S 1-2

## Macrophage screen identifies *Francisella* genes required for intracellular replication

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**Aims:** To determine which genes, previously identified as being required for *Francisella* pathogenesis *in vivo*, contribute to virulence by directly facilitating replication in macrophages.
**Background:** *Francisella tularensis* is a highly infectious bacterial pathogen that causes tularemia, a potentially life-threatening disease in humans. Due to the ease of aerosol dissemination of this organism and the minimal inoculum (≤10 bacteria) necessary to cause severe disease, *F. tularensis* has been weaponized for use in biowarfare. Unfortunately, we do not fully understand the genetic requirements for *F. tularensis* pathogenesis within the mammalian host. We recently employed a powerful global *in vivo* negative selection screen (TraSH) in mice to identify genes required for the pathogenesis of *F. novicida*, a subspecies of *Francisella* that is >95 % identical to *F. tularensis* at the DNA level, encodes many of the same virulence genes, and causes a tularemia-like disease in mice but not humans. This approach resulted in the identification of 164 genes that are required for virulence *in vivo*, 44 of which encode novel hypothetical genes of unknown function. Since the ability to replicate within macrophages, thought to be the primary niche for replication *in vivo*, is a critical virulence attribute of *Francisella*, we set out to determine which of the 164 genes required for virulence *in vivo* are required for replication within macrophages or contribute to virulence through other processes.
**Methods:** We performed a bacterial replication screen in RAW264.7 macrophages, making use of a panel of transposon insertion mutants.
**Results:** Sixty-five genes were identified as being critical for bacterial replication in macrophages, including 20 of the 44 novel hypothetical genes. Many of the genes in the *Francisella* pathogenicity island were identified, validating the screen. Testing of a deletion mutant lacking one of the hypothetical genes demonstrated its role in replication in bone marrow-derived macrophages and in virulence in mice. Interestingly, 99 of the 164 genes were not required for intracellular replication in macrophages.
**Conclusions:** These results identify a panel of novel genes, which will be the basis for future study, as being critical for replication of *Francisella* in macrophages. The results also highlight the requirement in pathogenesis of a large number of genes that likely contribute to replication in other cell types or other processes during *in vivo* infection.

S 1-3

## Determining the function of the cytoplasmic membrane protein RipA

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**Aims:** To determine the function of RipA, a cytoplasmic membrane protein that is conserved among *Francisella* and is required for growth within host cells.
**Methods:** We generated alanine substitution mutants at conserved residues within RipA. These mutants were assayed for intracellular replication and IL-1β production. Secondly, pull-down assays were performed using HA-tagged RipA expressed in LVS to identify proteins that physically interact with RipA. We then generated the deletion strain lacking *iclR*, the gene encoding the RipA-interacting protein IclR, and examined the effects on gene expression, intracellular growth, cytokine induction by infected cells and virulence in a mouse model of pulmonary Tularemia.
**Results:** Pull-down assays yielded 3 RipA-interacting proteins, the strongest interaction being with the transcriptional regulator IclR (FTL_1364). Growth of LVSΔ*iclR* within alveolar macrophages was indistinguishable from wild-type LVS and the virulence of LVSΔ*iclR* was equivalent to wild-type in the mouse model. Likewise, IL-1b expression by LVSΔ*iclR* infected BMMs was not significantly different from wild-type infected cells. Transcriptional profiling of LVSΔ*iclR* by microarray revealed that IclR regulated the expression of a limited number of genes, including some putative and known virulence factors. Using the RipA point mutants, we identified residues that are involved in intracellular replication and expression of IL-1β.
**Conclusions:** Specific residues in RipA are important for the function of RipA as it applies to intracellular growth and cytokine expression in host cells. In *F. tularensis* subsp. *holarctica* LVS RipA appears to physically interact with the IclR transcriptional regulator. Gene expression and phenotypic analysis of LVSΔ*iclR* support the conclusion that IclR directly or indirectly suppresses the expression of a several virulence-associated genes. We hypothesize that under specific conditions RipA sequesters IclR, thereby alleviating repression of genes that are necessary for intracellular growth.

## VIRULENCE FACTORS ASSOCIATED WITH INTRACELLULAR GROWTH OF *FRANCISELLA*

**S 2-1**

### Intracellular nutrition and *F. tularensis* pathogenesis

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The role of nutrient acquisition systems in survival of intracellular bacterial pathogens within infected cells is yet poorly understood. We recently adapted a cefotaxime-based negative selection to isolate intracellular growth-deficient mutants of *F. tularensis* LVS. This procedure allowed us to select one mutant in a gene (*FTL_0766*) encoding a putative γ-glutamyl transpeptidase (GGT). The mutant strain showed severe intracellular growth defect and was strongly attenuated for virulence in mice.
*F. tularensis* requires cysteine for growth and the specific requirement for cysteine has been attributed to a nonfunctional pathway for sulfate assimilation. We found here that *F. tularensis* GGT activity allowed utilization of glutathione (γ-glutamyl-cysteinyl-glycine, GSH), and of γ-glutamyl-cysteine dipeptide as cysteine sources to ensure bacterial growth.
GSH is the most abundant source of cysteine in the host cytosol, and the cleavage of cysteine-containing peptide by GGT activity thus provides the essential source of cysteine required for intracellular multiplication. This is the first demonstration of the essential role of a nutrient acquisition system in the intracellular multiplication of *F. tularensis*.
These data will be integrated in the more general frame of how *F. tularensis* adapted its metabolic needs to the nutrients available (nature and concentration), to multiply efficiently and simultaneously to avoid a premature death of the host cell.

S 2-3

## A genetic screen identifies novel *Francisella tularensis* ssp. *novicida* genes involved in modulating the macrophage innate immune response

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Within the host, *Francisella* is engulfed by host macrophages where it escapes phagosomal degradation and replicates to high numbers in the cytosol. The host response to *Francisella* involves coordination of both vacuolar and cytosolic sensing of the pathogen. Sensing in the vacuole is dependent on TLR2 and the adapter MyD88, which leads to production of TNF-a, and pro IL-1b. Cytosolic bacteria are recognized by a cytosolic surveillance pathway, which leads to the production of type-I interferons (IFN), including IFN-β. These type-I IFNs act in an autocrine and paracrine fashion to initiate a signaling cascade resulting in inflammasome activation and the release of mature pro-inflammatory cytokines (IL-1β and IL-18) and macrophage cell death. We conducted a genome-wide forward genetic screen to identify *Francisella* genes involved in modulating the host innate immune response in macrophages. We identified 72 gene insertions that result in lower induction of the innate immune response. These include known virulence factors such as genes located in the *Francisella* Pathogenicity Island (FPI), which contains homologs of type VI secretion systems. These mutants showed decreased intracellular replication, and induced lower levels of IFN-b and host cell death relative to a wild-type strain. Additionally, 188 gene insertions were identified that resulted in increased induction of pro-inflammatory responses. These include genes involved in lipid A modification and LPS and O-antigen synthesis. Targeted deletions of FTN_1212, wbtA, and lpcC resulted in strains that induced a hyper inflammatory response indicated by increased kinetics of TNF-a, IFN-b, and IL-1b release as well as host cell death relative to a wild-type strain. This phenotype was not due to increased intracellular replication. Additionally, this hyper induction of pro-inflammatory cytokines was still dependent on the FPI genes, as LPS-FPI double mutants failed to induce the cytosolic response. Furthermore, TLR2 and Myd88/Trif signaling contribute to the increased inflammatory response to LPS mutants. Taken together these data suggest that genes in the FPI are critical for intracellular replication and induction of macrophage innate immune responses, and that *Francisella* uses lipid A modification to mask ligands that can be recognized by TLR2 as well as other intracellular pattern recognition receptors.

S 2-4

## Role of Type IV pilin genes in virulence of *Francisella tularensis* subspecies *holarctica* and *tularensis*

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The biogenesis and regulation of type IV pilus (Tfp) is complex and can involve up to 40 genes. Analysis and comparisons of genome sequences of different *Francisella tularensis* subspecies has revealed the presence of genes encoding a putative Tfp. This analysis has also revealed interesting differences between the subspecies. Two of the pilin genes and the gene encoding the PilT ATPase are non-functional in subsp. *holarctica* (type B). In addition *pilA*, that we previously have shown to be required for mouse virulence, is lacking in the type B vaccine strain LVS.
**Aims:** The aims were to study the role of Tfp encoding genes in the human pathogenic subsp. *holarctica* and *tularensis* (type A).
**Methods:** Tfp encoding genes in a virulent human type B isolate and the type A strain Schu S4 were targeted by mutagenesis and evaluated for their potential role in virulence. The different mutants were assessed for virulence in single strain infections as well as in competition with the isogenic wild-type strain.
**Results:** Of the pilin genes only PilA was found to be required for mouse virulence in the type B strain. In addition, we could also establish that two pseudopilins FTT1621-22 contributed to virulence. We also verified that PilA, as well as the inner membrane protein PilC and the PilQ secretin are required for full virulence of the type A strain Schu S4. Interestingly PilT was found not to be required for mouse virulence of Schu S4.
**Conclusions:** We have confirmed that PilA is essential for mouse virulence in both type A and type B strains and that PilC and PilQ, postulated to be required for assembly of Tfp, also are required for full virulence in Schu S4. PilT on the other hand, which is only functional in type A strains and postulated to have a role in pilus retraction, was not required for mouse virulence. Further evaluation of the importance of these genes in other infection models, more relevant for human infections, is needed to establish the role of Tfp genes in the pathogenesis of tularemia.

S 3-4

## A genome-scale phenotype map of *Francisella novicida*

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We constructed a genotype-phenotype “map” of *F. novicida* in which the genes required for growth under 35 different nutritional and stress conditions were identified. To do this, we developed methods for the large-scale analysis of a comprehensive, sequence-defined transposon mutant library of the organism. Mutants from 384-well source plates were robotically spotted onto test media, and subsequent growth was quantified using image analysis. Confirmed phenotypes were identified for mutations inactivating about a quarter of the organism’s nonessential genes. This analysis has provided experimental tests of genome annotation assignments, and has led to the discovery of new genes associated with nearly every phenotype examined. The map should help provide a foundation for understanding intrinsic antibiotic resistance, virulence, and other complex traits in terms of more fundamental processes of the organism.

## SHORT PRESENTATIONS 1

### S 4-1

### Heterologous expression of *Francisella tularensis* type IV pili genes in *Neisseria gonorrhoeae* confirms that PilA can form functional pili

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Accumulating evidence from a number of studies strongly suggests that proteins orthologous to those involved in type IV pili (Tfp) assembly and function are required for *Francisella* pathogenicity. However, the molecular mechanisms by which the components exert their influence on virulence remain poorly understood. In particular, the expression of Tfp filaments by these species has yet to be unambiguously demonstrated.
**Aim:** In this study we aimed to verify if any of the potential pilin genes identified in *F. tularensis* could form pili structures.
**Methods:** Trans-species complementation of various Tfp biogenesis mutants have been achieved in a number of cases, and strains expressing heterologous pilin subunits provide unique opportunities to examine the role of the pilus. In this study we expressed a number of *Francisella* pilin genes in the Tfp expressing pathogen *N. gonorrhoeae* lacking its endogenous pilin subunit.
**Results:** Two gene products, the orthologous PilA proteins from *Francisella* subsp. *tularensis* and *novicida*, were capable of restoring the expression of Tfp-like appendages that were shown to be dependent upon the neisserial Tfp biogenesis machinery for surface localization. Expression of *Francisella* PilA pilins also partially restored competence for natural transformation in *N. gonorrhoeae*, a phenotype that was not complemented by expression of the PulG and XcpT proteins that are equivalent components of related type II protein secretion systems (T2SS).
**Conclusion:** Our results provide evidence that pilin-like proteins from *Francisella* species are capable of interacting with components of an established Tfp system. These findings provide compelling but indirect evidence of the potential for *Francisella* to express functional Tfp.

S 4-2

## Identification of *F. tularensis* from environmental water specimens in tularemia epidemics in Turkey by both culture and real time TaqMan PCR methods

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**Aim:** Tularemia, of which oropharyngeal form occurs the most often, is an endemic disease in Turkey. In this study, it is aimed to isolate of *F. tularensis* from natural water supplies thought to cause tularemia epidemiologically.
**Methods:** A total of 29 water specimens (7 from Corum, 12 from Sivas, and 10 from Samsun), the volumes of which were between 0.3 to 1.5 liters, from 3 different epidemic areas were collected. Water specimens were filtered by 0.22-mm-diameter cellulose acetate membranes. The membranes were placed on antibiotic added (Oxoid SR147) Cysine Heart Agar Base with blood media and incubated at 37°C in a humidified atmosphere containing 5 % $CO_2$ for 4-10 days. Our test procedures including water filtration and culture were carried out in the class III biological safety cabinet in the properly installed room. After the incubation, the suspected colonies on the plate seen the growth was picked to subculture using single colony method. The subcultured colonies were confirmed by *F. tularensis* antiserum (BD) and Real Time TaqMan PCR method. The surfaces of filters were washed with sterile distilled water for 15 minutes in a shaker, to get all the suspected colonies. DNA was isolated from samples obtained from filters. The primer and probe sets targeting IS*Ftu2* genome were used for Real Time TaqMan PCR method.
**Results:** A total of three *F. tularensis* isolates were obtained from 29 water samples (1 isolate from Corum, 1 isolate from Sivas, and 1 isolate from Samsun) by culture method. At same time, the presence of *F. tularensis* DNA from four water specimens was shown by Real Time TaqMan PCR method.
**Conclusion:** Although DNA presence of *F. tularensis* has been detected from water sources by PCR method in Turkey, until now, there had no isolation from water specimens by culture. In our study, the isolation of *F. tularensis* from water sources has been exhibited as first data by both culture and Real Time TaqMan PCR methods.

S 4-3

## Characterization of a novel *Francisella* sp. from blood and urine of a patient with an unusual clinical presentation

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**Aims:** Characterization of a novel *Francisella* sp. (FnSp1) isolated from human blood and urine.
**Methodos:** Two isolates of a gram negative coccobacile with the same biochemical characteristics were obtained from blood and urine of a 43 year old male who was admitted to the hospital with the initial diagnosis of acute obstructive pyelonephritis. Empiric treatment was started with Aztreonam and Clavulanic acid/Amoxicillin. Five days later, the patient's condition worsened with severe sepsis. After a preliminary identification of the isolate (FNSp-1) as *Francisella* spp., the treatment was changed to Tobramycin and the patient recovered in five days. Subsequent analyses were performed by agglutination with specific anti-*F. tularensis tularensis* (*Ftt*) immune serum (BBL), serologic response of the patient against its own isolate (western blotting and microagglutination), protein and biochemical profiles, antimicrobial susceptibilities, PFGE, reactivity to different PCR protocols, including SSTR9 and pdpD, sequencing of 16S rRNA, *lpnA* and VNTR M19, and finally Multilocus Sequence Analysis (MLSA) using *tpiA*, *dnaA*, *mutS*, *prfB* and *putA.*
**Results:** Microagglutination was negative for Ftt (strain B38), *Ft holarctica* (strain LVS) and *Ft novicida* (strain UTAH-112). Western blotting with soluble fractions of the 3 subspecies and the patient's isolate disclosed a protein band specific of FnSp1. Biochemical profile was closer to Ftn than to Ftt or Fth and protein profile was unique for FnSp1. The PFGE pattern with *Pme*I was distinc from those for *Ftt*, Fth, Ftn and F. phylomiragia. The size of the SSTR9 was similar to that of *Ftn* Fx1, and no amplification was obtained for *pdp*D as happens with *Fth* LVS. The sequence of 16S rRNA showed a high homology to *Ftt* (99.9 %), the sequence of *lpnA* was close to that of *Ftn*-like strain 3523 and that of M19 showed similarities to both *Ftt* and *Ftn*. Interestingly, the MLSA pattern showed a high divergence of FnSp1 to any of the described subspecies.
**Conclusions:** Microbiological and molecular analyses of this strain indicate that it can represent a novel *Francisella* sp. Since the recognition of this agent can be mislead by routine identification methods, attention should be paid to identify additional isolates to assess the actual role of this microorganism in human disease. Due to the scarce number of strains and of clinical cases caused by this agent, this report provides additional data that could be of clinical and microbiological relevance.

S 4-4

## Description of *'Francisella novicida'* FSC454$^T$ as a novel species of the genus *Francisella*

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*'Francisella novicida'* FSC454$^T$ = FnSp1$^T$ = F62$^T$, isolated from human blood in Spain (2003), was examined in order to clarify its taxonomic position. By 16S rRNA and recA gene sequencing, this strain had been recently shown to belong to the genus *Francisella*, with similarity values greater than 99 % in the 16S rRNA gene to *F. tularensis* and 84–91 % in the recA gene to the other *Francisella* species. The genus affiliation was supported by a quinone system typical of *Francisella* (Q-8 as the major component), a complex polar lipid profile similar to *Francisella tularensis* with the major components phosphatidylethanolamine, phosphatidylcholine and an unknown aminophospholipid and a fatty acid profile consisting mainly of C10:0, C14:0, C16:0 and C18:1 ω9c and the hydroxy acid C18:0 3-OH. DNA-DNA hybridization unambiguously showed that FSC454$^T$ represents a novel species. Together with the results from examination of biochemical properties these observations allowed genotypic and phenotypic differentiation of the isolate from all hitherto described *Francisella* species. A multiplex-PCR developed in the course of this study discriminated FSC454$^T$ from representatives of all other *Francisella* species and subspecies, clades A.I and A.II of *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica* biovar japonica and between these representatives as well. Hence, from our point of view assignment of *'Francisella novicida'* FSC454$^T$ to a novel species is justified.

S 6-3

## Interaction of mosquito larvae with *Francisella tularensis* LVS biofilms

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**Aims:** *Francisella tularensis* is a highly virulent human pathogen responsible for causing the disease tularemia. *F. tularensis* LVS can form biofilms in aquatic environments which provides them a niche to survive harsh environmental conditions. Arthropods such as mosquitoes, ticks and deer flies are vectors for *F. tularensis.* These arthropods can transmit the infection to various mammalian hosts including humans; however the method and source of acquisition of *F. tularensis* by arthropods is unclear. We hypothesize that aquatic mosquito larvae feed on *F. tularensis* biofilms and play important roles in persistence and dissemination of this bacterium in the environment.
**Methods:** Persistence of *F. tularensis* LVS in mosquito larvae was measured following oral uptake of suspended cultures and biofilms. Larvae were exposed to either planktonic *F. tularensis* or biofilms grown on glass slides for 24h. Larvae were collected at days 1, 3 and 9 post exposure for analyses by fluorescence microscopy and Real Time PCR.
**Results:** Real time PCR and fluorescence microscopy confirm the presence of *F. tularensis* LVS within the larval gut for up to nine days post exposure. Real time reverse transcription PCR demonstrates that the mosquito antimicrobial peptides defensin, gambicin and cecropinA are altered suggesting manipulation of the larvae immune response by the bacteria.
**Conclusions:** Our results show the potential of *F. tularensis* to be acquired by a known vector during larval feeding. Uptake and persistence of *F. tularensis* by mosquito larvae have not previously been shown although the interaction has the potential for significant consequences on transmission of the disease. Future studies will examine the localization of the *F. tularensis* within the larvae gut as well as possible dissemination of the bacteria across the gut epithelium. It is essential to examine the environmental factors and biotic interactions affecting transmission of *F. tularensis* in order to understand and optimize outbreak response and prevention.

## SHORT PRESENTATIONS 2

### S 8-1

### Fate of the complex formed between nucleolin present on membrane of human cells and LVS surface elongation factor Tu (EF-Tu) during LVS infection

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*Francisella tularensis,* the causative agent of tularemia, is one of the most infectious human bacterial pathogens. Participation of C3, CR3, class A scavenger receptors and mannose receptor in bacterial uptake have been already reported in initial bacterial uptake. However, contribution of an additional, as-yet-unidentified receptor for *F. tularensis* internalization has also been suggested. We previously demonstrated that cell-surface expressed nucleolin is a receptor for *F. tularensis* Live Vaccine Strain (LVS). Nucleolin interacts with bacterial ligand EF-Tu. This interaction allows cell infection (Barel et al., *BMC Microbiol.*, 2008).
**Aim:** Our goal was to determine the fate of nucleolin-EF-Tu interaction after adhesion of bacteria on human cell surface.
**Methods:** Human THP-1 monocyte-like cells were infected for 30 min, washed with gentamycine and further incubated for 24 h. Presence of the complex formed between bacterial EF-Tu and human nucleolin was analyzed at 30 min, 5 h and 24 h, after permeabilizing cells and staining with specific antibodies by fluorescent and confocal microscopy. SiRNA were used to abolish nucleolin expression.
**Results:** After 30 min infection by LVS, EF-Tu / nucleolin complex was visible on internalized bacteria, as previously described (Barel et al. 2008). Strikingly, after 5 h, when LVS has started to actively multiply in cytosol, bacteria were still interacting with nucleolin. After 24 h, when cells begin to undergo apoptosis, nucleolin was recovered in the nucleus and EF-Tu was no more able to form complex with it. As these results suggest that intracellular nucleolin may also play a role in LVS infection, we studied the effect of its knocking-down with siRNA targeting nucleolin. Cells were transfected with specific or scrambled siRNAs for 72 h then infected with LVS. CFU number was counted at 24 h. Bacterial infection was decreased by 90 % with siRNA targeting nucleolin.
**Conclusions:** These data suggest that *F. tularensis* hijacks human nucleolin to perform a dual role in infection:

I) at the cell surface, nucleolin allows LVS entry through its surface-exposed EF-Tu,
II) in the cytosol, where it remains transiently bound to bacteria and may participate to *F. tularensis* intracellular survival.

This is the first report of an interaction between *Francisella* proteins and intracellular proteins of its host. This intracellular interaction may be important for triggering signaling pathways, which may facilitate intracellular infection.

S 8-2

## Differences in virulence between subspecies of *Francisella tularensis* are reflected in different gene expression profiles of human neutrophils

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**Aims:** Neutrophils are the first line of cellular defense against pathogenic microorganisms, playing a crucial role in innate immunity. It is known that LVS is able to evade killing by human neutrophils. By in vitro infection of human granulocytes with *Francisella* (*F.*) *tularensis* (*t.*) strains of different virulence we aimed to identify cellular process within the granulocytes which are interfered by infection and to identify those interferences exclusively mediated by virulent strains of *F. t. tularensis* and *F. t. holcarctica*.
**Methods:** Human neutrophils were isolated from heparinized venous blood of three healthy individuals using density gradient centrifugation. Five different *F. tularensis strains* (a highly virulent *F. t. tularensis* an attenuated *F. t. tularensis*, a virulent *F. t. holarctica,* an attenuated *F. t. holarctica* vaccine strain (LVS) and a *F. t. novicida* were grown to exponential phase and used to infect the isolated neutrophils. RNA isolated 30 and 120 min post infection was employed for gene expression profiling using Human Genome U133 Plus 2.0 arrays (Affymetrix) according to standard protocols. The microarray data was validated by quantification of several rRNAs from these experiments by qPCR. Cytokine release was measured in culture supernatants using Luminex-based bead array technology.
**Results:** In general, the host-pathogen interaction resulted in modulation of transcriptional maschinery, dysregulation of neutrophil bioenergetics, host immune responses and cell death processes. Differences in PMN gene expression induced by distinct strains could also be observed. The most striking difference was the lack of increased levels of IL-6, IL-8 and TNFa mRNA in neutrophils infected with the virulent strains of *F. t. tularensis* and *F. t. holarctica* respectively, compared to the increased levels in the lesser or avirulent *F. tularensis* strains (0 versus 1.7 to 6.45 fold change). Cytokine levels in cell culture supernatants support the micro array results.
**Conclusions:** We could find differences in the gene expression of human neutrophils infected with different *F. tularensis* strains of varying virulence. The lack of increased levels of IL-6, IL-8 and TNFa suggests that virulent *F. t. tularensis* and *F. t. holarctica* interfere with the inflamatory response of neutrophils, thereby possibly dysregulating processes as chemoattraction of neutrophils, respiratory response and apoptosis.

S 8-5

## The 58-kDa major virulence factor, FTT0918, of *Francisella tularensis* is required for utilization of iron

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**Aim:** This study aimed to characterize the phenotype of Δ*FTT0918* and thereby obtain an understanding of the role of FTT0918 that could explain its important contribution to the virulence of *F. tularensis*. *FTT0918* is highly similar to the siderophore receptor *fslE*. To this end, we asked if FTT0918 performs a function in the iron metabolism of Schu S4.
**Methods:** Schu S4, a strain of *F. tularensis* subsp. *tularensis*, and the isogenic mutants Δ*FTT0918* and Δ*fslA* were characterized with regard to growth, gene expression, siderophore production and uptake.
**Results:** Δ*fslA* did not produce siderophores and showed impaired growth under iron limiting conditions. Δ*FTT0918* produced siderophores but showed impaired siderophore uptake and reduced growth under iron limiting conditions. After depletion of the intracellular iron pool, Δ*FTT0918* showed impaired growth even in high iron concentrations.
**Conclusions:** The results demonstrate that FTT0918 plays an important role in the utilization of iron by the highly virulent Schu S4 strain. This occurs under iron-deplete, and likely also under iron-replete conditions and these multiple roles may be an important reason for its major contribution to the virulence of *F. tularensis*.

S 9-3

## Integrating *Francisella's* intracellular lifestyle

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*Francisella* is a formidable intracellular pathogen with a high infectious efficiency that is determined by its interaction with its respective host cell. We attempted to integrate experimental data starting at *Francisella's* entry into the host cell into comprehensive computational models in order to understand *Francisella's* pathogenesis and intracellular life style. Constraint-based analysis of *Francisella's* metabolic capabilities demonstrate preferential amino acid catabolism and dynamic carbohydrate utilization patterns with associated changes in energy homeostasis. With a two-compartment agent-based model that simulates inhalational tularemia with subsequent dissemination to the liver we attempted to provide a broader framework for infection with *Francisella*. Such models underline the importance of the initial *Francisella*-host interaction for the outcome of the infection.

S 9-4

## Mast cell-mediated signaling in reduction of *Francisella tularensis* Schu S4 intramacrophage replication and apoptosis

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**Aims:** To determine the effector mechanism(s) by which mast cells mediate the inhibition of intramacrophage *F. tularensis* subsp. tularensis Schu S4 or LVS replication and induction of apoptosis.
**Methods:** An in vitro bone-marrow derived mast cell/macrophage co-culture system, in addition to confocal and scanning electron microscopy were utilized. Flow cytometry was used for analysis of activation markers and expression of apoptotic proteins.
**Results:** Macrophages infected with *F. tularensis* Schu S4 or LVS and co-cultured with mast cells exhibited significant reduction of intramacrophage growth and caspase-3 expression compared to macrophages cultured alone. This reduction was dependent on mast cell secreted products, including IL-4, and contact-dependent events. Mast cells co-cultured with macrophages exhibited up-regulation of MHC II (12.7 % to 87.1 %), and c-Kit (17.0 % to 61.0 %) expression associated with cellular activation. Since mast cells express toll-like receptor-2 (TLR2), which is important for pathogen recognition and IL-4 production, TLR2 deficient ($TLR2^{-/-}$) mast cells additionally were analyzed. $TLR2^{-/-}$ mast cells exhibited a significant increase (2-3.0 $log_{10}$) in LVS replication and enhanced expression of caspase-3 (10 % to 28 %) in contrast to wild type (WT) mast cells. Whereas, LVS-infected $TLR2^{-/-}$ mast cells did not produce IL-4; these cells cultured in the presence of WT macrophages restored IL-4 production and inhibition of apoptosis and *Francisella* intramacrophage growth.
**Conclusions:** The inhibitory effect of mast cells on intramacrophage bacterial replication and apoptosis also is evident with a type A human strain. Moreover, the TLR2 signaling pathway within mast cells may be an important component in control of *Francisella* replication within the mast cell itself. Together, our results demonstrate that cross-activation events are critical for mast cell production of IL-4 and control of *Francisella* intramacrophage replication and induction of apoptosis.
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S 10-5

## Quantitative proteomic profiling of host – pathogen interaction: The interaction of *Francisella tularensis* LVS with macrophage using J774.2 cell line

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*Francisella tularensis* is a facultative intracellular pathogen that can invade and replicate inside macrophages. The bacterium evades antimicrobial mechanisms of macrophages by escaping from phagosome into cytosol. There is growing evidence that various intracellular bacteria exploit specialized plasma membrane microdomains, commonly called membrane rafts, as an infectious strategy to mediate an alternative endocytic pathway avoiding fusion with lysosomes. Inhibition of phagosome-lysosome fusion has been proposed as a mechanism for survival inside host cells. Here, we present evidence that plasma membrane organization in membrane rafts is critical to *Francisella tularensis* uptake. Cholesterol depletion by methyl-β-cyclodextrin and filipin significantly inhibited *Francisella tularensis* LVS uptake. Contemporary, we analyzed membrane raft proteins of macrophages that were recruited at the bacterial entry using proteomic quantitative approach. The response of the macrophage proteome to *Francisella tularensis* LVS internalization reflects the host immunity reaction as well as bacterial induced mechanisms that may be beneficial for pathogenic microbe.

## STATUS OF NON-HUMAN PRIMATE MODELS OF *FRANCISELLA* INFECTION

### S 11-1

# Humoral immunity to *Francisella tularensis* strain LVS fails to uniformly protect Cynomolgus macaques from disease induced by aerosol infection with strain Schu S4

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Vaccination with *Francisella tularensis* strain LVS has been shown to protect non-human primates from the mortality induced by aerosol exposure to *F. tularensis* Schu S4.
**Aims:** Determine the mechanism(s) of protection induced by the LVS vaccine in Cynomolgus macaques.
**Methods:** Macaques were vaccinated with $10^4$ – $10^7$ LVS via the intradermal or sub-cutaneous route or by scarification. Five weeks to 27 months later, vaccinated animals and non-vaccinated controls were challenged with 27 – 1780 Schu S4 CFU by aerosol. The immune response was characterized by measuring plasma IgG anti-LVS levels, as well as the ability of peripheral blood mononuclear cells to secrete IFNγ in response to LVS.
**Results:** Although all the animals produced IgG specific to LVS (titers $> 1 \times 10^5$) , only 2 of 9 vaccinated primates survived to day 21 post-aerosol challenge and none remained disease free. Seven vaccinated animals succumbed to the Schu S4 aerosol between day 4 and 16, whereas two of three non- vaccinated controls succumbed on days 5 and 6 and one was euthanized on day 21. LVS-induced IFNγ production was variable, with a trend toward higher responsiveness being correlated with a protracted time to death. Schu S4 organisms were recovered from the lungs and tracheobronchial lymph nodes despite vaccination status but dissemination to the liver and spleen appeared diminished in LVS-vaccinated animals.
**Conclusions:** Data suggest that LVS vaccination induces a uniformly strong humoral immune response and a variable cellular immune response, the former of which is unable to predict protection of macaques from mortality induced by Schu S4 aerosol challenge.
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S 11-2

## Exposure of cynomolgus macaques to aerosolized *Francisella tularensis* Schu S4

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**Objectives:** *Francisella tularensis* is a highly virulent intracellular pathogen, the etiologic agent of tularemia, and is classified as a category A priority biodefense pathogen. Due to the low natural incidence of tularemia, FDA licensure of tularemia countermeasures will require well developed animal models of disease. To establish a non-human primate tularemia model, we defined several parameters of inhalational tularemia in cynomolgus macaques.
**Methods:** Mauritian origin cynomolgus macaques (*Macaca fascicularis*) were exposed to various dosages of aerosolized *F. tularensis* Schu S4. Macaques were exposed to a range of dosages to estimate the median lethal dose ($LD_{50}$). To document the progression of disease, animals were observed for clinical signs of disease as well as implanted with radiotelemetry transmitters to obtain temperature, activity, pulmonary and arterial pressures, and electrocardiograph parameters. Blood samples were drawn periodically to measure clinical laboratory parameters, bacteremia, and immune responses.
**Results:** The preliminary aerosol $LD_{50}$ for *F. tularensis* Schu S4 in cynomolgus macaques is 294 cfu/animal. Animals were commonly observed to be lethargic with hunched posture post exposure. All exposed animals experienced temperatures above 40°C between two and four days post exposure. Some animals experienced reduced temperatures (< 34 °C) prior to death. Animal activity was reduced beginning 1-2 days post exposure. Elevated heart rates and abnormal blood and pulmonary pressures were also observed. Bacteremia was observed in some animals prior to death. Animals were observed to have increases in numbers of neutrophils and large unstained cells and a decrease in numbers of eosinophils within one week post-exposure.
**Conclusions:** We have characterized a number of parameters for cynomolgus macaques infected with *F. tularensis* Schu S4 following aerosol exposure. The cynomolgus macaque is a good model of inhalational tularemia.
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S 11-3

## Comparison of aerosolized *F. tularensis* in three species of nonhuman primates

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Tularemia, a zoonotic disease caused by *Francisella tularensis*, is most commonly transmitted to humans by contact with infected animals, tick bites, or inhalation of aerosolized bacteria. *F. tularensis* is highly infectious via the aerosol route with less than 10-50 organisms able to cause disease. The pneumonic form of the disease occurs after inhalation of bacteria and has a 30 % case-fatality rate if left untreated, which can be reduced to 3 % if treated early with antibiotics. Therefore, development of effective antibacterial therapeutics and vaccines against *F. tularensis* is necessary to protect our military and civilian populations against this potential biothreat agent. Potency and efficacy testing of new vaccines or antibacterial therapeutics require animal models that imitate human disease as closely as possible.
**Aims:** In this study, three species of nonhuman primates (African green monkey (AGM), cynomolgus and rhesus macaque) were exposed to various doses of aerosolized *F. tularensis* Schu 4 utilizing a staircase method to determine the $LD_{50}$ and $ID_{50}$.
**Methods:** Blood samples were taken after exposure to assess complete blood count (CBC), clinical chemistry, bacterial load, and cytokines.
**Results:** While AGMs and cynomolgus macaques succumbed to disease with 100 CFU of aerosolized Schu 4, the target dose that caused lethality for rhesus was $10^5$ CFU.
**Conclusion:** Overall results indicated that the cynomolgus macaque model for aerosolized tularemia was the best model representing human tularemia among three species.

## MECHANISMS OF IMMUNITY, VACCINATION AND IMMUNOTHERAPY

### S 12-1

# Novel live vaccine candidates against airborne *Francisella tularensis*

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*Francisella tularensis* LVS remains the sole vaccine shown to have efficacy in humans against systemic or airborne challenge with the type A subspecies of the pathogen. In an attempt to produce a better-defined and more efficacious vaccine than LVS, we have been producing targeted deletion mutants of the virulent type A strain, Schu S4. Until recently, we had made deletion mutants that were either at least as attenuated as or as effective as LVS, but not both in a murine intradermal (ID) vaccination and aerosol challenge model. However, deleting the *clpB* gene alone or both the *FTT0918* and *capB* gene has generated mutants that fulfill or surpass both criteria. In BALB/c mice, ID inoculation of LVS generates protective immunity against a subsequent challenge via the same route with $> 10^3$ $LD_{50}$ of Schu S4. However, in our hands, the same vaccination regimen only prolongs life by a couple of days in mice challenged with an aerosol of Schu S4. In contrast, the two new deletion mutants elicited significantly longer survival times ($P< 0.05$) following aerosol challenge. To try and determine the basis for this enhanced survival, we have been comparing various immune responses in mice following immunization with LVS or Schu S4 Δ*FTT0918*Δ*capB* or Schu S4 Δ*clpB* or following immunization and aerosol challenge. Immunoproteomic analysis revealed distinct patterns of serum antibody response, and FACS and multiplex cytokine and chemokine analysis revealed distinct patterns of cell-mediated responses in the lungs, spleen and serum. The implications of these findings for defining mechanisms and correlates of live vaccine-induced protection will be presented along with a brief overview of progress with other vaccine efforts within the tularemia field.
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S 12-2

## Type A *F. tularensis* induces caspase-3-dependent macrophage death in infected tissues

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**Aims:** Although *Francisella tularensis* subsp. *tularensis* is known to cause extensive tissue necrosis, the mechanism of in situ cell death is not known.
**Methods:** Using a mouse respiratory challenge model of tularemia, we have defined the pathological responses that occur in infected tissues during the first 4 days after infection with the type A *F. tularensis* strain KU49.
**Results:** Three days post-infection, well organized inflammatory infiltrates developed in the spleen and liver that resembled those found in mice infected with the Live Vaccine Strain of *F. tularensis* subsp. *holarctica*. However, by day 4 of infection with KU49, numerous cells with double strand DNA breaks appeared throughout these inflammatory foci. Dying cells within KU49-infected tissues expressed activated caspase-3, but very little activated caspase-1. Moreover, infected caspase-1-deficient mice showed the same pathological changes as infected wild type mice, including extensive cell death and foci of necrosis in the liver and spleen. KU49-infected caspase-3-deficient mice showed diminished pathology and much less death among their splenic F4/80-positive cells. These infected mutant mice also retained the ability to express splenic tumor necrosis factor-α and inducible NO synthase, responses that were completely lost in infected wild type mice. Ly-6G-positive cells in the spleen and lungs were not spared death in caspase-3-deficient infected mice, indicating that death of myeloid cells occurs by a caspase-3-independent mechanism. With the destruction of hepatic granulomas on day 4 of infection with KU49, *Francisella* antigens were found disseminated throughout the liver, rather than confined to the granulomas. In infected caspase-3-deficient mice, this effect was significantly decreased, indicating that caspase-3-dependent cell death results in a decreased ability to limit dissemination of the infection.
**Conclusions:** These findings suggest that Type A *F. tularensis* benefits from caspase-3-dependent macrophage apoptosis, which dampens potentially important innate immune responses to the pathogen and permits its dissemination throughout infected organs.
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S 12-3

## Molecular mechanisms responsible for antibody-mediated clearance of *F. tularensis*

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There is a growing appreciation for the role of antibodies in immunity against intracellular bacteria including that of *Francisella tularensis*. We have previously demonstrated that serum antibodies provide protection against pneumonic tularemia and have therapeutic benefits. The antibody-mediated protection was dependent on FcgγR-bearing phagocytes such as alveolar macrophages (AMs) and their activation by IFN-γ. Here, we further elucidate the molecular mechanism of synergy between FcγR and IFN-γR signaling that results in the rapid intracellular killing of *F. tularensis*. Activated AMs produced significantly higher levels of nitric oxide (NO) upon infection with antibody-opsonized *F. tularensis*. Chemical inhibition of NO production resulted in the abrogation of intracellular killing of opsonized bacteria by AMs. Increased NO production and rapid clearance of opsonized bacteria by AMs was independent of TLR2 and TNF-α stimulation suggesting a higher order of complexity to the „prime and trigger" model of macrophage activation. In fact, our preliminary studies suggest that differential trafficking of opsonized bacteria within IFN-γ activated AMs may contribute to the rapid clearance of *F. tularensis*. We are currently establishing the dynamic interaction of the bacteria with the activated phagocytes in order to design effective strategies for sterilizing prophylaxis and therapeutics.

S 12-4

## Essential elements of protective immunity to *Francisella*

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Understanding the fundamental nature of mammalian immune responses to infections with virulent species of *Francisella* is of interest from both basic science and practical applications perspectives. Many types of different innate and adaptive immune responses to *Francisella* have been described through observations of disease and vaccination in humans, as well as studies in various animal models. Despite this, it remains challenging to discriminate between those that are critical to control of infection, those that contribute partially, and those that may only be epiphenomena. Here we focus on studies using parenteral vaccination of mice coupled with *in vivo* manipulations and *in vitro* studies to determine the most critical cell types and mediators, particularly with regard to development predictive correlates of vaccine-induced protection. Innate immune responses leading to production of TNF-α, Interferon-γ, and IL-12 p40, as well as processes requiring the cell-associated molecules MyD88 and CCR2, are critical, but none of these are useful individually in predicting protection. Because T cells are clearly crucial effector cells during adaptive responses, we have evaluated the utility of an *in vitro* co-culture assay that measures the ability of *Francisella*-immune T cells to control the intramacrophage growth of *Francisella* as a functional correlate of protection. T cells were obtained from mice vaccinated with a panel of qualitatively different *Francisella* vaccine candidates that provide strong protection, modest partial protection, and little to no protection. To date, there is excellent agreement between the magnitude and hierarchy of protection provided by this panel of *Francisella* vaccine candidates against *in vivo* LVS challenge, and the magnitude of *in vitro* activity of T cells obtained from vaccinated mice in controlling intramacrophage replication of *Francisella* LVS. Further, studies comparing gene expression between naive T cells and T cells primed by various vaccine candidates have defined a tentative panel of differentially upregulated gene products that track with *in vivo* protection, both quantitatively and qualitatively. Future studies will therefore explore the usefulness of both the functional assay and the panel in predicting survival of challenge with fully virulent *Francisella*, in other animal models, and human vaccination.

S 12-5

## Derivation of a panel of potential correlates of vaccine-induced protection against *Francisella tularensis* LVS

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**Aims:** Ideally, protection against *Francisella tularensis* would be provided by the development of a vaccine that could be safely used on a large population to prevent tularemia. Our goal is the identification of a panel of immunologic and biologic correlates that are essential for predicting protection provided by immunization with any live attenuated *Francisella* strain. Such a panel might further be applied to *in vitro* screening of potential vaccines prior to more extensive animal studies. Ultimately, we wish to predict the outcome of vaccination in humans.
**Methods:** We have coupled an *in vitro* co-culture assay with genomic methodologies to identify biomarkers that correlate with vaccine efficacy. Using the *in vitro* assay, we compared LVS-infected bone marrow macrophages co-cultured with lymphocytes from naive control mice to those from LVS-vaccinated mice. The assessment of control of intramacrophage LVS growth provided correlations with vaccine efficacy, while analysis of purified mRNA from both recovered splenocytes and macrophages by real-time PCR provided information about genes involved in these immune interactions.
**Results:** Initially, we analyzed protein and gene expression of LVS-infected bone marrow macrophages to differentiate their contributions in controlling *in vitro* bacteria growth from the effects of splenocytes of vaccinated mice that were added to the culture. Macrophage expression of IL-6, IL-12 and TNF-α was partially independent of vaccine efficacy. In contrast, IFN-γ was produced entirely by splenocytes from LVS-vaccinated mice. Subsequently, we extended the analyses to a large panel of genes of immunologic interest. Comparisons of either total splenocytes or splenic T cells from LVS-vaccinated mice to those from non-vaccinated mice indicated that IFN-γ was up-regulated, as would be expected. However, other genes such as CCR3, TNF-α, IL-12Rβ2, IL-9, IL-6, Gf1, T-box 21 and CXCR3 were reproducibly up-regulated as well. The most notable down-regulated genes were Spp1, IL-18R1, Tnfrsf8 and Tyk2.
**Conclusions:** Our model appears to be a valid tool for identifying genes that predict protection against *Francisella tularensis*. Ongoing studies are extending these data, comparing responses to LVS with good, fair, and poor lots and derivatives of LVS, as well as qualitatively different new *Francisella* vaccines.

## POSTER PART 1 – MOLECULAR ANALYSES AND EPIDEMIOLOGY, PERSISTANCE AND DISEASE CONTROL

### P1-01

## A constraints-based systems approach to metabolic analysis of *Francisella tularensis* during infection

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*Francisella tularensis* is an ill-characterized pathogen and a potential biowarfare agent necessitating a better understanding of its metabolism and virulence mechanisms. Genome scale constraints based metabolic models built for several microorganisms including *S. typhimurium* and *H. influenzae* provide a systems framework to study metabolism, infection and pathogenesis. We have developed a metabolic network reconstruction for *F. tularensis* using the current genome sequence annotation and biochemical legacy data. Mathematical techniques like Flux balance and flux variability analysis (FBA, FVA) were used to explore the metabolic capabilities of the *in silico* pathogen under *in vitro* and *in vivo* conditions. Gene expression, growth and physiological data were obtained experimentally and used as additional constraints in the model to compute and predict functional states of the pathogen. Integrating this data into the model as constraints allow us to reduce the solution space representing cell behavior and predict cellular function with more accuracy. *Francisella* was capable of growth with different specific growth rates on glucose and alternate carbon sources including glycerol, xylose, ribose, fructose and arabinose in a chemically defined medium with differential and preferential consumption of amino acids thus validating our model prediction that they can make up the bulk of the biomass. Gene expression of selected metabolic genes at specific time points of *francisella* during infection provide a snapshot and implicate certain metabolic pathways as critical to growth and survival in the host cell. Some observations include operation of TCA cycle in a branched mode; several functional amino acid pathways. Fatty acid gene transcript levels indicate their role as preferred gluconeogenic substrates during infection. Several gene transcripts detected in this data set were present in the optimal reactome calculated using FVA. Such an analysis provides insights into the lifestyle of the bacterium and the metabolic dependence on the host. Iterative model building can further help refine our understanding of the intracellular milieu encountered by the bacterium inside the host-cell and point towards genes essential in growth, survival and pathogenesis. Collectively, the results presented herein suggest an effective systems biology strategy of combining *in silico* modeling with experimental technologies to discover novel drugs and targets.

**P1-03**

## *Francisella novicida* as a model to study tick transmission

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**Aims:** Many bacterial pathogens that impact human and animal health are transmitted by ticks. However, little is known about the mechanisms by which these pathogens are able to transition from the mammalian to the vertebrate host. Using a well-characterized *Francisella novicida* transposon mutant library and Dermacentor andersoni ticks, the goal of this research was to develop an in vivo negative selection assay to identify the genes required for tick colonization.
**Methods:** BALB/c mice were inoculated subcutaneously or intraperitoneally with one or more *F. novicida* mutants. Dermacentor andersoni nymphs were then fed on the bacteremic mice. Blood and ticks were cultured on selective media. Culture results were confirmed by mutant-specific PCR. Quantitation of the relative amounts of each *F. novicida* mutant in the mouse or tick was done by real-time PCR.
**Results:** The infection rate in D. andersoni nymphs that fed on *F. novicida*-infected mice varied from 12 to 100 %, depending on the level of bacteremia during the time of feeding. The presence of *F. novicida* within the ticks reflects colonization rather than transient infection because the number of colony forming units within the tick increases after feeding, ticks remain infected after molting to adults, and *F. novicida* colonies can be visualized in midgut epithelial cells. When mice are infected with two *F. novicida* mutants, the proportion of each mutant comprising the bacteremia in the mouse is reflected in the tick infection. To further validate the utility of this model, *F. novicida* strains with mutations in outer membrane proteins or chitinase will be screened for their ability to colonize the tick.
**Conclusions:** This is the first work demonstrating the ability of *F. novicida* to colonize D. andersoni ticks. The *F. novicida* mutant library provides a powerful tool to identify the genes required for transition from the mammalian host to the tick vector, which is essential for maintenance of many tick-borne bacterial pathogens.

**P1-04**

## Molecular typing of *Francisella tularensis* strains isolated in Georgia

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**Aim:** The National Center for Disease Control and Public Health of Georgia (NCDC) carries out surveillance on especially dangerous pathogens throughout the country. Seasonal outbreaks of tularemia in Georgia represent a public health concern. From 2006 to 2008, two outbreaks occurred in addition to nine sporadic cases. The aim of this study was
1) to subtype both new isolates of *Francisella tularensis* and archived strains from NCDC collection,
2) to create a reference genotype database for Georgia, and
3) to identify epidemiological relationships between geographically diverse environmental strains and clinical samples.

**Methods:** Pulse Field Gel Electrophoresis (PFGE) methodology was performed on archived strains of *F. tularensis* and LVS. A 25 marker Multiple Locus Variable Number Tandem Repeat Analysis (MLVA) system was used to identify genetic relationships among 17 from 87 Georgian *F. tularensis* isolates. Based on observed data a 3-marker MLVA multiplex assay was developed to provide a convenient and rapid subtyping system using markers which showed diversity on a country-wide strain panel. These 3 markers were used for preliminary subtyping of the remaining 70 *F. tularensis* strains, bubo aspirates and tick emulsions.

**Results:** PFGE profiles for all strains were the same. All Georgian isolates of *F. tularensis* typed as *holarctica* (TypeB) using C1-C4 marker. Two MLVA markers (M3-M4) revealed a total of six unique genotypes across all Georgian strains. The genotypes from water samples isolated during the outbreak were identical to those of clinical samples taken in the same region. The multiplex assay containing the two diverse markers and TypeB control (C1-C4) showed robust amplification of all three markers in a single reaction mix.

**Conclusions:**
a) *F. tularensis* type B is the prevalent subspecies in Georgia and shows little genetic diversity as previously observed in MLVA analyses across globally diverse isolates;
b) MLVA is useful for subtyping archival isolates, for direct detection and subtyping of clinical diagnostic material, and in subtyping samples directly from vector species preparations;
c) This multiplex technique can be used in the future for fast and precise typing of clinical and environmental samples as well as newly isolated strains. This assay thus assists in epidemiological analyses and in making predictions regarding the relationships among outbreaks that occurred at different times and different locations.

P1-05

## Phenotypical analysis of the putative 2nd polysaccharide gene cluster of *Francisella tularensis*

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**Aims:** *Francisella tularensis* has one polysaccharide gene cluster, which encodes the O-antigen, and one putative polysaccharide gene cluster. The function and product of the putative cluster is unclear. The aim of this study is to dissrupt the putative polysaccharide gene cluster by deleting a gene and analyse the resultant mutant's phenotype.
**Methods:** Deletion mutants of the polysaccharide gene cluster were made from subspecies *novicida* and *tularensis* and the virulence of these strains was determined. Sensitivity to guinea pig serum in addition to the uptake and survival in macrophages was compared to that of the wild-type strains.
**Results:** The galE gene was deleted in both subspecies. In the mouse model the subspecies *tularensis* mutant was virulent, whereas in subspecies *novicida* the mutant was attenuated and afforded protection against a *novicida* challenge. Both mutants were impaired in their ability to replicate in vitro and showed no difference to serum sensitivity compared to the wild-type strains.
**Conclusions:** Our study shows that the putative second polysaccharide gene cluster products for subspecies *novicida* and *tularensis* differ in their role in virulence. © Crown Copyright, Dstl, 2009.

P1-06

## Effect of Hfq on *Francisella tularensis* growth and stress resistance

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**Aims:** The goal of this research was to determine the impact of the RNA chaperone protein Hfq on *Francisella tularensis* growth and survival.
**Methods:** The Hfq protein has been shown to play an important role in small RNA-mediated regulation of gene expression in a wide range of bacteria. An *hfq* knockout mutant of *F. tularensis* subsp. *novicida* strain U112 was constructed and growth rates were monitored under a number of stress conditions including low pH, high salt content, and high temperature. Expression levels of *hfq* were analyzed via quantitative real-time PCR under these same conditions. Because *hfq* mutants in other bacteria have exhibited altered cell morphology, both the wild-type and mutant strain were viewed under transmission electron microscopy.
**Results:** The *hfq* mutant exhibited a slower growth rate compared to wild-type under all conditions tested, particularly at 42 °C. Data obtained from qRT-PCR analysis of the wild-type showed minor fluctuations in *hfq* expression during growth under both optimal and stress conditions. TEM images showed no distinct difference in cell morphology between wild-type U112 and the *hfq* mutant strain.
**Conclusions:** The Hfq protein appears to play an important role in *F. novicida* growth and survival under a variety of different stress conditions. The minor changes in *hfq* expression suggest that a certain concentration of Hfq is ideal for cell growth and survival. The lack of altered cell morphology in the *hfq* mutant of *F. novicida* is consistent with previous studies of *hfq* in *F. tularensis*. Hfq often assists regulation of gene expression by small RNAs in other bacteria and, though no small RNAs have been found to date in *F. tularensis*, a similar relationship could account for the observed impact of Hfq on cell growth.

## P1-08

# Production of outer membrane vesicles and tube-like structures by *Francisella tularensis*

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**Aims:** *Francisella tularensis* is a Gram-negative bacterium and the causative agent of tularemia. This organism lacks many of the virulence-associated secretion systems typically found in other intracellular pathogens, such as the type III and type IV pathways. Outer membrane vesicles (OMVs) are constitutively shed from growing Gram-negative bacteria and have been shown to act as secretory vehicles for toxins in other pathogenic bacteria. We examined the production of OMVs by *F. tularensis* to assess their potential as a virulence factor secretion system.
**Methods:** We isolated OMVs from *F. tularensis* subsp. *novicida* U112 using high-speed centrifugation of filtered culture supernatant fractions. Intact bacterial cells and isolated OMVs were visualized by transmission electron microscopy. Vesicle fractions were further purified via flotation through a density gradient. Purified vesicles were analyzed for protein content using SDS-PAGE and mass spectrometry.
**Results:** We were able to isolate OMVs from strain U112. Examination of purified vesicles revealed the presence of tube-like structures in addition to spherical OMVs. The tube-like structures were also observed protruding from the surface of intact bacteria. Using mass spectrometry we identified several proteins enriched in the OMV/tubes. Examination of various U112 mutants identified genes associated with type IV pilus production as important for secretion of some vesicle-associated proteins and for OMV/tube production.
**Conclusion:** *F. tularensis* subsp. *novicida* U112 produces OMVs as well as novel, tube-like structures. The tube-like structures are both secreted into the culture medium as well as present on the bacterial surface. Genes associated with type IV pilus biogenesis appear to play a role in OMV/tube production. The function of the OMVs and tube-like structures is currently unknown, but some of the proteins identified as vesicle-associated were previously shown to be important for *Francisella* virulence.

P1-09

## The phosphoproteome of pathogenic bacterium *Francisella tularensis*

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**Aims:** The purpose of this study is to compare the distribution of phosphorylated proteins in *F. tularensis* FSC155, FSC200 and Schu S4 strains differing in virulence and further to identify the phosphorylated proteins and characterize their site of post-translational modification.
**Methods:** *F. tularensis* strains were cultivated under standard or stress conditions in Chamberlain medium to late logarithmic phase. The whole cell lysates and the membrane fractions were used for *in vitro* kinase assay with $\gamma^{32}$P-ATP to label *F. tularensis* phosphoproteins. In order to distinguish between N-and O-phosphates, we tested the chemical stability of bound phospho-group in acidic or alkalic environment. Control reactions were incubated on ice without acid or alkali. The samples were separated on 2D gels which were exposed to BioMax MS films. The protein spots corresponding to phosphoproteins were excised and after trypsin digestion analyzed on 4800 MALDI TOF/TOF analyzer. The phosphopeptides were enriched using $TiO_2$ and IMAC.
**Results:** We found out that the strains differ in a number of phosphoproteins. The most phosphorylated proteins were found in highly virulent Schu S4 strain. As expected, under standard conditions mainly proteins participating in glycolysis (e.g. glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate mutase), tricarboxylic acid cycle (e.g. pyruvate 2-oxo-glutarate dehydrogenase) and protein biosynthesis metabolism (e.g. elongation factor EF-Tu) were detected. For several proteins more than one spot differing in the pI was found. This might indicate differentially phosphorylated or otherwise covalently modified forms of the same protein. Further studies concerning phosphoproteins detected under stress conditions (heat stress, iron starvation, stationary phase and oxidative stress) are underway aimed at detailed characterization of Schu S4 phosphoproteome in relation to the proteins function.
**Conclusions:** Phosphorylation is a key regulatory mechanism in bacteria often connected with the virulence. So far there are no consistent data about presence of phosphorylated proteins and their function in *F. tularensis*. This work is one of the first dealing with this problem.
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## P1-10

## Comparative proteome analysis of *Francisella tularensis* LVS and DsbA deletion mutant: identification of potential substrate proteins for the DsbA

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**Aims:** The conserved hypothetical lipoprotein (FTL_1096) exhibits high homology with proteins of the disulfide oxidoreductase DsbA family. In our previous studies the gene encoding this DsbA homologue was verified to be required for survival and replication in macrophages and also for *in vivo* virulence in the mouse infection model for tularemia. The DsbA proteins are known to be important for toxin secretion or proper folding of outer membrane adhesions in many pathogens. To recognize *F. tularensis* proteins whose folding might be dependent on the DsbA protein activity we investigated the possible membrane accumulation of misfolded proteins in the *dsbA* mutant.
**Methods:** To examine the potential substrate proteins for the *F. tularensis* DsbA homologue, we performed comparative proteomic analyses of fractions enriched in membrane proteins of LVS and ΔFTL1096 mutants. The fractions enriched in membrane proteins of bacteria grown in defined Chamberlain medium were prepared using carbonate extraction and ultracentrifugation. The obtained protein samples were analyzed using both classical (two-dimensional gel electrophoresis + mass spectrometry identifications) and shotgun (iTRAQ labeling + LC-MS/MS) proteome approaches.
**Results:** Using the both mentioned proteomic approaches we were able to identify in total seven up-regulated proteins in fractions of the mutant strain. Five proteins do not exhibit any homology to known bacterial proteins and their function has to be further investigated. In addition to the DsbA protein, two other proteins were found down-regulated in *dsbA* mutant.
**Conclusions:** The proteins differentially expressed in the mutant strain appear to depend on DsbA for localization and function and are candidates for being more directly responsible for its virulence attenuation. Further studies are under way to address the role of these genes in the pathogenesis of tularemia.

**P1-11**

## Analysis of the phenotype associated with the disruption of *mglA* in *Francisella tularensis* LVS underlines its central role in manifestation of virulence

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**Aims:** MglA is a pleiotropic transcription factor controlling the expression of more than 100 genes in *F. novicida*, including the ORFs located on the pathogenicity island. In this study, we further probe the role of MglA in the pathogenicity of the related strain *F. tularensis* LVS.
**Method:** A mutant strain in which the *mglA* locus was disrupted was generated in the LVS background, by insertion of a non-polar selectable marker cassette. The phenotype associated with the *mglA* null mutation in comparison to the isogenic parental strain was analysed *in-vitro* and *in-vivo*.
**Results:** The Δ*mglA* mutant:
(I) cannot multiply *in-vitro* in macrophages,
(II) is severely attenuated in a murine model of infection, exhibiting over $10^4$ fold decrease in virulence by intra-nasal (IN) administration, and over $10^7$ fold decrease by the intra-peritoneal (IP) route of infection,
(III) unlike the wild type LVS strain, following IP administration, the mutant cannot multiply in the lungs, liver and spleen of infected animals,
(IV) following IN administration Δ*mglA* bacteria do not disseminate to target organs (e.g. liver and spleen). Infection by Δ*mglA* bacteria elicit a significant humoral response, but does not appear to induce a cellular immune response, judging by the levels of INFγ and IL-2 in an *in-vitro* stimulation assay. High doses of Δ*mglA* bacteria can protect mice against a lethal IP challenge of the wild-type LVS strain.
**Conclusion:** The results indicate that MglA plays a major role in *F. tularensis* LVS virulence as shown previously for *F. novicida*. Our studies suggest that inactivation of *mglA* may serve as a platform for the development of improved attenuated vaccine of virulent *F. tularensis* strains.

P1-12

## Mapping the presence of *Francisella tularensis* glycoproteins

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**Aim:** Our study seeks to discover the presence of glycoproteins in the bacterium *Francisella tularensis (F. tularensis)*, in which the molecular mechanisms of virulence are still not understood.
**Methods:** Both detection and enrichment glycoproteomic strategies were undertaken to explore the occurrence of glycoproteins *in F. tularensis*.
The presence of glycoproteins was investigated using two detection systems, applied to 2D-PAGE-separated samples. First, carbohydrate-specific staining was used. *Cis*-diol carbohydrate groups of glycoproteins were oxidized, followed by reaction of the resulting aldehydes with the fluorescent dye. In the second method, various digoxigenin-labeled lectins were utilized. 2D-PAGE-separated proteins were blotted onto nitrocellulose membranes where putative glycoprotein-capturing lectins were detected with anti-digoxigenin-labeled alkaline phosphatase. The detected proteins were excised from gels, tryptically digested, and identified by various types of mass spectrometers.
Once the presence of glycosylation was disclosed, enrichment techniques, such as lectin-affinity chromatography and chromatography on phenylboronic acid-based resin, were employed. In lectin-affinity approach, the lectins Concanavalin A, *Sambucus nigra* agglutinin, Peanut agglutinin, and *Datura stramonium* agglutinin were utilized. Isolated glycoproteins were subjected to trypsin digestion and the digests were analyzed by cap RP-HPLC interfaced to a mass spectrometer. By using phenylboronic acid-based resin, glycoproteins containing *cis*-diol groups were bound to *m*-aminophenylboronic acid. Eluted glycoproteins were then tryptically digested and identified by mass spectrometry.
**Results:** Several putative glycoproteins were detected by the fluorescent reagent and identified as FTL1096, FTL0112, and FTL1328, etc. Moreover, putative glycoproteins FTL0112, FTH1463, FTL0949, and FTL1328 were found out by using labeled lectins. Lectin-affinity chromatography revealed the presence of several putative glycoproteins, e.g. FTL1096, FTH0414, and FTH0384. Finally, FTH0414 was observed as glycosylated using the phenylboronic acid-based resin.
**Conclusions:** In this study, the presence of glycosylation-modified proteins was determined. The differences in occurrence of glycosylation among the analyzed *F. tularensis* subspecies were observed.

**P1-13**

## The only alternative σ factor of *Francisella tularensis* is a genuine heat shock σ factor

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The ability of *Francisella tularensis* to replicate within macrophages relies on the tightly regulated expression of a series of genes. Regulation of gene expression in bacteria occurs primarily at the transcriptional level. The association of dedicated alternative sigma factors to the core of the RNA polymerase (RNAP) provides a simple and efficient way for bacteria to rapidly adapt to various environmental changes. The RNAP holoenzyme contains the subunits of the core molecule (two α subunits, the β, β' and ω subunits) and a sigma factor enabling the holoenzyme to specifically recognize promoter elements and initiate transcription at these sites. The regulon of a single sigma factor can comprise hundreds of genes and the number of sigma factors encoded by different bacterial species varies considerably. *In silico* analysis of the *F. tularensis* LVS genome led us to identify, in addition to the genes encoding the RNAP core, one gene encoding the major sigma factor $\sigma^{70}$, and a unique gene (*FTL_0851*) encoding a putative alternative sigma factor homolog of the $\sigma^{32}$ heat shock family (designated *rpoH*). Hence, *F. tularensis* represents one of the minorities of bacterial species that possess only one or no alternative sigma factor in addition to the main factor $\sigma^{70}$. We have shown that *FTL_0851* encodes a genuine $\sigma^{32}$ factor. Transcriptomic analyses of the *F. tularensis* LVS heat stress response allowed the identification of a series of orthologs of known heat shock genes and a number of genes implicated in *Francisella* virulence. A bioinformatic analysis was used to identify genes preceded by a putative $\sigma^{32}$-binding site. Our results suggest that RpoH is an essential protein of *F. tularensis*, which positively regulates a subset of genes involved in heat shock response.

**P1-14**

## Genotyping of the Health Protection Agency *Francisella* strain collection

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**Aim:** The Health Protection Agency (HPA) *Francisella* strain collection consists of 17 *Francisella* isolates collected from North America and Europe over the last 56 years. The aim of this study was to investigate the genotype of the isolates in the *Francisella* strain collection.
**Methods:** DNA was extracted from the 17 isolates in the *Francisella* strain collection. RD1 analysis was carried out as described by Broekhuijsen *et al*, 2003 to discriminate between the four subspecies. Multi-locus variable-number tandem repeat analysis (MLVA) has been used to further investigate their phylogeny in relation to other isolated strains.
**Results:** The isolates have been identified to subspecies level using RD1 analysis. MLVA analysis has allowed the genetic relationships to be further broken down, and a comparison to be made with other known characterized strains.
**Conclusions:** The collection contains isolates belonging to *F. tularensis* subspecies *tularensis*, *F. tularensis* subspecies *holarctica* and *F. tularensis* subspecies *novicida.* The identified subspecies and the locations they were isolated from are in agreement with the current geographical distribution models of *Francisella tularensis.*

**P1-18**

## Non-antibiotic selectable markers for *Francisella tularensis* strains LVS and Schu S4

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**Aims:** The aim of this project was to identify genes that could serve as non-antibiotic selectable markers for genetic manipulation of *F. tularensis* LVS and Schu S4.

**Methods:**

1) A review of the annotated genomes revealed that both organisms lack critical genes in the biosynthetic pathways for each of 12 amino acids found to be absolutely required for growth in Chamberlain's defined medium (CDM). From the isoleucine-leucine-valine pathway, *ilvD*, encoding dihydroxy acid dehydratase, is present in Schu S4 but not in LVS, while *ilvC*, encoding ketol acid reductoisomerase, is present in LVS but not Schu S4. We tested with reformulated CDM whether the metabolic intermediates, 2-isovalerate and 3-methyl-2-oxopentanoate could rescue the dependency on valine and isoleucine, respectively, which would be highly suggestive that *ilvD* and *ilvC* could serve as non-antibiotic selectable markers.
2) In a second line of experimentation we tested whether the Calvin Cycle enzyme phosphoribulokinase (PRK) would interfere with the pentose phosphate pathway, leading to a casamino acid (CAA)-dependent phenotype in CDM. The hypothesis was that the PRK phenotype could be rescued by a second key enzyme from the Calvin Cycle, ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO). PRK from the cyanobacterium *Synechococcus* PCC7942 was codon optimized and expressed from the *Francisella groE* promoter, and it was also co-expressed with codon-optimized RubisCO from the bacterium *Rhodospirillum rubrum.*

**Results:**

1) When added to reformulated CDM, 2-isovalerate relieved the requirement for valine, and 3-methyl-2-oxopentanoate rescued the dependency upon isoleucine, for both LVS and Schu S4.
2) PRK transformants could not be obtained on rich medium, even when co-transformed with RubisCO. As such, the phenotype on CDM could not be tested.

**Conclusions:**

1) The outcomes with 2-isovalerate and 3-methyl-2-oxopentanoate suggest that *ilvD* and *ilvC* will serve as non-antibiotic selectable markers in *Francisella*. The genes have been amplified and cloned for further testing.
2) The use of RubisCO as a selectable marker could not be tested, due to the toxicity of PRK even in rich medium.

This outcome suggests that tighter control of PRK expression will be required to test RubisCO as a selectable marker. As such, we are now examining use of the *Francisella trp* promoter as an inducible promoter for better control of PRK expression.

**P1-21**

## Results from screening the *F. novicida* transposon two-allele mutant library

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The availability of the *F. novicida* Tn 2-allele mutant library provides opportunity to study genes of interest in parallel at a genomic level for the first time. We aimed to study the cytopathogenicity of each mutant by screening this library in several steps. Firstly, J774 macrophage-like cells were seeded in 24-well plates and infected with $OD_{600}$-unnormalized overnight cultures. Changes of J774 cell morphology were followed since infection. Mutants were put into different categories such as faster killing, slower killing and normal killing groups per their killing kinetics. To verify some of those observations, individual mutant culture was OD600 normalized and used to infect J774 cells for morphology observation, CFU counting, and LDH measurement in 6-, 24-, 96-well plates respectively. To test in vivo significance, mutants were selected to infect Balb/C mice and animal survival was monitored.
In summary, some mutants have differential in vitro growth defect, and some mutants are faster or slower in killing J774 cells than U112. In vitro results of host cell cytopathogenicity and bacterial intracellular growth ability are the two major criteria to judge the potential of a mutant for attenuation. Utilizing host cell morphology change as an initial screening standard has several advantages over the use of intracellular bacterial growth in that it makes possible examination of one experimental sample at multiple time points, thereby reducing the chance of overlooking mutants that could be normal in intracellular growth. The strategy could also be useful for screening libraries of human virulent *F. tularensis* strains.

**P1-22**

## Identification of *Francisella* loci that impact expression of *rip*A

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**Aim:** The goal of this study was to identify loci in *F. tularensis* that impact expression of RipA, a known virulence determinant in LVS.
**Methods:** We created a *ripA-lacZ* reporter and integrated it onto the chromosome of *Ft* LVS. This reporter strain, JF127, was subjected to transposon mutagenesis. Transformed bacteria were selected on media containing X-gal for blue/white screening. Mutants exhibiting an altered colony phenotype compared to JF127 were subjected to further study. Expression of *rip*A in these mutants was compared to the parent strain using a standard β-galactosidase assay. Transposon insertion sites were identified by amplifying adjacent genomic sequence using semi-degenerate PCR. Mutants that exhibited a decrease in *rip*A expression were evaluated for their ability to replicate within J774.1 and TC-1 cell lines.
**Results:** 77 of roughly 7000 total mutants were selected for further analysis based on their blue/white phenotype. Of these, 22 harbored transposon insertions in *lac*Z. Transposon insertions into FTL0329, FTL1306, FTL1929, FTL0895 (*hup*B) and FTL1810 (*nus*A) each resulted in a ≥ 2-fold decrease in *rip*A expression. Transposon insertions into FTL0439 (hypothetical), FTL0957 (*bla*A), and FTL0707 all resulted in a ≥ 2-fold increase in expression of *rip*A. Interestingly, we obtained 3 total insertions in FTL0439 and 2 insertions in FTL0073. Deletion of FTL0073 in *Ft* LVS resulted in a mutant strain with reduced intracellular growth in both J774.1 and TC-1 cells. Additionally, this mutant also demonstrated reduced virulence in a mouse model of inhalation tularemia.
**Conclusions:** The use of a *ripA-lacZ* reporter allowed us to rapidly screen a transposon library for loci that impacted expression of *rip*A. β-galactosidase activity of mutants vs the parent strain were in agreement the observed colony phenotype. Additionally, one of the mutants, FTL0073, was found to be defective for growth in both *in vitro* and *in vivo* models of *F. tularensis* infection.

**P1-24**

## Siderophore utilization in *Francisella tularensis* Live Vaccine Strain (LVS)

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Under conditions of iron-limitation, strains of *F. tularensis* secrete a siderophore whose production is dependent on the genes of the *fsl* operon which is highly conserved across the different strains. *fslE* encodes a potential siderophore receptor that is essential for siderophore utilization in Type A strain Schu S4 and in *novicida* strain U112.
**Aims:** A study was undertaken to ascertain if the mechanism for siderophore utilization in the Live Vaccine Strain (LVS) of *F. tularensis* was similar to that in Schu S4.
**Methods:** An in-frame deletion was generated in the *fslE* gene of LVS using a *sacB* suicide vector. Immunoblotting was used to test for presence of FslE in cell lysates of LVS, a *fur* mutant derivative of LVS and the *fslE* mutant. The deletion mutant was tested for ability to grow in iron-limiting medium in liquid and on plates in paired comparison with the parent LVS. It was also tested for ability to produce and to utilize siderophore in a plate-based assay.
**Results:** We confirmed loss of the band corresponding to FslE in immunoblots of *fslE* mutant lysates. Growth of the mutant was similar to parent LVS in iron-limiting medium. The mutant also produced siderophore and was able to utilize siderophore equally as well as the parent.
**Conclusions:** These studies indicate that FslE does not play a critical role in siderophore utilization by LVS. Although siderophore production is similar in LVS and Schu S4, alternate uptake mechanisms appear to be functional in the vaccine strain. Studies directed at identifying these mechanisms will assist in understanding if iron acquisition impacts virulence traits in *F. tularensis*.

**P1-28**

## Type IV pilin-like proteins of *Francisella tularensis* contribute to virulence in a context-dependent manner

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**Aims:** To discern a role for the type IV pilus in virulence of *Francisella tularensis* ssp*. tularensis* (Schu S4) and a derivative of *F. tularensis* ssp. *holarctica* (LVS).
**Methods:** In *Francisella* spp. there are six proteins with significant homology to type IV pilins and the potential to encode the major subunit of a type IV pilus. In LVS, only three of these genes encode full length proteins. We used homologous recombination to generate independent deletions of these three genes in Schu S4 (designated FTT0861c, FTT0230c, and FTT1314c), as well as their counterparts in LVS, and performed transmission electron microscopy (TEM) to determine whether these mutations altered the production of extracellular fibrous appendages. To determine the potential role of these genes in adherence and invasion to host cells, all knockout strains were subjected to *in vitro* adherence assays to compare adherence and invasion phenotypes relative to their wild-type parent strains. *In vivo* mouse studies are ongoing to characterize what, if any, roles these proteins have in virulence.
**Results:** TEM data suggests that two of the type IV pilin homologs are not involved in the production of pili, while the third protein is indispensible and participates either as the major structural subunit or part of the assembly apparatus. Counterpart mutations in Schu S4 and LVS exhibited similar piliated phenotypes. However, *in vitro* adherence and *in vivo* virulence studies indicate the three proteins may play different roles in these processes in their respective strains.
**Conclusions:** Taken together, the data suggest that subtle contextual differences in type IV pilin systems contribute to the different infectious capacities of virulent and avirulent *Francisella* strains.

**P1-34**

## Validation of Etest® for the determination of antibiotic susceptibilities of *Francisella tularensis*

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**Aims:** To assess the antibiotic susceptibility of a panel of *Francisella tularensis* subsp. *holarctica* strains isolated in France and to evaluate the accuracy of the Etest® technique by comparison with a reference method.
**Methods:** We determined the MICs of eight antibiotics for 71 strains of *F. tularensis* subsp. *holarctica* isolated in France, by agar dilution and Etest® methods on IsoVitaleX™-supplemented Mueller-Hinton II agar. We assessed susceptibility to doxycycline, nalidixic acid, ciprofloxacin, rifampicin, streptomycin, gentamicin, telithromycin and chloramphenicol.
**Results:** All isolates were fully susceptible to all antibiotics tested. The two methods gave similar MICs in more than 85 % of cases for ciprofloxacin, doxycycline and streptomycin. Susceptibility classification was identical with both methods for all antibiotics, even those for which poor agreement was observed between MIC values. Antibiotic susceptibilities to gentamicin, ciprofloxacin and doxycycline were also determined by these two methods on supplemented chocolate medium. On this medium, more than 80 % agreement was observed for the MICs of ciprofloxacin and doxycycline and 77 % agreement was observed for gentamicin.
**Conclusions:** All French isolates of *F. tularensis* subsp. *holarctica* tested were susceptible to the main antibiotics proposed for treatment or prophylaxis of tularaemia. Etest® appears to be a practical and reliable method for studying the antimicrobial susceptibility of *F. tularensis,* including the subspecies *tularensis*, on supplemented Mueller-Hinton II reference medium. This technique can also be applied by routine or field laboratories with ready-to-use, commercially available, supplemented chocolate agar medium.

P1-35

## Tulateremia wet-lab exercise

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**Aims:**
1) compare and exchange working knowledge of tularemia disease in endemic countries and,
2) improve the connection among the network of laboratories charged with the responsibility to detect, assess and confirm tularemia disease in humans, animals and its presence in the environment,
3) opportunity to examine different diagnostic techniques and algorithms.

**Methods:** Since 2000, the World Health Organization (WHO) has been working with an advisory group of tularemia experts to develop a comprehensive tularemia guidance (www.who.int/csr/resources/publications/deliberate/WHO_CDS_EPR_2007_7/en) and organizing periodic reviews and updates. As an outcome of these activities, it was proposed that a tularemia wet-lab exercise, jointly hosted by WHO and by tularemia expert laboratories, would be of value. Therefore, as an initiative of the Tularemia International Society (TULARINS), FOI (Swedish Defence Research Agency) with support from the World Health Organization hosted a comparative exercise workshop on the detection of *Francisella tularensis* and the diagnosis of tularemia. The focus of the workshop was to perform molecular, culture and serology methods for the detection of *F. tularensis* and the clinical diagnosis of human specimens. Each participating laboratory had the opportunity to compare and validate their methods using unknown samples provided at the workshop. There were also demonstrations of other techniques as determined by the participants request.

**Conclusions:** This exercise was very successful in bringing together 8 countries to compare and evaluate their diagnostic tests for the detection of *F. tularensis* and related organisms.
As the exercise workshop format requires hands-on participation, only a limited number of teams may be hosted at any one time. Therefore, a series of workshops may be organized on a continual basis under the auspices of various volunteer hosting sites in order to make the opportunity available to all qualified laboratory that wishes to participate.

**P1-36**

## Development of reference material to perform EU-wide proficiency tests for the diagnosis of highly pathogenic bacteria, including *Francisella tularensis*

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**Aims:** Within the framework of an EU project 21 participants in 18 different countries will have the opportunity to assess their quality of diagnostics of highly pathogenic bacteria, including *F. tularensis*. One major aim is to build up a permanent network.
**Methods:** Several work packages ensure the exchange of information, material and training among participants in order to establish different tools, for example network shared reference stocks for internal and external quality control. Furthermore, in support of diagnostic methods several external quality exercises with increasing degree of difficulty were and will be organized.
During the first exercise each laboratory received 15 different DNA samples for detection, including *F. tularensis ssp tularensis*, *F. tularensis* ssp*. holarctica* and *F. philomiragia*, as well as 15 samples of diverse species of inactivated bacteria, incl. *F. tularensis,* the latter of which were diluted in three matrices, PBS and water and mouse myeloma-cells as a clinical substitute specimen. To avoid any deviation of results deriving from the preparation of the test items, particular carefulness was attributed to planning, design, marking, packaging and transport. All samples were tested several times by molecular methods, immunological performance, sterility testing and microscopy to ensure high quality and homogeneity of the test items and to prevent cross contamination.
**Results:** The overall evaluation of the first exercise revealed the high quality of the provided reference material and various approaches in working procedures, equipment and preparedness of the participating laboratories. However, there is obvious need for further improvement of laboratory diagnostics because the differentiation of the subspecies as well as the contamination by *Francisella tularensis* and other coccobacilli during DNA extraction of inactivated samples produced false positive results of *F. tularensis.*
**Conclusion:** The best testing methods and working procedures according to quality management guidelines evaluated will be standardized after having proven practicable for all laboratories being involved.

P1-39

## Therapy approach and problems in treatment tularemic patients in Serbia

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**Introduction:** The first epidemic of tularemia in South-eastern Serbia happened in 1999. During 10 years period, from 1999. till 2008, there were hospitalized and treated 151 patients.
**Aims:** The purpose of the study was to analise and determine the best therapy choices in patients suffered from tularemia.
**Methods:** Before hospitalization 16.5 % were not treated, 58.8 % were inadequately treated, 10.3 % were adequately treated and 14.4 % unknown.An average duration of disease before hospitalization was 35 days. Antibiotic monotherapy was administrated to 53 patients: gentamicin (27 patients), ciprofloxacin (11), amikacin (8), streptomycin (5) and doxycycline (2 patients). Combined (polyvalent or successive) antibiotic therapy was administrated to 98 patients: gentamicin and ciprofloxacin (66 patients), gentamicin and doxycycline (18), ciprofloksacin and doxycycline (2), gentamicin, ciprofloxacin and doxycycline (12 patients).
**Results:** The biggest success in treatment (75 % cured) was remarked in patients after combination of gentamicin, ciprofloxacin and doxycycline, then combination of gentamicin and ciprofloxacin (71%), then monotherapy of gentamicine (68 % ) and combination of gentamycin and doxycycline (67% cured). In comparison to number of treated patients, the biggest success in treatment (71% ) had combination of gentamicin and ciprofloxacin, then monotherapy of gentamicine (68 %).
Outcome of medicamentosus treatment: 92 patients (61%) of 151 were totally cured. The complications happened at 59 patients (39 %): 1.abscessing of lymph nodes with or without fistulisation at 36 patients ( 23.84 %), 2. relapse -7 patients (4.63 %), 3. both complications at 11 patients (11%). The relapses included repeated lymph node enlargement or intake more than one lymph nodes. Final outome after conservative and radical treatment was total curing at 143 patients (94.7%) and residual persistant lymphadenopathy at 8 patients (5.3 %).
**Conclusion:** Reasons for appearance of complications during therapy of tularemic patients are:

1. inadequatelly initial antibiotic treatment,
2. late oncet of adequate antibiotic therapy as well as possible appearence of resistant species of *F.tularensis* in the region of South-eastern Serbia.
   We can conclude that early, forehand treatment restrains the colliquation of lymph nodes, recurrence of disease and dissemination of infection. Therefore, the early diagnosis and treatment are crucial for prevention of complication appearence and complete curing.

**P1-40**

## Detection of *Francisella tularensis* in the European hare by real-time TaqMan PCR

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Tularemia in lagomorphs (European brown hare [*Lepus europaeus*] and European wild rabbit [*Oryctolagus cuniculus*]), particularly due to *Francisella tularensis* subsp. *holarctica*, is historically enzootic in continental Europe. In France, the surveillance of tularemia is carried out almost exclusively on lagomorphs found dead or dying within the National Surveillance Network of Game Death Causes (SAGIR).
Bacterium sensitivity to temperature variations, as well as frequent putrefaction of the dead bodies, hamper diagnosis of tularemia in lagomorphs' tissue samples. A multitarget Real time TaqMan PCR assay, with primers and probes targeting the *tul4*, *fopA* and *ISFtu2* genes, has been developed in this laboratory, for *F. tularensis* detection and for isolate identification. In this study, conventional bacteriology, nested-PCR and real-time PCR were compared for their reliability in the detection of *F. tularensis.* DNA was extracted using a commercial kit. Analytical specificity was first confirmed on 30 other bacterial strains, frequently isolated from wild animals, particularly from hares. Moreover, the diagnostic specificity was 100 %, when evaluated on 635 spleen samples of hunted wild-boars, apparently healthy.
Diagnostic sensitivity was assessed on 298 hare tissue samples (spleen or liver) collected in France from 2004 to 2007. Our results showed that real-time PCR is significantly more sensitive than conventional bacteriology and nested-PCR (positive samples: 52 % *vs*. 9 % and 25 % respectively). Real-time PCR detected all culture-positive samples and all nested-PCR positive samples. Moreover, 27 % of the samples including especially those putrefied or stored for long periods of time, were detected only by real-time-PCR.
Sensitivity, fastness, simplicity and reliability of this multitarget real-time TaqMan PCR assay make it a useful tool for tularemia routine diagnosis, which appears to be more appropriate than conventional bacteriology and PCR tools for an adequate surveillance of tularemia in wildlife.

**P1-42**

## Prolonged course of tick-borne ulceroglandular tularemia in a 20-year-old patient in Germany – case report

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**Introduction:** Tularemia has become a rare zoonosis in Germany after world war II. Between 1974 and 2005 German health authorities recorded three cases per year on an average. In 2007 and 2008 annual recordings grew to 21 and 15 respectively. The number of estimated unknown cases is considered high. Transmission to humans occurs mainly from rabbits. Ectoparasites like ticks have not been reported as a vector for years though relevant transmission is well-known from other countries.
**Investigations and methods:** A 20-year-old female patient presented in our hospital with painful axillary lymphadenopathy. She reported a tick bite in her right hand five months ago in Saxony-Anhalt, Eastern Germany, followed by fever, chills and regional lymphadenopathy. Empiric antibiotic treatment with doxycyclin and ciprofloxacin had led to defervescence but no change in painful lymph node swellings. Surgical exstirpation of a cubital lymph node had already been performed three months after the tick bite.
Laboratory findings were normal apart from moderate elevation of C-reactive protein. Serology (ELISA and Westernblot) confirmed the suspected clinical diagnosis of ulceroglandular tularemia. Retrospective real-time PCR (markers *fopA* and *tul4*) for *Francisella tularensis* in lymph node histology preparations taken two months before was negative.
**Results:** Clinical presentation and serologic test results were consistent with a prolonged course of tick-borne ulceroglandular tularemia and sustained reactive lymph node swelling. The patient desired surgical exstirpation of the aching axillary lymph node. Histology showed reticulocytic, abscess forming lymphadenitis with pseudotuberculosis type granulomatosis and negative acid-fast staining. Culture, capture ELISA and real-time PCR for *Francisella tularensis* performed in native lymph node preparations were negative. A complete recovery was achieved without renewed antibiotic treatment.
**Conclusions:** According to recent seroprevalence studies, the emergence of tularemia as a rare zoonosis in Germany is clinically underestimated. Our case report illustrates possible appearance of the disease apart from risk groups (e.g. hunters, lumbermen). Ectoparasites like infected ticks have to be considered as vectors even in non endemic regions.

P1-43

## First isolation of *Francisella tularensis* subspecies *holarctica* from foxes (*Vulpes vulpes*) in Germany

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**Aims:** Serological investigations have shown that foxes may well serve as indicators for the occurrence of *Francisella* (*F.) tularensis* (Höflechner-Pöltl et al., 2000). Our serological and bacteriological examinations of fox samples were supposed to produce up-to-date epidemiological data on the prevalence of tularemia in the federal state of Brandenburg.
**Methods:** Between 2007 and 2008, organ and serum samples of numerous foxes in the federal state of Brandenburg (around Berlin) have been collected during rabies monitoring and stored at -80°C. 610 organ samples were homogenised immediately after defrosting. 30 µl of the homogenates of each organ sample were applied on Cystine heart blood agar (QUELAB) and Neisseria selective medium PLUS (OXOID) by means of 3 loop streaks. Additionally, a selective enrichment medium (modified medium T) was inoculated.
The identification of suspected colonies as *F. tularensis* was carried out by microscopical, serological and molecular-biological methods. For further characterisation of the isolate, the biochemical properties as well as the sensitivity to erythromycin and further antibiotics were tested. In order to identify the subspecies, an RD1 (region of difference) PCR was carried out and eight variable number tandem repeat (VNTR) locis were analysed.
**Results:** After examination of organ samples of 305 foxes, only from one liver *F. tularensis* ssp. *holarctica* biovar II could be cultivated so far. The bacterium was isolated from a fox from the region with the highest frequency of positive serological results.
**Conclusions:** *F. tularensis* ssp. *holarctica* definitely occurs in the federal state of Brandenburg. The gained epidemiological results suggest the existence of endemic regions. Our results confirm, that the fox is well suited as indicator animal for further epidemiological investigations of this significant zoonosis.

**P1-45**

## Enhanced growth of *Francisella tularensis* in a liquid nutrient medium (medium T)

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**Aim:** Enrichment and isolation of *Francisella tularensis* from environmental or clinical samples possibly containing disturbing flora.
**Methods:** The liquid nutrient medium T (Pavlovich and Mishańkin, 1987) based on heart-brain infusion, bactotryptone, technical casamino acids and a salt-glucose-cysteine-solution was tested for confirmation of growth and enrichment of *F. tularensis.* For selective cultivation of *F. tularensis* from environmental samples or samples with high concentration of contaminating flora, antibiotics and a fungicide (colistin, trimethoprim, vancomycin, sulfamethoxazol, amphotericin B) were added to the medium (modified medium T). The cell amount of *F. tularensis* was determined over four days on cysteine heart blood agar.
**Results:** Enhanced growth of *F. tularensis* strain LVS could be shown by inoculating a defined amount of the bacteria (2 x $10^2$ cfu/ml) into medium T. After three days of incubation, a growth up to 5 x $10^8$ cfu/ml could be detected independently from the addition of antibiotics to the media. Growth of other highly pathogenic bacteria like *Bacillus anthracis*, *Yersinia pestis, Burkholderia mallei* and *Burkholderia pseudomallei* could also be demonstrated in medium T. On the other hand, it could be shown that the modified medium T inhibited the growth of selected adulterating bacteria like *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, *Bacillus cereus* and *Yersinia pseudotuberculosis.* The application of modified medium T for the analysis of organ samples from foxes for an epidemiological investigation on distribution of *F. tularensis* in the German federal state of Brandenburg confirmed an inhibition of contaminating flora.
**Conclusions:** Cultivation of the fastidious bacteria *F. tularensis* is known to be difficult and sometimes the recovery of the bacteria from samples with high concentration of contaminating flora is a diagnostic challenge. Therefore, the nutrient medium T can be highly recommended for the cultivation of *F. tularensis* from environmental or clinical samples and also for other highly pathogenic bacteria.

**P1-46**

## Prevalence of *Francisella tularensis* in European brown hare populations in Lower Saxony, Germany

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Tularemia is a notifiable disease in Germany in consequence of its contagiousness and zoonotic potential. Recently tularemia got into the public focus in Germany due to cases of human infections after contact with European brown hares. The knowledge about the occurrence and spreading of tularemia is a precondition of a successful diagnosis, treatment and management of human infections. However, in Lower Saxony as in many other German states and European countries there is no information about the incidence or prevalence of *Francisella tularensis* within the European brown hare population. Therefore in an ongoing study of the LAVES – Veterinary Institute Hannover in collaboration with the IWFo, the FLI and the Bundeswehr Institute of Microbiology we try to evaluate this situation. To date more than 1,700 European brown hares were investigated.

## POSTER PART 2 – EPIDEMIOLOGY AND ECOLOGY, CELL BIOLOGY, HOST RESPONSE AND VACCINES

### P2-01

### *Francisella* Sec secretion contributes to attachment during biofilm formation and chitin colonization

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*Francisella tularensis*, the etiologic agent of tularemia, can infect numerous small mammals and arthropod vectors in nature, and may persist in non-host environments. Although *F. tularensis* pathogenesis is essential to comprehending the infection state of this bacterium, it is notable that mammalian infection is just one step in the ecology of *Francisella* species and mammals are most likely not the environmental reservoir for this pathogen. Since *F. tularensis* may be associated with chitin-based surfaces in nature, a substrate conducive for biofilm formation, we tested the ability of *Francisella* species to form these bacterial communities. *F. tularensis* subsp*. tularensis*, the most virulent subspecies, *F. tularenisis* subsp. *novicida,* and *F. tularenis* subsp. *holarctica* live-vaccine strain (LVS) all formed these surface-associated bacterial populations on abiotic surfaces. We also observed *F. novicida* biofilms on chitin-containing crab shells and synthetic chitin indicating that biofilms represent a potential mechanism of environmental persistence for *F. tularensis*. A forward genetic screen for biofilm mutants identified 88 genes that contribute to biofilm formation; including the Sec translocon apparatus, as well as 14 putative secreted proteins. We mutated two chaperone genes (*secB1* and *secB2*) involved in Sec-dependent secretion and 4 genes that encode for putative secreted proteins. All mutants were deficient for attachment to both polystyrene and chitin surfaces and for biofilm formation compared to wild-type *F. novicida*. Furthermore, these mutants were virulent in macrophages and mice. These data suggest that biofilm formation may be a non-host survival mechanism for *F. tularensis* and indicates that *Francisella* species likely utilize a different set of genes to colonize environmental surfaces and persist as biofilms in nature compared to those needed for infecting a mammalian host.

## P2-03

### **Evaluation of the vector competence of *Dermacentor variabilis* (American dog tick) for transmission of *Francisella tularensis***

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Ticks have historically been considered important biological vectors of *Francisella tularensis*, the etiological agent of tularemia, because they can transmit the bacteria to humans as well as propagate the organism in zoonotic cycles. In the United States, the American dog tick, *Dermacentor variabilis* is considered among the most important species involved in enzootic maintenance and for bridging *F. tularensis* from zoonotic cycles to humans. The geographic distribution of this tick is concordant with states reporting the highest incidence of tick-borne tularemia (Missouri, Arkansas, and Oklahoma). Immature life stages of *D. variabilis* (e.g., larvae and nymphs) are thought to be associated with enzootic transmission, whereas adult *D. variabilis* have been implicated in outbreaks of human tularemia in South Dakota and endemic transmission in other parts of the United States. Although there is strong historical evidence implicating *D. variabilis* with tick-borne tularemia cases, there have not been any recent studies comparing the vector efficiency of *D. variabilis* subsequent to the recognition of epidemiologically significant differences among *F. tularensis* subspecies and clades (A1, A2 and type B). In this study we compared nymphal transmission rates of *D. variabilis* infected as larvae with wild type strains of A1, A2 and type B as well as bacterial kinetics of the infection in ticks, and the fitness cost of infection was compared to uninfected ticks. Transmission of *F. tularensis* to 6 week old Swiss Webster mice was not observed with clade A1 (n = 37), and low rates were observed with clade A2 (8.0 %; n = 25) and type B (13.5 %, n = 37). Bacterial loads determined at specific time periods (1 day post-infection, pre-molt, post-molt, day of nymphal feed and post-nymphal feed) suggest that A1, A2 and type B *F. tularensis* replicate significantly during the nymphal bloodmeal. On average, we observed a 2.2 log increase in bacterial loads between unfed and replete nymphs. We also observed a significant difference in the survivorship of uninfected nymphs (68.3 %) compared to A1- (31.7 %), A2- (18.0 %) and type B-infected (29.8 %) nymphs 63-70 days post-larval blood meal. Our results provide evidence for a high fitness cost and low transmission rates during the immature stages, suggesting that *D. variabilis* may play a limited role in enzootic maintenance of *F. tularensis*.

P2-05

## Isolation of *Francisella tularensis* from mandibular lymph nodes of red foxes indicates active natural foci in Austria

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**Aims:** The aim of this study was to clarify whether the red fox (*Vulpes vulpes*) could be an appropriate indicator for active endemic regions, since this predator covers a large area and prefers preying on European brown hares (*Lepus europaeus*).
**Methods:** 669 red foxes from 6 districts in well-known endemic regions in the north-eastern part of Austria were provided for the study between June 2007 and March 2009, in line with the rabies abatement program. A selective agar medium was inoculated with a section plane of the mandibular lymph nodes and incubated for 7 days. Identification and characterisation of the isolates was done by standard bacteriological methods.
**Results:** In autumn/winter of the years 2007/08, 4 out of 51 investigated foxes from Waidhofen/Thaya, where tularemia was detected in 14 brown hares ten years before, showed a positive result in culture. In spring/summer 2008, 1 out of 11 investigated foxes from Mistelbach proved to be infected. In that district *Francisella tularensis* was isolated from 29 brown hares from 1994 until 1998, when two epidemics with 86 reported human cases occured in the north-eastern part of Austria. Furthermore *Francisella tularensis* was isolated from 10 out of 494 investigated foxes in 4 districts of Burgenland from summer 2007 until winter 2009. Altogether 15 strains of *Francisella tularensis* subspecies *holarctica* biovar II could be isolated from mandibular lymph nodes with no visible lesions.
**Conclusions:** Our findings clearly demonstrate that isolation of *Francisella tularensis* from the mandibular lymph nodes of red foxes with latent tularemia infection can indicate active endemic foci. Besides testing for rabies, additional screening of red foxes for the presence of *Francisella tularensis* should therefore be recommended in endemic regions.

P2-08

## Extra- and intracellular proliferation of *Francisella tularensis* (LVS) in presence of amoebae

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**Aims:** The purpose of the study was to investigate whether *Francisella tularensis (F.t.)* is able to use amoebae as a reservoir for survival under environmental conditions.
**Methods:** Cultures of *Acanthamoeba castellanii* in ATCC medium no. 712 (PYG) were infected with *F.t. ssp. holarctica* Live Vaccine Strain (LVS). The co-cultures were incubated at different temperatures in medium, PBS, or natural river water and subsequently analysed by means of immunofluorescence, electron microscopy, determination of colony forming units (CFU), and RTq- PCR over different time periods up to several weeks.
**Results:** CFU determination in co-cultures with amoebae over five days showed a rapid proliferation of LVS from an initial concentration of approximately $10^2$ CFU/ml on day 0 up to $10^9$ CFU/ml on day 5. In culture medium alone without amoebae, no bacteria could be re-cultured from an inoculum of $10^3$ and $10^4$ CFU/ml after 3 days.Analysis of immune-stained samples showed an accumulation of *F.t.* around and on the surface of the amoebae. Preliminary results from electron microscopy could support these observations for cultures after 4 days. Association between bacteria and amoebae was tight at focal points of the contact area suggesting the formation of particular contact structures.
**Conclusions:** Proliferation rates of *F.t.* in presence of amoebae and the finding that the bacteria are mainly located extracellularly indicate that *F.t.* might be able to use metabolic products of the amoebae to facilitate proliferation and survival. The close structural contact between the cells might be used for nutrition of bacteria and could be important for an eventual fast entry of *F.t.* as reaction to negative changes of environmental conditions. Further investigations are carried out in order to determine how long *F.t.* can survive under different conditions in presence of amoebae and whether the localisation of the bacteria changes from mainly extra- to intracellular compartment under sub-optimal environmental conditions.

P2-09

## Application of multiple-locus variable number tandem repeat analysis (MLVA) for subtypin g of Swedish isolates of *Francisella tularensis*

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Tularaemia is a zoonotic disease caused by the gram-negative coccobacillus *Francisella tularensis*. Its wide distribution in Sweden poses a challenge for understanding the transmission, ecology and epidemiology of the disease. The aim of this study was to evaluate MLVA as a typing system for practical use in epidemiological investigation i.e. the possibility to use molecular epidemiology to predict where patients have been infected. MLVA based on five previously described VNTR:s was applied on *F. tularensis* isolates collected during1995-2007 from 95 Swedish patients with ulcero-glandular tularaemia. MLVA types were compared to detailed epidemiological information including map locations.

P2-10

## An outbreak of tularaemia in Tuscany, Central Italy, linked to a natural spring water

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**Aims:** We report an outbreak of tularaemia occurred in the Italian province of Pistoia, Tuscany, linked to a natural spring water.
**Methods:** Between April 2007 and March 2008, 43 cases of tularaemia were confirmed by clinical signs and serological analysis by tube agglutination test. Most cases (n = 38) occurred between December 2007 and March 2008. The probable source of infection was natural spring water collected from a small cement basin at about 950 m of altitude. Water from the spring was usually collected by resident people and tourists, stored in containers and consumed at home. Water samples from the spring were collected twice, on February 15th and March 4th, 2008 and tested for *Francisella tularensis* by PCR, microbiogical methods and mouse inoculation.
**Results:** Out of the 43 confirmed cases, 34 (79 %) had been exposed to a common source of water. The most frequently clinical presentation observed was a cervical lymphadenopathy and tonsillitis and sore throat were observed in a few cases. Antibodies titres of confirmed cases ranged from 1:50 to 1:1,600. *Fransisella tularensis* subsp. *holarctica* (type B) was demonstrated both times in the natural spring water by PCR and mice inoculation. Direct culture of the organism from water was unsuccessfull. The outbreak was brought under control after the demolition of the cement basin and the restoration of the water source as stream.
**Conclusions:** This large epidemic occurred about 20 years after two last important outbreak of Tularaemia recorded in northern and central Italy (Liguria and Tuscany regions) and confirms the circulation of *Fransisella tularensis* in Tuscany. Nevertheless further investigations are needed to explain the route of contamination and the environmental characteristics that could have maintained or supported the presence or the replication of *Francisella tularensis* in the natural spring waters.

**P2-11**

## Development of a Multiple-Locus Variable number of tandem repeat Analysis (MLVA) for Chinese *Francisella tularensis* and its application to some strains

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**Objective:** To develop Multiple-Locus Variable number of tandem repeat Analysis for Chinese *Francisella tularensis* (*F. tularensis*) and study the genotypes of some Chinese strains.
**Methods:** Primers sequences of 25 loci obtained from GPMS (Genomes, Polymorphism and Minisatellites) database, the method was optimized and evaluated. Using established method to explore the genetic diversity within Chinese strains.
**Results:** 20 strains used for the development of the method showed good results. The 25 loci displayed repeat sizes from 2 bp (M-25) to 23 bp (M-23) in length. 6 loci showed some genetic diversity**,** among which M-3 locus containing seven genotypes had highest diversity value (0.91). Based on the results from MLVA**,** phylogenetic analysis was done. It revealed 5 groups among Chinese strains, i.e. the group of Tibet, Xinjiang, Heilongjiang, Inner Mongolia and vaccine, which was concordant with the distribution pattern of tularemia in China.
**Conclusion:** The MLVA described in this report was proved to be a powerful tool for Chinese *F. tularensis*, the strains used in the study showed high genetic diversity and conservation among different loci.

**P2-12**

## *Francisella*-like endosymbiont in *Dermacentor reticulatus* collected in North of Portugal

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*Francisella tularensis* can cause a wide variety of clinical syndromes including severe, sometimes fatal disease. Regarding the extraordinary potential of *F. tularensis* as an agent of bioterrorism and the frequent occurrence of outbreaks in several European countries, the precise characterization of the epizootiology and epidemiology of the infection is essential to the implementation of prevention measures. In Portugal, recent studies have confirmed the presence of this bacteria in 1,1% of the Dermacentor reticulatus analysed. Bacterial endosymbionts with significant homology to *F. tularensis* have been described in several species of ticks. Knowing that the presence of endosymbionts may influence the prevalence of *F. tularensis* in ticks, we tried to confirm if they are also present in our country and therefore could have some impact in the referred prevalence. Using a PCR approach, a total of 59 D. reticulatus collected in wolfe (Canis lupus) and dogs (Canis familiaris) in the North of Portugal were analysed by PCR, targeting the TUL-4 gene of *Francisella* spp. that encodes the 17 kDa lipoprotein. In all the positive results, a partial sequence screened for sequence similarity by BLASTN. The obtained sequence data was analyzed and phylogenetic relationships were assessed by tree construction using different tree building methods within the PAUP software.This study reported the first molecular detection of *Francisella*-like endosymbiont (FLE) in Portuguese D. reticulatus ticks (40 %), confirming the presence of these bacteria in Portugal and the need to take this into account in ticks and environmental samples detection. The importance of these findings is related to the hypothesis that the presence of FLE may lead to a reduction in vertical transmission and maintenance of infectious bacteria by the arthropod host, influencing the natural cycle of *F. tularensis*.

P2-13

## Longterm follow up of a natural focus of tularemia in south-west Slovakia

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**Aims:** Identification of potential rise in epizootic activity of a tularemia natural focus for to signal the risk of disease occurrence in humans.
**Methods:** Locality in a lowland of south-west Slovakia identified as a natural focus of tularemia in 1964, when *Francisella tularensis* was isolated from small mammals *Microtus arvalis*, *Mus musculus* and *Rattus norvegicus* during an epidemic outbreak, was selected for ecological studies and followed from 1994 to 2008. Ectoparasites and organs of animals were examined by inoculation into white mice, partially by cultivation and PCR method.
**Results:** Circulation of *F. tularensis* was confirmed by an isolation from *Ixodes ricinus* tick adults collected from vegetation in April 1996 – prevalence of infection 0.2 %. Activation of the natural focus was proved by isolations of the agent from a dominant species of *Apodemus flavicollis* mice live-trapped in October 2000 and November 2002 – prevalence of infection in animals being 2.2 % and 9.1%, respectively. All strains were typed as *F. tularensis subsp. holarctica, biovar II.* Ectoparasites collected from small mammals were found negative in biological experiments. Results of longterm systematic studies correlated with epidemic occurrence of tularemia in the endemic region of south-west Slovakia.
**Conclusions:** Study showed that longterm follow up of selected localities being able to detect an activation of the epizootic process and epidemiologically important changes. Methods of molecular biology tested were proved promising and can extend potential of classical techniques in the surveillance of tularemia natural foci.

P2-14

## The course of tularemia in Kosovo since the first outbreak in 1999

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**Aims:** The purpose of this study is to provide a follow-up on the prevalence and incidence of tularemia in Kosovo after the occurrence of two post-war outbreaks in 1999 and 2001.
**Methods:** For surveillance, all ambulances and medical centers were obliged to fill in special reporting forms every week to report aggregate and individual data of a number of diseases, including tularemia, to the regional IPH who, subsequently, pass them to the central IPH. Here, the data were included in the central data base to be regularly analyzed by means of EPI-INFO software. Diagnostic sera were analysed with regard to specific antibodies to *Francisella tularensis* routinely using a micro-agglutination assay. Suspect sera were checked by highly specific ELISA and Western blot techniques directed against LPS of *F. tularensis*.
**Results:** During the tularemia outbreaks 1999 and 2001, 327 and 353 cases, respectively, were serologically confirmed as tularemia. It can be assumed from earlier statistics that the disease was not observed in humans in the region of Kosovo before. From 2003 to 2008, the number of reported tularemia cases decreased to a still relatively high level of approximately 100 cases per year with an incidence of over 4/100.000 inhabitants. The most affected age group was the one between 20-40 years, of which about 61% were female. Housewives (59%) and pupils (23%) were the main target population. The most important sources of infection were contaminated drinking water and food. The glandular (79%) and ulcero-glandular (21%) forms were now the dominant clinical manifestation. Today, the disease is spread over the entire territory of Kosovo.
**Conclusions:** As a result of the war and the subsequent environmental disruption, mass population displacements and a breakdown of sanitation and hygiene, two major outbreaks of tularemia preceded the establishment of an active endemic area of emerging or re-emerging tularemia in Kosovo.

P2-15

## Epidemiologic situation in the occurrence of tularemia in Slovakia, 1997–2008

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**Aims:** To analyse the development of epidemiologic situation in the occurrence of tularemia in Slovakia during a time period of the last twelve years and changes of some epidemiologic characteristics in relation to sources of infection and routes of transmission.
**Methods:** Basis for the epidemiologic analysis being data from published results of surveillance of tularemia in Slovakia and records of epidemiologic examination of reported cases as well as information from longterm surveillance of natural foci.
**Results:** In the years 1997 – 2008 tularemia was reported from all counties of Slovakia, in total 453 cases – mean incidence rate 0.71 per 100 000 population, out of it 95.4 % in the west of the country, predominantly in County Nitra – mean incidence rate 4/100 000. During an epidemic in 2002 the incidence rate reached 18/100 000 in this region. Cases occurred in all age groups 1.9 x more frequently in men compared with women. Seasonal occurrence peaked in summer, month of July. More prevalent were cases transmitted from other sources – 58.5 % as from hares – 16.3 %, which was in correlation with marked decrease of hare tularemia foci in this time period. Transmission of the disease resulted most frequently from manipulation with contaminated feed, litter and working in dusty environment with increased occurrence of rodents. Proportion of cases transmitted by ticks and biting insect comprised 12.8 % and 12.4 % were epidemiologically unclarified cases. Most frequent clinical manifestations were ulceroglandular and glandular forms – 55.6 %, pulmonary, oroglandular and others represented 21.2 %, 18.8 % and 4.4 %, respectively.
**Conclusions:** Changed dominance of sources of infection and routes of transmission conditioned changes in epidemiology of tularemia in Slovakia, proven mainly by decreased number of professional infections, rise of cases acquired during leisure activities in summer, infections in lower age groups, as well as rise of pulmonary and oroglandular clinical forms. Importance of surveillance of tularemia being pointed out.

P2-16

## Tularaemia seroprevalence studies under foxes and raccoon dogs in Germany

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**Aims:** The prevalence of *Francisella tularensis* in humans and animals in Germany is not yet well studied. To find out more about the distribution of the pathogen especially in eastern Germany, we investigated the seroprevalence of tularaemia in foxes (*Vulpes vulpes*) and raccoon dogs (*Nyctereutes procyonoides*) in the federal state of Brandenburg (around Berlin).
**Methods:** Sera of animals (n = 315 and n = 32, respectively) from the years 2007 and 2008 were tested for *F. tularensis* – LPS antibodies in an indirect ELISA and reactive samples were confirmed by Western blot for LPS ladder recognition using Protein G – POD conjugate. Furthermore, we investigated the serum samples by a competitive ELISA using a peroxidase-conjugated anti *F. tularensis* – LPS monoclonal antibody.
**Results:** From the serum collection, 32 (10.2 %) foxes and 2 raccoon dogs (6.3 %) were positive for specific *F. tularensis* antibodies. The geographical distribution showed several hot spots in the investigated region west of Berlin.
**Conclusions:** Our results indicate a higher seroprevalence in wild carnivores for tularaemia in eastern regions of Germany than assumed. Since the occurrence of tularaemia in humans seems to be underestimated for the last decade, the real prevalence of the pathogen is unknown. The high number of tularaemia antibody positive foxes and raccoon dogs indicates that this zoonosis is present in wildlife in eastern Germany. However, the impact of transmission of zoonotic pathogens including *F. tularensis* from wildlife to domestic animals and humans is not yet well studied.

**P2-17**

## Monitoring the survival of fish *Francisella* pathogens in aquatic microcosms

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**Aims:** To investigate the survival or persistence behavior of fish *Francisella* in aquatic environments under different temperature conditions.
**Methods:** Two fish *Francisella* isolates (*F. philomiragia* subsp. *noatunensis*) were inoculated into non-sterile (natural) and sterile freshwater and seawater microcosms and monitored under different temperatures (4 °C, 8 °C, 12 °C) over a period of 60 days. No nutrients were added to the microcosms, simulating conditions in their natural ecological niches where available nutrients may be limiting. Plate culturing, DNA-based real time PCR and in-situ fluorescence hybridisation (FISH) techniques were used to identify and quantify the inoculated strains recovered from the water samples.
**Results:** The persistence of culturable cells was greater in sterile than in natural water samples. In the non-sterile microcosms, *Francisella* remained culturable no longer than 5-12 days on plates. On other hand, culturability of the bacterium was prolonged (>20 days) in sterile microcosms, especially under low temperature conditions. These results were confirmed by quantitative PCR analysis targeting the succinate dehydrogenes (sdhA) gene. Regardless, of the water sample used, PCR quantification of cell numbers (estimated from gene copy numbers) remained at the same level (ca. $10^6$ CFU) throughout the experiment. A cell viability assay coupled with FISH analyses showed that the bacterium enters into Viable but not Culturable (VBNC) state after a period in water.
**Conclusions:** Fish *Francisella* may have low survivability in the natural water environments. The data contribute to a better understanding of the behaviour of *F. philomiragia* subsp. *noatunensis* in natural seawater and freshwater environments and suggest that the aquatic bacterial population play an important role in the survival of this fish pathogen. The ability to enter into VBNC state may have some ecological relevance for its spread, after disease outbreaks.

**P2-21**

### A real-time PCR array for hierarchal identification of environmental and human pathogenic *Francisella*

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A robust, rapid and flexible real-time PCR assay for hierarchal genetic typing of clinical and environmental isolates of *Francisella* is presented. Typing markers were found by multiple genome and gene comparisons. Twenty-three canonical single nucleotide polymorphisms and 11 canonical insertion-deletion mutations were selected to serve as phylogenetic guidelines for classification ranging from genus to isolate level. Specificity was assessed using 62 *Francisella* isolates of a diverse genetic and geographical origin. The final assay is in a 96-well plate real-time PCR format and was successfully used for typing of 14 *F. tularensis* subsp. *holarctica* isolates from tularemia patients in Sweden year 2008 and five genetically more diverse *Francisella* isolates of a global origin. The detection limit of the 96-well assay was 100 pg. When applied to human ulcer specimens for direct pathogen detection, the results were incomplete. Nevertheless, a selected subset of markers could be analyzed to expose fine resolution among *F. tularensis* subsp. *holarctica* causing infection in the patients.
In contrast to other real-time PCR assays for *Francisella* that are typically designed for specific detection of a species, subspecies, or strain; this type of assay can easily be tailored to match a preferred phylogenetic and geographical resolution level.

## P2-23

### The efficacy of oral vaccination with a defined *Francisella* vaccine strain in inducing protective immunity against pneumonic tularemia

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**Aims:** The oral route of vaccination has been shown to be preferential in inducing mucosal immunity. In this study, we sought to examine the protective immunity induced by oral immunization with a defined *F. tularensis* subsp. *novicida iglB* mutant strain (KKF235) in a murine model of pneumonic tularemia.
**Methods:** Confocal microscopy was used to examine interaction of mCherry-expressing KKF235 with intestinal M-cells following oral delivery. Antigen-specific IFN-γ induction and humoral responses in the systemic and mucosal compartment stimulated by oral vaccination with KKF235 were measured. The protective efficacy of vaccination was examined by pulmonary challenge with *F. tularensis* subsp. *novicida*, LVS or *F. tularensis* subsp. *tularensis* Schu S4.
**Results:** Confocal microscopy revealed the co-localization of orally delivered KKF235 to M-cells within the Peyer's patches. Mice immunized orally with KKF235 induced robust antigen-specific splenic IFN-γ recall responses, as well as the production of systemic and mucosal antibodies including IgA. Mice vaccinated orally with KKF235 were highly protected against pulmonary *F. tularensis* subsp. *novicida* challenge, and exhibited protection against pulmonary LVS or *F. tularensis* Type A challenge as well.
**Conclusions:** The oral route of vaccination may be an appropriate and efficacious mode of antigen delivery to induce protective immunity against pulmonary *Francisella* infection.
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P2-24

## *Francisella tularensis* suppresses the proinflammatory response of endothelial cells via the endothelial protein C receptor

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**Aim:** To investigate the ability of *F. tularensis* to inhibit the proinflammatory response of human umbilical vein endothelial cells (HUVEC).
**Methods:** Using both the LVS and Schu S4 strains, HUVEC were exposed to living bacteria and material released from heat-killed *F. tularensis*. Pre-incubation times and multiplicities of infection of the living bacteria were varied. For other experiments, the living bacteria were separated from the HUVEC by Transwell inserts or exposed to the endothelial cells in the presence of a monoclonal antibody blocking the endothelial protein C receptor (EPCR). Conditioned media were assayed for the chemokine CCL2 by ELISA to assess proinflammatory activation of the HUVEC.
**Results:** Living *F. tularensis* LVS and Schu S4 did not stimulate secretion of the chemokine CCL2 by HUVEC, while material released from heat-killed bacteria did. Furthermore, the living bacteria suppressed secretion of CCL2 in response to heat-killed *F. tularensis*. This phenomenon was dose- and contact-dependent and occurred relatively rapidly. The living bacteria did not inhibit the activation of HUVEC by *E. coli* LPS, highlighting the relative specificity of this suppression. The EPCR confers anti-inflammatory properties when bound by activated protein C. When the EPCR was blocked, *F. tularensis* lost the ability to suppress activation of HUVEC.
**Conclusions:** The endothelium, when activated by various pathogens, secretes proinflammatory cytokines and recruits circulating leukocytes. These data demonstrate that *F. tularensis* actively inhibits proinflammatory activation of HUVEC. To our knowledge, this is the first report that a bacterial pathogen inhibits the host immune response via the EPCR. Endothelial cells are a critical component of the innate immune response, and suppression of their activation by *F. tularensis* is likely a mechanism that aids in bacterial dissemination and evasion of host defenses.

P2-25

## Early immune responses to the Live vaccine strain and Schu S4 infection in cynomolgus macaques

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**Aim:** The early host response to pulmonary infection with *Francisella tularensis* was characterized in non-human primates.
**Methods:** Cynomolgus macaques received either 1x10$^5$ cfu of the Live vaccine strain (LVS) or 1100 cfu of the Schu S4 strain of *F. tularensis* by pulmonary instillation and the bacterial growth, dissemination, cell recruitment, cytokine responses, and histology were examined 1, 4 and 7 days after infection.
**Results:** No growth of LVS in the lungs was detected post inoculation. LVS was detected in draining lung associated lymph nodes (LALN) on day 1 with an increase in LVS colonies found on days 4 and 7 post inoculation with clearance by day 28. No LVS colonies were detected in blood, spleen, or liver at any time points. In contrast, Schu S4 demonstrated an increase of up to 6 logs within 7 days in the lungs, LALN, spleen, and liver. LVS induced a robust mononuclear infiltrate, consisting of lymphocytes, monocytes, and dendritic cells expressing MHC class II in lungs and LALN. The LVS-induced cytokine profile consisted predominantly of increased levels of chemokines. The cellular infiltrate associated with Schu S4 inoculation was similar qualitatively but was decreased quantitatively as compared to LVS and there were additional increases in TNFα and IL-2. Histolopathology comparisons on days 1, 4 and 7 demonstrated that LVS inoculation induced a mild, multifocal, granulomatous bronchiolitis and bronchopneumonia with infiltrates consisting primarily of epithelioid macrophages and lymphocytes. The pathology at identical time points associated with Schu S4 infection demonstrated a moderate to severe, multifocal to coalescing, necrotizing and pyogranulomatous bronchitis, bronchiolitis, and bronchopneumonia.
**Conclusions:** Understanding the basis for the difference in host response to the attenuated LVS strain versus the Schu S4 strain may provide insight into the impact that Schu S4 virulence factors have on the host immune response.
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P2-26

## The pathology of *Francisella tularensis* Schu S4 in Cynomolgus macaques

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*F. tularensis* is considered a biothreat agent due to the high degree of infectivity and virulence of specific strains. As such, well-characterized animal models are needed to test vaccines and therapeutic agents.
**Aim:** Our aim was to characterize the pathophysiology of aerosolized Schu S4 in *Cynomolgus macaques.*
**Methods:** As part of a study to determine the lethal dose 50 % for aerosolized *F. tularensis*, *Cynomolgus macaques* were exposed to varying aerosol doses of Schu S4, and tissues were collected for pathologic evaluation at the time of death.
**Results:** Doses of Schu S4 ranged from 1 to greater than $10^6$ CFUs. Animals died or were euthanized due to severe clinical disease over the course of several weeks, with a time to death that depended upon inoculum dose. Lesions were detected primarily in the nasal cavity, lungs, pleura/thoracic cavity, spleen, liver, lymph nodes and bone marrow, and less commonly in other tissues. Lesions in most organs consisted of macrophage and neutrophilic infiltrates, with prominent necrosis of parenchyma and infiltrating cells, vasculitis, fibrin exudation and hemorrhage. Higher doses were associated with miliary lung lesions and minimal lesions outside of the respiratory tract/thoracic cavity. Mid-range doses were associated with multifocal, nodular to cavitating lung lesions and development of necrotizing inflammation in the spleen and peripheral lymph nodes. Low doses were associated with sparse, nodular to cavitating lung lesions, prominent necrotizing inflammation in the spleen and peripheral lymph nodes, and sporadic necrotizing inflammation in other tissues (brain, kidneys, eyes).
**Conclusions:** The pathology of aerosolized *F. tularensis* in *Cynomolgus macaques* is similar to humans. The widespread distribution of lesions in the animals exposed to lower inoculum doses is interpreted to be a consequence of their prolonged post-exposure survival.
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P2-28

## T cell Epitope Identification in *F. tularensis*

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**Aims:** *F. tularensis* is a facultative intracellular pathogen that infects a range of vertebrate hosts. The innate immune system is critical for the resistance to infection and the adaptive immune system is required for bacterial clearance. We have defined T cell epitopes derived from *F. tularensis* to facilitate the study of the adaptive T cell immune response. We developed a series of T cell hybridomas from *F. tularensis* infected animals using standard hybridoma technology. These hybrids have been used to define *F. tularensis* proteins able to stimulate T cells.
**Methods:** Recently we have developed a novel method to screen nearly all open reading frames from *F. tularensis*. We recloned the Venter Institute Schu S4 ORF library into the pBAD-DEST49 expression vector. We expressed and purified each product and arrayed the recombinant proteins in a high throughput 96 well arrayed format.
**Results:** We expanded this method by producing a sub-array of 45 gene products identified by a combination of seroreactivity on western blots and previously described immune targets. This set of expression constructs has been dubbed "Greatest Hits." We used these to screen 6 hybridomas derived from infected B6 mice and identified one hybridoma reactive with pathogenicity island encoded protein, IglB.
In addition we have described four epitopes in B6 mice. Two map in LpnA (Tul4) and two in IglC. We have also described one epitope in BALB/c mice mapping in L3 ribosomal subunit protein. Finally we have prepared hybrids from transgenic HLA-DR4, $IA^{-/-}$ mice. We have identified approximately 60 hybridomas that respond to *F. tularensis* extracts presented on $DR4^{+}$ but not $DR4^{-}$, B6 APC, indicating they are human DR4 restricted.
**Conclusions:** We have devised a novel method for the identification of *F. tularensis* epitopes and have been able to use it to provide information on T cell reactivity.

P2-31

## Pyrin enhances IL-1β processing and release in response to *Francisella*

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**Aims:** Bacterial recognition by TLR and NLR sensors of mononuclear cells often initiates assembly of an inflammasome resulting in activation of caspase-1 and processing of IL-1β, a key pro-inflammatory cytokine. However, the *Francisella*-sensing inflammasome remains uncharacterized. The purpose of this study was to investigate pyrin as a potential sensor of *Francisella* and its effect on caspase-1 activity.
**Methods:** Human monocytes (Mo), monocyte-derived macrophages (MDM), THP-1 and HEK293 cell lines, stably expressing either siRNA against pyrin or YFP-pyrin, were infected with *F. novicida* and analyzed for inflammasome activation. To confirm the effect of pyrin on caspase-1 activation, recombinant pyrin was analyzed for its effect on caspase-1 using an *in vitro* inflammasome.
**Results:** Using *F. novicida* as a model organism, we have shown that *Francisella* efficiently activates the inflammasome and IL-1β release in Mo but not in MDM. In this context expression profiling showed a dramatic down regulation of pyrin levels in MDM, as compared to Mo (270 fold). Suppression of pyrin levels in Mo and THP-1 cells reduced caspase-1 activation and ability to process and release IL-1β by *Francisella* challenge. In contrast, induction of pyrin in MDM (with M-CSF) and THP-1 (by overexpression) corrected the robust IL-1β response to *Francisella.* Confocal microscopy showed a co-localization of YFP-pyrin with the inflammasome adaptor CFP-ASC and RFP-caspase-1 after cell stimulation, where ASC formed a central core and pyrin forms ring outside of the speck. Finally, *in-vitro* pyrin inflammasome assembly on ASC cross-linked to maltose beads, showed that pyrin and caspase-1 formed a complex with ASC and that pyrin directly enhanced caspase-1 activity.
**Conclusions:** Intracellular pyrin upregulates mononuclear cell IL-1β processing and release. Thus, pyrin is potential intracellular sensor for *Francisella*.

P2-32

## A partial screen for *Francisella tularensis* virulence determinants using *Drosophila melanogaster*

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**Aims:** Identification of new virulence determinants has so far mainly been performed in mammalian systems. But, gene-by-gene *in vivo* testing in mice is expensive and ethically questionable. To enable high-throughput screening of single gene knockouts we developed a protocol using *Drosophila melanogaster* as a screening host and a well-characterized transposon mutant library of *Ft novicida*.
**Methods:** By using fly survival as readout we tested 249 *F. tularensis* genes which had previously been implicated in virulence in mouse infection models. Candidates were further characterized in additional experiments like viable count analysis in flies and in mouse macrophages.
**Results:** We identified 43 transposon insertion mutants that were attenuated also in flies. These include almost all loci in the *Francisella* pathogenicity island plus other genes required for intracellular survival and responses to environmental stress. Additional testing further splits these mutants into four functional groups, of which one seems to be involved in inducing a chronic inflammation-like state. Interestingly, we found seven mutants to be hypervirulent in *Drosophila*.
**Conclusions:** Our screening model probably best resembles the intraperitoneal route of infection in mammals. The overlap of "hits" with conventional negative selection screens in mice was similar to the overlap between such studies. Our results reiterate the importance of intracellular bacterial growth for virulence, but they also demonstrate a role of conserved *in vivo* physiological reactions for the survival of such diverse hosts as flies and mice.

P2-35

## A novel role for *Francisella tularensis* antioxidant enzymes in modulating human macrophage signaling and activation

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*Fransiella tularensis* strains demonstrate differential regulation of host immune responses and sensitivity to redox cycling drugs.
**Aims:** In this study, we test the hypothesis that *F. tularensis* antioxidants play a key role in regulating host immune signaling events.
**Methods:** In our experimental model we examined the effects of infection with *F. tularensis* Live Vaccine Strain (LVS), a mutant lacking the catalase gene (LVSΔ*katG*), as well as, the highly virulent Schu S4, on macrophage activation and function. Following infection of primary human monocytic derived macrophages (hMDM), we examined intracellular growth, cytokine production, NFκB activation and phosphoinositide-3-kinase (PI3K) signaling pathway by ELISA and immunoblotting. PI3K activates Akt, a key component of the macrophage signaling network, which drives inflammatory cytokine gene expression.
**Results:** Infection of inactivated hMDM with LVS suppresses cytokine production, while infection of IFNγ-activated hMDM elicits a significant increase in cytokine production. Schu S4 infection severely inhibits proinflammatory cytokines secretion from both inactivated and IFNγ-activated hMDM when compared to LVS. Interestingly, infection with LVSΔ*katG* fails to suppress macrophage cytokine production. In addition, NFκB activation is suppressed following infection with Schu S4, but not with LVS. Schu S4 infection also suppressed AKT phosphorylation which correlates with increased expression of its antagonist the dual lipid protein phosphatase PTEN. Oxidation of PTEN can lead to its inactivation and a subsequent increase in Akt signaling. Schu S4 infection prevented $H_2O_2$-dependent PTEN oxidation, while LVS infection only partially blocked PTEN oxidation and infection with LVSΔ*katG* was unable to block oxidation.
**Conclusions:** Together, these findings suggest a novel mechanism, by which, *F. tularensis* antioxidants restrict macrophage signaling by preserving phosphatase activity and tempering kinase signaling.

P2-36

# A capsule-deficient mutant of *Francisella tularensis* live vaccine strain is significantly more attenuated than LVS yet induces comparable protection in mice against *F. tularensis* challenge

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**Aim:** To develop a live attenuated vaccine against *Francisella tularensis* that is safer than LVS and comparably potent.
**Methods:** A putative capsule-deficient antibiotic marker-free mutant (LVSΔcapB) of *F. tularensis* Live Vaccine Strain (LVS) was constructed by allelic exchange. LVSΔcapB was evaluated for serum sensitivity, virulence in human macrophage-like THP-1 cells, and virulence and protective immunity in BALB/c mice after intranasal (i.n.) and intradermal (i.d.) immunization. BALB/c mice were immunized with LVSΔcapB at doses ranging from $10^3$ to $10^7$ CFU i.n. and $10^6$ to $10^8$ CFU i.d. Four weeks later, the mice were challenged i.n. with $10^4$ CFU LVS ($>5 \times LD_{50}$), and at 5 days post-challenge, the bacterial burden in the lung, liver and spleen was assayed. Sham-immunized mice and mice immunized with LVS at doses ranging from $3 \times 10^2$ to $10^4$ CFU i.n. and $10^5$ to $10^7$ CFU i.d. served as controls.
**Results:**
(I) LVSΔcapB, which retains the O-Antigen, was serum resistant.
(II) In a competition experiment, LVSΔcapB was outgrown by parental LVS in THP-1 cells.
(III) LVSΔcapB was significantly attenuated in mice and caused no weight loss, obvious signs of illness, or deaths at any dose tested; hence the $LD_{50}$ i.n. was $>10^7$ CFU vs. $1.8 \times 10^3$ CFU for LVS and the $LD_{50}$ i.d. was $>10^8$ CFU vs. $3.2 \times 10^7$ CFU for LVS.
(IV) Mice immunized with LVSΔcapB i.n. or i.d. and then challenged 4 weeks later with a lethal dose of LVS i.n. were 100 % protected from illness and death.
In mice immunized with LVSΔcapB, the bacterial burden in the lung was 3 – 5 logs lower than in sham-immunized animals, and the bacterial burden in the spleen and liver was 3 – 4 logs lower than in sham-immunized animals, comparable to that in mice immunized with LVS. This indicated that both local replication in the lung and systemic dissemination of *F. tularensis* was strongly inhibited by immunization with LVSΔcapB.
**Conclusions and discussion:**
(I) LVSΔcapB is serum resistant but significantly attenuated in both human macrophages and mice, providing a safer vaccine candidate than LVS.
(II) Immunization with LVSΔcapB by the i.d. or i.n. route induces strong protective immunity, comparable to that induced by LVS at maximum tolerated doses, against lethal i.n. challenge with *F. tularensis*.
Future studies will examine the capacity of LVSΔcapB to protect against aerosol challenge with *F. tularensis* subsp. *tularensis* Schu S4.

P2-40

## Analyses of phagocytic ability of rat macrophages for *Francisella tularensis*

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**Aims:** The Fisher 344 rat, in comparison to the mouse, may represent an infection model which is more representative of human *F. tularensis* infections. In this study, we sought to examine the phagocytic activity and inflammatory cytokine production of bone marrow derived macrophages (BMDM) from F344 rats exposed to different *F. tularensis* strains.
**Methods:** F344 rat BMDM were infected with various MOIs of LVS, *F. tularensis* subsp. *holarctica* (Type B) or *F. novicida*. At specified intervals, cells were lysed and bacteria enumerated by serial dilution plating. Culture supernatants were analyzed for the production of nitric oxide and TNF-α. Virulence of the different *Francisella* species also was ascertained by intra-tracheal pulmonary challenges.
**Results:** Using inert fluorescent microbeads (100 beads:1 cell), it was determined that the basal phagocytic uptake of rat BMDM was greater than that of mouse BMDM (87.1% vs. 33%). There was minimal uptake/replication of LVS in rat macrophages, and marginal replication (1-2 $log_{10}$) of *F. novicida, i*n contrast to the 3-4 $log_{10}$ increase of Type B at 72 hr. Levels of nitric oxide (LVS 25 μM, *F. novicida* 50 μM, Type B < 10 μM) and TNF-α(LVS 930 pg/ml, *F. novicida* 2040 pg/ml, Type B 72 pg/ml) production were inversely correlated with intramacrophage replication. Finally, *in vivo* pulmonary challenges revealed that both LVS ($LD_{50} > 10^7$ CFU) and *F. novicida* ($LD_{50} > 10^5$ CFU) were highly attenuated for virulence in rats as in humans.
**Conclusions:** LVS and *F. novicida* exhibit minimal replication in rat macrophages. Moreover, rats were greatly resistant to pulmonary challenge with LVS and *F. novicida*, correlating with *in vitro* production of nitric oxide and TNF-α. Collectively, the *in vitro* phagocytic analyses and rat model of pulmonary tularemia may serve to extend our current understanding of the pathogenesis of *Francisella tularensis*.
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**P2-41**

## Activation of apoptotic pathways in the course of B cell infection with *Francisella tularensis*

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*Francisella tularensis* is able to adhere and entry B lymphocytes and cause their apoptosis. We infected human B cell line Ramos with *F. tularensis* FSC200 *in vitro*. Killed bacteria *F. tularensis* FSC200, uninfected Ramos cells, cells influenced by 10 mM Staurosporine, and 200 ng/ml anti-CD95 antibodies were used as controls. Only live bacteria induced apoptosis measured by flow cytometry using AnnexinV in time and MOI manner. We measured activation of Caspase 8, Caspase 9 (western blot, colorimetric assay), caspase 3 (colorimetric assay), presence of Bcl-2 family proteins Bax and Bcl-xL in whole cell lysate, tBid, Cytochrome C and AIF (Western blot), in mitochondrial and cytosolic fraction and low-molecular form of protein PARP-1 that is cleaved by active caspase 3 in whole cell lysate by Western blot. To detect the changes on mitochondrial membrane potential we used flow cytometry and probe JC-1 that is specific for mitochondrial membrane depolarization. Live bacteria *F. tularensis* FSC200 activate both caspase pathways – receptor mediated and mitochondrial – as well as caspase independent pathway. Low level of pro-apoptotic protein Bax was expressed in the course of infection with or live either killed bacteria and vice versa huge amount of Bcl-xL was stimulated particularly after infection of B cells with killed bacteria. Killed bacteria also stimulated Caspase 3 and PARP but caspase 8, 9, tBid, AIF and cytochrome C were not activated and killed bacteria did not cause apoptosis measured by Annexin and JC-1. Results show that only live bacteria *F. tularensis* induce apoptotic process terminate by cell death.

P2-43

## Depletion of dendritic cells impairs the innate response to *Francisella tularensis* LVS

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**Aims:** Relatively little is known about the *in vivo* role of dendritic cells (DCs) during *Francisella tularensis* infection. Our goal is to determine whether DCs are required for the initial innate immune response to *F. tularensis* and for the subsequent development of an adaptive response.
**Methods:** We have used a transgenic mouse model in which DCs can be transiently depleted. In this model, mice express the simian receptor for diphtheria toxin (DT) under the control of the CD11c promoter. Because CD11c is a cell-surface marker expressed primarily on DCs, administration of DT to these mice results in specific and transient depletion of DCs, allowing infection with the live vaccine strain of *F. tularensis* (LVS) to be studied in their absence. Wild-type (WT) and DC-depleted mice were infected with LVS via the intradermal (i.d.) route and observed for survival, bacterial burden, and antibody responses. Some survivors of this primary infection were assayed for the development of a memory response by a secondary intraperitoneal (i.p.) challenge. Others were sacrificed for use in an *in vitro* co-culture assay in which the ability of splenocytes to control intramacrophage LVS growth is measured.
**Results:** DC-depleted mice are very susceptible to LVS. While the $LD_{50}$ of LVS i.d. infection is on the order of $10^5$ colony forming units (CFU) for WT mice, DC-depleted mice succumb to i.d. infection with an LVS dose as low as $10^1$ CFU. DC-depleted mice infected with $10^2$ LVS i.d. have ten-fold greater bacterial burden in the spleen before dying than do WT mice given the same dose. DC-depleted mice typically die from LVS i.d. infection within about five days, indicating a deficiency in the innate immune response. However, those mice that do survive a primary LVS infection while depleted of DCs develop a memory response. These mice survive a secondary LVS i.p. infection dose of $10^4$ CFU, which is 1000 $LD_{50}$s for naïve mice. DC-depleted mice that are primed with LVS develop substantial LVS-specific antibodies, as well as memory T-cells that are capable of controlling intramacrophage LVS growth.
**Conclusion:** We have found that dendritic cells are necessary for the innate immune response to i.d. LVS but that their reduction does not lead to an absolute deficiency in the adaptive immune response. This suggests that other cells may compensate for the lack of DCs or that small numbers of DCs are sufficient to prime adaptive responses.

P2-44

## Passive immunization with immunoglobulin G protects against a lethal respiratory infection with type A *Francisella tularensis* in Fischer 344 rats

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**Aim:** To determine the protective role of antibodies against a lethal pulmonary infection with type A *Francisella tularensis* (Schu S4) in Fisher 344 rats.
**Methods:** Naïve rats were treated with live vaccine strain (LVS)-immune rat serum (IRS) or immunoglobulin G (IgG) purified from IRS or normal rat serum (NRS) and subsequently challenged intratracheally with 200-360 colony-forming units of Schu S4. Survival of the infected rats and bacterial burden and histology in the lungs, liver and spleen were examined.
**Results:** Naïve rats treated i.p. with IRS survived a lethal respiratory infection with Schu S4 while NRS treatment failed to protect. Compared to NRS-treated rats, which exhibited increasingly severe clinical symptoms, the IRS-treated rats recovered within two weeks after displaying lower levels of disease symptoms, while rats vaccinated with LVS did not display any overt signs. Bacterial growth kinetics in lungs, spleen and liver of IRS-treated rats revealed that the Schu S4 growth rate and dissemination was intermediate between rats treated with NRS and LVS-vaccinated animals, suggesting an early control of Schu S4 numbers. Interestingly, after a brief arrest in the escalating bacterial growth rate in IRS-treated rats, the bacterial counts reached a peak number that was similar to NRS-treated rats when they died. However, animals receiving IRS treatment cleared the infection. Finally, treatment with IgG purified from IRS conferred 100 % protection.
**Conclusions:** Passive immunization of Fischer 344 rats with IgG generated during LVS vaccination limits bacterial growth rate and dissemination during a respiratory challenge with Schu S4, ultimately resulting in protection.
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**P2-45**

## Importance of B cells in parenteral murine infection with *Francisella novicida*

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**Aims:** *Francisella tularensis* is an intracellular pathogen that infects macrophages and causes the acute febrile disease tularemia. *Francisella novicida* is a subspecies considerably less virulent in humans but has retained its virulence in mouse models. Previous studies have looked at *in vivo* infection of mice with the attenuated live vaccine strain (LVS). Protection against LVS infection is predominantly achieved through T cell mediated pathways. However, some literature suggests an important role for antibodies as well as B cells. Relatively little is known about protection against *F. novicida*. This study examines the role of B cells and antibody responses to intradermal infection with *F. novicida*, using wild type C57BL/6J and B-cell knock out (BKO) mice. Information from these studies is especially useful for facilitating the characterization and investigation of mutant bacterial strains which have been made on *F. novicida* background.
**Methods:** C57BL/6J mice and BKO mice were infected intradermally with a sublethal dose of *F. novicida* strain U112 (FnU112) and followed for survival. Mice were sacrificed to determine bacterial dissemination, organ burdens, clearance, and serum analyses. Spleens from surviving mice were harvested for study using an *in vitro* co-culture assay of FnU112 infected bone marrow macrophages to determine ability to control bacterial infection. Other survivors were challenged with a lethal dose of FnU112 intraperitoneally.
**Results:** FnU112 appeared to be highly virulent in both C57BL/6J and BKO mice. BKO mice were more sensitive to primary infection, and exhibited an estimated $LD_{50}$ of 25 CFU (compared to $LD_{50}$ of 100 CFU in C57BL/6J). Bacterial burdens were greater and persisted longer in infected BKO mice compared with wild type mice. Although BKO mice were able to withstand a moderate secondary challenge dose, vaccinated BKO mice were much more susceptible to secondary i.p. challenge with FnU112 than vaccinated C57BL/6J mice.
**Conclusions:** B cells play an important role in the ability to survive primary i.d. infection with FnU112. Using transfer studies, the relative contributions of immune serum and B cells to protect against *F. novicida* challenge is under active investigation.

**P2-47**

## Full virulence is restored by reintroduction of two virulence loci into the live vaccine strain (LVS) of *Francisella tularensis*

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The live vaccine strain, LVS, is one of the most studied strains of *Francisella tularensis*. It has previously been used as a vaccine and found to protect staff from laboratory acquired tularemia. The genetic events leading to the attenuation are unknown and this has hampered licensing of LVS as a vaccine. Interestingly, LVS lacks two regions linked to virulence, RD18 and RD19. The latter encodes a putative type IV pilin (PilA), which contributes to virulence in type B strains, while RD18, encoding the outer membrane protein FTT0918 has been verified to be required for virulence of the highly pathogenic type A strains.
**Aim:** The aim was to determine to what extent these gene deletions contributed to the attenuation of LVS.
**Methods:** Each of the deleted regions was restored by genetic complementation *in cis* and the complemented strains were assessed for virulence in a mouse infection model.
**Results:** Complementation of both RD18 (FTT0918) and RD19 (*pilA*) fully restored virulence of LVS to a level indistinguishable from virulent clinical isolates of type B strains. Reintroduction of a functional *pilA* gene partially restored the ability of LVS to cause infection by the peripheral subcutaneous route in mice, while reintroduction of FTT0918 had a major impact on virulence of LVS in mice both by subcutaneous and intraperitoneal route of infection.
**Conclusions:** Our work support that the major genetic events resulting in the attenuation of LVS was deletion of the two direct repeat flanked regions RD18 and RD19. As the two attenuating deletion events are irreversible, our work supports that LVS can not revert to become more virulent which could facilitate the licensing of LVS for use in humans.

P2-48

## Advanced phage display – perspective approach for preparing of *Francisella tularensis* monoclonal antibodies

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**Aims:** In recent years, the view of antibody-mediated immunity against intracelullar pathogens has been relatively fully described. At the present time, there are no doubts about the antibody contribution in the protective immunity during the infection caused by *Francisella tularensis* (*F. tularensis*). It was described that polyclonal antibodies, as well as monoclonal antibodies are efficacious. The aim of this construed study is to demonstrate the application of phage display libraries for construction of monoclonal antibodies against *F. tularensis*. So prepared antibodies could be applicable for the alternative prophylaxis and possible therapy of infections caused by this pathogen.
**Methods:** The technique of phage display is used for producing antibody-like molecules. Gene segments encoding the antigen-binding variable of V domains of antibodies are fused to genes encoding the coat protein of a bacteriophage. Bacteriophage containing such gene fusions are used to infect bacteria, and the resulting phage particles have coats that express the antibody-like fusion protein, with the antigen-binding domain displayed on the outside of the bacteriophage. We are used the human single fold scFv library I (Tomlinson I+J) which is based on a single human framework for VH and Vκ with side chain diversity and comprises over 100 milion different scFv fragments cloned in an ampicillin resistant phagemid vector and transformed into TG1 E. Coli cells.
**Results:** Applied library contains at least twenty clones which can react with bacterial cell lysate of *F. tularensis* live vaccine strain. Further this library contains two clones react with recombinant protein FTT 1103 Δ signal of *F. tularensis* which was constructed without signal sequence.
**Conclusion:** Regarding our results, we sought to demostrate that the phage display can be perspectively used for development of monoclonal antibodies for both research and diagnostics, eventually for prophylaxis and therapeutics of tularemic infection.

P2-49

## Optimization of aerosol generation techniques for *Francisella tularensis* LVS and Schu S4 strains

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**Aims:** Testing was performed to develop methods for aerosolization of viable *Francisella tularensis* strains LVS and Schu S4 to allow future animal inhalation exposure studies. Aerosolization of viable microbes is a challenging process because of microbial susceptibilities to drying, temperature, and shear forces exerted during the aerosolization process. This process is even more challenging when using vegetative microbes such as *Francisella tularensis*.
**Methods:** Aerosolization methods were developed for both *F. tularensis* LVS (LVS) and *F. tularensis* Schu S4 (Schu S4) using a 3-jet Collison generator that allowed non-human primates to be challenged with doses ranging from 1 to $10^6$ cfu. Bacteria were grown in a defined medium (Chamberlain's broth) and harvested in log phase for aerosol challenge.
**Results:** Collison-generated bioaerosols have consistent particle sizes, but the microbes are subjected to considerable shear forces. Initial bioaerosol characterization was performed with LVS with the assumption that Schu S4 would exhibit similar properties. Stability, spray factor, and virulence were determined. LVS spray titers dropped approximately 0.17 log during sprays while Schu S4 spray titers dropped approximately 0.34 logs. Average spray factors for LVS were -6.52 ($log_{10}$) while spray factors for Schu S4 were -6.97. 30 % of mice exposed to $6.95x10^3$ cfu LVS or $4.37x10^2$ cfu Schu S4 survived. Mice exposed to higher doses of Schu S4 had 0 % survival by Day 5.
**Conclusions:** LVS and Schu S4 have different susceptibilities to aerosolization with LVS having higher spray factor values and greater stability, but lower virulence than Schu S4 in mice.
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P2-50

## *Drosophila* RNAi screen for host factors required for *Francisella tularensis* infection

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*Francisella tularensis* is a facultative intracellular pathogen that causes tularemia. The virulence of this pathogen depends on its ability to replicate within the cytosol of the host after escaping from its vacuole. To identify the host factors required for bacterial entry, vacuolar escape and cytosolic replication, we performed a genome-wide RNA interference screen in *Drosophila* macrophage-like cells, using *F. tularensis subspecies novicida*. We screened a library of ~21,300 dsRNA representing the *drosophila* genome. We identified more than a hundred host factors with various biological activities, including those required for vesicular trafficking, mostly common to other intracellular pathogens, as well as other factors specific for *F. tularensis* infection. Interestingly, some of the identified host factors were found to increase intracellular replication of *iglC, iglD and mglA,* mutants defective for intracellular growth, indicating a possible role in the intracellular defect associated with those mutants.

P2-54

## Susceptibility to disease following intraperitoneal infection with *Francisella tularensis* differs between Fisher 344 and Sprague-Dawley rat strains

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With it's high capacity to cause severe disease and mortality, high infectivity via various routes, and ease of dissemination by aerosol *F. tularensis* represents a major biodefence threat.
**Aims:** In most commonly used animal models of tularemia the experimental host is extremely susceptible to infection with very low doses of type A strains of the pathogen making it difficult to study protective innate immune mechanisms. In an attempt to address this issue, we have examined the susceptibility of two rat strains to intraperitoneal (IP) challenge with various clinical strains of type A and type B *F. tularensis*. Although not natural, the IP route was chosen as other experimental rodents appear to be most susceptible when tularemia is initiated by this route.
**Methods:** The current study evaluates host response and disease resistance in Fisher 344 and Sprague-Dawley rats following low dose IP infection with virulent type A (Schu S4), type B (strain FSC108) and high dose IP infection with the attenuated live vaccine strain (LVS) of *F. tularensis*.
**Results:** Fisher 344 rats displayed higher levels of morbidity following low dose challenges with type A and B *F. tularensis* or high doses of LVS. The low dose type A infected Fisher rats developed irreversible morbidity but most Fischer rats infected with low dose type B or a high dose of LVS recovered from disease. In contrast, Sprague-Dawley rats were resistant to low dose challenges with type A and B *F. tularensis* strains and high dose challenge with LVS. These susceptibility differences were also associated with differences in pathology, cytokine production and bacterial burden in the tissues of the infected rat strains.
**Conclusions:** These response and susceptibility differences will now be used to explore the basis for the innate resistance of Sprague Dawley rats in the expectation that this information will be useful in the development of immunotherapeutics against *F. tularensis*.

P2-60

## Virulence comparisons between Schu S4 (A1a) and A1a, A1b, A2 and type B strains using temperature as a surrogate endpoint for death

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Murine models are commonly used for efficacy testing of new vaccines and drugs for treatment of tularemia, as well as for pathogenesis studies. Since tularemia causes an acute febrile illness in mice, we hypothesized that changes in temperature could be exploited as a measurement of disease progression and for determination of an ethical endpoint correlating to death. In this study, subcutaneous temperature was recorded in C57BL/6 mice infected with low passage human clinical isolates identified as A1a, A1b, A2 and type B, as well as the passaged laboratory strain Schu S4 (an A1a strain). Differences in subcutaneous mouse temperature over the time course of infection identified three distinct phases (normal phase, febrile phase, and hypothermic phase), as well as a temperature highly correlative with death. This temperature point, termed drop point, occurs prior to entry of mice into the hypothermic phase and offers an unbiased surrogate endpoint for death and ethical euthanasia. Survival curves based on drop point were generated for all *F. tularensis* infections and fit using a Weibull distribution. The survival curve for Schu S4 differed significantly from A1a, A1b and A2 strains, but not type B, when comparing the scale parameter (e.g. the shift in the curve reflected that Schu S4 infected mice reached their drop point later). The Schu S4 survival curve also differed significantly from A1a, A1b, A2 and type B when the shape parameter was compared (e.g. mice infected with Schu S4 dropped within a much narrower range). Furthermore, the probability of a mouse infected with a low passage A1a strain reaching its drop point before a mouse infected with Schu S4 was found to be 99 %. Our results indicate that the drop point temperature can serve as a marker that allows for accurate comparative analyses between animals and studies. Significant differences in survival curves were demonstrated between infections caused by Schu S4 as compared to low passage clinical isolates of A1a, A1b and A2, suggesting that Schu S4 is less virulent than low passage clinical isolates of A1a, A1b, and A2 and more similar in virulence to type B. Furthermore, the shape of the survival curve for Schu S4 infected mice provides evidence for Schu S4 being highly clonal in comparison to the low passage isolates tested. The inclusion of low passaged type A isolates, in addition to Schu S4, may therefore be of importance when testing the efficacy of new vaccines and drugs against tularemia.

P2-61

## Evaluation of defined *Francisella tularensis* subsp. *tularensis* attenuated strains for vaccine efficacy in a murine model

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**Aims:** We sought to create attenuated strains of *F. tularensis* subsp. *tularensis* strain Schuh4 by inactivating genes within the *Francisella* Pathogenicity Island (FPI), and then evaluate the efficacy of these strains to protect against Schuh4 challenge in a murine pulmonary model of tularemia.
**Methods:** FPI genes *iglC*, *iglD*, and *vgrG* were inactivated in Schuh4 utilizing retargeted Group II introns. Resulting mutant Schuh4 strains were evaluated for virulence attenuation via intramacrophage growth and virulence in BALB/c mice via intranasal inoculation. Immune responses of surviving mice were measured. Mice surviving initial infection with mutant Schuh4 strains were challenged intranasally 30 days later with lethal doses of Schuh4.
**Results:** *F. tularensis* subsp. *tularensis iglC1 iglC2*, *iglD1 iglD2*, and *vgrG1 vgrG2* Schuh4 strains were successfully constructed utilizing Group II intron insertion. All three strains are highly defective at intramacrophage replication, and highly attenuated for virulence in mice via intranasal route ($LD_{50}$ $> 10^6$ CFU). However, mice surviving infection with attenuated strains failed to survive a subsequent lethal dose (20-400 CFU) of Schuh4 delivered intranasally. Vaccinated animals demonstrated a robust antibody response (Th1/Th2) to *F. tularensis* subsp. *tularensis*.
**Conclusions:** FPI mutants of *F. tularensis* subsp. *tularensis* are highly attenuated for virulence through the pulmonary route, but fail to induce protective immunity against homologous pulmonary challenge in mice.
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P2-65

## Characterization of a nonhuman primate aerosol exposure system for generation, delivery, and collection of *Francisella tularensis* DVC Schu S4

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**Aims:** Studies were conducted to develop a *Francisella tularensis* aerosol exposure model for non-human primates. A critical step in model development is gaining an understanding and documenting the performance characteristics of the aerosol system to be used. The aerosol Spray Factor (SF) value and its reproducibility were evaluated in the aerosol system.
**Methods:** Four different starting concentrations (nebulizer) were tested in triplicate over a three day period. *F. tularensis* nebulizer solutions were diluted in sterile brain heart infusion broth (BHIB) and approximately 8 mL were used for each test. Impinger samples were collected in BHIB containing Antifoam A using an impinger model 7541 (Ace Glass Inc, Vineland NJ). The nebulizer (pre and post challenge) and impinger concentrations were quantified using filters and the spread plate method.
**Results:** Analysis of the SF results indicated that there was a regression relationship between the SF and the pre-challenge nebulizer concentrations indicating that as the nebulizer concentration increased the SF became worse (i.e. smaller). Although a regression relationship was observed, it was not statistically significant at the nebulizer concentrations tested and as a result the predicted mean SF was $1.74 \times 10^{-7}$ with a standard deviation of $6.76 \times 10^{-9}$ and 95 % confidence interval of $(6.29 \times 10^{-8}, 5.46 \times 10^{-7})$.
**Conclusions:** The consistency of *F. tularensis* SF in the nonhuman primate aerosol exposure system was established for future aerosol challenge studies. Advanced knowledge of the SF and its variability allows determination of theoretical aerosol concentrations for any starting nebulizer concentration. Thus, by knowing an animal's respiratory volume along with the aerosol concentration and the SF, the amount of atmosphere an animal must breathe to reach as desired dose can be determined.
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