growth on cholesterol are conserved within a large gene cluster in the human pathogen *Mycobacterium tuberculosis* H37Rv. Several of these conserved genes are critical for the survival of *M. tuberculosis* in macrophages. Cholesterol metabolism thus is related to the survival of intracellular pathogens in macrophages. Clearly, steroid catabolism is a highly interesting research topic from both biotechnological and medical points of view. Genetic engineering of steroid catabolism in *Rhodococcus* strains is hampered by the presence of multiple pathways and/or gene homologues. This increases *Rhodococcus* catabolic versatility and efficiency, but initially prevented the construction of metabolically blocked mutant strains. The *sacB* counter-selection based method for generating unmarked gene deletions in several *Rhodococcus* species [2] has allowed construction of a range of mutant strains. Surprisingly, this method is not applicable in *R. equi*. A novel and efficient method for the construction of unmarked in-frame gene deletions in *R. equi* was developed and successfully applied to study cholesterol degradation by this micro-organism.

[1] Van der Geize *et al*. (2007) *Proc. Natl. Acad. Sci U.S.A*. 104: 1947-1952.
[2] Van der Geize *et al*. (2001) *FEMS Microbiol. Lett.* 205:197-202.

SESSION 1

Membrane anchored molecules of *Rhodococcus equi*: insights from the genome

I.C. Sutcliffe [1], M. Letek [2], J.A. Vazquez-Boland [2] and The R. equi Genome Consortium. [1]School of Applied Science, Northumbria University, Newcastle upon Tyne NE1 8ST, and [2]Microbial Pathogenesis Unit, Centre for Infectious Diseases, University of Edinburgh, U.K.*

There are two major classes of membrane-anchored macromolecule in *R. equi*: lipoproteins and the previously describe lipoarabinomannan, ReqLAM (Garton et al., J Biol Chem 2001). Bioinformatic analysis of the *R. equi* genome has allowed the reconstruction of the lipoprotein biosynthesis and identified >140 putative lipoproteins. Functional analyses of these suggest several interesting targets for future studies. As in other Gram-positive bacterial genomes, ca 40% of the putative lipoproteins are predicted to be substrate binding proteins in ABC transport systems, with peptides and amino acids likely to represent important nutrient sources. Other putative lipoproteins are likely to carry out novel enzyme activities and participate in cell envelope homeostasis. Bioinformatic analyses have also allowed the reconstruction of many key steps in the pathway predicted to lead to ReqLAM biosynthesis, with both cytoplasmic and extracytoplasmic stages predicted. As ReqLAM is a significant immunomodulator, biochemical studies of this pathway are warranted. These studies suggest that membrane-anchored macromolecules make an important contribution to the biology of *R. equi*.

SESSION 1

Iron acquisition by *Rhodococcus equi*

R. Miranda-CasoLuengo and W.G. Meijer. School of Biomolecular and Biomedical Science, Conway Institute, University College Dublin, Dublin 4, Ireland.*

Bacteria have specialized systems to maintain the homeostasis of iron under different environmental conditions. *Rhodococcus equi* is not the exception but little is known about the iron metabolism of this facultative pathogen. Using a transposome mutagenesis approach, we isolated two mutants, called a5 and a6, sensitive to subinhibitory doses of 2,2'-dipyridyl. These mutants led to the discovery of the first siderophore-based iron acquisition system for *R. equi* and a novel signal transduction system unique to mycolic acid containing bacteria. The a5 mutant was affected in the IupABC transport system and showed accumulation of a catecholate-type siderophore. Targeted mutagenesis of the IupS non-ribosomal peptide synthetase allowed the finding of the gene cluster required for the biosynthesis of this siderophore which was showed primarily involved in uptake during saprophytic life. The a6 mutant was mutated in the second gene of the discistronic operon: *iupIR*. The IupIR system is analogous, but not homologous, to the BlaRI signal transduction system of *Staphylococcus aureus* which controls expression of b-lactamase. IupR is a transmembrane protein with an intracellular Zn^{2+}-protease domain and IupI is likely to be a repressor. When activated, most likely due to the presence of a siderophore-iron complex, IupR proteolytically inactivates the putative repressor IupI. The regulon controlled by this system is currently under investigation.

SESSION 1

Definition of *Rhodococcus equi* secreted proteins by electrospray mass spectrometry and sequence analysis *in silico*

C. Barbey [1], A. Budin-Verneuil [2], S. Cauchard [1], A. Hartke [2], C. Laugier [1], V. Pichereau [2], and S. Petry[1]. AFSSA, Laboratoire d'Etudes et de Recherches en Pathologie Equine, IPC, Goustranville, 14430 Dozulé, France. [2]Laboratoire de Microbiologie de l'Environnement, USC INRA 2017, EA956, IFR ICORE 146, Université de Caen, 14032 Caen Cedex, France*

The objective of the present study is to identify and characterize secreted proteins released from *R. equi* ATCC 33701 during *in vitro* growth at 30°C or 37°C and at mid-exponential or early-stationary phase. A systematic proteomic approach was undertaken using SDS PAGE combined with mass spectrometry or analyzing directly the unfractionated protein extract by mass spectrometry. A homology research in gene bank database allowed us to identify 48 proteins. Identified proteins belong to several functional categories: pathogenicity, stress adaptation, export, cell wall biosynthesis, energy metabolism and general cell processes. The cholesterol oxidase ChoE appears to be the major secretory protein. The sequence analysis predicted that 24 proteins are transported by a signal peptide-dependent pathway (21 with secretory signal peptides and three with twin-arginine signal peptides). By taking account of the growth conditions, no significant difference was revealed between the growth phases whatever the temperature. On the other hand, the Virulence associated proteins VapA and VapD are expressed at 37°C only. In conclusion, a comprehensive analysis of the secretome of *R. equi* ATCC 33701 revealed several proteins that are known to mediate the establishment of infection and many additional candidates which exact role need to be further characterized.

SESSION 1

Bacteriophages infecting *Rhodococcus equi* – diversity and genome analysis

S. Foley, M. Olesen, S. Dunbar, and S. Salifu. School of Life Sciences, Napier University, 10 Colinton Rd, Edinburgh EH10 5DT, UK.*

The objective of this study is the characterisation of bacteriophages infecting *R. equi*, including genome analysis of selected phage(s). Considering the significance of *R. equi* as a pathogen of foals and other animals, and recent emergence as a human pathogen, the exploitation of phages infecting *R. equi*, as a therapeutic agent and in the control of reservoirs of infection, is of potential interest. Difficulties encountered with diagnosis and potential emergence of multi-drug resistance also provide justification for exploitation of phages. Soil samples, ranging from agricultural to garden soil, were screened for the presence of phages infecting *R. equi*. This revealed that phages could be readily isolated at titres of 10^3-10^6 plaque forming units.g^{-1} of soil. Eight phage isolates were propagated, purified, and their host-range determined as a preliminary indicator of diversity. Based on host-range alone, phages could be divided into 4 distinct groups. This was further supported by a comparison of restriction profiles of genomic DNA. From these, phage E3 was selected for genome sequence determination – selection criteria included (i) the ability to infect *R. equi* NCIMB10027 and a number of clinical isolates, and (ii) the availability of restriction endonucleases capable of digesting the phage DNA yielding fragment sizes best-suited for cloning. The genome sequence is currently being determined. Preliminary analysis reveals striking similarity to phages infecting the closely related genus *Mycobacterium*. Further analysis of *R. equi* phage genomes may provide insights not only to phage diversity and evolution but also to the host, of particular significance in light of increasing recognition of the contribution of phages to pathogen evolution.

SESSION 1

Identification of a sensor kinase and arylsulfatase interacting with the orphan response regulator VirS (Orf8) encoded by the virulence plasmid of *Rhodococcus equi* 103

C. Finn, X. Chen, J. Yang, and W. G. Meijer, School of Biomolecular & Biomedical Science and Conway Institute, University College Dublin, Dublin 4, Ireland.*

The pathogenicity island of the virulence plasmid of *Rhodococcus equi* encodes a response regulator VirS but not a cognate sensor kinase, suggesting that VirS interacts with a chromosomally encoded sensor kinase. The yeast two-hybrid system was used to screen a chromosomal library using VirS as bait. Two interacting proteins, VirX and AstA, were identified. VirX is similar to a sensor kinase whereas AstA is similar to an arylsulfatase. $AstA^{585-775}$ and $VirX^{337-652}$ were precipitated by c-Myc tagged VirS using Myc antibodies, validating the results from the yeast two-hybrid screen. Using the yeast two-hybrid system and immune precipitation it was shown that VirX and AstA interact with each other. Disruption of *virS* resulted in strongly reduced transcription of the pathogenicity island genes *vapG*, *orf9*, *orf10* and *vapA* but not *orf5*. Furthermore, the *virS* mutant did not proliferate in macrophages and was not cytotoxic. Disruption of *virX* and *astA* had no effect on transcription of the pathogenicity island genes. The cytotoxicity and ability to proliferate in macrophages was also not affected. The results show that the virulence plasmid encoded orphan response regulator VirS interacts with at least two chromosomally encoded sensor kinases, one of which is VirX.

SESSION 1

Enumeration of proliferating *Rhodococcus equi* inside macrophage cells

A. Gnojska, R. Fahey, R. Miranda-CasoLuengo, X.X. Chen and W.G. Meijer. School of Biomolecular and Biomedical Science, Conway Institute, University College Dublin, Dublin 4, Ireland.*

Infection of macrophages by *Rhodococcus equi* depends on interfering with maturation of endosomes, thus establishing a permissive environment for survival and proliferation. Nevertheless, studying intracellular proliferation is a challenging task. Intracellular proliferation of *R. equi* has been inferred by enumeration of CFU and visualization by immunofluorescence at different times post-infection (pi). These approaches are time-consuming and sensitive to bacterial clumping, thus reducing their practical and quantitative significance. To overcome these limitations, the number of the virulent 103S and avirulent 103p- strains inside the macrophage was followed by qPCR of 16S rDNA. While the number of 16S rDNA was similar between both strains 2hpi, the virulent strain was 16 times higher after 48hpi. Interestingly, 48hpi a 5-fold drop in bacteria was seen relative to 2hpi, suggesting that not all the internalized bacteria were able to survive. Surviving bacteria actively transcribe *vapA*, *gyrB*, and *16S rRNA*. Interestingly, transcription of *vapA* is constant while *gyrB* transcripts are 10-fold higher 48hpi than 2hpi. Since *gyrB* is actively expressed during logarithmic growth in culture but dramatically drops at early stationary phase, the expression of this gene relative to that of 16S rRNA can be used as a marker of proliferation.

SESSION 1

The cholesterol response of mycobacteria is controlled by two independent transcriptional regulators, KstR and KstR2"

S.L. Kendall, A. ten Bokum, M.J. Withers, P. Burgess, R. Balhana, N. Mokhtar, N. Owoh, J.S. Lott, N.G. Stoker. Department of Pathology and Infectious Diseases, Royal Veterinary College, London.*

We previously reported the characterization of a TetR-like transcriptional regulator, KstR, which controls 83 genes in *Mycobacterium smegmatis* (Kendall et al. 2007). Many of these genes lie clustered within a small region of the chromosome, and their orthologues are induced in *Rhodococcus* by growth in cholesterol (van der Geize 2007). The predicted functions of the genes suggest that the genes are involved in cholesterol catabolism, and there is experimental supporting this for a small number of proteins. A bioinformatic analysis indicated that many of these genes have orthologues in the M. tuberculosis genome, and we confirmed that the *M. tuberculosis* KstR (Rv3574) protein binds specifically to several promoters within this regulon containing a palindromic motif, which we believe to be the binding site. From this analysis we predicted that the M. tuberculosis regulon contains 74 genes. There is however a group of genes within this cluster that is not controlled by KstR, although these genes are induced by cholesterol in *Rhodococcus*. One of these genes (Rv3557c) is also a TetR-like transcriptional regulator. In this study we demonstrate that this regulator (which we call KstR2) controls transcription of the group of genes described.

References:

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SESSION 2

Overview of *Rhodococcus equi* pathogenesis and virulence

M. Hondalus, University of Georgia, Athens Georgia, USA

A snapshot of the current state of knowledge regarding the pathogenesis and virulence of the opportunistic bacterium *R.equi* will be presented. The strengths and limitations of existing models of virulence will be discussed. Development of new molecular tools allowing genetic manipulation and thus the dissection of virulence pathways will be highlighted.

SESSION 2

Microarray experiments: Some lessons from mycobacteria

N. Stoker. Dept. of Pathology and Infectious Diseases, Royal Veterinary College, London, UK.

The availability of genome sequences has led to many technological developments in the field of functional genomics. Probably the most widely used of the is microarray analysis, where DNA from each gene is represented on a glass slide or chip, and global patterns of gene expression can be determined. In this talk I will discuss how microarrays have been used in studying mycobacteria over the last nine years, and share some perspectives on how they can be used effectively, and difficulties encountered. I will also discuss rhodococcal-mycobacterial synergies in analysing the shared cholesterol degradation pathway, and the potential for further interactions between researchers in the two fields.

SESSION 2

The virulence plasmid of *R. equi*: how does it work?

W.G. Meijer, School of Biomolecular and Biomedical Science and Conway Institute, University College Dublin, Dublin 4, Ireland.

Rhodococcus equi induces a set of genes located in a putative pathogenicity island (PI) under growth conditions resembling those occuring in the host. A number of environmental parameters, including temperature, pH and oxidative stress, control the expression levels of the genes located in the pathogenicity island including those encoding the virulence associated proteins (Vap). The regulation of virulence plasmid gene expression is complex and dependent on many parameters. The PI only encodes two transcriptional regulators, one of which is an orphan response regulator (VirS). Using a yeast two hybrid approach the cognate chromosomally encoded sensor kinase protein was identified. The second regulatory protein (VirR) in the PI is a LysR-type-transcriptional regulator. VirR and VirS are encoded within a five cistronic operon. The first gene of the operon, *virR*, is constitutively transcribed from P_{virR}, whereas the four downstream genes are transcribed from both P_{virR} and the regulated internal promoter P_{orf5}. Evidence will be provided that the *virR* operon appears to function as a masterswitch, controlling the expression of other PI genes, including those located in the *vapA* and *orf9* operons. It has been conclusively demonstrated that the PI island encoded proteins are essential for virulence. However, little is known beyond this. Many of the PI encoded proteins are unique to *R. equi*, and do not have any obvious functional domains. In addition, the PI encodes a family of Vap proteins. Whereas VapA has been shown to be essential for virulence, the role other Vap proteins play and why more than one Vap protein is encoded in the PI remains unclear. In order to functionally link proteins, we have carried out a protein interaction analyses, revealing that VapA sits at the centre of a small protein interaction network. The implications of these findings for virulence will be discussed.

SESSION 2

Rhodococcus equi phagocytosis: when macrophages suffer a digestive disorder

A. Haas [1], K. von Bargen [1], J. F. Prescott [2], T. Sydor [1], M. Polidori 1. [1] Cell Biology Institute, University of Bonn, Ulrich-Haberland-Str. 61a, 53121 Bonn, Germany and [2]Department of Pathobiology, University of Guelph, Guelph, Ontario.*

Like most intracellular pathogens, *R. equi* inhabits a non-canonical endocytic compartment in macrophages. This does not mature into a fully degrading phagolysosome, as it would with harmless particles, but is arrested at an intermediate step between an early endocytic and a late endocytic stage. The '2 hour-phagosome' is neutral in pH, does not contain cathepsin D or lysosomal β-galactosidase, but has lost all tested early endocytic marker molecules (Rab5, transferrin receptor and others). To identify bacterial factors that support diversion from the normal route, we have established and screened a library of more than 3,000 *R. equi* mutants for normalised phagosome development and we have isolated several clones. One of them, coding for a β-ketoacyl-acyl carrier protein-synthase (kasA) homologue, was considered particularly interesting, as kasA is a central enzyme in the synthesis of long mycolic acid compounds in mycobacteria and may participate in diversion of phagosome maturation. Data on phagosome maturation, cytotoxicity, and bacterial multiplication will be presented. Furthermore, we investigated maturation of phagosomes containing a deletion mutant in *vapA* which, early in macrophage infection, matured like phagosomes containing the virulent wild type, while trafficking was as with the avirulent sister strain by 24 h of infection. This could mean that VapA's major role is required only after several hours into the infection.

SESSION 2

The *Rhodococcus equi* PhoP-PhoR two-component system and its involvement with phosphate starvation

V. R. Parreira, J. F. Prescott. Department of Pathobiology, Ontario Veterinary College, University of Guelph, Guelph, Ontario N1G 2W1, Canada.*

Environmental regulation of bacterial gene expression is usually mediated by two-component signal transduction systems. A Δ*phoPR* mutant of *R. equi* was hypervirulent in mice. To test the role of the two-component regulatory system (TCS) PhoP-PhoR in the regulation of virulence genes of the virulence plasmid and its involvement in the response to phosphate (Pi) starvation, we constructed transcriptional promoter fusions using a promoterless *lacZ* vector of two genes of the virulence plasmid (*orf8:* an orphan 2CS response regulator; and *vapA:* virulence-associated protein A) and three genes of the phosphate starvation-specific Pho regulon (*phoA*: an alkaline phosphatase gene; *pst*: phosphate-specific transporter; and *phoPR:* TCS). The results suggest that *orf8*, which is a DNA-binding protein, might be aiding in the regulation of *vapA*, which encodes VapA that is essential for virulence and that *orf8* is in turn under partial control by PhoPR. Analysis of *phoA* and *pstS–lacZ* reporter gene assays showed their expression to be up-regulated under phosphate starvation conditions. The PhoP-PhoR TCS transduction system is believed to be activated by phosphate limitation. Taken together, these results indicate that transcription initiation of the genes of virulence plasmid, especially the *vapA* promoter, is complex but includes partial control by phosphate concentration.

SESSION 2

VapA is not all there is

G. B. Coulson, V. N. Tripathi, S. Jain, M. K. Hondalus. Department of Infectious Diseases, University of Georgia, Athens, Georgia, USA.*

Rhodococcus equi is a facultative intracellular pathogen of the immunologically naive or immunodeficient. Virulence of *R. equi* is dependent on the presence of the ~81Kb virulence plasmid, which contains a ~27Kb pathogenicity island harboring a novel family of proteins known as the virulence associated (Vap) proteins. One member of this family in particular, *vapA*, has been shown to be essential for growth *in vitro* in macrophages and for virulence *in vivo* (Jain et al., 2003). However, although *vapA* is necessary for virulence, it alone is not sufficient (Giguere et al., 1999) and the identity of additional plasmid-encoded virulence genes remains to be determined. In our present study, we have created a pathogenicity island (PI) deletion mutant through homologous recombination, replacing it with a selectable apramycin resistance marker. Complementation of the PI-mutant, a *vapA* mutant, and the virulence plasmid cured (103-) strain with a recombinant plasmid expressing *vapA* from a constitutive mycobacterial *hsp*60 promoter, allowed us to assess the contribution of *vapA* to intracellular growth and to virulence *in vivo*. Although complementation with *vapA* restored the virulence phenotype of the *vapA* mutant it had no significant effect on the 103- and PI- strains which were unable to replicate in murine macrophages or *in vivo* in the mouse model of *R. equi* infection. The data demonstrate that although expression of *vapA* could slightly delay *in vivo* clearance of the PI-mutant, it did not restore virulence, thus suggesting that additional genes located within the pathogenicity island (and presumably not outside of this region) are required for virulence. The identification of those additional virulence determinants is currently under investigation.

SESSION 2

Which two-component system sensor kinase phosphorylates the Orf8 response regulator of *Rhodococcus equi*?

I. MacArthur [1], V. R. Parreira [1], D. Lepp [2], J.A. Vazquez-Boland [3], J. F. Prescott [1]. [1]Department of Pathobiology, University of Guelph, Guelph, Canada. [2]Agriculture and Agri-Food Canada, Guelph, Canada. [3]Microbial Pathogenesis Unit, Centre for Infectious Disease, University of Edinburgh, Edinburgh, UK.*

Rhodococcus equi survival in macrophages and mice is dependent on the expression of the virulence plasmid-encoded protein known as virulence-associated protein A (VapA), the function of which is unknown. A response regulator protein (Orf8), which is part of a two-component system, is encoded on the virulence plasmid just upstream of *vapA* and has been shown to regulate the expression of *vapA*, thus playing an essential role in the regulation of *R. equi* virulence. To further understand the regulation of *vapA*, the chromosomally encoded sensor kinase(s) that is responsible for the activation of Orf8 needs to be identified. A small DNA microarray has been developed using 70bp oligonucleotide probes corresponding to unique regions of each of 23 two-component systems (TCSs), plus an orphan sensor kinase and 4 orphan response regulators, *vapA*, *orf8* and 3 housekeeping genes. By comparing the gene expression levels of the bacteria in macrophages and in broth culture, the TCSs that are up-regulated during intracellular growth will be targeted for deletion mutation. Each mutant will be tested for virulence, and the effect of the mutation on *vapA* expression assessed. Reporter gene fusion(s) will be used to identify the stimulus detected by the sensor kinase by examining the expression level in the presence of different stimuli. In future, the transcriptome of the Orf8 two-component system will be determined using a genome scale DNA microarray.

SESSION 2

A long way down – The intracellular pathogen *Rhodococcus equi* in activated macrophages

K. von Bargen, A. Haas. Cell Biology Institute, University of Bonn, Germany*

The actinomycete *Rhodococcus equi* is a facultative intracellular pathogen. After ingestion by macrophages, *R. equi* blocks the normal pathway of phagosome maturation in between the stages of an early and a late endosomal compartment. The bacteria multiply in their unusual vacuoles and the host cells are finally destroyed in a necrotic process. However, bacterial multiplication can be competely averted by immunological activation of macrophages prior to infection. It has been presumed that this might be due to the bactericidal effects of peroxynitrite generated by the NADPH oxidase and the inducible nitric oxide synthase (NOS2) (Darrah *et al.*, 2000). Non-canonical cellular compartments of some intracellular pathogens are forced to become phagolysosomes upon activation of their host cells. We have therefore analyzed the effect of macrophage activation on maturation of *R. equi* containing vacuoles. Although we comfirmed the previously demonstrated inhibition of bacterial multiplication in mouse and horse macrophage cell lines upon activation, we saw no evident changes in the *R. equi* containing vacuole. There was no enhanced fusion with lysosomes and accessibility to fluid phase markers up to 5 hours post infection remained unchanged. Macrophage death at 24 hours post infection was significantly reduced upon activation, yet only to about 80%. Phagosome pH was barely affected by activation of macrophages prior to infection. Since killed *R. equi* encounter a delayed but normal phagosome maturation, we checked for the viability of *R. equi* in activated macrophages by determination of colony forming units. The bacteria survived for at least 24 hours before they were killed. These experiments indicate that the reason for the lack of *R. equi* multiplication in activated macrophages is not a rapid killing by bactericidal compounds, but rather a slow process which is under investigation.

SESSION 2

Real time PCR analysis of *Rhodococcus equi* virulence plasmid genes under inducing and non-inducing conditons

R.J. Fahey, W.G. Meijer. School of Biomolecular and Biomedical Science, UCD Dublin, Ireland.*

The virulence plasmid of *Rhodococcus equi* contains several operons encoding virulence associated proteins or Vaps in addition to various regulatory genes. VapA has been shown to be regulated by both pH and temperature. Real time PCR analysis was used to detect in vitro transcription levels of Orf 5, 10, VapA and VapG under both inducing and non inducing conditions. VapA has been shown to be regulated by both pH and temperature. Orf5 is located within the VirR operon, containing VirS, which has been shown to regulate both VapA and Orf10. VapG is known to interact with VapA. pH and temperature were analysed separately to determine the individual effects on inducement. *R.equi* was grown in minimal media at either pH 5.5 or pH 8 and 37 or 30, to late logarithmic phase. Real time expression analysis was used to determine absolute transcript levels under individual conditions. Temperature was shown to have a stronger individual effect on expression levels than pH alone.

SESSION 2

Study of C4 dicarboxylic acid transport in *Rhodococcus equi*

V.N. Tripathi, I. Azuonye, A. Rogovskyy, G. Coulson, M.K. Hondalus. Department of Infectious Diseases, University of Georgia, Athens, Georgia, USA*

Microenvironments around pathogenic bacteria change frequently during the infectious cycle and the ability to efficiently adapt to different conditions both inside and outside of the host is most often mediated by two-component response systems (TCS). These regulatory systems, characterized by sensor kinase and response regulator protein partners, can be considered an essential prerequisite for pathogenicity. Although significant progress has been made in the elucidation of virulence factors in *R. equi*, very little work has been done to understand the role of TCS in this bacterium. With the help of the available genome sequence and different *in silico* programs, we searched for TCS in *R. equi*. To facilitate this analysis, we used well studied TCS from the closely related organism, *M. tuberculosis*, as a reference. Twenty putative TCS were identified, which were found to be widely distributed across the genome. To begin to assign a function to these systems (ie determine the genes regulated by them), we closely analyzed the genes in adjacent areas of the genome. One putative TCS (HK18/RR18) was identified next to an ORF showing high homology to a potential C4 carbon transporter. Carbon utilization is a very important aspect for survival, particularly so in harsh conditions (ie inside of host macrophages) and there are several reports where carbon utilization enzymes, especially those of the glyoxalate cycle, have been shown to be important for virulence. Although at present, there is no report for a direct role for C4 carbon transport in virulence, these carbon substrates are part of glyoxalate pathways and the possibility of their indirect role in virulence under some conditions can not be ruled out. Therefore, we chose to study these genes through mutagenesis and observation of the effect of mutation on bacterial growth on defined medium and in macrophages. Uptake of the toxic precursor 5'fluoro orotic acid (FOA) has been reported to involve a C4 carbon transporter, and mutation thereof led to FOA resistance. Thus, we used the FOA resistance phenotype as a selection marker for mutation. Wild type FOA sensitive *R. equi* cells were randomly mutagenized by *Himar*1 transposon insertion and then selected for FOA resistance. In so doing, we obtained mutants with insertions in the predicted C4 carbon transporter as well as the putative histidine kinase (HK18) gene. Characterization of these mutants is currently underway.

SESSION 3

Current understanding of immunity to *Rhodococcus equi* pneumonia in foals

S.A. Hines, Washington State University, Pullman, Washington, USA

This presentation will briefly review what is known about immunity to *R. equi*, notably the "correlates to protective immunity" and the types of immune responses that an effective vaccine will need to elicit. A focus will be on the barriers to immunization in foals and the critical gaps in our knowledge regarding protective immunity and the immunologic capabilities of neonatal horses. Effective immunization of foals is likely to require a novel approach.

SESSION 3

Foal dendritic cells become activated upon *Rhodococcus equi* infection *in vitro*

M.J.B.F. Flaminio[1], D.V. Nydam[2], H. Marquis[3], M.B. Matychak[1], S. Giguère[4], [1]Dept. of Clinical Sciences, [2]Dept. Population Medicine & Diagnostic Sciences, [3]Dept. of Microbiology and Immunology, College of Veterinary Medicine, Cornell University, Ithaca, NY, USA; and [4]Dept. of Large Animal Clinical Sciences, College of Veterinary Medicine, University of Florida, Gainesville, FL, USA.*

Susceptibility of foals to *R. equi* pneumonia is exclusive to the first few weeks of life. The objective of this study was to investigate immediate immunologic response of foal and adult horse antigen presenting cells upon *R. equi* infection. We measured the activation of transcriptional factor IRF-7, antigen presenting molecule MHC class II, and co-stimulatory molecules CD40 and CD86 in monocyte-derived macrophages or dendritic cells of adult horses or foals of different ages (from birth to 3 months of life) infected in vitro with virulent *R. equi* or its avirulent plasmid-cured derivative. Foal macrophages cultured with avirulent *R. equi* for 24hrs revealed an age-dependent increase in CD86 co-stimulatory molecule expression, and foals at 3 months of age had greater expression than adult horses ($p = 0.03$). In contrast, adult horse dendritic cells, but not macrophages, revealed a trend for greater MHC class II expression than foal cells at most ages ($p = 0.06$). Virulent *R. equi*-infected foal macrophages and dendritic cells responded with greater IL-12p40 and IRF-7 mRNA expression than adult horse cells ($p \leq 0.003$ and $p = 0.01$, respectively). These data suggest that foal dendritic cells can respond to virulent *R. equi* infection and develop signals for activation of the acquired immune system. Nevertheless, the limitation in the expression of MHC class II raises the question if additional activation stimulus (e.g. from lymphocytes) is necessary to make those cells signal the acquired immune system more efficiently.

SESSION 3

Identification of immunologically relevant genes in equine dendritic cells infected with *Rhodoccocus equi* and a potential role for indoleamine 2,3 dioxygenase (INDO) in *Rhodoccocus equi* infection

M.C. Heller, K.A. Jackson, J.L. Watson, Department of Medicine and Epidemiology, School of Veterinary Medicine, University of California, Davis, CA 95616*

The first study objective was to identify differentially expressed genes in *Rhodococcus equi* infected dendritic cells (DCs). Monocyte-derived DCs from three mares and foals were infected with *R. equi* for 24 hours. Six suppressive subtractive hybridization experiments were performed, yielding 200 clones comprised of 40 unique sequences. Thirteen genes were deemed immunorelevant candidates for further investigation. One of these genes, indoleamine 2,3 dioxygenase (INDO), was previously identified as differentially expressed in *R. equi* infected alveolar macrophages. The second study objective was to determine the role of INDO in *R. equi* infection. Bacterial growth curves were obtained for *R. equi* grown in minimal media with 0µg/ml to 200µg/ml tryptophan and cultivated in equine alveolar macrophages incubated with and without 3-methyl tryptophan. No difference in bacterial growth was observed in either system. To investigate the role of INDO in *R. equi* infection *in vivo,* $INDO^{-/-}$ and control mice were used. At 6 days post-infection, organ weights and bacterial counts for liver and spleen were compared. $INDO^{-/-}$ mice had significantly heavier livers than wild type mice. Spleen weights and bacterial CFUs from spleen and liver did not differ between mouse strains. In conclusion, *R. equi* does not require tryptophan, inhibition of INDO does not effect *R. equi* intracellular growth *in vitro* and INDO may have an immunomodulatory role in *R. equi* infection *in vivo*.

SESSION 3

TLR9-mediated cytokine production in neutrophils of new born foals and its therapeutic potential in preventing *Rhodococcus equi* infection

M. Liu[1], J. R. Nerren[1], T. Liu[1], N. D. Cohen[1], V. Juillard[2], J. Minke[2]. [1]Department of Large Animal Clinical Sciences, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, TX, USA. [2]Merial SAS Discovery, Lyon, France.*

Intracellular Toll-like receptor-9 (TLR9) responds to bacterial DNA, and stimulates protective immune responses against intracellular pathogens such as *R. equi* by phagocytes, including neutrophils. The focus of this study was maturation of TLR9-mediated neutrophil responses in neonatal foals. Unmethylated CpG-oligodeoxynucleotides (CpG-ODNs), ligands for TLR9, were used to stimulate peripheral blood leukocytes from 8 Quarter Horse foals in vitro, within 12 hours of birth (at birth), and at 8 weeks of age. For comparison, *R. equi* was used as another stimulus in parallel. Neutrophils were then isolated from samples and examined for expression of TLR9 and a panel of T_h-1 promoting cytokines (IFN-γ, TNFα, IL-6, IL-8, IL-23p19, IL-12p40, and IL-12p35) by real-time RT-PCR. TLR9 mRNA was expressed in neutrophils at birth and at 8 weeks of age, and expression was unaffected by all stimuli tested. TLR9-mediated cytokine production, however, was evident at birth. Neutrophils showed significantly up-regulated ($P < 0.05$) mRNA production of IL-6, IL-8 and IL-12p40 in response to B class CpG-ODNs. Except for IL-8, there was no age-related increase in the sensitivity to CpG-ODN stimulation, suggesting that TLR9 is functional at birth. In contrast, when stimulated by *R. equi*, both IL-8 and IL-12p40 production were increased in 8-week-old foals as compared to foals at birth. This finding of age-dependent cytokine production may partially account for the age-related susceptibility of foals to *R. equi* infection. The ability of CpG-ODNs to trigger age-dependent cytokines indicates therapeutic potential for modulating TLR9 in neonatal foals to prevent *R. equi* pneumonia.

SESSION 3

Immunization with *Salmonella enterica* expressing the VapA protein of *Rhodococcus equi* induces a Th1 immune response, associated with a long-term protection

A.F. Oliveira[1], S.G. Soares[1], S.A. Cardoso[1], L.P. Ruas[1], M.C. Roque-Barreira[1]. [1]Department of Cellular and Molecular Biology. School of Medicine of Ribeirão Preto-USP, Ribeirão Preto-SP, Brazil.*

Rhodococcus equi is a leading cause of pneumonia in foals; however conventional vaccines to prevent rhodococcosis have not been successfully obtained. Since VapA protein corresponds to an important virulence factor of *R. equi*, we developed an attenuated *Salmonella enterica* strain expressing this protein, able to protect mice against *R. equi* infection (Oliveira *et al*., 2007). Th1 immune response is crucial in host defence against rhodococcosis. This study aimed to evaluate the immune response induced and the long-term protection conferred by vaccination. BALB/c mice were orally vaccinated with two doses of *S. enterica vapA*$^{+}$, whereas control groups received *S. enterica vapA*$^{-}$ or PBS. Following intravenous challenge with *R. equi*, the vaccinated mice presented higher levels of IL-12, IFN-γ, IL-10, nitric oxide and T-bet, and lower levels of TNF-α than the control groups. In addition, lower *R. equi* CFU was recovered from tissues of vaccinated mice, when they were challenged 5 months after immunization. These data indicate that the vaccination induces a long-term protection against *R. equi* infection, allowing the development of vigorous Th1 protective immune response, toward bacterial challenge. Moreover, high IL-10 and low TNF-α levels account for the milder inflammation developed by immunized mice.

Acknowledgements: FAPESP and CNPq.

SESSION 3

Recognition of *Rhodococcus equi* lipid antigens by cytotoxic T-lymphocytes

S.P. Harris, M.T. Hines, D. Alperin, R.H. Mealey, S.A. Hines. Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, WA.*

As an intracellular pathogen, cytotoxic T-lymphocytes which recognize and lyse *R. equi* infected macrophages are thought to be important to immunity. These CTL are present in all immune adult horses, and conversely are absent during the peak susceptibility period of neonatal foals. After the first 6-8 weeks of life, foals develop near adult levels of CTL corresponding to their development of natural immunity. A unique feature of these CTL is their lysis of *R. equi* infected macrophages in an MHC unrestricted manner. This research evaluates whether MHC independent antigen presentation is occurring via the CD1 system. CD1 is a group of conserved MHC-class I like molecules which are known to present lipid antigens to T-lymphocytes. *R. equi's* unique lipid-rich cell wall makes it a likely candidate for similar antigen presentation. Investigation was performed using four adult horses. PBMC from each horse were stimulated in cell culture with virulent *R. equi* and then tested for the ability to lyse MHC mismatched blood-derived macrophages presenting either protease digested soluble *R. equi* antigen or an *R. equi* lipid extract, derived through the Folch procedure. In all horses, statistically significant cytotoxicity was observed for both proteases digested or lipid pulsed groups, thereby supporting the premise that MHC-unrestricted CTL are recognizing *R. equi* lipids presented via the CD1 system.

SESSION 3

Activation of cells of the monocyte lineage results in more robust production of IL-10 in neonatal foals compared to adult horses

B. A. Sponseller[1,2,], M. M. de Macedo[3], S. K. Clark[1], D. E. Jones[3,1] Department of Veterinary Microbiology and Preventive Medicine, [2] Department of Veterinary Clinical Sciences, [3] Department of Veterinary Pathology, College of Veterinary Medicine, Iowa State University, Ames, IA 50011*

Foals are particularly vulnerable to infection by *Rhodococcus equi* during the first two weeks of life whereas mature horses are not. While an innate immunodeficiency likely accounts for this clinically relevant vulnerability, the factors that contribute to infection by *R. equi* have not been fully elucidated. In this study, we demonstrate that cells of the monocyte lineage, including monocytes, macrophages, and dendritic cells, that have been activated with LPS and IFN-γ, respond with a statistically significant, greater amount of cytokine mRNA production of IL-10, IL-12p35, and IL-12p40 than unstimulated control cells. Interestingly, activation of neonatal cells resulted in a two-fold log increase in baseline cytokine mRNA expression of IL-10 compared with adult cells. In contrast, no significant differences in mean cytokine mRNA expression of IL-12p35 and IL-12p40 were detected, suggesting that the defect in chromosomal remodeling that prevents IL-12p35 gene transcription as a cause for decreased IL-12 synthesis in human neonates is not a likely occurrence in equine neonates. Collectively, these differences indicate that *in vivo* activation of equine cells of the monocyte lineage may result in different autocrine and paracrine cellular responses that vary according to age, with potential impact on regulation of adaptive and innate immune responses.

SESSION 3

Interferon-gamma expression in young foals when treated with an immunostimulant or plasma

S. Wright, D.W. Horohov. Gluck Equine Research Center, University of Kentucky, Lexington, KY.*

Neonatal foals exhibit decreased expression of interferon-gamma (IFNg) during the first months of life. This period of time corresponds to when they are at greatest risk for infection with *Rhodococcus equi*. We hypothesize that decreased IFNg expression contributes to the susceptibility of these foals to infection and that enhancing IFNg expression could increase their resistance to *R. equi*. We have tested various commercially available immunostimulants and other treatments for their effect on IFNg expression in the foal. Foals were treated within the first week and of life and *in vivo* expression of IFN-g was determined using RT-PCR analysis of Paxgene blood samples. Peripheral blood mononuclear cells and bronchoalveolar lavage cells were stimulated *in vitro* with phorbol 12-myristate 13-acetate, ionomycin and brefeldin A. The cells were subsequently stained for intracellular IFNg using an anti-bovine IFNg antibody. None of the treatments had a significant effect on IFNg production by PBMC or BAL cells in the youngest foals. Older foals did respond to the immunostimulants. While disappointing, these results are consistent with the observations that neonatal cells exhibit altered responses to pathogen-associated molecular patterns, presumably as a result of defective toll-like receptor signaling. The nature of this underlying defect in the foal remains unknown.

SESSION 3

Experimental infection of neonatal foals with *Rhodococcus equi* triggers adult-like gamma interferon induction

S. Jacks, S. Giguère, P.C. Crawford, and W.L. Castleman. University of Florida, Gainesville, Florida 32610.*

Rhodococcus equi is a facultative intracellular pathogen that causes pneumonia in young foals but does not typically induce disease in immunocompetent adult horses. Clearance of *R. equi* depends mainly on gamma interferon (IFN-g) production by T lymphocytes, whereas the predominance of interleukin 4 (IL-4) is detrimental. Young foals, like neonates of many other species, are generally deficient in the ability to produce IFN-g. The objective of this study was to compare the cytokine profiles, as well as cell-mediated and antibody responses, of young foals to those of adult horses following intrabronchial challenge with *R. equi*. The lymphoproliferative responses of bronchial lymph node (BLN) cells to concanavalin A were significantly higher in foals than in adult horses. In contrast, adult horses had significantly higher lymphoproliferative responses to *R. equi* antigens than did foals. Infected foals had significantly lower IL-4 mRNA expression but significantly higher IFN-g expression and IFN-g/IL-4 ratio in *R. equi*-stimulated BLN lymphocytes than did infected adults. Infection with *R. equi* in foals resulted in a significant increase in the percentage of T lymphocytes and $CD4^+$ T lymphocytes in bronchoalveolar lavage fluid in association with a significant decrease in the percentage of these cell populations in BLNs. Infection of foals also resulted in a marked increase in serum IgGa and IgGb levels, resulting in concentrations in serum that were significantly higher than those of adult horses. This study demonstrates that the immune response to *R. equi* in foals is not biased toward IL-4 and is characterized by the predominant induction of IFN-g.

SESSION 3

Differential gene expression of equine pulmonary alveolar macrophages and monocyte-derived dendritic cells in response to *Rhodococcus equi* infection *in vitro*

J. L. Watson, K.A. Jackson, M. C. Heller, W. D. Wilson, S. K. Hietala*
Department of Medicine and Epidemiology, School of Veterinary Medicine, University of California, Davis, CA, 95616.

The objective of this study was to investigate differential gene expression in equine antigen presenting cells (APCs) during *Rhodococcus equi* infection. Primary alveolar macrophages (AMs) and monocyte-derived dendritic cells (mDCs), harvested from seven foals and their dams, were infected with *R. equi,* and real time PCR was used to quantify mRNA transcripts for the cytokines/chemokines IL-1β, IL-6, CXCL-8, IL-12p40 and TNF-α at 6 and 24 hours after infection. Using a linear mixed effect model of cytokine transcript expression, the main effect of age was significant for all cytokines measured in AM cultures, with foals showing 2-3 fold less up-regulation than mares. Infection of mDCs induced significant up-regulation of IL-1β, IL-6, CXCL-8, IL-12p40 and TNF-α in cells from mares and foals. The main effect of age was significant for IL-6, IL-12p40 and TNF-α measured in mDC cultures, with foals showing 2-3 fold less up-regulation than mares. To identify novel transcripts, differentially expressed during infection, cDNA libraries were constructed using mRNA derived from foal and mare alveolar macrophages at 24 hours post-infection and used for suppression subtractive hybridization (SSH). A total of 81 clones were obtained from 2 reciprocal SSH experiments and 30 unique cDNAs were identified. In conclusion, there are significant differences in cytokine/chemokine transcript expression of *R. equi* infected APCs between adults and foals, and SSH can be used to identify novel genes involved in *R. equi* infection.

SESSION 3

Treatment of neonatal foals with immunostimulants enhances phagocytic cell activity against *ex vivo* infection with *Rhodococcus equi*

C. Ryan, S. Giguère. University of Florida, College of Veterinary Medicine, Gainesville, FL.*

Our objective was to determine the effect of immunostimulants on neutrophil and macrophage activity against *ex vivo* infection with *R. equi*. Seventeen neonatal foals were treated with Zylexis®, EqStim®, or saline on days 7, 9, and 15 of life. Peripheral blood mononuclear and BAL cells were collected on days 7 (pre-treatment), 19, 31, and 43. Neutrophil phagocytosis and oxidative burst after *R. equi* infection was assessed by flow cytometry. Intracellular proliferation of *R. equi* within macrophages was assessed by light microscopy for day 7 and 19 samples. Neutrophils from Zylexis® treated foals had significantly greater ability to phagocytize opsonized *R. equi* and had higher oxidative burst on day 19 and day 31 (post-treatment) compared to baseline ($P < 0.05$). On day 31, Zylexis® treated foals had significantly greater phagocytosis and oxidative burst than EqStim® treated foals ($P < 0.05$). There was no significant effect of time on neutrophil activity in control and EqStim® treated foals. Treatment with EqStim® resulted in significantly less intracellular proliferation of *R. equi* within monocyte-derived and BAL macrophages on day 19 compared to control foals ($P <0.05$) but not compared to Zylexis® treated foals. In conclusion, treatment of neonatal foals with Zylexis® enhances the activity of neutrophils whereas EqStim® enhances activity of macrophages following *ex vivo* infection with *R. equi*.

SESSION 3

CpG-induced stimulation of cytokine expression by peripheral blood mononuclear cells of foals and their dams.

Tong Liu[1], Jessica Nerren[1], Angela Bordin[1+], Jennifer Murrell[1], Veronique Juillard[2], Hanane El Garch[1], Ronald Martens[1], Noah Cohen[1]. [1]Dept. of Large Animal Clinical Sciences, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, Texas. [2]Merial SAS Discovery, Lyon, France[2]*

A relative immunodeficiency of young foals may account for the increased susceptibility of foals to pneumonia caused by *Rhodococcus equi*. In this report, peripheral blood mononuclear cells (PBMC) from healthy foals at 14 and 56 days of age or from their dams, were incubated with 3 stimulatory and 1 non- stimulatory (control) synthetic cytosine-phosphate-guanosine oligodeoxynucleotides (CpG-ODNs), and mRNA expression of TNF-α, IFN- γ, IL-6, IL-8, IL12-p 35, and IL-12p40 were determined. Results indicated that synthetic CpG-ODNs can induce strong, rapid cytokine responses in healthy foals and adult horses. B-class CpG-ODNs 2135 and 2142 induced greater mRNA expression of IFN- γ, IL-6, and IL-12p40 than the C-class CpG-ODN 2395 in foal PBMCs. In foals, B-class CpG-ODNs induced IFN-γ, IL-6 and IL12P40 mRNA expression that was similar or higher in magnitude to that observed in adult horses. These observations indicate that CpG-ODNs might be useful as immunomodulators or as potential adjuvants for vaccines to aid in preventing *R. equi* pneumonia and other bacterial diseases of foals.

[+] Graduate student at Texas A&M University, Veterinary Medicine & Biomedical Sciences PhD Program

SESSION 3

Antibacterial properties of the equine α-defensin DEFA1 against *Rhodococcus equi*

*J. Cauchard[1] *, O. Bruhn[2], C. Gelhaus[3], E. Kalm[1], C. Laugier[1], J. Grötzinger[4] [1] Laboratoire d´Etudes et de Recherches en Pathologie Equine – French Food Safety Agency, France[2]; Institute of Animal Breeding and Husbandry – University of Kiel, Germany; [3] Institute of Zoophysiology – University of Kiel, Germany; [4] Biochemical Institute – University of Kiel, Germany. Underlined : contributed equally to this work.*

Defensins are a predominant class of antimicrobial peptides, which act as endogenous antibiotics. Defensins are classified into three distinct sub-families, θ-, β-, and α-defensins. An α-defensin synthesis is confirmed only in primates and glires to date and is presumably unique for a few tissues including neutrophils and Paneth cells of the small intestine. Antimicrobial activities of these peptides were shown against a wide variety of microbes including bacteria, fungi, viruses, and eukaryotic pathogens. In horses, transcription analysis revealed that the transcript of the DEFA1 peptide is present in the small intestine only and membrane permeabilization is at least an essential part of the peptide´s mode of action. Here, we report the antimicrobial effect of the equine α-defensin DEFA1 on two strains of *Rhodococcus equi* (85F and ATCC 33701). To examine the antibacterial properties, assays were performed to determine the LD90 and MBC values. DEFA1 had been tested only on human pathogens and had shown an antimicrobial activity against different gram positive and gram negative bacteria and *Candida albicans*. DEFA1 was tested in this work for the first time on a typical equine pathogen, *Rhodococcus equi*, and demonstrated its highest efficiency on it.

SESSION 3

Susceptibility of *Rhodococcus equi* to equine antimicrobial peptides

Marianela Lopez[1], Volker Gerdts[1], Sam Attah-Poku[1], George Mutwiri[1], Hugh Townsend[1,2,3]. [1]Vaccine and Infectious Disease Organization, University of Saskatchewan. [2]Department of Veterinary Microbiology, and [3]Department of Large Animal Clinical Sciences, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK, Canada.*

Differences in the innate immunity may represent important immunologic mechanisms associated with increased susceptibility of individual foals to infection with *R. equi*. To gain understanding of these mechanisms, in this study we synthesized equine β-defensin and characterized *in vitro* its effect on *R. equi.* We determined the microbicidal effect of synthetic β-defensin in growth inhibition assays, and demonstrated that β-defensin inhibits *R. equi* growth in a dose dependent manner. Expression levels of toll-like receptor 9 (TLR9), and antimicrobial peptides (β-defensin, CATH-1, -2, and -3) in bronchoalveolar lavages (BAL) cells from foals and adult horses were determined by qRT-PCR. Variation between individuals was detected; however expression of these genes was lower in foal BAL samples than in adult horse BAL samples. We analyzed the antimicrobial effect of BAL fluids in foals and adult horses. BAL fluid from adult horses had a bactericidal effect, which was not present in BAL fluid from foals. We are currently trying to identify the BAL components which are responsible for this effect.

SESSION 3

Bronchoalveolar lavage component comparison between foals and adult horses

Marianela Lopez[1], Katharina Lohmann[2], Fernando Marques[2], Sam Attah-Poku[1], George Mutwiri[1], Volker Gerdts[1], Hugh Townsend[1,2,3]. [1]Vaccine and Infectious Disease Organization, University of Saskatchewan. [2]Department of Large Animal Clinical Sciences, and [3]Department of Veterinary Microbiology, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK, Canada.*

Our understanding of the mechanisms that differentiate neonatal immune responses from those of adult horses is limited by the scarce information on equine pulmonary components participating in defense against pathogens. In this study we analyzed bronchoalveolar lavage (BAL) samples biweekly in 11 foals over the first two months of age (five time points in total) and compared them with those from 10 adult horses (sampled once). The BAL fluids were analyzed using HPLC. The chromatograms of the horse samples had an identical pattern; likewise, the chromatograms of the foal samples were similar between foals and did not change over time. Interestingly, adult horse BAL chromatograms showed a peak which was absent or negligible in the foal samples. We are analyzing this peak by HPLC, SDS-PAGE, and mass spectrometry. We characterized BAL cells by qRT-PCR for expression of TLR-9, β-defensin, cathelicidins, and cytokines (IFN-γ, IL-4, IL-5, IL-12, IL-17). Whereas gene expressions did not change significantly in the foal samples over time, most of them were lower than those in adult horses. Given the role of these molecules in microbe killing and activation of immune responses, these results suggest that their low levels might contribute to the foal susceptibility to pulmonary infections.

SESSION 3

Analysis and genomic structure of the equine CD1 gene family.

R.G. Dossa[1], D.C. Alperin[1], S.A. Hines[1].[1]Department of Veterinary Microbiology and Pathology, Washington State University, College of Veterinary Medicine, Pullman, WA, USA.*

The CD1 family is a group of MHC-class I like molecules that are able to present lipid-based antigens to T cells, including a variety of mycobacterial lipid antigens. Recent work in our lab has shown the potential importance of MHC Class I-unrestricted cytotoxic T lymphocytes in the immune control of *Rhodococcus equi*. Understanding the roles played by the equine CD1 family may be crucial in the prevention of *R. equi* induced pneumonia. In this study, 13 distinct CD1 clones were identified in the equine genome; seven genes were classified as homologues of human CD1a, two were homologues of human CD1b, one was a homologue of human CD1c, one was a homologue of human CD1d, and two were homologues of human CD1e. Five pseudogenes were also identified. As in other species, the CD1 genes are located in clusters between KIRREL and olfactory receptor (OR). A phylogenetic analysis with the equine CD1 genes shows that these genes are segregated by isoform and not by species. Major amino acids differences appear in the alpha-1 and alpha-2 region, which is the antigen binding site in previously described CD1 proteins. As in other species, the alpha-3 region is highly conserved. These data provide evidence that *Equus caballus* is well equipped to present lipid-antigen to T cells. The unusually large number of CD1a (the largest found until now), leads us suggest that a high number of different lipid-antigens from the late phagosome can be presented to T-cells.

SESSION 3

Antibody mediated regulation of immunity to *Rhodococcus equi*

C Montoya, D Alperin, and SA Hines. Dept. of Veterinary Microbiology and Pathology, College of Veterinary Medicine, Washington State University, Pullman, WA.*

The ability to survive and replicate in macrophages is considered the key event in the pathogenesis of *Rhodococcus equi* infection. As an intracellular pathogen cell-mediated immunity, notably a Type 1 immune response characterized by the production of IFN-gamma and cytotoxic T lymphocytes, is considered the primary mechanism of immune clearance. However, antibody likely also plays a role – possibly by altering the route of cell entry and thereby enhancing phagosome maturation. In this study we hypothesized that antibody mediated uptake of *R. equi* enhances intracellular killing and decreases the cytotoxic effects produced by virulent strains (i.e. decreases necrotic cell death). We further hypothesized that antibody also has effects on ensuing T lymphocytes responses – specifically that the uptake of *R. equi* via Fc receptors enhances the production of Type 1 cytokine involved in immune clearance. Thus far, we have shown that monocyte derived macrophages (MΦ) infected with *R. equi* previously incubated with immune horse serum (IHS) contain significantly fewer viable bacteria when compared to MΦ infected with *R. equi* incubated with non-immune horse serum (NHS). MΦ infected with IHS opsonized *R. equi* also had significantly increased viability. Using real time RT-PCR, we are now looking at the ability of *R. equi* opsonization to alter production of a variety of Type 1 cytokines, including TNF-α, IL-12, IL-18, and IL-23, as well as the Type 2 cytokine IL-10.

SESSION 3

Development of live-attenuated metabolic drift mutant strains of a virulent *Rhodococcus equi* strain

Linde, Klaus; Sandra Schwarz, Anke Grosse –Herrenthey and Monika Krueger. Institute of Bacteriology and Mycology, Faculty of Veterinary Medicine, University of Leipzig*

Based on our experience in developing live-attenuated vaccines we developed metabolic drift (MD) mutant strains of a virulent *R. equi* –isolate bearing the 85- 90 kD plasmid .
MD mutants are clones of microorganisms bearing drift mutations in different compartments of metabolism leading to reduced growth (slightly smaller or even dwarf colonies, virulence and increased generation times. Such MD mutants we find with a low frequency between mutants resistant to the antibiotics streptomycin, fusidin acid (Fus), chloramphenicol (Cam) and to the dyestuff crystal violet (Kv). The wild *R. equi* strain (soil isolate) and the two *R. equi* mutant strains were investigated for generation time, stability, virulence plasmid and fatty acid pattern. The generation time of the wild *R. equi* strain was about 86 min., the mutant strain *R. equi* Fus 14/Cam 1 about 103 min. and the mutant strain *R. equi* Kv1/Brg1 about 119 min. The mutant strains were unchanged in their generation time and colony size after 20 passages in a liquid medium. The fatty acid pattern of the wild strain and of the mutant strains was equal. All the strains bear the 85-90 kD virulence plasmid. Virulence experiments will be carried out in 7 d old chicken embryos.

SESSION 3

Immune response anti-*Rhodococcus equi* after immunization of neonate foals with live attenuated *Salmonella* vaccine strain expressing the VapA protein

A. C. R. C. Porto[1], S. G. Soares[2], A. F. Oliveira[2], P. E. Vendruscolo[2], L. E. Ferraz[2], W. R. Fernandes[1], M. C. Roque-Barreira[2]. [1]Dept. Clínica Médica, Faculdade de Medicina Veterinária e Zootecnia - USP, Brazil. [2]Dept. Biologia Celular e Molecular e Bioagentes Patogênicos, Faculdade de Medicina de Ribeirão Preto – USP, Brazil.*

No effective vaccine against *R. equi* disease in foals is available. Recently, we expressed the VapA antigen from *R. equi* in a *Salmonella enterica* Typhimurium strain, which was able to colonize and persist in the lymphoid tissue of BALB/c mice. Attenuated *Salmonella* strain carrying *vapA* antigen innoculated by oral or nasal pathway induced protection in mice, associated with the detection of high levels of anti-VapA IgG2a and high production of IL-12 and IFN-γ. Here, we evaluated the immune response induced by vaccination in neonate foals. Six animals were inoculated with attenuated Salmonella carrying *vapA* antigen and three with attenuated *Salmonella* carrying plasmid only. The inoculation was performed by nasal route at 12 hours after birth and 14 days later. *R. equi* challenge was intranasal preceded with inoculum of 2 x 10^7 CFU of *R. equi* ATCC 33701. Cytokines, antibodies, lymphoproliferative responses and clinical aspects were evaluated. Higher levels of IgGa and IgGb specific antibodies than IgGT were detected in immunized foals. High levels of Th1 cytokines and lymphoproliferative response were observed in 50% of animals immunized. These results indicate that the immune response to *R. equi* in foals can be modulated by the inoculation of attenuated *Salmonella* Typhimurium carrying *vapA* antigen.

This work was supported by FAPESP and CNPq.

SESSION 3

Mice inoculation by nasal route of a live attenuated *Salmonella* vaccine strain expressing the VapA protein induces a long-term protection against *R. equi* infection

S. A. Cardoso, S. G. Soares, A. F. Oliveira, L. L. Oliveira, M. C. Roque-Barreira. Dept. Biologia Celular e Molecular e Bioagentes Patogênicos, Faculdade de Medicina de Ribeirão Preto – USP, Brazil.*

Rhodococcus equi is an important pathogen of young foals aged up to 3-5 months old, which has emerged as causing an opportunistic infection in immunocompromised humans. Recently, our group has developed a novel oral vaccine against *Rhodococcus equi* based in the expression of VapA antigen by *S. enterica* Typhimurium strain using an *asd*-balanced-lethal vector-host system. Here, we evaluated the effect of nasal vaccination with the *Salmonella enterica* expressing the VapA protein on the modulation of immune response toward *R. equi* challenge, performed at 35 or 133 days after immunization. When challenged 35 days after vaccination, the mice produced higher IL-12p40 and IL-4 levels and lower IFN-γ, TNF-α and IL-10 levels than the control animals. However, only TNF-α down-regulation was still observed when challenge was carried out 133 days after vaccination. Sections of spleen and liver showed fewer focal infiltrates of leukocytes in vaccinated mice than in control mice. Our results show that vaccination with an intranasal single-dose of *S. enterica* Typhimurium expressing the VapA promotes an efficiently modulated Th1 response, able to confer long-term protection against infection and to avoid exacerbated inflammatory response.

This work was supported by FAPESP and CNPq

SESSION 3

Development of a live attenuated VapA-producing *Rhodococcus equi* oral vaccine to protect neonatal foals against pneumonia.

A. E. Whitehead[1], V. R. Parreira[2], J. Hewson[1], J. F. Prescott[2], [1]Department of Clinical Studies, [2] Department of Pathobiology, University of Guelph, Guelph, Ontario, Canada*.

The objective of this study is to develop a safe and efficacious attenuated strain of *Rhodococcus equi* for use in oral immunization of foals. The approach involves expression of VapA in a live, virulence-plasmid negative strain of *R. equi* (strain 103-), with assessment of virulence in mice and macrophages. This will be followed by assessment of immunogenicity in mice and, if appropriate, in foals. PCR-amplified fragments of entire *vapA* gene with and without the upstream virulence-plasmid genes *virR*, *orf5*, *vapH*, *orf7* and *orf8* (orf4-8) was cloned into a shuttle vector pNBV1. These plasmids, named pAWVapA and pAW48A, were electroporated into strain 103-. The presence of the recombinant vectors in the attenuated strain (103-) and the integrity of the inserted genes was confirmed by antibiotic selection, PCR and DNA sequencing. SDS-PAGE and Western blotting showed that both constructs expressed VapA, but 103-/pAW48A, the plasmid with the two regulatory genes (*virR*, ORF8), had slightly stronger expression of VapA. Expression of VapA was approximately one-third to one-half that of wild type *R. equi* 103+ as assessed by Western blot. The virulence for mice of the two recombinant *R. equi* was compared to that of wild type *R. equi* 103+ by intravenous inoculation of mice with the organisms and examination of liver clearance 4 days later. Mice inoculated with *R. equi* 103-, 103-/pAWVapA and 103-/pNBV1 completely cleared infection. Strain 103-/pAW48A persisted in mice but at levels approximately 1 % of wild-type 103+. The low level persistence of this construct suggests that it might have value in immunization. Further work will be done to assess the persistence of this strain in macrophages.

SESSION 4

Introduction to Clinical Session: *R. equi* - The priorities of the industry that we serve

Des Leadon, Irish Equine Centre, Johnstown, Naas, Co.Kildare, Ireland.

There are an estimated 58,372,106 horses in the world. The USA has a horse population of 9,500,000 (which includes Texas 978,822; California 698,345; Florida 500,124; Oklahoma 326,134; Kentucky 320,173; Ohio 306,898; Missouri 281,255). The states with the fewest horses are Rhode Island (3,509) and the District of Columbia, which reports a fluctuating total of around 33. Each of the primary use segments of the industry, (recreational horse use being the largest segment with 3.9 million horses in this classification) creates an immense contribution to the overall economy, having a direct impact of $39 billion and an overall impact of $102 billion, when indirect and induced spending are included. The US horse industry supports 1.4 million equivalent full-time jobs. Countries with horse population totals in excess of one million include: China (7,402,450); Mexico (6,260,000); Brazil (5,787,249); Argentina (3,655,000); Columbia (2,533,621); Mongolia (2,029,100); Ethiopia (1,655,383); Russian Federation (1,319,358); and Kazakhstan (1,163,500). Guam (20) and Grenada (30) had the lowest population totals. Two countries, Rwanda and Saint Helena, report a zero horse population. The EU has an estimated horse population of 3.7 million (including - Romania 834,000; Germany 500,000; France 422,872; Poland 306,992; Italy 300,000; Spain 245,000; UK 185,000; Netherlands 128,500; Bulgaria 125,000; Sweden 95,700; Austria 85,000 and Ireland 79,900). The horse industry is one of Australia's biggest industries and is worth more than Aus$8 billion a year with about 1.2 million horses used for racing, equestrian sports and recreation

Although these are impressive totals, only a small fraction of total horse populations are of sufficient economic value to justify investment in diagnosis, treatment and attempted control of *R. equi* disease. (It is estimated that 60% of the world's horses are working equidae, based in developing countries.) The thoroughbred sector contains the highest, individual, foal value segment of the horse industry. The highest producers of thoroughbred foals are the USA - 36,317; Australia 17,640 and Ireland 12,633. Ireland produced more thoroughbred foals in 2007 than the combined total of the UK (5,843) and France (5,269) and more than 40% of all EU thoroughbred foal are Irish. Italy (2,130) and Germany (1,224) are the other principal EU producers. More than 40% of all thoroughbred foals born in the USA are sired by Kentucky-based stallions – in 2006 - 14,801 live foals, which when placed in the above Kentucky total horse population context of 320,173 represents just 4.6% of the total. Other leading states ranked by number of state-sired live foals in 2007 include Florida, 4,063; California, 3,131; Louisiana, 2,016, and New York 1,316. The Japan thoroughbred foal crop was 7,655.

In Australia generally 1-10% of foals are affected by *R, equi* disease and although mortalities are usually maintained below 1% by early, aggressive therapy, on some studs mortalities of 20% or higher occur in some years. The estimated annual cost of this disease to the Australian industry may be somewhere between Aus$2-4 million. This figure when multiplied in estimates of the potential global horse industry costs of *R. equi* disease becomes many millions in any currency. However, every stud farm is different and has its own economic priorities. The costs to the end-user of improved laboratory diagnosis of *R. equi* disease will be evaluated by both the farms and the attending clinicians against the costs and immediate on-farm, "live" information provided by thoracic ultrasonography. Although ultrasonic evidence of pulmonary abscessation is not pathognomonic for *R equi* disease, the steeply increasing cost:benefit ratio of pursuit of "gold standard" diagnosis is likely to remain unattractive to many, especially in times of recession.. The industry has been clamoring for an effective *R equi* vaccine from the first recognition of endemic *R equi* disease. Advances in genomic technology and vaccine efficacy will be balanced by potential manufacturers against the high costs of production and product registration, the market size constraints referred to above and the present economic and horse industry climate. Even the most efficacious vaccines are not and never will be a substitute for management hygiene / control strategies.

SESSION 4

R. equi disease in Ireland's temperate climate – Clinical studies 2002 to 2007

Mariann Klay[1], Des Leadon[2], Ursula Fogarty[2] and Tom Buckley[2]. Swordlestown Little[1] and [2]The Irish Equine Centre, Johnstown, Naas, Co Kildare, Ireland.*

We examined 584 foals on 10 thoroughbred studfarms in Ireland's temperate climate, from 2002 to 2007. Pulmonary abscessation was evident on thoracic ultrasonography in 159 (27.2%) of these foals. The incidence of pulmonary abscessation on endemic farms ranged from 37 of 78 foals (47.4%), to 60 of 129 (46.5%), and 10 of 33 (30.3%). Sporadic / rare case farms incidence ranged from 24 of 205 (0.5%), 1 of 20 (5%), 5 of 33 (15%), 2 of 9 (22%), 15 of 58 (25.9%), 8 of 12 (66.6%) and 5 of 7 (71%) The relatively high incidence figures in the sporadic / rare category reflect astute case selection for investigation by management and the attending clinicians. On one farm, serial examinations over the entire five year period saw a decrease in the incidence of pulmonary abscessation from 6 of 14 foals (42.8%) in 2002, to 3 of 12 (25%) in 2003, 7 of 10 (70%) in 2004, 0 of 7 in 2005, 0 of 6 in 2006 and 0 of 9 in 2007 perhaps resulting from increased awareness and improved management on this farm. The mean age on the day of diagnosis in these 159 foals was 85 days (min 24 days, max 252). Initial presentations varied from mild respiratory disease to peracute onset, dramatic respiratory disease to chronic ill-thrift. WBC counts ($x10^9$/l) ranged from 6.6 to 47, mean 15.9 on endemic farms and from 3.5 to 19.7, mean 12.9 on non-endemic farms. Plasma fibrinogen concentration (g/l – modified Clauss method) ranged from 2.03 to 6.54, mean 3.5 on endemic farms and from 1.79 to 4.88, mean 2.59 on non-endemic farms. There were 8 *R. equi* related fatalities in total, 7 on the endemic farms directly associated with *R. equi* disease and 1 fatality on a non-endemic farm possibly due to an adverse drug reaction (enteritis) to the *R. equi* treatment regime. The arguably sub-clinical status of early *R. equi* cases is reflected in the difficulty of obtaining *R. equi* isolates (8 of 58 = 13.8%) from the tracheal secretions. PCR became available in 2003/2004 and 16 of 38 tracheal secretions were *R. equi* PCR-positive, while 22 were PCR-negative. Treatment records were available from 88 foals. They received antibiotic treatment (Azithromycin and Rifampicin or Tulathromycin and Rifampicin or Clarithromycin and Rifampicin) for a mean duration of 27.5 days (min 4, max 90).

SESSION 4

Diagnostic of pulmonary abscesses in foals: Comparison of sonographic and radiographic examination

M. Venner[1], S. M. Walther[1], P. Stadler[1], B. Muenzer[2]. [1]Equine Clinic University of Veterinary Medicine Hannover, Foundation, Bischofsholer Damm 15, D-30173 Hannover, Germany, [1]Equine Clinic, Depart for Surgery and Radiology, Oertzenweg 19b, D-14163 Berlin, Germany.*

The aim of this study was to assess the diagnostic value of ultrasonographic and radiographic examination in abscess-forming pneumonia in foals. The main focus was to evaluate whether one or both of these techniques can be used to detect pulmonary abscesses under field conditions in an early stages of the disease. Clinical and hematological examination as well as thoracic ultrasonography and radiography was performed on 61 foals on a stud with endemic *Rhodococcus equi* pneumonia. 19 foals were clinically healthy and showed no haematological and no ultrasonographic evidence of pneumonia. In 42 foals, clinical signs of a respiratory disease were present or hematologic findings indicated an infection and at least one pulmonary abscess were observed by ultrasonography. Radiographic evaluation revealed an abscessing pneumonia in 20 of the 42 sick foals. Furthermore a discrepancy was noted in the interpretation of pulmonary radiographs by three experienced equine internists. Finally a good concordance between radiographic and ultrasonographic findings of abscesses was observed only in the central region of the lung. During the study it was noticed that thoracic radiography under field conditions is more time-consuming and more expensive than ultrasonography. Ultrasonographic examination appears to be more sensitive for the diagnosis of pulmonary abscesses in foals.

SESSION 4

Effectiveness of Azithromycin in preventing pulmonary abscesses in foals of a *Rhodococcus equi* endemic breeding farm

M. Venner, B. Reinhold, K. Feige. Equine Clinic, University of Veterinary Medicine Hannover, Foundation, Bischofsholer Damm 15, D-30173 Hannover, Germany.*

The prophylactic application of azithromycin in order to prevent pulmonary abscesses in foals was evaluated on a stud with endemic *Rhodococcus equi* pneumonia. Forty five foals served as untreated controls in two groups. Twenty five foals were given azithromycin (10 mg/kg bwt) orally once daily for four weeks. The foals were examined once a week from birth to the age of five months. If clinical signs of respiratory disorders or leukocytosis were noted in association with pulmonary lesions detected by ultrasonography (diameter larger than 10 millimetres), a diagnosis of abscessing pneumonia was made. The number of affected foals and of pulmonary abscesses in the patients were similar in the control groups (31 out of 45 foals), and in the azithromycin group (15 out of 25 foals), but the foals in the azithromycin group were affected significantly later (median: day 83; min.-max: 67-123) (control groups: day 54 [52-82] and 46 [28-86] of age). It was concluded that the application of azithromycin (10 mg/kg BW orally) for the first 28 days following birth does not reduce the prevalence of pulmonary abscesses in foals on a stud with endemic *R. equi* pneumonia.

SESSION 4

Chemoprophylactic effects of azithromycin against *Rhodococcus equi* pneumonia among foals at endemic equine breeding farms

M. K. Chaffin, N. D. Cohen, R. J. Martens. Equine Infectious Disease Laboratory, Department of Large Animal Clinical Sciences, College of Veterinary Medicine & Biomedical Sciences, Texas A&M University, College Station, TX 77843.*

Rhodococcus equi causes severe pneumonia in foals worldwide. Most affected foals likely become infected very early in life. A novel strategy for prevention of *R. equi* pneumonia is the administration of effective antimicrobials to foals during the first few days of life. The objective of this study was to determine the effect of azithromycin (AZ) chemoprophylaxis on the incidence of *R. equi* pneumonia among foals at *R. equi* endemic equine breeding farms. A controlled, randomized, clinical trial was performed at 10 equine breeding farms with history of endemic *R. equi* infections. Group 1 foals were untreated controls and group 2 foals were treated with AZ (10 mg/kg PO q 48 hrs) during the first 2 weeks of life. Enrolled foals were monitored for development of *R. equi* pneumonia. Fecal and tracheobronchial isolates of *R. equi* were tested for susceptibility to AZ. Data were compared between treatment groups. There were 338 foals studied. The proportion of *R. equi* - affected foals was significantly higher for control foals (21%) than for AZ-treated foals (5%). The estimated protective efficacy of AZ chemoprophylaxis was > 85%. Neither adverse effects of AZ therapy nor AZ-resistant isolates were observed. The results of this study demonstrate that AZ chemoprophylaxis is an effective strategy for reducing the prevalence of *R. equi* pneumonia among foals at endemic breeding farms. Adverse effects of AZ chemoprophylaxis were not detected and resistance to AZ was not detected in isolates of *R. equi.* Nonetheless, resistance could develop if indiscrimnant wide-spread use of azithromycin in foals as a preventive therapy. The investigators failed to identify resistance in this limited study but that absolutely should not be interpreted to mean that resistance would not develop if this practice was widely adopted.

SESSION 4

Safety of enteral gallium maltolate in neonatal foals

R.J. Martens[1], N.D. Cohen[1], M.K. Chaffin[1], T.L. Blanchard[1], V.R. Fajt[2], R.J. Taylor[3], L.R. Bernstein[4].*
[1]Dept.of Large Animal Clinical Sciences, [2]Dept. of Physiology and Pharmacology and [3] Department of Veterinary Integrative Biosciences, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, TX.[4]Terrametrix, 285 Willow Rd. Menlo Park, CA.

Rhodococcus equi requires adequate iron for survival. Gallium interferes with iron availability and causes the death of *R. equi* . Most foals with spontaneous disease become infected as neonates. Enteral gallium maltolate (GaM) attains therapeutic concentrations in neonatal foals and has been proposed for disease prevention. These experimental and observational studies were conducted to assess whether it was safe to administer GaM enterally to neonatal foals.
Experimental study: Nine, 1- to 2-day old Quarter Horse foals (6 principal; 3 controls) received GaM (20 mg/Kg) or dH_2O, respectively, via nasogastric tube, daily for 5 days. Foals were monitored daily for 7 days via physical examination (clinical score), weight gain, CBC, serum biochemical profile, serum iron-binding capacity, total serum iron concentration and serum gallium concentrations. There were no significant differences in any of the physical or clinical pathologic variables measured between principal foals and controls. Observational study**:** Ten Thoroughbred foals were administered an oral GaM-methylcellulose paste, at approximately 20 mg GaM/Kg, daily for the first 28 days of life. Foals were monitored daily by a resident veterinarian for wellness, and CBC and serum biochemical profiles and gallium concentrations were monitored weekly. Foals did not manifest any health issues attributable to GaM, and clinical pathologic parameters were within normal ranges. Oral or intragastric administration of GaM (20 mg/Kg) to foals during the first month of life does not appear to cause adverse health effects.

SESSION 4

Pharmacokinetics of a methylcellulose oral formulation of gallium maltolate in neonatal foals

M.K. Chaffin,[1] V. Fajt,[2] N.D. Cohen,[1] R.J. Martens,[1] L.R. Bernstein.[3] [1]Dept.of Large Animal Clinical Sciences, and [2]Dept. of Physiology and Pharmacology, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, TX, and [3]Terrametrix, 285 Willow Rd. Menlo Park, CA.

Gallium has been proposed as a novel agent for prophylaxis of *Rhodococcus equi* infections in foals. Gallium has antimicrobial activity against R. equi due to its ability to interfere with bacterial iron metabolism. Gallium reduces *R. equi* tissue concentrations in mice experimentally-infected with R. equi. Gallium is well absorbed following intragastric administration of 20 mg/kg of gallium maltolate (GaM) to 1 day-old foals. The purpose of this study was to evaluate the dose-related pharmacokinetic properties, in neonatal foals, of orally-administered GaM in a methycellulose gel formulation (GaM-MCGF). Twelve normal, neonatal (4 to 8 days old) Quarter Horse foals were studied. Six foals received a single oral dose of 20 mg/kg of GaM-MCGF and 6 foals received a single oral dose of 40 mg/kg of GaM-MCGF. Blood samples were collected at 0, 15, 30 minutes, and at 1, 2, 3, 4, 5, 6, 8, 12, 24, 48 and 72 hours following drug administration. Serum was harvested and gallium concentrations were determined. Gallium was well absorbed in a dose-dependent manner following oral administration of GaM-MCGF. Marked variability existed among foals in the maximum serum concentrations (Cmax) of gallium. No adverse effects were recognized. Gallium is absorbed in a dose-dependent manner following oral administration of GaM-MCGF to neonatal foals. Due to the marked variability of Cmax, dosages higher than 20 mg/kg may be needed to consistently achieve adequate serum concentrations to have antimicrobial effects against *R. equi.*

SESSION 4

Rhodococcus equi in adult animals

Ursula Fogarty [1], Patrick Mc Grath [2], Tom Buckley [1]. [1]The Irish Equine Centre, Johnstown, Naas, Co. Kildare, Ireland. [2] Somerton Equine Hospital, Friarstown, Co. Kildare, Ireland.*

Rhodococcus equi was detected in adult Thoroughbred race horses in training. Detection was based on cytopathological examination of gram stained smears, Polymerase chain reaction and isolation in culture. A number of horses on a number of premises were detected using the above methodology. Details of one premises are described in more detail. Horse ranged in age from two to four years of age, presented with persistent respiratory tract problems, and were associated with excessive mucopurulent secretions on endoscopic examination ranging to pulmonary abscessation in five animals. Not all animals were positive by all methods of detection. Animals with pulmonary abscessation were more likely to be PCR and culture positive. Affected animals responded to antimicrobial therapy with either erythromycin and rifampin or a combination of sulphonamide and rifampin. Bronchoalveolar lavage findings included an increase in the percentage of neutrophils with detection of multinucleated cells in some animals. Mast cell percentages were also elevated. *Rhodococcus equi* was usually identified in the environment of these horses and environmental conditions where the organisms were found were sub-optimal. In less severely affected animals removal of the source of infection appeared to be effective in controlling the exposure.

SESSION 4

Resistance studies of erythromycin and rifampin for *Rhodococcus equi* over a 10-year period

T.C., Buckley, E. McManamon and S. Stanbridge. Irish Equine Centre, Johnstown, Naas, Co. Kildare, Ireland.*

A study carried out at the Irish Equine Centre sought to determine whether an increase in resistance of *Rhodococcus equi* to the antibiotics rifampin and erythromycin occurred over a 10-year period. This was carried out by the use of E test strips for rifampin and erythromycin to determine the MIC (minimum inhibitory concentration) values of *Rhodococcus equi* to this combination of antibiotics. The findings of this study indicated that there was an increase in resistance of *Rhodococcus equi* to rifampin and erythromycin over the 10-year period. The MIC for rifampin increased from 0.081 µg/ml in 1996 to 0.187 µg/ml in 2006 and from 0.258 µg/ml for erythromycin during the years prior to 2000 to 0.583 µg/ml in 2006. This finding suggests that there may be a problem in the treatment of *Rhodococcus equi* infections in foals in the future, particularly as the number of drugs available for treatment of *Rhodococcus equi* infection is limited because of the intracellular capabilities of this bacterium. Antibiotics used in its treatment have to be able to penetrate the polysaccharide cell wall of *Rhodococcus equi* as well as the alveolar macrophages in which the bacterium is capable of surviving.

SESSION 4

Prevalence of *Rhodococcus equi* isolates resistant to macrolides or rifampin and outcome of infected foals

S Giguère[1], E Lee[1], ND Cohen[2], MK Chaffin[2], N Halbert[2], RJ Martens[2]. [1]University of Florida, Gainesville, FL. [2]Texas A&M University, College Station, TX.*

The objectives of this study were to establish the prevalence of *R. equi* isolates resistant to macrolides or rifampin and to determine the outcome of foals infected with resistant isolates. Thirty-four isolates classified as resistant to erythromycin, clarithromycin, azithromycin, or rifampin, were obtained from 6 laboratories between 1997 and 2007. For each isolate, minimum inhibitory concentration of the 4 antimicrobial agents was determined using both the E test and broth macrodilution. Each isolate confirmed to be resistant to at least one antimicrobial agent was also evaluated for presence of the virulence plasmid using PCR amplification of the *vapA* gene. Only 19 of the 34 isolates (55.9%) submitted as resistant were *R. equi* isolates resistant to at least one drug. Two isolates were resistant to rifampin only whereas 17 isolates were resistant to all 3 macrolides and rifampin. Two of the 19 resistant isolates were avirulent. The prevalence of resistant isolates at the University of Florida was not significantly different from that of Texas A&M University. The overall prevalence of *R. equi* isolates resistant to macrolides or rifampin was 3.7%. The survival rate of foals infected with resistant isolates (25%) was significantly lower ($P < 0.004$) than that of foals from which susceptible isolates were cultured (69.6%). In conclusion, approximately half the *R. equi* isolates identified as resistant to a macrolide or rifampin by diagnostic laboratories are either not *R. equi*, not resistant, or avirulent. Foals infected with resistant isolates are less likely to survive than foals infected with susceptible isolates.

SESSION 4

Studies on the persistence of transfused *Rhodococcus equi* antibodies in Thoroughbred foals

L. Palmer[1], C.D. Cooke[1], M.A. Holmes[3] and S.J. Stoneham[2]. [1]Rossdale and Partners, Beaufort Cottage Stables, High Street, Newmarket, CB8 8JS. [2]Lone Oak Stud, East Green, Great Bradley, Newmarket, UK [3]University of Cambridge, Department of Veterinary Medicine, Madingley Road, Cambridge, CB3 0ES*

The objective of this study was to use an enzyme-linked immunosorbent assay (ELISA) based on VapA for *R. equi* serology in foals (Prescott, J.F., personal communication) to determine colostrum derived antibody levels and to monitor the efficacy of *R. equi* plasma transfusion in raising these levels and their subsequent decline.Transfused foal group: A litre of commercially available hyperimmune plasma obtained from donors vaccinated against *R. equi* was administered intravenously to foals at 2 days of age and a second transfusion given at 28 days of age. A sample of the hyperimmune plasma was retained for analysis. Study 1 (2007 foaling season): Nineteen Thoroughbred mares and foals from a stud farm where *R. equi* associated pneumonia is endemic were used in this study (transfused group n=19). Jugular blood samples were taken from foals at 2 days of age (prior to the first transfusion), 3 days (24h post transfusion), 28 days (prior to the second transfusion), 29, 42 and 62 days. Colostrum samples were collected prior to nursing. Results: Serum IgG levels were <4g/l in 3 foals (16% failure of passive transfer). *R. equi* antibody titres in Mare's colostrum ranged from 1:10 – 1:1280. Foal *R. equi* antibody titres at 2 days of age ranged from 1:10 – 1:1280. Foal antibody titres were appeared to be dependent on colostrum titres. There was a wide variation in *R. equi* titres in the donor hyperimmune plasma (7 donors, median range 1:1280 – 1:6400). Median *R. equi* antibody titres at age 2, 3, 28, 29, 42 and 62 days were 1:40, 1:320, 1:120, 1:320, 1:320 and 1:240 respectively. There was a statistically significant ($P<0.01$) increase in *R. equi* antibodies 24 hours post transfusions, followed by a statistically significant decline ($P<0.01$) in *R. equi* antibodies 26 days after the first transfusion and persistence in *R. equi* antibodies until the end of the measurement period (33 days post transfusion 2). Disease associated with *R. equi* was seen in two foals during this study period. Study 2 (2008 foaling season): 21 Thoroughbred mares and foals were used in this study (transfused group n=12 and control group n=9). Jugular blood samples were taken from all foals at 2, 3, 7, 14, 21, 28, 29, 42, and 62 days. Colostrum samples were collected prior to nursing. Sample collection and analysis will be complete by 1st June. All samples will be analysed for routine biochemistry/haematology and *R. equi* antibodies. Conclusion: Results from study 1 support previous studies showing that the incidence of disease is reduced when hyperimmune plasma is administered at 2 days and 28 days. It demonstrates the further benefits of monitoring antibody levels in hyperimmune plasma and foal serum in relation to reducing the incidence of disease. The efficacy of *R. equi* hyperimmune plasma in raising the foal's *R. equi* antibody levels was dependent on the level of *R. equi* antibodies in the hyperimmune plasma.

SESSION 4

Immune-mediated arthritis and uveitis associated to *Rhodococcus equi* infection in foals

Corradini I,[1] Tarancón I,[1] Fonseca C,[1] Leiva M,[2] Peña T[2] y Monreal L[1] [1]Servei de Medicina Interna Equina y [2]Servei d'Oftalmologia, Facultat de Veterinària, Universitat Autònoma de Barcelona, Barcelona, Spain.

The objective of this retrospective study was to assess the incidence of the immune-mediated arthritis and uveitis, as well as other clinical complications, in hospitalized foals, in which a clinical diagnosis of *Rhodococcus equi* pneumonia was achieved. The medical records of 29 foals (1-5 months of age) with a clinical diagnosis of *Rhodococcus equi* bronchopneumonia from the Equine Teaching Hospital of Barcelona were reviewed to assess complications associated with the disease. Nine out of 29 foals (31%) had one or more joints distended. All these cases had clinical findings consistent with a non-septic, immune-mediated arthritis. Regarding ocular findings, 15 out of the 17 foals examined by ophtalmologists (88%) had a diagnosis of exudative anterior uveitis and/or corioretinitis, among other ocular problems. Other detected complications were: renal failure (n=4), diarrhea (n=2), osteomyelitis (n=1), severe pleuritis due to *Salmonella* infection (n=1), and strangles (n=1). Foals were treated with systemic antibiotics orally, like rifampin plus a macrolide (erythromycin, azithromycin or clarithromycin), and some of them with additional ocular therapy. Most foals (69%) showed a good progression, and the evolution of the uveitis and arthritis was always associated to the general progression of the systemic disease. Finally, 2 out of 9 euthanized/death foals had a big abdominal abscess with subsequent peritonitis. In conclusion, ocular and articular findings consistent with immune-mediated uveitis and arthritis may be commonly diagnosed in foals with *R. equi* pneumonia.

SESSION 4

Detection of *Rhodococcus equi* by microbiological culture and by polymerase chain reaction in samples of tracheobronchial secretions of foals

M. Venner[1], P. Heyers[1], K. Strutzberg-Minder[2], N. Lorenz[2,] J. Verspohl[3],. [1]Equine Clinic, University of Veterinary Medicine Hannover, Foundation, Bischofsholer Damm 15, D-30173 Hannover, Germany. [2]Gesellschaft für Innovative Veterinärdiagnostik mbH, Ahlem, Germany. [3]Departement for Microbiology, University of Veterinary Medicine Hannover, Foundation*

The goal of the present study was to investigate whether new PCR-methods would improve the diagnosis of pneumonia caused by *R. equi* in foals. The tracheobronchial secretions of 48 foals of a *R. equi* endemic farm with pulmonary abscesses and of 37 healthy foals were evaluated by bacteriological culture as well as by four PCR-methods: *aceA*-, *ideR*-, *vapA*- and VP-PCR. In respect to the "gold standard" microbiological culture, the sensitivity of most PCR methods ranged between 63.9 and 69.4% except the *vapA*-PCR (27.8%). The specificity of all four PCR methods in this comparison was between 98 to 100%. In this analysis, clinical diagnosis had a low sensitivity (66.7%) and a low specificity (51.0%). In respect to the clinical diagnosis, sensitivity of microbiologic culture was 50.0% and specificity 67.7%. In this analysis, sensitivity rates of *aceA*-, *ideR* and VP-PCR methods lay between 33.3 and 37.5% and sensitivity of the *vapA*-PCR was lower (10.4%). The specificity of all PCR methods was between 78.4 and 86.5%. In conclusion, these results show that the diagnostic potential of both microbiologic methods is different and that for the diagnosis of *R. equi*–pneumonia in foals the combination of microbiologic culture with PCR should be used for examination of samples of the airways of foals.

SESSION 4

Evaluation of tulathromycin in the treatment of pulmonary abscesses in foals.

*M. Venner *, R. Kerth. Equine, University of Veterinary Medicine Hanover, Foundation, Bischofsholer Damm 15, D-30173 Hannover, Germany*

Tulathromycin is a new injectable macrolide antibiotic for the treatment of pulmonary diseases of swine and cattle. This study was conducted on a breeding farm with endemic *Rhodococcus equi* pneumonia to an incidence up to 80% each year for several years. 37 foals with ultrasonographic evidence of lung abscesses were treated with tulathromycin (2.5 mg/kg BW i.m. once weekly, group 1) and 33 foals (group 2) with a combination of azithromycin (10 mg/kg BW oral once daily for the first seven days of therapy, thereafter every other day) and rifampin (10 mg/kg BW oral twice daily). The bacterial aetiological agent was not determined. The foals were only mildly sick and the median number of pulmonary abscesses was 1.4 (group 1) and 1.6 (group 2). 30 foals in each group were successfully treated without modifying therapy protocols until all clinical signs of disease had subsided. Tulathromycin was administered for a mean 53 days, azithromycin/rifampin for 42 days. The following side effects were associated with tulathromycin (279 intramuscular injections): self-limiting diarrhoea in eleven foals, transient elevated temperature in six foals, and moderate swellings at the injection site in twelve foals. This study provides some evidence that tulathromycin is well tolerated and appears promising for the treatment of pulmonary abscesses in foals.

SESSION 4

A double blind study comparing the effect of hyperimmune plasma and standard equine plasma on reducing the incidence of *Rhodococcus equi* infection and requirement for treatment on an endemic stud farm

T.R.M.Y. Dawson[1&2], A.C. Cunningham[2], N. Carter[2]. [1]Veterinary Immunogenics Ltd, Cumbria, [2]University of Sunderland, Tyne and Wear, UK.*

Veterinary Immunogenics Ltd produces an equine plasma product containing elevated levels of anti-*Rhodococcus equi* antibodies (Hypermune-RE). The effect of Hypermune-RE on reducing the incidence of *R. equi* infection and requirement for antibiotic treatment was investigated on one endemic stud farm (n=90 foals). A comparison was made to standard equine plasma treated and non-treated foals. Pulmonary abscesses and other clinical criteria were monitored for a period of 150 days from birth. Specific serum antibody levels were monitored by *R. equi* (IgG) and VapA (IgG, IgGa and IgGb) ELISA. Hypermune-RE significantly reduced the number of pulmonary abscesses ($p<0.05$) and requirement for antibiotic treatment ($p<0.01$) compared to untreated foals. A significant increase ($p<0.0001$) in anti-*R. equi* antibodies was measured post transfusion of Hypermune-RE, and to a lesser extent ($p<0.05$) post transfusion of standard equine plasma. Standard equine plasma provided some benefit in reducing clinical abscesses and requirement for treatment compared to untreated foals, but not as well as Hypermune-RE. Furthermore, a significant correlation ($p<0.05$) between high anti-VapA IgGa (Th1) antibodies in Hypermune-RE and protection in recipient foals was found. To continue assessing the humoral immune profile of *R. equi* infection, it may be beneficial to identify an International Standard for *R. equi* and VapA antibody.

SESSION 5

Epidemiology of *Rhodococcus equi* disease: facts and thoughts

Gary Muscatello[1], James R Gilkerson[2] and Glenn F Browning[2] . [1] Faculty of Veterinary Science, The University of Sydney, NSW 2006, Australia. [2] Equine Infectious Disease Laboratory, Faculty of Veterinary Science. The University of Melbourne, Victoria 3010, Australia*

Rhodococcus equi causes disease in a variety of animal species, predominantly horses and pigs. *R. equi* pneumonia is also seen in humans, and the intermediately virulent *R. equi* strains found in pigs may have important public health consequences. The economic significance of *R. equi* pneumonia in the equine breeding industry worldwide has driven investigations into the underlying ecology and epidemiology of this important disease. Virulent *R. equi* survives well in soil, particularly in soil contaminated with horse faeces, but there is no relationship between the abundance of the organism in soil and the prevalence of *R. equi* pneumonia on horse breeding farms. Aerosolisation of the virulent organism from its environmental niche is an essential component of the pathogenesis of *R. equi* pneumonia in foals, and clear associations have been demonstrated between airborne concentrations of virulent *R. equi* and disease prevalence. The potential for airborne transmission between foals has been suggested recently, and similar concentrations of virulent *R. equi* have been detected in samples of expired air from symptomatic and asymptomatic foals. The possibility of a contagious route of transmission may explain why dust-reduction strategies do not always reduce the prevalence of disease, especially on farms with large foal populations. The interactions between the host, the pathogen and the environment that underpin the epidemiology of *R. equi* disease are highly complex. There are gaps in our knowledge in these areas that need to be filled before we can fully understand the epidemiology of *R. equi* disease.

SESSION 5

Foal and farm level factors associated with *Rhodococcus equi* foal pneumonia

Noah D. Cohen, M. Keith Chaffin, and Ronald J. Martens. Department of Large Animal Clinical Sciences, College of Veterinary Medicine & Biomedical Sciences, Texas A&M University, College Station, TX, 77843-4475, USA*

The purpose of this presentation will be to review risk factors for *Rhodoccus equi* pneumonia at the levels of individual foal and farm. At the foal level, gene polymorphisms and correlates of immunity have been associated with increased risk of disease; however, other management practices or month of birth have not been statistically associated with risk of disease. In the United States, the disease appears to be most prevalent in farms that use desirable management practices. Larger farms and farms with higher density of mares and foals appear to be more commonly affected. The airborne, but not soil, concentrations of virulent *R. equi* have been correlated with disease incidence at farms. To date, management practices that differentiate farms with endemic *R. equi* from unaffected farms have not been definitively identified. The relationship between host, environment, and agent appears to be complex for *R. equi* foal pneumonia. Further work is needed to elucidate causal factors for this disease.

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Epidemiology and evolution of *R. equi*: new insights from molecular studies

J.A. Vazquez-Boland [1,2,3], D.A. Lewis [1], A.A. Ocampo-Sosa [1,4], M. Letek [1,2,4], R. Mainar [5], P. Gonzalez [1,4], J. Navas [6], M. Scortti [1,2,3], W. Meijer [7], S. Bentley [8], J. Parkhill [8], D. Leadon [4]. T. Buckley [4], U. Fogarty [4].*
[1]Microbial Pathogenesis Unit, Centre for Infectious Diseases, University of Edinburgh, UK; [2]Veterinary Molecular Microbiology Section, Faculty of Medical and Veterinary Sciences, University of Bristol, UK; [3]Grupo de Patogenómica Bacteriana, Instituto de Biología Molecular y Genómica, Universidad de León, Spain; [4]Irish Equine Centre, Johnstown, Naas, Ireland; [5]CITA-Unidad Sanidad Animal, Gobierno de Aragón, Zaragoza, Spain; [6]Departamento de Biología Molecular, Universidad de Cantabria, Spain; [7]School of Biomolecular and Biomedical Sciences, University College Dublin, Ireland; [8]Pathogen Genomics, Wellcome Trust Sanger Institute, Genome Campus, Hinxton, UK .

The genome sequence of a single isolate is just a snapshot in a bacterium's evolution and it is important to place this information in a population and evolutionary perspective. We therefore carried out a population genetics study in which we genotyped a global collection of >1,000 *R. equi* isolates from a diversity of geographical origins (25 countries) and sources (horse, human, pig, cattle, small ruminants, dog, cat, soil) by pulsed field gel electrophoresis (PFGE). The PFGE data revealed a high level of genetic variability, but traces of a same genomic backbone were evident in all the isolates, indicating that *R. equi* is genetically homogeneous and evolved by clonal diversification.

Associations between certain PFGE genogroups and specific animal hosts were observed. Characterization of the virulence plasmids using a novel PCR-based typing tool combined with mulvariate analysis demonstrated, however, that these associations were spurious and were in fact dictated by the plasmid carried by the isolates. The new plasmid typing scheme, TRAVAP, classifies *R. equi* into four categories: $traA^{+}/vapA^{+}B^{-}$, $traA^{+}/vapA^{-}B^{+}$, $traA^{+}/vapAB^{-}$, and $traA^{-}/vapAB^{-}$ (plasmidless). Plasmid type $traA^{+}/vapA^{+}B^{-}$ was characteristic of horse isolates, $traA^{+}/vapA^{-}B^{+}$ was associated with pig isolates, and $traA^{+}/vapAB^{-}$ was characteristic of bovine isolates, defining a new host-specific virulence plasmid type in *R. equi*.

Among plasmid-positive ($traA^{+}$) strains, no $vapB^{+}$ marker was found among equine isolates, no $vapA^{+}$ marker was found in bovine isolates, and no $vapAB^{-}$ plasmids were identified among porcine isolates, suggesting host-driven exclusion of specific plasmid types. All plasmid types were common in human isolates, possibly reflecting the predominantly opportunistic nature of *R. equi* infection in this host. The presence in humans of plasmid types characteristic of specific animal hosts clearly points to the existence of a transmission flux between animals and man, suggesting that human *R. equi* infection is zoonotic.

Comparative genomic studies of the $vapA^{+}$- and $vapB^{+}$-type virulence plasmids, each associated with a specific non-human animal host, revealed that both share a conserved backbone but differ in their *vap* pathogenicity island. The "*vapB* PAI" carries five *vap* genes (*vapB* and *vapJ* to *M, vapK* present in two copies) encoding proteins differing by 24 to 84% in amino acid sequence from those encoded in the *vapA* (horse-type) plasmid, suggesting that the specific *vap* multigene family complement carried by the virulence plasmid plays a critical role in *R. equi* host tropism. Sequence analyses, including interpolated variable order motifs for detection of "alien" DNA, and the reconstruction of Vap family phylogenetic relationships, suggested that the *vap* PAI was acquired by a common ancestor plasmid via lateral gene transfer, subsequently evolving by *vap* gene duplication and sequence diversification to give different host-adapted plasmids.

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Proliferation of virulent *Rhodococcus equi* in the gastrointestinal tract of foals.

*C. Chicken[1,2,3] *, J. R. Gilkerson[1], A. P. Begg[4], G. F. Browning[1], and G. Muscatello[5] . [1]Equine Infectious Disease Laboratory, Faculty of Veterinary Science, The University of Melbourne, Parkville, Victoria 3010. [2]Scone Veterinary Hospital, Scone, NSW 2337. [3]Hunter Valley Equine Research Centre, Scone, NSW 2337. [4]Symbion Vetnostics, North Ryde, NSW 2113. [5]Faculty of Veterinary Science, The University of Sydney, NSW 2006.*

Objectives
This study aimed to determine where virulent *R. equi* proliferated within the gastrointestinal tract (GIT) of foals and whether the distribution of virulent *R. equi* in the foal GIT was affected by age and/or clinical disease status.
Methods
Gastrointestinal contents from six pre-determined sites were collected during post mortem examination from 25 Thoroughbred foals of varying ages and disease status. Quantitative bacterial culture of *R. equi* was performed using selective media. Resulting cultures underwent colony blotting and DNA hybridization techniques to determine the concentration and proportion of virulent *R. equi* within the *R. equi* gastrointestinal population at each site sampled.
Results
There was a significant increase in the median number of total and virulent *R. equi* at, and beyond, the level of the caecum. Age and disease status affected the distribution of virulent *R. equi* throughout the GIT. Neonatal foals (0-7 days of age) had negligible growth of virulent *R. equi* in their GIT. Foals that died from *R. equi* disease had higher levels of virulent *R. equi* in more proximal regions of their GIT.
Conclusions
This data indicate that *R. equi* and virulent *R. equi* replicate primarily in the caecum of foals. There may therefore be factors in the caecal environment of foals which are important for gastrointestinal replication of virulent *R. equi*. Further investigation into the caecal environment may result in methods for modification of the caecal micro-environment with an aim of reducing or preventing virulent *R. equi* proliferation.

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Shedding of *Rhodococcus equi* in faecal and tracheal secretions of foals with pulmonary abscesses

*M. Lämmer * and M. Venner. Equine Clinic, University of Veterinary Medicine Hannover, Foundation, Bischofsholer Damm 15, D-30173 Hannover, Germany*

Objective
The aim of this study was to evaluate the amount of *R. equi* shed from foals on a breeding farm with a high prevalence of pulmonary abscesses in the foal population.
Methods
Sixty foals were evenly distributed into 3 groups based on their health and treatment status (healthy foals, foals with pulmonary abscesses receiving treatment and untreated foals with pulmonary abscesses). Foals were defined as healthy if they showed no clinical, hematologic, sonographic, or radiographic evidence of pulmonary disorders. Diagnosis of pulmonary abscessation was made by sonographic examination of the thorax. Foals with few or small abscesses (abscess diameter under 50 mm) were not treated but were examined twice weekly for signs of deterioration. Foals with many abscesses were treated with rifampin (10 mg/kg BW, p.o., b.i.d.) in combination with tulathromycin (2.5 mg/kg BW, i.m., once weekly) or azithromycin (10 mg/kg BW, p.o., s.i.d.).
These foals were sampled repeatedly over a period of 2 weeks after diagnosis (Table 1). Faecal and tracheal secretions from these foals were evaluated by microbiological culture for presence of *R. equi*.

Table 1: Sample types and days on which samples were collected from foals in this study.

Samples	Day 0	Day 3	Day 7	Day 14
Tracheal secretions	X		X	X
Faeces	X	X	X	X

Results
R. equi was cultured on the day of diagnosis of pulmonary abscessation in the faeces of 31% of sick foals and in the tracheal secretions in 35% of these foals. Only 5% of healthy foals were *R. equi* positive on faecal samples and 10% of these were positive for *R. equi* on culture of tracheal secretions.
From a total of 185 paired faecal and tracheal secretions samples of the same foals (healthy and sick) culture results were identical in both sample types in 158 cases. The faecal and tracheal culture results therefore where in agreement for 85% of the paired samples.
Forty-three percent (43%) of foals on the day therapy for pulmonary abscesses commenced shed *R. equi* in their faeces and tracheal secretions. On day seven of treatment, *R. equi* was isolated in 24% of the fecal samples and in 33% of the tracheal samples. On day fourteen of treatment, *R. equi* was cultured in 5% of the fecal samples and in 19% of the tracheal samples in the 20 treated foals.
Conclusion
This data suggests that *R. equi* shedding in faecal and tracheal secretion does occur in the sick and healthy foal. Given that faecal samples are practically easier to perform and their culture results were similar to those seen in tracheal secretions, faecal culture should be considered as a diagnostic option. The notion, that faecal and respiratory secretions of foals can be seen to be a source of contamination even after periods of therapy is an important consideration farm managers need to consider when devising strategies to minimize the impact of *R. equi* pneumonia.

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Assessment of the genetic diversity of the virulence plasmid present in *Rhodococcus equi*, the agent of equine rhodococcosis

*F. Duquesne[1] *, C. Sevin[1], M-F Breuil[1], S. Pronost[2], C. Laugier[1], S. Petry[1]. [1]AFSSA, Laboratory for Studies and Research on Equine Disease, IPC, Goustranville, 14430 Dozulé, France. [2]Frank Duncombe Départemental Laboratory, 1 Route de Rosel, 14053 Caen Cedex 4, France*

Objective
Rhodococcus equi is a facultative, intracellular, Gram-positive coccobacillus that causes chronic, suppurative bronchopneumonia and enteritis. Several studies have associated the pathogenicity of the bacterium with the presence of an 80- to 90-kb virulence plasmid classified according to restriction fragment length polymorphisms (RFLP) patterns. These plasmid types appear to have some degree of geographical specificity. As a result, and given the major international trade in horses, the typing of *R. equi* virulence plasmids by RFLP may be an effective tool for tracing virulent strains. In this context, our main objective was to characterise and then compare the plasmids extracted from a representative population of our *R. equi* collection.
Methods
We selected around 100 strains depending on the aetiology of the disease (pulmonary, digestive, osteoarticular, cause of abortion) and ecological niche (strains isolated from autopsies or collected in the environment in horse farms in the form of faeces, straw, soil or dust samples). Plasmids from isolates were typed using the technique of Takai *et al*. (1999).
Results
The vast majority of strains had plasmid-types previously described (91%), 3/4 of which had plasmids that were already known to be present in Normandy: 85-kb type I, type II and 87-kb type I. The remaining 1/4 was comprised of at least 3 new types of plasmid which would be worth characterising in greater detail. A small percentage of strains examined were plasmid-less (9%) and therefore considered to be avirulent. We also showed that a relative small proportion of isolates with plasmids were from the equine environment (30%) and that all strains (100%) without a plasmid were environmentally derived.
Conclusion
This study supports the notion that the 80- to 90-kb plasmid is indeed a key element for the virulence of the bacterium and highlights the environment of horse farms as a contamination reservoir. This study found no relationship between the etiology of the disease and a specific plasmid type. We plan to sequence several types of plasmid with a view to revealing the genetic variabilities responsible for this plasmid diversity.
References
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Mycobacterium avium subsp. *paratuberculosis*: another challenging pathogenic actinomycete

K Stevenson. Moredun Research Institute, Pentlands Science Park, Penicuik, Scotland, U.K.

As another actinomycete, *Mycobacterium avium* subspecies *paratuberculosis* (Map) has commonalities with *Rhodococcus equi*. This presentation will be a brief overview of aspects of Map epidemiology, ecology and virulence factors to promote exchange of knowledge and ideas between researchers working on these organisms and increase our understanding of this challenging group of bacteria.
Map causes Johne's disease, a chronic fatal granulomatous enteritis predominantly affecting ruminants. The disease is spread mainly by the faecal-oral route but also via colostrums, milk and *in utero*. Infection occurs via M cells in the small intestine. The bacteria cross the epithelial layer and are phagocytosed by epithelioid macrophages where they persist and multiply. Following infection there is a prolonged incubation period of 2-4 years during which time the animal shows no clinical signs but sheds Map intermittently. Map can infect a broad range of hosts and there is evidence for a role for wildlife in the epidemiology of the disease. Map also has been associated with Crohn's disease raising public health concerns regarding the presence of Map in milk and water supplies. Despite being an intracellular pathogen Map is very resilient and can withstand pasteurisation, chlorination and survive for long periods in the environment.

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Comparison of *Rhodococcus equi* strains isolated from swine, wild boars and humans in Hungary

*L. Makrai[1] *, L. Fodor[1], J. Varga[1], B. Dénes[2], I. Hajtós[3], K. Jánosi[1], M. Gyuranecz[1], A. Kobayashi[4], M. Matsuoka[4], S. Takayama[4], S. Tsubaki[4], Y. Sasaki[4], T. Kakuda[4] S. Takai[4]. [1]Dept. of Microbiology and Infectious Diseases, Szent István University, Budapest, Hungary. [2]Central Veterinary Institute, Budapest, Hungary. [3]Animal Health and Food Control Station, Miskolc, Hungary. [4]Dept. of Animal Hygiene, School of Veterinary Medicine and Animal Sciences, Kitasato University, Towada, Aomori, Japan.*

Objective
This study aimed to examine and compare the serological features and plasmid profiles of *R. equi* isolates from swine, wild boars and humans.
Methods
Two hundred and forty-six (246) *R. equi* strains from 1655 submaxillary lymph nodes of pigs and wild boars and 7 strains from humans were examined. The isolates were serotyped using the Prescott system and tested for the presence of the genes encoding the 15-17-kDa (VapA) and 20-kDa virulence-associated protein (VapB) by PCR. Size and polymorphisms of plasmids were analysed using restriction endonuclease- generated RFLP (restriction fragment length polymorphism).
Results
Most isolates (swine 72%, wild boar 54%, human 85%) belonged to serotype 2. None of the isolates contained *vapA* but 70 isolates (27.7%) harbored *vapB*. No virulence plasmids were in 183 strains (72.3%). Plasmid types 5 (57 strains), 1 (4 strains), 7 (2 strains), 4, 6, 17, 21, 25, 26 and 27 (1 strain for each) were detected in *vapB*-positive intermediately virulent isolates. The prevalence of intermediately virulent *R. equi* strains in wild boars (25.6%) and pigs (26.8%) were very similar, plasmid type 5 was prominent in both the porcine and human-derived isolates.
Conclusions
Our results suggest epidemiological relationship between human *R. equi* isolates and those obtained from pigs and wild boars. The route of infection in humans is unclear; however food-borne transmission is presumed. Since *R. equi* is a human pathogen, extending our knowledge of *R. equi* infection in pigs and wild boars should be a priority.

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Retrospective study of *Rhodococcus equi* infection from a population of 1352 autopsied foals in France

J. Tapprest [a], C. Laugier [a], C. Mauger [a], N. Foucher [a], C. Sevin [a], K. Maillard[b] . [a] AFSSA, Laboratory for Studies and Research on Equine Disease, IPC 14430 Goustranville, France. [b] Frank Duncombe Départemental Laboratory 1, route de Rosel 14280 Saint Contest, France*

Objective
The objectives of this study were to:
- pinpoint the prevalence of rhodococcosis among the causes of morbidity/mortality identified during autopsy of foals
- investigate foal factors that may influence the occurrence of rhodococcosis
- describe the different lesions of the disease and their clinical characteristics
Methods
This retrospective study spanned an 18-year period during which 1352 foals aged from 1 day to <1 year were autopsied at AFSSA Dozulé. The following data were gathered: medical history, date of death, age, breed, sex, anatomopathological, histological and bacteriological results. Statistical analyses were performed using χ^2 and analysis of variance.
Results
Of the 1352 foals autopsied, 161 were affected with rhodococcosis, an overall prevalence of 11.9%. A significant breed effect was found. No sex-related associations were observed. There were significantly more cases in the spring and summer.
The distribution and frequency of typical lesions associated with the infection were: 60.9% of isolates were obtained from pulmonary lesions, 1.9% of isolates were obtained from digestive lesions, 26.1% of infected foals had isolated obtained from both pulmonary and digestive lesions and 11.1% of isolates were obtained from musculoskeletal lesions.
The clinical characteristics recorded depended on the different lesional forms**Conclusion**s
The insidious nature of the disease can results in lengthy delays between the onset of pathological lesions and the identification of symptoms. “Sudden deaths” associated with pulmonary forms are not uncommon. The clinical expression of digestive lesions seems to be particularly inconsistent. Spinal forms, although rare, mean that rhodococcosis should be included in the possible aetiology of signs of ataxia and/or paresis in foals <1 year of age.

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Rhodococcus equi infection in foals in Poland

L. Witkowski[1], M. Rzewuska[2], J. Kaba[1], J. Kita[1]. [1]Division of Infectious Diseases and Epidemiology, Dept. of Clinical Sciences, [2]Division of Microbiology and Molecular Biology, Dept. of Preclinical Sciences, Faculty of Veterinary Medicine, Warsaw University of Life Sciences.*

Objectives
For many years Polish equine veterinarian have seen *Rhodococcus equi* infection in foals. Virulent bacterial strains have been isolated from clinical samples and soil, but there has not been any epidemiological research of rhodococcal infection conducted in Poland. Thus the aim of this study was to determine the prevalence of *R. equi* infection in Polish horse-breeding farms.
Methods
Interviews were carried out on 23 state-owned horse-breeding farms (in existence for over 30 years) and 6 privately owned farms (in existence for less than 15 years). General information about the farm and detailed information about respiratory tract disorders of foals were collected. Samples were collected from foals with suspected rhodococcosis for microbiological investigations. In case of death, anatomopathological examination was performed.
Results
Cases of *R. equi* infection in foals were diagnosed in 20 of 29 farms investigated. At 18 farms, the diagnosis was confirmed by isolation of virulent *R. equi* from clinical samples (64 strains). At 13 of the farms with cases, the disease was observed sporadically (not every season). On 7 farms rhodococcosis was a considered a serious problem. Prevalence of the rhodococcosis on farms ranged from 10% to 50% with case fatality of about 5-15%. In a few seasons rhodococcosis prevalence on some farms was observed to be close to 100% with case fatality close to 30%..
Conclusions
Rhodococcosis is a serious problem on some horse breeding farms in Poland. Infection is present in many farms but clinical cases and fatalities are usually low.
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PARTICIPANTS

Debra Alperin
Dept. Veterinary Microbiology & Pathology
Washington State University
Pullman, WA 99164 – 7040
USA
Tel: 509 335 6096
Fax: 509 335 8529
dalperin@vetmed.wsu.edu

Jean Jacques Ballet
AFSSA Lerpe
Goustranville
Dozule, 14430
FRANCE
Tel: 02 31 79 22 68
Fax: 02 31 39 21 37
ballet-jj@chu-caen.fr

Corinne Barbey
AFSSA Lerpe
Goustranville
Dozule, 14430
FRANCE
Tel: 02 31 79 22 68
Fax: 02 31 39 21 37
j.barbey@dozule.afssa.fr

Thomas Buckley
Irish Equine Centre
Johnstown
County Kildaire
IRELAND
Tel: 353 45 866 266
Fax: 353 45 866 273
tbuckley@equine-centre.ie

Silvia Cardoso
Dept. Cellular and Molecular Biology.
University of Sao Paulo
São Paulo, 14049-900
BRAZIL
Tel: 55 16 36023066
cardososil@usp.br

Julien Cauchard
AFSSA Lerpe
Goustranville
Dozule, 14430
FRANCE
Tel: 02 31 79 22 68
Fax: 02 31 39 21 37
j.chauchard@dozule.afssa.fr

Severine Cauchard
AFSSA Lerpe
Goustranville
Dozule, 14430
FRANCE
Tel: 02 31 79 22 68
Fax: 02 31 39 21 37
s.cauchard@dozule.afssa.fr

Morgan Keith Chaffin
Dept. of Large Animal Clinical Sciences
Texas A & M University
Texas 77843-4475
USA
Tel: 979 845 4890
Fax: 979 847 8863
k.chaffin@cvm.tamu.edu

Catherine Chicken
Faculty of Veterinary Science
The University of Melbourne
Victoria 3010.
AUSTRALIA
Tel: 61 407015751
Fax: 61 265452903
c.chicken@gobushmail.com.au

Noah Cohen
Dept. of Large Animal Clinical Sciences
Texas A & M University
Texas 77843-4475
USA
Tel: 979 845 3541
Fax: 979 847 8863
n.cohen@cvm.tamu.edu

Garry Coulson
Dept. of Infectious Diseases
University of Georgia
Athens, GA 30602
USA
Tel: 706 542 5777
Fax: 706 542 5771
gcoulson@uga.edu

Anne Cunningham
Health, Natural & Social Sciences
University of Sunderland
Sunderland, SR1 3SD
UNITED KINGDOM
Tel: 0191 515 2979
Fax: 0191 515 3405
anne.cunningham@sunderland.ac.uk

Tamsin Dawson
Veterinary Immunogenics Limited
Carleton Hill
Penrith, Cumbria, CA11 8TZ
UNITED KINGDOM
Tel: 01768 863881
Fax: 01768 891389
tamsindawson@veteinaryimmunogenics.com

Willie Donachie
Moredun Research Institute
Pentlands Science Park
Penicuik, EH26 0PZ
UNITED KINGDOM
Tel: 020 7259 8363
Fax: 020 7333 0041
willie.donachie@moredun.ac.uk

Robson Dossa
Dept. Veterinary Microbiology & Pathology
Washington State University
Pullman, 99164-7040
USA
Tel: 509 335 6311
Fax: 509 335 8529
robsondossa@vetmed.wsu.edu

Fabien Duquesne
AFSSA Lerpe
Goustranville
Dozule, 14430
FRANCE
Tel: 02 31 79 22 68
Fax: 02 31 39 21 37
f.duquesne@dozule.afssa.fr

Ruth Fahey
School of Biomolecular & Biomedical
Scs. University College Dublin
Dublin 4
IRELAND
Tel: 35317161625
ruth.fahey@ucd.ie

Ciaran Finn
School of Biomolecular & Biomedical
Scs. University College Dublin
Dublin 4
IRELAND
Tel: + 353 1 7161625
ciaran.finn@ucd.ie

Julia Maria Flaminio
College of Veterinary Medicine
Cornell University
Ithaca, NY 14853
USA
Tel: 607 253 3100
Fax: 607 253 3787
mbf6@cornell.edu

Ursula Fogarty
Irish Equine Centre
Johnstown
County Kildaire
IRELAND
Tel: 353 45 866 266
Fax: 353 45 866 273
ufogarty@equine-centre.ie

Sophie Foley
School of Life Sciences
Napier University
Edinburgh, EH10 5DT
UNITED KINGDOM
Tel: 0131 455 2626
s.foley@napier.ac.uk

Steeve Giguere
Dept. of Large Animal Clinical Sciences
University of Florida
Gainesville, FL 32610
USA
Tel: 352 392 4700
Fax: 208-460-3930
gigueres@mail.vetmed.ufl.edu

Aleksandra Gnojska
School of Biomolecular & Biomedical
Science University College Dublin
Dublin 4
IRELAND
Tel: + 353 1 7161625
Email:
Aleksandra.Gnojska@ucdconnect.ie

Albert Haas
Cell Biology Institute
University of Bonn
Bonn, 53121
GERMANY
Tel: 49 228 73 63 40
Fax: 49 228 73 63 04
albert.haas@uni-bonn.de

Richard Harland
Novartis Animal Health Canada Inc
Calgary
Alberta, T2V 3P8
CANADA
Tel: 14036140947
richard.harland@novartis.com

Seth P. Harris
Dept. Veterinary Microbiology & Pathology
Washington State University
Pullman, WA 99164
USA
Tel: 509 335-6339
sharris@vetmed.wsu.edu

Meera Helle
School of Veterinary Medicine
University of California
Davis, CA 95616
USA
Tel: 530-752-0290
mcheller@ucdavis.edu

Steven Hines
College of Veterinary Medicine
Washington State University
Pullman, WA 99164-7040
USA
Tel: 509 335 6069
Fax: 509 335 8529
shines@vetmed.wsu.edu

Mary Hondalus
College of Veterinary Medicine
University of Georgia
Athens, GA 30602
USA
Tel: 706 542 5778
Fax: 706 542 5771
hondalus@vet.uga.edu

David W. Horohov
Maxwell H. Gluck Equine Research Center
University of Kentucky
Lexington, KY 40546-0099
USA
Tel: 859 257-4757 x 81098
Fax: 859 323-3981
David.Horohov@uky.edu

Patricia González Iglesias
Centre for Infectious Diseases
University of Edinburgh
Edinburgh, EH9 3JT
UNITED KINGDOM
Tel: 44 131 6508656
Fax: 44 131 6513619
pgiglesi@staffmail.ed.ac.uk

Antonius Jacobs
Intervet Internacional BV
PO Box 31
Boxmeer, 5830 AA
THE NETHERLANDS
Tel: 31 485 587 586
Fax: 31 485 587 490
ton.jacobs@intervet.com

Mariann Klay
Irish Equine Centre
Johnstown
County Kildaire
IRELAND
Tel: 353 45 866 266
Fax: 353 45 866 273
mklay@equine-centre.ie

Monika Krueger
Faculty of Veterinary Medicine
University of Leipzig
Leipzig D-04103
GERMANY
Tel: 49 341 973 8180
Fax: 49 341 973 8199
mkrueger@vmf.uni-leipzig.de

Marc Lammer
Lewitz Farm
Lewitzhof 1
Neustadt-Glewe, D-19306
GERMANY
Tel: 49 387 575 4370
marclaemmer@gmx.de

Laszlo Makrai
Faculty of Veterinary Science
Istvan University
Budapest, H-1148
HUNGARY
Tel: 36 1 251 99 00
Fax: 36 1 25 1 92 60
makrai.laszlo@aotk.szie.hu

Des Leadon
Irish Equine Centre
Johnstown
County Kildaire
IRELAND
Tel: 353 45 866 266
Fax: 353 45 866 273
dleadon@equine-centre.ie

Michal Letek Polberg
Centre for Infectious Diseases
University of Edinburgh
Edinburgh, EH9 3JT
UNITED KINGDOM
Tel: 44 131 6508656
Fax: 44 131 6513619
mlpolber@staffmail.ed.ac.uk>

Marianela Lopez
Vaccine and Infectious Disease Organization
University of Saskatchewan
Saskatoon SK, S7N 5E3
CANADA
Tel: 306 966 1517
Fax: 306 966 7478
marianela.lopez@usask.ca

Raul Miranda Caso Luengo
School of Biomolecular & Biomedical Scs.
University College Dublin
Dublin D4
IRELAND
Tel: 353 1 716 1625
miranda.raul@ucd.ie

Paul Lunn
Dept. of Clinical Sciences
Colorado State University
Colorado, 80523-1620
USA
Tel: 970 297-1274
Fax: 970 297-1275
lunnp@colostate.edu

Iain MacArthur
Department of Pathobiology
University of Guelph
Guelph, Ontario, N1G 2W1
CANADA
Tel: 519 824 4120 x 54717
Fax: 519 824 5930
imacarth@uoguelph.ca

Celia Marr
Rossdales & Partners
Beaufort Cottage Stables
Newmarket, CB8 8JS
UNITED KINGDOM
Tel: +44 (0)1638 663150
Fax: +44 (0)1638 660157
celia.marr@rossdales.com

Ronald Martens
9544 County Road 275
Somerville, 77879
Texas
USA
Tel: 979 272 1171
rmartens@cvm.tamu.edu

Mei Liu
College Station
Texas A & M University
Texas 77843-4475
USA
Tel: 979 845 3541
Fax: 979 847 8863
mliu@cvm.tamu.edu

Adrián Martinez Vieira
Centre for Infectious Diseases
University of Edinburgh
Edinburgh, EH9 3JT
UNITED KINGDOM
Tel: (0)131 6508656
Fax: (0)131 6513619
tritom29@hotmail.com

Wim Meijer
School of Biomolecular & Biomedical
Scs University College Dublin
Dublin 4
IRELAND
Tel: 353 1 716 1374
Fax: 353 1 716 1183
Wim.Meijer@ucd.ie

Mark Meyer
MG Biologics
2366 270th Street
Ames, 50014
USA
Tel: 515 769 2340
Fax: 515 769 2390
jodi@mgbiologics.com

Sarah Meyer
MG Biologics
2366 270th Street
Ames, 50014
USA
Tel: 515 769 2340
Fax: 515 769 2390
jodi@mgbiologics.com

Crystal Montoya
Veterinary Microbiology & Pathology
Washington State University
Pullman, 99164-7040
USA
Tel: 509 335 6311
Fax: 509 335 8529
cmontoya@vetmed.wsu.edu

Gary Muscatello
Faculty of Veterinary Science
University of Sydney
Sydney 2006
AUSTRALIA
Tel: 612 9351 5074
Fax: 612 9351 8025
g.muscatello@usyd.edu.au

Vivian Nicholson
Ontario Veterinary College
University of Guelph
Guelph, Ontario, N1G 2W1
CANADA
Tel: 519 824 4120 x 54717
Fax: 519 824 5930
vivianni@uoguelph.ca

Aline Oliveira
University of Sao Paulo
Ribeirão Preto
São Paulo, 14049-900
BRAZIL
Tel: 55 16 3602-3066
alinefopereira@usp.br

Lorraine Palmer
Rossdales & partnes
Beaufort Cottage Stables
Newmarket, CB8 8JS
UNITED KINGDOM
Tel: +44 (0)1638 663150
Fax: +44 (0)1638 660157
lorraine.palmer@rossdales.com

Valeria Parreira
Ontario Veterinary College
University of Guelph
Guelph, Ontario, N1G 2W1
CANADA
Tel: 519 824 4120
Fax: 519 824 5930
vparreira@uoguelph.ca

Nicholas Petrovitch
Centre for Infectious Diseases
University of Edinburgh
Edinburgh, EH9 3JT
UNITED KINGDOM
Tel: 44 131 6508656
Fax: 44 131 6513619
starn101@yahoo.com

Gene M. Pranzo
The Dorothy Russell Havemeyer Foundation
60 East 42nd Street, 40th Floor
New York, NY 10165-0006
USA
Tel: 212 682 3700
Fax: 212 682 3704
drh.fdtn@verizon.net>

John Prescott
Dept. of Pathobiology
University of Guelph
Guelph, Ontario, N1G 2W1
CANADA
Tel: 519 824 4120 x 54453
Fax: 519 824 5930
jprescott@uoguelph.ca

Dragan Rogan
Bioniche Animal Health Canada Inc.
Belleville
Ontario, K8N 5J2
CANADA
Tel: 613 966 8058
Fax: 613 966 4177
Roslyn.Brown@Bioniche.com>

Artem Rogovskyy
Dept. of Infectious Diseases
University of Georgia
Athens, GA 30602
USA
Tel: 706 201-8354
artem@uga.edu

Ana Carolina Rusca Correa Porto
University of Sao Paulo
Ribeirao Preto, 14049-900
Sao Paulo
BRASIL
Tel: 55 163 602 3066
Fax: 55 163 602 4590
carolporto@usp.br

Clare Ryan
College of Veterinary Medicine
University of Florida
Gainesville, FL 32610
USA
Tel: 352 392 2670
Fax: 208 460 3930
ryanc@vetmed.ufl.edu

Mariela Scorrti
Centre for Infectious Diseases
University of Edinburgh
Edinburgh, EH9 3JT
UNITED KINGDOM
Tel: (0)131 6513624
Fax: (0)131 6513619
m.scortti@ed.ac.uk

Brett Sponseller
College of Veterinary Medicine
Iowa State University
Ames, IA 50011
USA
Tel: 515 294 2237
Fax: 515 294 8500
baspon@iastate.edu

Karen Stevenson
Moredun Research Institute
Pentlands Science Park, Bush Loan
Penicuik, EH26 0PZ
UNITED KINGDOM
Tel: 44 131 445 5111
Fax: 44 131 445 6111
karen.stevenson@moredun.ac.uk

Neil Stoker
Pathology and Infectious Diseases
Royal Veterinary College
London, NW1 0TU
UNITED KINGDOM
Tel: 020 7468 5272
Fax: 020 7468 5306
nstoker@rvc.ac.uk

Sarah Stoneham
Lone Oak Stud
Great Bradley
Newmarket, CB8 9LU
UNITED KINGDOM
Tel: 07764 575517
sarah@loneoakstud.co.uk

Iain Sutcliffe
School of Applied Sciences
Northumbria University
Newcastle Upon Tyne, NE1 8ST
UNITED KINGDOM
Tel: 0191 227 4071
Fax: 0191 227 3519
iain.sutcliffe@unn.ac.uk

Shinji Takai
School of Veterinary Medicine
Kitasato University
Aomori 034-8628
JAPAN
Tel: 81 176 24 9458
Fax: 81 176 23 8703
takai@vmas.kitasato-u.ac.jp

Hugh G.G. Townsend
Dept. of Veterinary Microbiology
University of Saskatchewan
Saskatoon SK, S7N 5B4
CANADA
Tel: 306 966 1514
Fax: 306 966 7478
hugh.townsend@usask.ca

Robert Van der Geize
Biomolecular Sciences & Biotechnology
University of Groningen
Haren, 9751 NN
THE NETHERLANDS
Tel: 31 50 363 2257
Fax: 31 50 363 2154
r.van.der.geize@rug.nl

Jose Vazquez Boland
Centre for Infectious Diseases
University of Edinburgh
Edinburgh, EH9 3JT
UNITED KINGDOM
Tel: 44 131 6508656
Fax: 44 131 6513619
v.boland@ed.ac.uk

Monica Venner
Equine Clinic
Veterinary School of Hanover
Hannover, D – 30173
GERMANY
Tel: 49 151 11595947
MVenner@gmx.de

Tripathi Vibhay
Dept. of Infectious Diseases
University of Georgia
Athens, GA 30602
USA
Tel: 706 542 5777
Fax: 706 542 5771
vibhay@vet.uga.edu

Kristine Von Bargen
Cell Biology Institute
University of Bonn
Bonn, 53121
GERMANY
Tel: 49 (0)228736339
Fax: 49 (0)228736340
kristine.vonbargen@uni-bonn.de

Johanna Watson
Dept. of Medicine and Epidemiology
University of California
Davis, CA 95616
USA
Tel: 530 754 8693
Fax: 530 752 0292
jlwatson@ucdavis.edu

Ashley Whitehead
University of Guelph
Ontario Veterinary College
Guelph, Ontario, N1G 1E5
CANADA
Tel: 519 835 9218
awhitehe@uoguelph.ca

Lucjan Witkowski
Faculty of Veterinary Medicine
Warsaw University of Live Sciences
Warsaw, 02-776
POLAND
Tel: 48 225 936 111
Fax: 48 228 457 818
lucekwit@o2.pl