57th Annual Brucellosis Research Conference
November 13-14, 2004
Congress Plaza Hotel - Chicago, IL

Saturday

7:00 – 8:00 am	Registration
8:00 am	**Welcome** – Jean-Pierre Liautard, Chair
8:15 am	**Genomics** Jean-Pierre Liautard, moderator
9:15 am	**Virulence** Phil Elzer, moderator
10:15 am	Break and Posters
10:45 am	**Virulence** continues
12:00 -1:30 pm	Lunch
1:30 pm	**Cellular Microbiology** Marty Roop, moderator
2:30 pm	**Immunology** Don Davis, moderator
3:30 pm	Break and Posters
4:00 pm	**Vaccine** Sally Cutler, moderator

Sunday

8:30 am	**Epidemiology** Tom Ficht, moderator
9:00 am	**USDA Eradication Report**
9:30 am	Break
10:00am	**USDA Regulatory Information**
11:00 am	**Business Meeting**

Front Cover image: "The Maltese Goat" by E. Caruana Dingli (1876-1950)

Saturday, November 13, 2004

Genomics:

Moderator – Jean-Pierre Liautard

8:15 am

The *Brucella* Bioinformatics Portal and *Brucella* Genome Curation in the Bioinformatics Resource Center at the Virginia Bioinformatics Institute

Yongqun He[1], Wenjie Zheng[1], Stephen Boyle[2], Oswald Crasta[1], Joao Setubal[1], Bruno Sobral[1]. 1. Virginia Bioinformatics Institute, Virginia Polytechnic Institute and State University, 1880 Pratt Drive, Blacksburg, VA 24061-0477; 2. Center for Molecular Medicine and Infectious Diseases, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Polytechnic Institute and State University, 1410 Price's Fork Road, Blacksburg, VA 24061-0342

8:30 am

Do Brucella have pathogenicity Islands?

Gireesh Rajashekara, Jeremy Glasner, David Glover, Mike Krepps, Dana Tackes and Gary Splitter. Department of Animal Health and Biomedical Sciences, University of Wisconsin-Madison, Madison, WI

8:45 am

Improved Expression Vector for *Brucella* species

Mohamed N. Seleem, [1] Neelima Sanakkayala, [2] Ramesh Vemulapalli, [2] Stephen M. Boyle,[1] Gerhardt G. Schurig, [1] and Nammalwar Sriranganathan [1]. [1] Virginia-Maryland Regional College of Veterinary Medicine, Virginia Polytechnic and State University, Blacksburg, Virginia 24061-0342; [2] School of Veterinary Medicine, Purdue University, West Lafayette, IN

9:00 am

The genome of *Brucella abortus* 2308: comparison with *B. suis* and *B. melitensis*

Comerci D[1], F Agüero[1], P Chain[2], M Tolmasky[3], E García[2] and R Ugalde[1].
[1]Instituto de Investigaciones Biotecnológicas (IIB-UNSAM), Universidad de Gral. San Martín, San Martín, Buenos Aires, Argentina; [2]Lawrence Livermore Nacional Laboratory, Livermore, CA; [3]California State University, Fullerton, CA, USA

Virulence:

Moderator – Phil Elzer

9:15 am

Biophotonic imaging to identify attenuated Brucella

Gary Splitter, David Glover, Gireesh Rajashekara, Menachem Banai, Mike Krepps, Department of Animal Health and Biomedical Sciences, University of Wisconsin-Madison, Madison, WI

9:30 am

Brucella suis* requires MgtC for intramacrophage survival; a mechanism shared *Salmonella enterica* and *Mycobacterium tuberculosis

Jean-Philippe Lavigne, David O'Callaghan and Anne-Béatrice Blanc-Potard
Institut National de la Santé et de la Recherche Médicale, Unité 431, UFR de Médecine, CS 83021, Avenue J.F. Kennedy, 30908 NIMES Cedex 02, France

9:45 am

The *Brucella abortus* sodA gene product is required for virulence in the C57BL/6J mouse model of infection

Daniel W. Martin, Jason M. Gee, and RM Roop II; East Carolina Universtiy, Greenville, NC 27834

10:00 am

The Role of DhbR, an AraC-like transcriptional activator, in *Brucella abortus* iron acquisition.

Eric S. Anderson, James T. Paulley and RM Roop II; East Carolina University, Greenville, NC 27834

10:15 – 10:45 am Break and Posters

10:45 am

The RelA/SpoT homolog (Rsh) of *Brucella* spp., involved in stringent response, is essential for the virulence of the pathogen

Marie Dozot[1], Rose-Anne Boigegrain[2], Rose-May Delrue[1], Régis Hallez[1], Jean-Jacques Letesson[1], Xavier DeBolle[1], and Stephan Köhler[2]. [1] Unite de Recherche en Biologie Moleculaire, Facultes Universitaires Notre-Dame-de-la-Paix, Namur, Belgium; [2] INSERM U-431, Universite Montpellier II, Montpellier, France.

11:00 am

The *Brucella abortus* xthA2 gene product contributes to resistance to reactive oxygen intermediates

Michael L. Hornback, and RM RoopII; The Brody School of Medicine, East Carolina University, Greenville, NC 27834

11:15 am

The bhuA Gene Product Of *Brucella abortus* Is Required For The Utilization Of Hemin And The Maintenance Of Chronic Infection In BALB/c Mice

James T. Paulley, Eric S. Anderson, and RM RoopII; East Carolina University, Greenville, NC 27834

11:30 am

Characterization of a small molecule inhibitor of the VirB11 family of type IV secretion components

Christoph Höppner[1], Anna Carle[1], Durga Sivanesan[2], Christian Baron[1,2] and members of the European Union-funded TFSS consortium. [1]Ludwig-Maximilians-Universität München, Department Biologie I, Bereich Mikrobiologie, Maria-Ward-Str. 1a, D-80638 München, Germany; [2]McMaster University, Department of Biology, 1280 Main St. West, Hamilton, ON LS8 4K1, Canada

11:45 am

The quorum-sensing related transcriptional regulator VjbR controls expression of the type IV secretion and the flagellar genes in *Brucella melitensis* 16M

J.J. Letesson, R-M. Delrue, S. Bonnot, C. Deschamps, S. Leonard, X.Debolle, URBM, University of Namur Belgium

12:00 – 1:30 pm Lunch

Cellular Microbiology:

Moderator – Marty Roop

1:30 pm

O-antigen controls macrophage uptake and the ultimate fate of *Brucella*

Jianwu Pei, Joshua E. Turse, and Thomas A. Ficht. Department of Veterinary Pathobiology, Texas A&M University and Texas Agricultural Experiment Station, College Station, TX 77843-4467

1:45 pm

Absence of evidence for the participation of the macrophage cellular prion protein in infection by *Brucella suis*

Fontes P, Alvarez-Martinez MT, Gross A, Carnaud C*, Kohler S and Liautard JP. INSERM U431, Université Montpellier II, place Eugène Bataillon, 34000 Montpellier. France * INSERM and Université Pierre et Marie Curie, Hôpital Saint-Antoine, Paris, France

2:00 pm

Microarray Analysis of Macrophage Gene Transcription in Response to Infection *in vitro* with Virulent *Brucella melitensis* at Different Time Points

Yongqun He[1], Sherry Poff[2], Sheela Ramamoorthy[2], Hanna Craig[1], Bruno Sobral[1], Gerhardt Schurig[2], Nammalwar Sriranganathan[2], Stephen Boyle[2]. 1-Virginia Bioinformatics Institute; 2-Virginia-Maryland Regional College of Veterinary Medicine; Virginia Polytechnic Institute and State University, Blacksburg, VA

2:15 pm

Macrophage transcript levels altered by Brucella infection reveal host mechanisms specific to pathogenic *B. melitensis*

Angie Mathison, Linda Eskra, and Gary Splitter- Department of Animal Health and Biomedical Sciences, University of Wisconsin-Madison, Madison, WI

Immunology:

Moderator – Don Davis

2:30 pm

The *B. abortus* Type IV secretion system contributes to evasion of CD4-dependent adaptive immunity

Hortensia García Rolán and Renée Tsolis, Texas A&M University College of Medicine

2:45 pm

The Dendritic Cell: A Highly Permissive Host Cell for *Brucella* Development
Elisabeth Billard, Jacques Dornand and Antoine Gross. INSERM U431 University of Montpellier 2, Place E Bataillon, 34095 Montpellier, France

3:00 pm

A *B. abortus* B-lymphocyte mitogen is a proline racemase
Juan M. Spera, D. Comerci, J. E. Ugalde and Rodolfo A Ugalde
Instituto de Investigaciones Biotecnológicas (IIB-UNSAM), Universidad de Gral. San Martín, San Martín, Buenos Aires, Argentina.

3:15 pm

Molecular and morphological study of the bovine ileal Peyer's patch response to *Brucella abortus*. C. A. Rossetti*, J. Nunes, S. Lawhon, J. Figueiredo, T. Gull, S. Khare and L. G. Adams. Dept. of Veterinary Pathobiology, College of Veterinary Medicine, Texas A&M University, College Station, TX.

3:30 – 4:00 pm Break and Posters

Vaccine:

Moderator – Sally Cutler

4:00 pm

Attenuation and protective efficacy in mice of a *Brucella melitensis* hfq mutant
M.P. Nikolich[1], M.J. Izadjoo[1], R.M. Roop II[2], and D.L. Hoover[1]. [1]Department of Bacterial Diseases, Walter Reed Army Institute of Research, Silver Spring, MD 20910;[2]Department of Microbiology and Immunology, Eastern Carolina University School of Medicine, Greenville, NC 27858

4:15 pm

***Brucella melitensis* candidate vaccine MNPH1 performance in rhesus macaques challenged by conjunctival route with virulent *Brucella melitensis* strain 16M**
R.H. Borschel[1], M.P. Nikolich[1], R.M. Roop II[2], and D.L. Hoover[1]. [1]Department of Bacterial Diseases, Walter Reed Army Institute of Research, Silver Spring, MD 20910;[2]Department of Microbiology and Immunology, Eastern Carolina University School of Medicine, Greenville, NC 27858

4:30 pm

Carboxyl-Terminal Protease Deficient *Brucella suis* is Attenuated and Protects Mice Against *B. suis* Infection
Aloka B. Bandara[1], Victor Dobrean[1], Ian T. Paulsen[2], Rekha Seshadri[2], Nammalwar Sriranganathan[1], Gerhardt G. Schurig[1], and Stephen M. Boyle[1]
[1]Center for Molecular Medicine and Infectious Diseases, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061, [2]The Institute for Genomic Research, Rockville, Maryland 20850

4:45 pm

Multivalent Swine Brucellosis Vaccines
Philip H. Elzer, Lorraine Molin, Joel W. Walker and Sue D. Hagius.
Louisiana State University AgCenter, Baton Rouge, LA

POSTERS

Presenters: Please have your posters displayed prior to the 10:15 am break on Saturday. **P1** through **P8** will be presented during the morning; **P9-P15** in the afternoon. Authors (or coauthors) should remain in the vicinity during the designated break on Saturday to answer any questions about their posters. Thank you.

P1. Brucella co-opts the small GTPase Sar1 for intracellular replication
Jean Celli[1,2] and Jean-Pierre Gorvel[1]. [1]Centre d'Immunologie INSERM-CNRS-Université de la Méditerranée de Marseille-Luminy, Parc scientifique et technologique de Luminy, case 906, 13288 Marseille cedex 09, France. [2]Present address: Laboratory of Intracellular Parasites, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, MT 59840, USA

P2. Cytopathic effects on J774.A1 macrophages by rough *Brucella abortus wboA* mutants
Xicheng Ding[1], Stephen Boyle[2], Nammalwar Sriranganathan[2], Gerhardt Schurig[2], Yongqun He[1] 1-Virginia Bioinformatics Institute; 2-Virginia-Maryland Regional College of Veterinary Medicine; Virginia Polytechnic Institute and State University, Blacksburg, VA

P3. Brucella genomic islands and pathogenesis
Mike Krepps, Gireesh Rajashekara, David Glover, and Gary Splitter. Department of Animal Health and Biomedical Sciences, University of Wisconsin-Madison, Madison, WI

P4. Induction of Specific Mucosal and Systemic Immune Responses by Intranasal Inoculation of Mice with Live and Gamma-Irradiated Recombinant *Brucella abortus* RB51
Neelima Sanakkayala, Jatinder Gulani, and Ramesh Vemulapalli. Department of Veterinary Pathobiology, School of Veterinary Medicine, Purdue University, West Lafayette, IN

P5. Protection conferred by *B. ovis* outer membrane proteins (OMP), vesicles and a bacterin in a murine model.
Salas-Téllez, E.[1] Núñez del Arco, A.[1] Tenorio-Gutiérrez, V.[2] Díaz-Aparicio, E.[2] de la Garza, M.[3] Suárez-Güemes F.[4*] [1] Facultad de Estudios Superiores Cuautitlán. UNAM. [2] CENID Microbiología, Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias. [3] Centro de Investigaciones y de Estudios Avanzados del IPN. [4] Departamento de Microbiología e Inmunología, Facultad de Medicina Veterinaria y Zootecnia, UNAM, México

P6. Preliminary anaylses of the alternative sigma factors of *Brucella abortus* 2308

Michael L. Hornback, John E. Baumgartner and RM Roop II; East Carolina University, Greenville, NC 27834

P7. The *Brucella abortus* Rhizobial Iron Regulator (RirA) homolog is necessary for virulence in the murine model of infection

Eric S. Anderson[1], James T. Paulley[1], Jonathan D. Todd[2], Andrew W. B. Johnston[2] and RM Roop II[1]; East Carolina University, Greenville, NC 27834[1], University of East Anglia, Norwich NR4 7TJ, UK[1]

P8. Isolation and Characterization of new Mexican Brucellaphages

N. Aréchiga-Ceballos, V. Flores-López, J. A. Quiroz-Limón and A. López-Merino. Departamento de Microbiología. Escuela Nacional de Ciencias Biológicas. Instituto Politécnico Nacional, México City, México

P9. Identification of *Brucella abortus* S19 in milk, during control program with *B. abortus* RB51

Luary Martínez-Chavarría., Antonio Verdugo-Rodríguez and Rigoberto Hernández-Castro. Departamento de Microbiología e Inmunología. Facultad de Medicina Veterinaria y Zootecnia. UNAM. México

P10. Macrophage screening of a mutant bank generated from *Brucella melitensis* by Mariner transposon mutagenesis

Qingmin Wu, Jianwu Pei, Carol Turse, and Thomas A. Ficht. Department of Veterinary Pathobiology, Texas A&M University and Texas Agricultural Experiment Station, College Station, TX 77843-4467

P11. Assessment of the stability of *Brucella* VNTR loci in outbreak situations and in experimental infection

A. M. Whatmore, S. J. Shankster, T.J. Murphy, L.L. Perrett, S. J. Cutler, and A. P. Macmillan. Dept. of Statutory and Exotic Bacterial Diseases, Veterinary Laboratories Agency, Addlestone, Surrey, United Kingdom, KT15 3NB

P12. Initial Characterization Of The Involvement FdtA and FiuA In The Transport Of Dihydroxybenzoic Acid By *Brucella abortus*

James T. Paulley, Eric S. Anderson, and RM RoopII; East Carolina University, Greenville, NC 27834

P13. Two *B. abortus* specific targets for real-time PCR assays

D.T. Newby, M.E. Gehring, and F.F. Roberto, Biotechnology Department, Idaho National Engineering and Environmental Laboratory, Idaho Falls, ID 83415-2203

P14. Expression of recombinant proteins of Brucella suis and evaluation of their activity

Xuan Z. Ding, Apurba K. Bhattacharjee, Mikeljon P. Nikolich, Ian Paulsen* Garry Myers*, Rekha Seshadri* and David L. Hoover. Walter Read Army Institute of Reseach, Silver Spring, MD 20910. *The Institute for Genomic Research, Gaithersburg, MD

P15. Non-replicative, gamma irradiated *B. abortus* vaccine strain RB51 is protective against virulent *Brucella* challenge in mice

Andrea Contreras[1], S.M. Boyle[1], N. Sriranganathan[1], R. Vemulapalli[2] and G.G. Schurig[1]. [1]Virginia-Maryland Regional College of Veterinary Medicine; Virginia Tech, Blacksburg, VA; [2] Department of Veterinary Pathobiology, School of Veterinary Medicine, Purdue University, West Lafayette, IN

Sunday, November 14, 2004

Epidemiology:

Moderator – Tom Ficht

8:30 am

Strain diversity identified by HOOF-Print genetic typing of a large collection of *B. abortus* field isolates

Betsy J. Bricker[1]* and Darla R. Ewalt[2]. [1]USDA, ARS, National Animal Disease Center, 2300 Dayton Rd, Ames, IA, 50010, USA; [2]USDA, APHIS, Veterinary Services, National Veterinary Services Laboratories, 1800 Dayton Rd, Ames, IA, 50010, USA

8:45 am

Application of HOOF-Print technology as an epidemiological tool in a natural outbreak of *Brucella abortus*

Betsy J. Bricker[1*], Darla R. Ewalt[2], Donald L. Montgomery[3], Kenneth W. Mills[3], Donal O'Toole[3], William H. Edwards[4], Walter E. Cook[5], Allen E. Jensen[1], James R. Logan[6], Bret A. Combs[7], Steven C. Olsen[1]. [1]USDA, ARS, National Animal Disease Center, 2300 Dayton Rd, Ames, IA, 50010; [2]USDA, APHIS, Veterinary Services, National Veterinary Services Laboratories, Ames, IA; [3]Wyoming State Veterinary Lab (WSVL), Dept. of Vet Science, University of Wyoming, Laramie, WY; [4]Wyoming Dept. of Game & Fish, Laramie, WY; [5]Assistant State Veterinarian, Cheyenne, WY; [6]State Veterinarian, Cheyenne, WY; [7]AVIC, USDA-APHIS, Cheyenne, WY

9:00 am

Current Status of the National Brucellosis Eradication Program

Dr. Debbi Donch, USDA-APHIS-VS, Riverdale, MD

9:30 – 10:00 am Break

10:00 am

USDA Regulatory Explanation

Dr. LeeAnn Thomas, USDA-APHIS

11:00 am

Business meeting

Oral Abstracts

The Genome

1. The *Brucella* Bioinformatics Portal and *Brucella* Genome Curation in the Bioinformatics Resource Center at the Virginia Bioinformatics Institute

Yongqun He[1], Wenjie Zheng[1], Stephen Boyle[2], Oswald Crasta[1], Joao Setubal[1], Bruno Sobral[1]. 1. Virginia Bioinformatics Institute, Virginia Polytechnic Institute and State University, 1880 Pratt Drive, Blacksburg, VA 24061-0477
2. Center for Molecular Medicine and Infectious Diseases, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Polytechnic Institute and State University, 1410 Price's Fork Road, Blacksburg, VA 24061-0342

Virginia Bioinformatics Institute (VBI) and its partners have been awarded a 5-year, $10.3 million contract from NIH-NIAID to establish a national Bioinformatics Resource Center (BRC) that consists of a multi-organism relational database in support of infectious disease research (http://brc.vbi.vt.edu/). *Brucella* is one of the targeted pathogens. Prior to the VBI BRC award, we had developed a web-based *Brucella* Bioinformatics Portal (BBP, http://bbp.vbi.vt.edu) using PHP and Oracle. BBP provides organized links to *Brucella* genomics, transcriptomics, and proteomics data and well-recognized tools for data analysis. The BBP InterBru database system is developed to make integrative connections between existing databases, store original and analyzed data, and allow users to query their interested *Brucella* gene and protein information. The BBP Publications page uses PHP scripts to weekly extract and display updated *Brucella* and brucellosis publications from PubMed, offers the email alert service for the registered members, and categorizes important *Brucella*-related publications and web links. The BBP Contacts database system is developed to store brucellosis researchers' contact information and allow users to query and enter their own information. We also provide an interactive forum for discussion of any *Brucella* related topics. BBP will be evolved by the VBI BRC team and as a component to serve the *Brucella* community. We welcome and expect the involvement and collaboration of the *Brucella* community in the development of the *Brucella* resource of the BRC. Any feedback can be emailed to: *Brucella*-BRC@vbi.vt.edu).

2. Do Brucella have pathogenicity Islands?

Gireesh Rajashekara, Jeremy Glasner, David Glover, Mike Krepps, Dana
Tackes and Gary Splitter Department of Animal Health and Biomedical Sciences, University of Wisconsin-Madison, Madison, WI

3. Improved Expression Vector for *Brucella* species.

Mohamed N. Seleem, [1] Neelima Sanakkayala, [2] Ramesh Vemulapalli, [2] Stephen M. Boyle,[1] Gerhardt G. Schurig, [1] and Nammalwar Sriranganathan [1]. [1] Virginia-Maryland Regional College of Veterinary Medicine, Virginia Polytechnic and State University, Blacksburg, Virginia 24061-0342, [2] School of Veterinary Medicine, Purdue University, West Lafayette, IN

An improved vector pNSGroE has been constructed for gene expression studies in *Brucella spp*. It is derived from the broad host range cloning vector pBBR1MCS. This new plasmid has several advantages over pBBR1MCS or its derivatives, it is (*i*) smaller in size, 2.9 kb, (*ii*) expresses proteins as His-tagged fusion for easy detection and purification, (*iii*) carries the *gro*E promoter for constitutive expression that is enhanced under conditions of stress in vitro and in vivo. Our expression studies using this improved vector in *B. abortus* strain RB51 indicated that the level of heterologous protein expression is higher with pNSGroE compared to pBBGroE vector. We have also demonstrated the ability of the new vector to express heterologous fusion proteins stably in *Brucella* species.

4. The genome of *Brucella abortus* 2308: comparison with *B. suis* and *B. melitensis*.

Comerci D[1], F Agüero[1], P Chain[2], M Tolmasky[3], E García[2] and R Ugalde[1].
[1]Instituto de Investigaciones Biotecnológicas (IIB-UNSAM), Universidad de Gral. San Martín, San Martín, Buenos Aires, Argentina. [2]Lawrence Livermore Nacional Laboratory, Livermore, CA. [3]California State University, Fullerton, CA, USA.

The complete sequence of the genome of *B. abortus* strain 2308 revealed the presence of two circular chromosomes, Chr I 2.121.359 bp and Chr II 1.156.950 bp. Both replicons have a G+C content of 58.2%. Chr I codes for 2,280 predicted ORFs, 41 tRNAs and 2 rRNAs operons. While Chr II codes for 1,214 predicted ORFs, 14 tRNAs and 1 rRNA operon. Comparison with *B. suis* and *B. melitensis* revealed a high degree of similarity among the three species and a different origin of both chromosomes. Chromosome I of the three species is highly conserved with a high degree syntheny. On the other hand Chr II revealed a plasmidic origin and has structural rearrangements as for example an important inversion of the central region present only in *B. abortus*. Most of the differences among the three species are the consequence of deletions and phage insertions. The pattern of inversion, deletions and insertions suggest that *B. abortus* and *B. melitensis* share a common ancestor which differ from that of *B. suis*, that seems to have evolved independently. Remarkably *B. suis* is the species that contains the highest number of unique genes (23) most of them as the result of horizontal transference. On the other hand *B melitensis* has only two unique genes that are transposases, thus it can be considered that as *B. abortus B. melitensis* has no species-specific genes. In additions to the already mentioned deletions, insertions and inversions, frameshift mutations and nonsense mutations are quite abundant in all the members of this group, with a total of 179 pseudogenes identified in *B. abortus*, 119 in *B. melitensis* and only 64 in *B. suis.* Given the absence of a large number of species-specific genes, gene inactivation rather than horizontal transfer, appear to play an important role in shaping the speciation of Brucellae.

Analysis of the functional categories of the inactivated genes shows that they are mainly involved in membrane transport, transcriptional regulation and synthesis of outer membrane structures. The process of reduction of transport complements could be the consequence of adaptation to intracellular lifestyle in a protected environment niche much more stable with a more constant supply of nutrients. Considering the high degree of gene conservation between the three species, the dissimilar accumulation of inactivated transcriptional regulators and outer membrane structures could be the result of the process of adaptation to a specific host. This suggests that virulence differences and host specificity observed between the three species may be related to divergence of regulatory functions.

Virulence

5. Biophotonic imaging to identify attenuated Brucella

Gary Splitter, David Glover, Gireesh Rajashekara, Menachem Banai, Mike Krepps
Department of Animal Health and Biomedical Sciences, University of Wisconsin-Madison, Madison, WI

6. *Brucella suis* requires MgtC for intramacrophage survival; a mechanism shared *Salmonella enterica* and *Mycobacterium tuberculosis*

Jean-Philippe Lavigne, David O'Callaghan and Anne-Béatrice Blanc-Potard
Institut National de la Santé et de la Recherche Médicale, Unité 431, UFR de Médecine, CS 83021, Avenue J.F. Kennedy, 30908 NIMES Cedex 02, France.

MgtC is a virulence factor required for intramacrophage survival and growth in low Mg^{2+} medium in *Salmonella enterica* and *Mycobacterium tuberculosis*, two pathogens that proliferate within phagosomes. A MgtC-like protein is present in *Brucella suis*, another intracellular pathogen that replicates within macrophages. We demonstrated that a *B. suis mgtC* mutant is deficient for survival within macrophages and growth in low Mg^{2+} medium. MgtC appears as a common virulence factor of intracellular pathogens that might be required to acquire magnesium in the phagosome.

7. The *Brucella abortus* sodA gene product is required for virulence in the C57BL/6J mouse model of infection.

Daniel W. Martin, Jason M. Gee, and RM Roop II; East Carolina Universtiy, Greenville, NC 27834

Brucella abortus is an intracellular pathogen of macrophages during chronic infections of man and cattle and must cope with reactive oxygen intermediates (ROIs) that act as a significant obstacle to growth within this niche. Both the oxidative burst from the host cell and the aerobic metabolism of *B. abortus* can produce superoxide anions ($O_2\cdot^-$). *B. abortus* encodes two superoxide dismutates (SOD) that function to detoxicify $O_2\cdot^-$. These include a Cu/Zn cofactored SOD, SodC, that is thought to be secreted into the periplasm and a Mn cofactored SOD, SodA, that is predicted to function within the cytosol. This study is focused on the characterization of the role of SodA in pathogenesis. A *sodA* mutant (JG1) was generated in the *B. abortus* 2308 background by allelic exchange. JG1 displayed a subtle growth defect in rich media. The *sodA* mutant was also found to be more sensitive than the wild-type 2308 to paraquat and menadione, known cytosolic $O_2\cdot^-$ generators. However, JG1 displayed wild-type sensitivity to the exogenous $O_2\cdot^-$ generator pyrogallol and hydrogen peroxide. These data support the notion that SodA functions to detoxify endogenous $O_2\cdot^-$ generated as a byproduct bacterial metabolism. The *sodA* mutant also displayed a pronounced attenuation in murine macrophages. Inhibition of the NADPH oxidase complex, which is responsible for generation of superoxide anions, failed to restore intracellular growth of the *sodA* mutant indicating that the reduced survival of JG1 within the macrophage could not be attributed to the oxidative burst. The defect in intracellular growth within macrophages was correlated to the inability of this strain to establish a chronic infection in experimentally infected C57BL6/J mice. These data suggest that SodA plays an

essential role in survival within macrophages and in the chronic model of infection. Currently, we are examining the nature of the defect resulting from *sodA* deletion and its relationship to protection from endogenously generated $O_2 \cdot^-$ and the establishment and maintenance of chronic infection in C57Bl6/J mice.

8. The Role of DhbR, an AraC-like transcriptional activator, in *Brucella abortus* iron acquisition

Eric S. Anderson, James T. Paulley and RM Roop II; East Carolina University, Greenville, NC 27834

Iron is essential to the survival of *Brucella*, but the mammalian host represents an extremely iron-restricted environment. In an effort to circumvent this restriction, *Brucella* synthesizes two catechol-type siderophores, 2, 3-dihydroxybenzoic acid (DHBA) and the more complex siderophore, brucebactin. Both are produced through the enzymatic activities of the products of the *dhb* operon, and expression of this operon is tightly regulated in response to environmental iron levels. Traditionally, iron-dependent regulation is under the control of the Ferric Iron Uptake Regulator (Fur). Preliminary analysis suggested typical Fur-dependent regulation, but an isogenic *fur* mutant constructed from *B. abortus* 2308 displays wild-type repression of *dhb* expression in response to iron-replete growth conditions, indicating that an alternate regulator controls expression of the *dhb* operon under low iron conditions.

One strategy employed by some bacteria to regulate siderophore biosynthesis is the use of AraC-like transcriptional activators. Examples of these activator proteins are YbtA (yersiniabactin A) of *Yersinia pestis* and AlcR (alcaligin biosynthesis regulator) of *Bordetella bronchiseptica*. In these organisms, the end product siderophore serves as a co-activator in conjunction with the AraC-like protein, and this activation is iron-responsive.

Brucella abortus 2308 possesses a homolog of the *B. bronchiseptica* AlcR. An isogenic *B. abortus alcR* mutant, BEA5, shows decreased catechol siderophore production under iron-deplete conditions, when compared to the parental 2308 strain, suggesting that the product of this gene, termed DhbR (dihydroxybenzoic acid regulator), functions as an activator of siderophore biosynthesis. Additional studies indicate that this regulation occurs at the level of transcription and is the result of direct interactions between DhbR and the *dhb* promoter region.

While DhbR is required to achieve maximal siderophore production, phenotypic studies suggest that DhbR may have additional regulatory functions. Current studies are focused on identifying these functions, and determining their contribute to iron homeostasis in *Brucella abortus*.

9. The RelA/SpoT homolog (Rsh) of *Brucella* spp., involved in stringent response, is essential for the virulence of the pathogen

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The stringent response, induced by nutrient stress such as amino acid depletion, results in physiological adaptation of the bacterium to the new environment. Recent studies

with intracellular pathogens suggest that the stringent response, via its effector molecule (p)ppGpp, is critical for timely interaction with eukaryotic host cells.

We have studied the role of the RelA/SpoT homolog of *Brucella* spp., named Rsh, in the virulence of the pathogen. *Rsh* deletion mutants in *B. suis* and *B. melitensis* grew normally in rich broth, but were characterized by altered morphology, and by the loss of growth capacity in minimal medium indicating that certain amino acid biosynthesis genes were not expressed. During stationary phase in rich broth, viability of the mutant rapidly declined as compared to the wild-type. In the macrophage and the HeLa cell models of infection, intracellular replication of the mutant strains was respectively abolished or considerably reduced. In the murine model, experiments with the *rsh* mutant of *B. melitensis* confirmed the reduced survival at 4 weeks post infection. Western blot analysis revealed the absence of VirB in the mutants under conditions allowing detection of VirB in the wild-type strains. *VirB*-promoter fusions to *gfp* confirmed these findings. Rsh therefore plays a key role in the setup of at least two major strategies adopted by *Brucella* to face the intracellular environment of the macrophage: adaptation to low-nutrient environment and escape from the endocytic pathway to a safe replicative niche via VirB.

In addition, RelA of *Sinorhizobium meliloti*, responsible for (p)ppGpp production in the plant symbiont and highly homologous to Rsh of *Brucella*, was able to functionally replace this *Brucella* virulence factor in a heterologous complementation assay performed in *B. suis*: Intramacrophagic multiplication and production of VirB were totally restored, describing the successful use of a gene from a plant symbiont to restore virulence in a facultatively intracellular pathogen of mammals.

10. The *Brucella abortus* xthA2 gene product contributes to resistance to reactive oxygen intermediates

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Upon phagocytosis, *Brucella abortus* is able to reside within host macrophages and survive the decrease in external pH, low nutrient availability, and exposure to reactive oxygen intermediates (ROIs) encountered in the phagosomal compartment. The reactive oxygen intermediates generated by the oxidative burst of host macrophages are toxic to bacterial cells because these ROIs can react with proteins, lipids, and DNA and the accumulation of these damaged molecules results in death. Bacteria, in general, are able to resist killing by synthesizing enzymes involved in repair of oxidatively damaged proteins, lipids, and DNA. Interestingly, studies involving *Salmonella* suggest that these repair mechanisms, specifically DNA repair, may play a more important role in protecting bacteria from the oxidative burst of host macrophages than primary anti-oxidants, such as catalase.

In *Escherichia coli*, the base excision repair pathway is involved with repair of oxidatively damaged DNA. A major component of the base excision repair pathway is exonuclease III, which is encoded by the *xthA* gene. In *E. coli*, *xthA* mutants have been shown to be hypersensitive to exposure of hydrogen peroxide suggesting this DNA repair pathway is necessary for survival of this organism in response to ROIs. Additionally, *Salmonella typhimurium* mutants that are AP endonuclease-deficient are killed in cultured macrophages and attenuated in the mouse model.

Analysis of the sequenced genome of *Brucella melitensis* 16M has revealed the presence of two *xthA* homologs which has been designated *xthA1* and *xthA2*. An isogenic *xthA2* mutant constructed from *B. abortus* 2308 exhibits increased sensitivity to DNA damaging agents and hydrogen peroxide *in vitro*, but displays wild-type virulence in cultured murine macrophages and experimentally infected mice. These findings indicate that the *xthA2* gene product contributes to the resistance of *B. abortus* 2308 to oxidative damage but is not required for wild-type virulence in the mouse model. Interestingly, all attempts to making an *xthA1* mutant have been unsuccessful. Current studies focus on determining if the *xthA1* gene is essential for viability of *B. abortus*, whether both *xthA1* and *xthA2* gene products have AP endonuclease activity, and determining the nature of gene transcription of these genes.

11. The bhuA Gene Product of *Brucella abortus* Is Required For The Utilization of Hemin and The Maintenance of Chronic Infection In BALB/c Mice

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The ability to survive within host macrophages is critical for the pathogenesis of *Brucella* spp. In order to live in their intracellular niche the brucellae must withstand a wide variety of assaults from the host macrophage. One such assault the brucellae must address is severe iron restriction. Within the macrophage heme and heme containing proteins are thought to be relevant iron sources that may serve as an iron source to the invading brucellae. To date, no outer membrane iron transport proteins have been characterized for *B. abortus*, however, mutations in the ferrochelatase gene (*hem*H) of *B. abortus* 2308 produce heme auxotroph mutants that can survive with the addition of exogenous hemin, indicating the presence of hemin transport machinery in *B. abortus* 2308. Searches of the *Brucella melitensis* 16M genome reveal the presence of an open reading frame with significant homology to genes encoding the outer membrane hemin receptors of other pathogenic bacteria. The analogous genetic locus was targeted for mutagenesis in *Brucella abortus* 2308 to evaluate the role the corresponding gene product plays in iron acquisition from heme and its contribution to the virulence of B. abortus 2308. Transcription of the gene designated *bhuA* (*B*rucella hemin utilization) appears to occur in all media conditions tested to date, however, the transcription appears to be iron dependent upon entry into stationary phase. Despite being transcribed in both rich and low iron minimal media mutation of the *bhu*A gene only leads to altered growth profiles under low iron conditions, demonstrating a dramatic decrease in viability during stationary phase. This decrease in the *B. abortus bhuA* mutant's viability can be eliminated by the addition of $FeCl_3$, but not the addition of hemin. Also, the *bhuA* mutant exhibits defective survival and replication in cultured murine macrophages and is unable to maintain chronic spleen infection in experimentally infected BALB/c mice. These findings indicate a role for the *bhuA* gene product in hemin utilization and suggest that hemin may represent an important iron source for the brucellae during establishment and maintenance of chronic infection in their mammalian hosts.

12. Characterization of a small molecule inhibitor of the VirB11 family of type IV secretion components

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Type IV secretion systems (T4SS) are required for the virulence of several Gram-negative pathogens of mammals and plants (1). They are typically composed of 10 to 12 protein components, which translocate virulence factors across the bacterial envelope into host cells. The T4SS of *Brucella suis* is essential for intracellular survival of the bacteria (2). It is believed to secrete one or more virulence factors, which modify endosomal trafficking to assure bacterial multiplication in a specialized vacuolar compartment (3). As T4SS are essential virulence factors they are ideal targets for the development of inhibitors, which could be applied as antimicrobial drugs. Such drugs would essentially disarm the pathogen permitting its clearance from the body by the immune system. VirB11 is an essential component of the *Brucella* T4SS, which resides in the cytoplasm and it is believed to energize T4SS assembly and/or substrate translocation. Its ATPase enzyme activity can be readily measured and this permits the setup of assays for high-throughput screening of small molecule compound libraries. We here report on the characterization of an inhibitor isolated from a combinatorial library. The molecule inhibits the ATPase activity of different VirB11 orthologs *in vitro* and T4SS function *in vivo*. The application of this molecule for basic research on T4SS function will be discussed.

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13. The quorum-sensing related transcriptional regulator VjbR controls expression of the type IV secretion and the flagellar genes in *Brucella melitensis* 16M

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The intracellular trafficking of *Brucella*, a pathogenic bacterium of the alpha-proteobacteria group, is essential for its infectious cycle. VirB, a type IV secretion system, is essential for the correct intracellular trafficking of Brucella. Here we report the identification of VjbR, a quorum sensing-related transcriptional regulator, that is acting as an activator of

the virB operon expression. In a cellular infection model, the vjbR and virB mutants associate with the same markers at the same times post-infection, which suggest they are stopped at the same stage during vacuole maturation. In the vjbR mutant, the virB expression is affected both in vitro and ex vivo. The vjbR mutant is also strongly attenuated in a mouse model of infection. Since C12-homoserine lactone pheromone is known to be involved in virB repression, we postulated that VjbR is mediating this effect. In fact, using a VjbR mutant complemented with a copy of the VjbR gene unable to bind HSL we were unable to show a repression of the virB expression by exogenous HSL.

The same regulator (VjbR) was also shown to activate the expression of flagellar genes (FliF, FlgE). The same genes were repressed by addition of C12-HSL. The effect of VjbR on the MS ring gene transcription is subordonned to the action of a other transcriptional regulator (FtcR) also necessary to express genes of the flagellar cascade. Actually while a FtcR mutant cannot be complemented by the VjbR gene in trans, the expression of the FliF gene in aVjbR mutant is fully complemented by a wild type copy of FtcR under the pLac promotor control.. Our data support a model in which VjbR acts as a major regulator of the two external appendages described in B. melitensis , the virB operon and the flagellum and as such play a major role both in the acute and the chronic phase of the infection in IP infected mice..

Cellular Microbiology

14. O-antigen controls macrophage uptake and the ultimate fate of *Brucella*

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Brucella infection reportedly inhibits monocyte and macrophage apoptosis while stimulating a proinflammatory response. However, experiments have revealed that the absence of O-antigen results in enhanced proinflammatory response suggesting that LPS prevents or limits macrophage activation. For these reasons we explored the initial interaction between *Brucella* and macrophages. Initially we determined the dynamics of *Brucella* uptake by J774.A1 using differential staining for intra- and extra-cellular bacteria. The internalization of smooth *Brucella* reached saturation in less than 3 minutes, while rough *Brucella* internalization continued up to 30 minutes, resulting in a 10-fold difference in overall uptake. These results were confirmed by F-actin and EEA1 colocalization. Treatment with various inhibitors (wortmannin, cytochalasin D, CTB and MβC) confirmed these and previous results by others suggesting that rough and smooth *Brucella* invade macrophages by different pathways. In addition, rough *Brucella* induced NF-κB p65 translocation, nitric oxide, cytokine production and osmotic cell death of the macrophages. Although this effect has been attributed to the increased uptake of rough *Brucella* reduction of MOI to provide equivalent uptake was shown to delay, but did not prevent macrophage death. Additional studies demonstrated that macrophage cell death caused by rough *Brucella* infection requires bacterial protein synthesis and direct interaction with the macrophages. We conclude that *Brucella* O-antigen is essential in establishing a successful infection by limiting invasion and preventing macrophage activation as a prelude to successful colonization of the host.

15. Absence of evidence for the participation of the macrophage cellular prion protein in infection by *Brucella suis*

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In a recent paper Watarai M et al. (*J Exp Med*. 2003 ; 198: 5-17) have published that "Cellular prion protein promotes *Brucella* infection into macrophages". Prion protein is anchored on the surface membrane and localizes within lipids rafts that have been demonstrated to be essential for *Brucella* phagocytosis. We thus decided to analyse the molecular mechanisms involved in this process. To our surprise, we cannot inhibit or modify *Brucella suis* internalisation using many different antibodies directed against the prion protein either in human (THP-1 cell line) or in mice (J774 cell line) macrophages.

It was also proposed that past phagocytosis, prion protein directs the bacteria towards a safe niche for replication. To test this hypothesis we compared the infection kinetics in macrophages derived from either wild-type C57BL/6 mice or from $PrP^{-/-}$ mice of the same genetic background (These mice were backcrossed 12 times from the Weissmann PrP-KO strain). Contrary to Watarai's results, we cannot evidence any differences neither in phagocytosis nor in *B. suis* intramacrophagic multiplication. Because *B. suis* regulates differently the VirB secretion system, we hypothesized that presentation of Hsp60, supposed to be the receptor for PrP^{C} on *Brucella*, could explain the discrepancies between our results and those published by Watarai's group. Indeed, we cannot evidence Hsp60 on the surface of *B. suis*, but the control on *B. abortus* was also negative. Temperature stress did not induce this presentation at the surface.

We thus decided to repeat the previous experiments using *B. abortus* .Our results were identical to those obtained with *B. suis*. Comparisons of $PrP^{-/-}$ and WT C57BL/6 infections are in progress.

16. Microarray Analysis of Macrophage Gene Transcription in Response to Infection *in vitro* with Virulent *Brucella melitensis* at Different Time Points

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Brucella spp. are facultative intracellular bacteria that cause chronic brucellosis in animals and humans. Virulent *Brucella* infect and replicate within host macrophages and the macrophage-*Brucella* interaction is the basis for establishment of chronic *Brucella* infections. After infection of murine macrophages, virulent *Brucella* undergo rapid clearance (>90% of *Brucella* killed) within the first 24 h followed by growth of the surviving bacteria. We utilized the Affymetrix mouse genome 430 2.0 array to analyze the mouse J774.A1 macrophage response against *B. melitensis* 16M infection at 0, 4, 24, and 48 hours post infection. A total of 12 microarray chips were used with 3 chips for each time point. The microarray image data was processed by Affymetrix GCOS program and GeneSpring (ver. 7.0) was used for microarray data analysis. Real-time RT-PCR was performed to confirm expression changes in selected genes of the microarray. Out of 45,101 mouse genes in the

array, 22,831 genes were selected for further analysis by filtering out genes either not exhibiting transcription in any one chip or exhibiting very low signals. By using the Welch t-test with Benjamini and Hochberg multiple testing correction, transcription of 1770 genes was significantly up- or down-regulated between 0 and 4 hours. Among these genes, 485 genes showed at least 2-fold changes. Only 12 genes were found with significant transcriptional differences at 0 and 24 hours and four of theses genes were differentially changed by at least 2 fold. No macrophage gene transcription was found to be significantly different at 0 and 48 hours. The differentially regulated genes observed cover a broad range of host molecules including proinflammatory molecules, receptors, signaling molecules, and transcription factors. These results indicate that the most significant macrophage changes happened during the very early infection stage but returned to normal by 24 hours post-infection. Overall, a rather small number of macrophage genes (i.e. app. 4%) change their expression level early following *in-vitro* infection, yet the vast majority of *Brucella* are killed. It is tempting to speculate that the killing of *Brucella* by macrophages may not be dependent on significant changes in gene expression but on activation and inhibition of existing mechanisms. The return to normal macrophage gene expression profiles at 24 and 48 hours post-infection correlates with intracellular replication of *Brucella* during this time. These results are consistent with the notion that the surviving *Brucella* mask themselves from their host by replicating in membrane-bound compartments.

17. Macrophage transcript levels altered by Brucella infection reveal host mechanisms specific to pathogenic *B. melitensis*.

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Immunology

18. The *B. abortus* Type IV secretion system contributes to evasion of CD4-dependent adaptive immunity

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19. The Dendritic Cell: A Highly Permissive Host Cell for *Brucella* Development.

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Dendritic cells (DCs) are sentinel cells which sense the presence of pathogenic microbes in the mucosal environment. They are the most potent professional antigen presenting cells and their normal function is essential for the induction of an efficient immune response. *Brucella* use macrophages as cellular niche in infected organisms. Disturbing several aspects of immune response, they can develop in these cells and persist chronically in the host. Macrophages and DCs are phylogenetically closely related and if they are functionally distant, these cells display some common properties (phagocytosis, cytokine secretion...). Therefore, because of their location in mucosal tissues, DCs appear as potential targets 1) to convey *Brucella* to peripheral organs and 2) to circumvent the immune system. To check these possibilities, we first assess whether *Brucella* are able to invade DCs. Microscopic analysis and CFU determination reveal that *B. suis* infects monocyte-derived

DCs at a much higher extent than monocyte-derived macrophages. Moreover, it was observed that during the first 48h following infection, *B. suis* replicated in DCs, exactly as in macrophages. Furthermore, the bacterial multiplication was VirB-dependent. Finally, DC infection with *Brucella* denoted a great permissiveness of these cells. These data were rather unexpected since other intracellular pathogenic bacteria are either destroyed or do not proliferate in DCs. *Brucella* penetration through lipid-rafts allows the avoidance of the phagosome/lysosome fusion which is required for the establishment of the bacteria. Comparative analysis of this initial step of infection showed differences in the phagocytosis process between DCs and macrophages. It demonstrated a peculiar interaction between *Brucella* and human DCs, compared to macrophages. Altogether, the results suggested that DCs might serve as a reservoir for host invasion by the pathogen. This novel information which concerns the spreading of *Brucella* within their host, seems of importance regarding the crucial role of DCs in the establishment of the specific immune response. Further studies should clarify now the way by which *Brucella* corrupt the crucial function of one of their host cells.

20. A *B. abortus* B-lymphocyte mitogen is a proline racemase

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Polyclonal activation is a general immunological feature following the infectious process. Mitogens and superantigens are part of the strategy used by some microorganisms to avoid host specific immune response and to ensure persistence.These molecules may be responsible for the initiation of non-specific (polyclonal) immune responses.

The complex antigenic composition of microorganisms might be the main cause of hyper-stimulation of the immune system thus leading to the expansion of lymphocyte clones specifically directed to the multitude of challenging antigens. The indiscriminate utilization of B- and T-cell genes encoding BCR and TCR is incompatible with oligoclonal responses. It have been proposed that the difficulties faced by immune system to eliminate micro-organisms may rely on the fact that parasites, viruses, fungi and bacteria trigger non-specific polyclonal B- and T- lymphocyte responses and ensuring evasion through avoiding efficient control at the beginning of the infection. A T-cell independent B-cell mitogen was recently identified and characterized in the pathogenic protozoa *T. cruzi.* The protein belongs to a family of proline-racemases present in a restricted number of bacteria and in humans. The *T. cruzi* protein is active on inducing B-lymphocytes proliferation and the mitogenic activity was found to depend on the integrity of the enzymatic activity.

We have recently cloned and expressed two *B. abortus* genes named RAC1 and RAC2 (for proline-racenase 1 and 2). RAC1 codes for a putative proline-racemase with a conserved active catalytic site and RAC2 codes for a protein with a partially conserved proline racemase active site in which a Cys residue of the active site is changed by a Thr. Both proteins were expressed in *E. coli*, purified to nearly homogeneity and lymphocyte mitogenic activity was determined. Our results indicate that RAC1 is an active T-cell independent B-cell mitogen while RAC2 is inactive. RAC1 mitogen activity depends on conservation of the enzymatic activity since site directed mutagenesis of the enzymatic active site abolished the activity.

The presence in *Brucella* of a gene that may induce a non-specific polyclonal B-lymphocyte response may ensure infection through avoiding efficient immune control at the beginning of the infection. In this context RAC-1 may be considered a virulence gene that prevents mounting, during the initial stage of infection, a specific immune response until the pathogen enters and colonize the intracellular niche.

21. Molecular and morphological study of the bovine ileal Peyer's patch response to *Brucella abortus*

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The objective of this study is to characterize the response of bovine Peyer's patches (PP) to infection with *Brucella abortus*. A 12 h ligated ileal loop non-survival surgical procedure was performed under general anesthesia in 3 week-old male, brucellosis free Holstein calf. Fourteen - 8 cm loops were ligated in the ileum and distal jejunum; seven were injected with 3ml containing 1.8 $x10^9$ *B. abortus* S19 CFU/ml and seven were injected with 3 ml of phosphate buffer solution as control loops. One infected loop and one control loop were removed, and tissue samples for bacteriology, histopathology, TEM, SEM and microarray gene expression profiling were collected at 0.25, 0.5, 1, 2, 4, 8 and 12 h post-inoculation (PI). *Brucella abortus* S19 was recovered from infected loops as early as 15 min PI at $1x10^6$ CFU/g intestinal tissue, higher numbers were recovered at 4 h PI ($1x10^7$ CFU/g), and then decreased to the initial value in the later time points. Neither fluid nor macroscopic lesions was observed in any loop during the surgery. Histological observation did not identify any differences between infected and non-infected loops. Microarray analysis revealed an up-regulation of pro-apoptotic genes and down-regulation of anti-apoptotic genes by 1 h PI, but an opposite tendency after 4 h PI; up-regulation of MHC I & II expression, Fe chelator and transcriptional and translational factors, and down-regulation of cell adhesion and cell-cell contact, intestinal mucous production and stability, and cell growth and cycle progression throughout the 7 time points. These results constitute a preliminary approach to characterize and understand the *B. abortus*:bovine host initial interactions.

Vaccine

22. Attenuation and protective efficacy in mice of a *Brucella melitensis* hfq mutant

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The majority of the *hfq* coding sequence in strain *Brucella melitensis* strain 16M was deleted and replaced with a kanamycin resistance marker. The resulting mutant, designated strain MNPH1, exhibited reduced survival inside human monocyte-derived macrophages and attenuated persistence in BALB/c mice after oral introduction. The attenuation of strain MNPH1 indicated by these results was comparable to that of *B. melitensis purEK* mutant WR201, though disseminated persistence appeared to be reduced relative to WR201 in a

male mouse model. Oral immunization of BALB/c mice with a single 10^{11} CFU dose of live strain MNPH1 did not provide significant protection to mice against intranasal challenge. However, two or three such doses of live vaccine MNPH1 did provide significant protection to mice against intranasal challenge in this model.

23. *Brucella melitensis* candidate vaccine MNPH1 performance in rhesus macaques challenged by conjunctival route with virulent *Brucella melitensis* strain 16M. R.H. Borschel[1], M.P. Nikolich[1], R.M. Roop II[2], and D.L. Hoover[1]. [1]Department of Bacterial Diseases, Walter Reed Army Institute of Research, Silver Spring, MD 20910;[2]Department of Microbiology and Immunology, Eastern Carolina University School of Medicine, Greenville, NC 27858.

Brucella melitensis human vaccine candidate strain MHPH1, an *hfq* deletion mutant, was tested for immunogenicity, persistence, safety, and efficacy in adult male rhesus macaques (*Macacca mulatta*) towards the development of a live attenuated vaccine for human use. All animals were surgically implanted with temperature data loggers, and bled twice weekly for blood cultures, complete blood counts, serum chemistries, and specific anti-Brucella LPS antibody. The vaccine was tested in three separate experiments beginning with a safety and immunogenicity study of the vaccine when given by gavage at a dose of $1x10^{12}$ and $1x10^{11}$ cfu in groups of 4 adult male macaques. The animals were sacrificed at 4 weeks and spleen, liver, lung, testis, epididymus, brain, bone marrow, kidney, and 6 lymph nodes cultured for viable *Brucella*, and tissues examined by histopathological techniques. No fever or histological evidence of acute brucellosis was observed; however, blood and fecal cultures indicated that the dose of *Brucella* was too large and that bacteremia and fecal shedding may present a problem. Additionally, all lymph nodes remained positive for viable vaccine organisms at 4 weeks following vaccination. In order to compare MNPH1 to the previously tested purine auxotrophic attenuation mutant WR201, the vaccine was tested for efficacy in macaques vaccinated with $1x10^{11}$ cfu of MNPH1and challenged 8 weeks after vaccination with $1x10^{7}$ cfu of strain 16M given to the conjunctiva in a manner identical to that used to test WR201. The vaccinated animals were bacteremic as seen previously and viable vaccine organisms were recovered from the feces up to 48 hours after gavage. The vaccine had 75% (3 of 4 protected) efficacy and the one animal having no protection had reduced disease. All animals had significant antibody titers. In an attempt to reduce bacteremia and fecal shedding and to increase efficacy, an additional efficacy study was conducted. In this study rhesus macaques were vaccinated twice 4 weeks apart using $1x10^{10}$ cfu of MNPH1 given by gavage. No fecal shedding was observed but one of the 6 vaccinated animals demonstrated bacteremia. None of the animals developed significant antibody titers and there was 0% efficacy when challenged 8 weeks following the second vaccine dose of $1x10^{7}$ cfu strain 16M. There were no differences in response between the vaccinated animals and the sham-vaccinated controls. The results from this study indicate that the vaccine candidate MNPH1 is inadequate for use as a candidate for human trials and has similarities to the previously rejected human vaccine candidate Rev1.

24. Carboxyl-Terminal Protease Deficient *Brucella suis* is Attenuated and Protects Mice Against *B. suis* Infection

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Carboxyl-terminal processing protease (CtpA) of *Brucella suis* 1330 is a member of a novel family of endoproteases shown to be involved in the maturation of proteins destined for the cell envelope. *B. suis* CtpA shared 31% homology with the C-terminal protease Prc of *Escherichia coli* and up to 77% homology with the C-terminal proteases of other bacteria. The 1.3-kb *ctpA* gene encoding CtpA of strain 1330 was cloned into a suicide plasmid and a 471-bp segment was replaced with a kanamycin resistance gene (Kan^R). The suicide plasmid was electroporated into wild type strain 1330 and a double-crossover recombination event selected that resulted in strain 1330Δ*ctpA*. The *ctpA* gene was also cloned into a broad host range plasmid and electroporated into the mutant 1330Δ*ctpA* to create a complemented strain 1330Δ*ctpA*[pBB*ctpA*]. On enriched trypticase soy agar, colonies of the CtpA-deficient strain appeared one-third to one-half the size compared to colonies of the wild type or complemented strains. The CtpA-deficient mutant also exhibited a 50% decrease in growth rate in enriched trypticase soy broth. In salt-free growth media, the CtpA-deficient strain exhibited zero growth exactly as reported for Prc-deficient *E. coli.* Prc is involved in C-terminal processing of penicillin-binding proteins (PBP), which determine the cell size, cell shape and cell division of *E. coli*. Based on these data, we hypothesize that *B. suis* CtpA is involved in processing of PBPs, and therefore, disruption of CtpA expression affected at least growth/cell division. In splenic clearance studies using BALB/c mice, 1, 3, and 6 weeks after intraperitoneal inoculation, the CtpA-deficient mutant was recovered at 2.4, 1.1, and 2.4 $\log_{10}$ cfu less than the wild type strain, indicating that CtpA is necessary for intracellular survival of *B. suis*. Mice vaccinated with the CtpA-deficient strain developed similar titers of immunoglobulin IgG1 and IgG2a suggesting a balanced Th1 and Th2 response. In BALB/c mice, relative to injection with PBS, immunization with the CtpA-deficient strain induced significant protection against challenge with virulent *B. abortus* strain 2308 or *B. suis* strain 1330 but not *B. melitensis* strain 16M. This study is the first report correlating an endoprotease deficiency in *Brucella* with attenuation.

Key words: *Brucella suis*, carboxyl-terminal protease, penicillin-binding proteins, virulence, protection, attenuation.

25. Multivalent Swine Brucellosis Vaccines

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Brucellosis and pseudorabies lead to abortion in pregnant sows and are perpetuated by feral swine reservoirs. A multivalent oral vaccine for these diseases would improve vaccination and eradication programs worldwide. Previous studies have shown that the rough attenuated *Brucella* strains RB51 and VTRS1, when administered subcutaneously to swine, stimulate host immune responses, transiently colonize tissues, and provide partial protection against virulent *B. suis* infection in pregnant sows. A plasmid encoding for the

pseudorabies virus glycoprotein D (PRV gD) has also been added to these strains as part of this project. This study evaluates the use of these strains as oral vaccines for swine brucellosis and investigates the ability of these vaccines to colonize lymph nodes and stimulate the production of antibodies against rough *Brucella* antigens and PRV gD.

Epidemiology

26. Strain diversity identified by HOOF-Print genetic typing of a large collection of *B. abortus* field isolates.

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HOOF-Printing is a new technique for genotyping *Brucella* strains. It exploits the accelerated mutation rates observed in genomic regions containing large numbers of tandemly repeated DNA motifs. Previously we reported that the *Brucella* genome contains tandem repeats of the octamer, AGGGCAGT, in eight independent chromosomal loci distributed on both chromosomes. A PCR test was developed to categorize the number of repeat units at each locus based on the size of the amplified fragment. This report describes the genetic typing of a collection of 97 B. abortus field isolates.

Among the 97 field isolates, 93 unique genotypes were identified. Among the individual loci, the array of alleles varied significantly, ranging from one allele for Locus-8 to 14 alleles for Locus-1. The allele frequencies among the eight loci were also variable. We looked for potential geographic clustering of alleles. The USA was divided into 5 regions and the alleles associated with each region were identified. No clustering of alleles was found. We conclude that HOOF-Print genotyping continues to look promising as an epidemiological tool from trace back.

27. Application of HOOF-Print technology as an epidemiological tool in a natural outbreak of *Brucella abortus*.

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In November of 2003, four cows from a herd from Sublette County, WY tested seropositive for *Brucella*. The results were confirmed by the culture of *B. abortus* biovar 1. Further testing of the 391 head in the herd revealed at least 31 seropositive animals. Tissues from the slaughtered animals were cultured at NVSL and NADC. *Brucella* was isolated from a variety of tissues collected from 21 animals. To date, 398 individual bacterial colonies (clones) of *B.* abortus were subcultured from the tissues of these animals.

The 398 clones were analyzed by HOOF-Print genotyping using 10 chromosomal loci. Thirty-one genotypes were identified. A dominant fingerprint profile accounted for 54.3% of all cattle derived clones. The genotypes were compared to 26 other clones of *B. abortus* derived from two elk found in the same area but at different times. The genotype profiles derived from the two elk were as similar to and different from each other as they were to the genotypes isolated from the cattle herd. Although no single genotype was shared among the three sources, there were similarities that suggest potential genetic links among the three groups.

The study illustrates several points: 1) the importance of examining multiple individual colonies from multiple animals to assess the full range of genotypes present in each infected population; 2) the value of testing numerous repeat loci; and 3) the need to identify the *B. abortus* HOOF-Print genotypes present in populations of infected wildlife.

Abstracts of Poster Presentations

P1. Brucella co-opts the small GTPase Sar1 for intracellular replication

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The pathogen *Brucella abortus* resides inside macrophages within a unique, replication-permissive organelle that is derived from the endoplasmic reticulum (ER). Although dependent upon the Brucella type IV secretion system VirB, the mechanisms governing the biogenesis of this compartment remain elusive. Here we have investigated a putative role of the early secretory pathway in ER membrane accretion by the Brucella-containing vacuoles (BCVs). We show that BCVs interact with ER exit sites (ERES) and blockade of Sar1 activity, which disrupts ERES, prevents intracellular replication of Brucella. In cells expressing the dominant interfering form Sar1[T39N], BCVs do not acquire ER membranes, suggesting they are unable to mature into replicative organelles. By contrast, treatments that block subsequent secretory events do not affect bacterial replication. We propose that Sar1-dependent ERES functions, but not subsequent secretory events, are essential for the biogenesis of the Brucella replicative compartment, and thus bacterial replication. These results assign an essential role for Sar1 in pathogenesis of an intracellular bacterium.

P2. Cytopathic effects on J774.A1 macrophages by rough *Brucella abortus wboA* mutants

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Pei and Ficht reported that *Brucella abortus* rough mutants are cytopathic for macrophages in culture and suggested the presence of O-side chain during the early stages of host-*Brucella* interaction is essential to maintain and support persistent intracellular infection. Pei and Ficht also indicated that *B. abortus* vaccine strain RB51 caused a mildly reduced cytopathic effect in macrophages compared to other rough mutants. The *wboA* gene in strain RB51 is interrupted by an IS711 element that prevents the production of O-side chain. Complementation of strain RB51 with a functional *wboA* gene (RB51WboA) results in O-side chain synthesis but no change in rough phenotype because of its inability to export the O-side chain. To determine the role of the interrupted *B. abortus wboA* gene, we compared the cytopathic effects induced by virulent smooth *B. abortus* 2308, strain RA1 (only the *wboA* gene is mutated) as well as strains RB51 and RB51WboA. Viable bacterial counts showed significantly more efficient uptake of all the three rough mutants than smooth strain 2308. Strain RA1 caused extensive macrophage cell death due to necrosis as estimated from the LDH release assay and annexin V/PI staining while strains RB51 caused very mild macrophage cell death only observed by the LDH release assay. It appears that strains RB51WboA and 2308 did not induce cytopathic cell death. The mechanism of significantly

higher macrophage cell death caused by strain RA1 relative to strain RB51 is unknown. We note that strain RA1 has a mutated *wbo*A gene while strain RB51 has in addition a mutated *rpo*B gene and at least one mutated, but unknown, gene affecting O-side chain transport (eg. *wzt*). We are currently exploring how the genetic difference and the O-side chain expression contribute to the phenomenon.

P3. Brucella genomic islands and pathogenesis

Mike Krepps, Gireesh Rajashekara, David Glover, and Gary Splitter. Department of Animal Health and Biomedical Sciences, University of Wisconsin-Madison, Madison, WI

P4. Induction of Specific Mucosal and Systemic Immune Responses by Intranasal Inoculation of Mice with Live and Gamma-Irradiated Recombinant *Brucella abortus* RB51

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Effective acquired resistance against several pathogens depends on both mucosal and systemic protective immune responses. We have previously demonstrated the feasibility of using *Brucella abortus* RB51 as a vaccine vector for the expression and delivery of homologous and heterologous protective proteins to host immune system. Recently, we have also demonstrated that exposure of recombinant RB51 to an optimal dose of gamma radiation renders the bacteria non-replicative without any negative effective on their ability to induce Th1 type immune responses when inoculated intraperitoneally into mice. In the present study, we examined if intranasal inoculation of mice with live and gamma-irradiated recombinant RB51 strains would lead to antigen-specific mucosal and systemic immune responses. Our results indicate that after intranasal immunization, both live and gamma-irradiated bacteria get dispersed to lung, spleen and liver, and the vaccinated mice develop both systemic and mucosal immune responses specific to the RB51 and the expressed heterologous antigens.

P5. Protection conferred by *B. ovis* outer membrane proteins (OMP), vesicles and a bacterin in a murine model.

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The objective was to evaluate the protection conferred by different *B.ovis* immunogens: a) *B ovis* OMP; b) *B. ovis* membrane vesicles; c) *B. ovis* bacterin. For the evaluation of the protection *in vivo* of the different immunogens, nine groups of 12 Balb/c mice were used: 1) OMP and Freund's complete adjuvant (FCA); 2) OMP and Freund's incomplete adjuvant (FIA); 3) vesicles and FCA; 4) vesicles and FIA; 5) bacterin and FCA; 6) bacterin and FIA; 7) FCA; 8) FIA; and 9) physiological saline (PS). The mice were inoculated subcutaneously with the immunogens as described above and a second

immunization was applied on day 10. On the 20th day after immunization, the mice were challenged, intravenously receiving *B. ovis* (3 x 10^5 CFU). The protection of the immunization was assessed by measuring *B. ovis* CFU/ml recovered from mice spleens. The spleens of three mice from each group were removed on days 7, 14 and 21 after the challenge. The data were statistically analyzed by ANOVA to determine the differences among the different groups. There were no significant differences on the CFU measured on day 7 among the different groups; however, on day 14, the groups of vesicles and bacterin showed significantly lower CFU counts than those in OMP group. On day 21, the vesicles showed a significant lower CFU counts compared to the other groups. Meanwhile, OMP only showed a significant difference vs. the bacterin group. In general, it was observed a decrease in CFU/ml during the experiment, probably due to a decreasing adjuvant effect. It is important to note, that the data analysis discriminated well the known effect of the adjuvants on the immune response. Therefore, by the end of the experiment, the final results depended solely on the protective effect of the immunogen. The results reported here using the murine model showed that even though *B. ovis* OMP show appreciable activity *in vitro*, it is not as efficient in the *in vivo* model. *B. ovis* vesicles showed a significantly different decrease in CFU/ml on day 21 after challenge. Further work is needed to elucidate *B.ovis* vesicles protective effect.

P6. Preliminary anaylses of the alternative sigma factors of *Brucella abortus* 2308

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The ability of *Brucella* to cause disease is directly related with its ability to survive within professional phagocytes. To date, little is known about how the brucellae survive within host macrophages. The conditions encountered by the brucellae within the papoose of host macrophages include low pH, reactive oxygen intermediates, and nutrient deprivation. To survive this wide range of unfavorable conditions, bacteria have evolved mechanisms for global induction of genes whose products are involved in resistance to stressful environments. In many cases, this global induction is directed by the alternative sigma factors of RNA polymerase.

The sequenced genomes of *Brucella melitensis* and *Brucella suis* have revealed that these organisms possess 6 putative sigma factors. Interestingly, 4 of these sigma factors have unassigned functions and are putatively designated *rpoE1*, *rpoE2*, *rpoH1*, and *rpoH2*. In other organisms, Ropy directs transcription of the heat shock region involved with cytoplasm stress response and the RpoE regulon is induced in response to misfolded proteins in the periplasm or the outer membrane. An *rpoH2* and *rpoE1* mutant has been constructed from *B. abortus* 2308 and the *rpoE1* mutant shows significant sensitivity to hydrogen peroxide and is unable to grow at 42°C. The *rpoH2* mutant also displays increased susceptibility upon exposure to hydrogen peroxide, however this mutant is not attenuated in the mouse model. Using intact *B. abortus rpoH2* and *rpoH1* genes for heat resistance complement assays in an *Escherichia coli rpoH* mutant strain revealed that the *B. abortus rpoH1* gene was able to restore growth of the *E. coli* mutant at 37°C and 42°C. These preliminary studies indicate that the *B. abortus rpoH1* gene product may be the authentic RpoH homolog with *rpoH2* gene product playing an auxiliary role and the *rpoE1* gene product appears to play a role in the general stress response. Current research involves understanding the roles of these

potentially overlapping gene products in terms of general stress response and virulence of *B. abortus*.

P7. **The *Brucella abortus* Rhizobial Iron Regulator (RirA) homolog is necessary for virulence in the murine model of infection.**

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The Ferric iron uptake regulator (Fur) is responsible for the iron-dependent regulation of siderophore biosynthesis genes in the majority of prokaryotes. However, in *Brucella abortus*, iron-dependent regulation of siderophore biosynthesis has been shown to be Fur-independent. This phenomenon of iron-responsive, but Fur-independent regulation has been noted in another member of the α-proteobacteria, the plant symbiont, *Rhizobium leguminosarum,* which utilizes the Rhizobial iron regulator (RirA) to control expression of iron-responsive genes. Mutations in this regulator in *R. legumniosarum* result in constitutive, iron-independent expression of a number of iron-responsive operons including those involved in heme uptake (*hmuPSTUV)*, vicibactin siderophore uptake (*fhuA* and *fhuCDB*) and vicibactin siderophore biosynthesis (*vbsC, vbsGSO* and *vbsADL).*

Brucella abortus possesses a homolog with greater than 70% identity to *rirA*, prompting the investigation of this gene for a possible role in the regulation of Brucella iron uptake. An isogenic *rirA* deletion mutant was generated in *B. abortus* 2308 via allelic exchange and analyzed for a role in the iron-dependent regulation of siderophore biosynthesis. Although wild-type repression of siderophore production was seen under iron-replete conditions, the *rirA* mutant displayed a growth defect in iron-restricted medium, as well as increased sensitivity to chelators, suggesting RirA may serve as a regulator for other iron-responsive genes. A *rirA* mutant also demonstrated significant attenuation in BALB/C mice, suggesting that RirA may be important for long-term survival during chronic infection. Studies are currently focused on identifying genes controlled by RirA, and their contribution to the phenotype displayed by a *rirA* mutant.

P8. Isolation and Characterization of new Mexican Brucellaphages

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Phage typing system has been used routinely in the identification of *Brucella* isolates. In general, *B. melitensis* strains isolated in México are not lysed by any of the brucellaphages reported. The aim of this investigation was to isolate and partially characterize new phages from regions where animal brucellosis has been reported as prevalent.

Five mexican brucellaphages were isolated: one from a sample of fresh cattle faeces and four from various fresh goat faeces samples. Four more phages were obtained by inducing several *Brucella* clinical isolates with mitomycin C, at different intervals of time (until 72 hours) aliquots of supernatants were placed onto lawns of indicator strains, plaques were collected and propagated on the appropriate *Brucella* strain. Near-lethal U.V. irradiation assays were performed as well, no phages were induced by this method.

The nine isolated phages produced two general types of plaques: from 1 – 5 mm clear halo plaques and point turbid plaques, characteristic of *B. melitensis* lytic phages.

The host range of each phage was checked by using representatives of all species and biovars of *Brucella*, phyllogenetically related species such as: *Rhizobium tropici* 299 and *Ochrobactrum anthropi*; *Yersinia enterocolitica 0:9* and *Escherichia coli* were also tested. None of the isolated or induced phages were able to lyse those bacteria or any *Brucella* rough variant at RTD. Therefore, we considered that the isolated brucellaphages were highly specific for *Brucella* smooth strains.

We tested the susceptibility of phages to several chemical and physical agents such as: surface active agents, organic solvents, reducing agents and enzymes, heat and freeze and thaw cycles. They presented a variable susceptibility to the chemical and physical agents, nevertheless, all of them were susceptible to chloroform and resistant to toluene.

Restriction endonuclease analysis of the DNA of brucellaphages was performed with the following enzymes: *Ava*I, *BamH*I, *Bgl*II, *Ecor*I, *Hind*III and *Pvu*II. For comparison purposes the reference Tbilisi *brucellaphage* was included. The new phages showed a new DNA restriction pattern, however, they could not be differentiate one from another with the enzymes assayed.

The ultrastructural morphology of the phages was examined by transmission electron microscopy All nine brucellaphages studied, were morphologically identical, they consist of an icosahedral head with a short tail. These features place them in the family Podoviridae.

P9. Identification of *Brucella abortus* S19 in milk, during control program with *B. abortus* RB51

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Brucellosis is an infectious disease caused by bacteria of the genus *Brucella* which affects several domestic and wild life animals, as well as humans. This disease causes economic and medical consequences. Significant efforts have been made to prevent the infection by the use of vaccines. Until the past decade, strain 19 (S19) of *B. abortus* was used as the vaccine for brucellosis in cattle in Mexico. *Brucella abortus* S19 carries a deletion in two genes of the operon *ery*, responsible for the erythritol catabolic pathway.

Since 1997, *B. abortus* strain RB51 was officially approved in Mexico for its use as a vaccine for cattle, replacing S19. This strain has an insertion sequence named *IS711,* which interrupts the *wboA* gen. Based on this knowledge, we used two PCR assays that identify strains S19 and RB51, and distinguish them from other *Brucella* species and strains. These assays were used for characterization of 11 field strains of *B. abortus* isolated from milk of cattle. A 456-bp DNA fragment was amplified from all strains when PCR that identifies RB51 was used, discarding the presence of vaccine strain RB51 among the field strains. In contrast, when the assay to identify strain S19 was performed, a 1063-bp DNA fragment was amplified from nine (9) strains, while the other two (2) were identified as vaccine strain S19 by amplification of a 361-bp DNA fragment. The finding of vaccine strains among this isolates is important to both epidemiological and government level, due to their direct and indirect implications on the National Campaign for Control and Eradication of Bovine Brucellosis.

P10. Macrophage screening of a mutant bank generated from *Brucella melitensis* by Mariner transposon mutagenesis

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P11. Assessment of the stability of *Brucella* VNTR loci in outbreak situations and in experimental infection

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VNTR (Variable Number of Tandem Repeat) based typing offers great promise as a tool to differentiate *Brucella* isolates and enhance the ability to use molecular approaches for epidemiological studies. One requirement that needs to be formally investigated is the stability of markers within an outbreak and within animals. We have now applied a VNTR typing scheme to some 400 *Brucella* isolates using the original eight 'Hoofprints' octamers and an additional seven novel loci of varying sizes. The genetic diversity of loci was found to differ markedly and it is clear that different loci may be required to assess diversity within different populations. Included in these studies were multiple isolates obtained from outbreak situations following importation of *Brucella* into the UK, as well as isolates obtained following experimental infection of pigs with *B. suis*. We have used these isolates to assess the stability of the various VNTR loci within such defined situations and report on our findings here.

P12. Initial Characterization Of The Involvement FdtA and FiuA In The Transport Of Dihydroxybenzoic Acid By *Brucella abortus*

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Iron is an essential element for almost all microorganisms. However, free iron is not readily available in nature. Therefore, bacteria must posses specialized mechanisms that aid in the iron acquisition under these iron-limiting circumstances. One of the ways in which bacteria cope with iron deprivation is through the production and excretion of siderophores. These low molecular weight, high affinity iron binding compounds bind extracellular iron and are then transported back into the bacterium through specific transport systems. *Brucella spp.* produce the monocatechol siderophore 2,3-dihydroxybenzoic acid (DHBA) and the related molecule brucbactin in response to iron limitation. Studies employing a genetically defined *B. abortus* mutant unable to produce DHBA have shown that the production of DHBA is required for virulence in pregnant cattle demonstrating the importance of DHBA production to the acute stage of *Brucella abortus* infection. To date, very little is known about how DHBA is transported back into the brucellae. However, evidence has shown that DHBA utilization requires the TonB/ExbB/ExbD energy coupling systems that is involved in the passage of ferric-siderophore complexes through the

outer membrane of Gram negative bacteria, indicating the involvement of an outer membrane receptor(s) in the transport of DHBA by *B. abortus*.

A survey of the genome sequence of *B. melitensis* 16M revealed the presence of two open reading frames with homology to the *cirA* and *fiuA* genes of *Escherichia coli* both of which encode outer membrane receptors capable of transporting DHBA. To analyze the role that cirA and fiuA gene homologues (designated ferric DHBA transport gene *fdtA* and Ferric iron uptake gene *fiuA*) play in DHBA transport, both were targeted for mutagenesis in *B. abortus* 2308. Mutations in either gene individually does not lead to altered growth in rich or low iron minimal media. Growth of single mutants can also be supplemented with exogenously added DHBA, indicating these mutants have not lost the ability to transport this siderophore. One striking phenotype observed for the single mutants is an increased sensitivity to the chelator ethylene diamine diacetic acid (EDDA). When the mutations are combined, to make a strain lacking both the *fdtA* and *fiuA* genes, the sensitivity to EDDA becomes greatly enhanced to similar levels observed for a mutant lacking the ability to produce DHBA. The cirA/fiuA double mutant is currently being evaluated for its ability to utilize ferric-DHBA under iron restrictive conditions.

P13. Two *B. abortus* specific targets for real-time PCR assays

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A variety of conventional PCR assays have been developed that can detect and differentiate *Brucella* species. Gene targets have included insertion sequences, outer membrane proteins, 16S rRNA or intergenic regions between the 16S and 23S rRNA genes, housekeeping genes, and erythritol utilization genes. We have focused on the development of real-time PCR assays for *Brucella abortus* to take advantage of the substantial reduction in analysis time (about 1 hour versus 2-3 hours) and elimination of a gel analysis step, in order to perform field or near-field diagnosis of the presence of *Brucella* in infected wildlife, such as bison and elk. Our first assay (Newby, et al., 2003, *Appl. Environ. Microbiol*. **69**, 4753-4759) targeted a region spanning *alkB* and IS711 (156 bp amplicon) in *B. abortus* which specifically detected the pathogen over 7 orders of magnitude, down to 2 genomic copies. In order to improve the reliability of detecting *Brucella* using real-time PCR, we have targeted a second locus within the *B. abortus* genome and incorporated an internal control, which combined together with the *alkB*:IS711 target increase the confidence in positive identification of the organism in a sample, and also provides a means for flagging samples that may contain inhibitory materials, thus helping to rule out false negatives. We are currently working with regional wildlife management agencies to evaluate the potential utility of our assays in dealing with brucellosis in the Greater Yellowstone Area (GYA).

P14.Expression of recombinant proteins of Brucella suis and evaluation of their activity

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Full-length genome sequences of *Brucella melitensis* and *B. suis*, the two most pathogenic species of *Brucella* for humans, were recently described (DelVecchio et al., 2002a; Paulsen et al., 2002). The *B. suis* genome contains 3,388 predicted open reading frames (ORFs) on 2 chromosomes. The description of the complete genomes of *Brucella* opens the way for genome-based analysis of the antigenicity of their proteins. Based on the genome of *B. suis*, we selected twenty ORFs for gene clone and protein expression by using our currently developed high efficiency clone technology. These proteins are located in both the cytosol and the outer membrane. Sizes of the proteins vary from 133 aa to 1078 aa. The twenty of *B. suis* genes were PCR synthesized and cloned into a destination vector, pET-DEST42, for protein expression. The protein translation was conducted in *E. coli* BL21 cells and induced by IPTG. The recombinant proteins were designed with 6-his and V5 epitope tags at their C termini to facilitate detection and purification. The expressed recombinant proteins were confirmed with western blot analysis using anti-6-his antibody conjugated with alkaline phosphatase. Eighteen out of twenty cloned proteins were successfully expressed. The expressed *B. suis* proteins were further purified by immuno-capture using a specific antibody against the V5 epitope in His-Grab 96 well plates. Purified proteins were produced with high efficiency in a microarray format conducive to analysis of their sero-reactivity against serum from an immunized rabbit. Antiserum binding activity was evaluated using a 1:2,000 dilution of rabbit pre-bleed serum, post-immunization serum and IgG purified from post-immunization serum. The negative control was recombinant LacZ protein and the positive control was *B.* cell lysate. The results indicate that outer membrane proteins of *B. suis* produced higher sero-reactivity than cytosol proteins.

P15. Non-replicative, gamma irradiated *B. abortus* vaccine strain RB51 is protective against virulent *Brucella* challenge in mice

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B. abortus strain RB51 is the official vaccine used to control cattle brucellosis in the US. This strain has been proven safe and effective in cattle, in which the disease has been virtually eradicated. Brucellosis is still endemic in elk and bison which may reintroduce the disease into cattle. Therefore, complete eradication of brucellosis requires its control in wildlife. Vaccination via the oral route using strain RB51 has been shown to induce protection in some animal species. However, oral route mass vaccination carries the risk of accidental inoculation of non-target species, in which the vaccine may be pathogenic.

In order to generate a fully attenuated vaccine, we studied the effect of gamma irradiation on strain RB51's replication and metabolic activity. The protective capacity of irradiated strains RB51 and RB51SODwboA was tested in the female BALB/c *Brucella* challenge model. We demonstrated that irradiation with 360,000 rads is able to abrogate

strain RB51's replication while retaining its metabolic activity. The irradiated vaccines induced serological responses and significant protection against virulent challenge at a level similar to that of non-irradiated strains. It is noteworthy that irradiated and non-irradiated strain RB51SODwboA induced almost complete protection against virulent challenge in mice.

These results suggest that irradiation of strain RB51 based vaccines may be a useful method to generate a fully attenuated and effective vaccines that could be used to protect wildlife.

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