Corynebacterium pseudotuberculosis biovar *equi* Infection in Horses: Transmission and Diagnosis

by

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Abstract

A recent re-emergence of equine disease caused by *C. pseudotuberculosis* has been observed including spread to non-endemic regions. *Corynebacterium pseudotuberculosis* is a Gram-positive bacterium with an equine (*equi)* and a small ruminant (*ovis*) biovar. The general purpose of our studies was to investigate the mode of transmission and diagnosis of *C. pseudotuberculosis* infection in horses in order to institute measures to control further disease spread. Our specific objectives were to determine the role of house flies (*Musca domestica* L.) as a potential vector of this bacterium, and to provide further information to support the development of a more accurate diagnostic serological assay.

Firstly, we demonstrated that house flies harbor live bacteria up to 24h following a 30min experimental exposure, providing support that this insect could be a plausible vector with the ability to spread the disease over a wide geographic area within a 24h period. Secondly, we demonstrated that house flies were mechanical vectors of *C. pseudotuberculosis* in an *in vivo* exposure study based on the development of clinical signs, positive culture results, increase in blood inflammatory markers, and increase in serological titers in ponies exposed to inoculated flies.

For the second objective, the current serological assay (synergistic hemolysis inhibition (SHI)) was evaluated in a non-endemic population (Alabama) and its performance was compared to an ELISA test. Results showed that the seroprevalence of detectable antibodies determined by SHI testing in Alabama was high and exposure to ruminants was associated with increasing SHI

titers. These findings could indicate possible false positive results caused by cross-reaction with antibodies against phospholipases from *C. pseudotuberculosis* biovar *ovis*.

An ELISA test, based on *C. pseudotuberculosis* biovar *ovis* exotoxin and cell wall antigens, was inferior to the SHI test for the diagnosis of *C. pseudotuberculosis* biovar *equi* in horses. Analysis of the immune-dominant proteins from *C. pseudotuberculosis* in horses showed that bacterial antigens recognized by antibodies from sera of experimentally infected horses differed from sera before inoculation and differed from reaction against biovar *ovis* antigens.

Our results provide evidence that house flies play a role in transmission of this disease in horses, which justifies further emphasizing insect control on equine farms to control its spread. In addition, the presence of detectable SHI titers in non-endemic populations, which was linked to ruminant exposure, and the preliminary analysis of immune-dominant proteins reiterates the need for further investigation to develop a more accurate diagnostic test.

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List of Abbreviations

AGID: agar gel immunodiffusion

BSA: bovine serum albumin

CDM: chemically defined medium

CLA: caseous lymphadenitis

CMNR: Corynebacterium, Mycobacterium, Nocardia and Rhodococcus

CP40: corynebacterial protease

DNA: deoxyribonucleic acid

ECL: enhanced chemiluminescence

EIV: equine influenza virus

ELISA: enzyme-linked immunosorbent assay

FBS: fetal bovine serum

FHK: fetal horse kidney

IFN: interferon

IL: interleukin

IQR: interquartile range

MIC: minimum inhibitory concentration

MS: mass spectrometry

NS1: non-structural protein 1

NO: nitric oxide

OD: optical density

PCR: polymerase chain reaction

PLD: phospholipase D

RNA: ribonucleic acid

SAA: serum amyloid A

SD: standard deviation

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

SERPA: serological proteome analysis

SEZ: *Streptococcus equi* subsp. *zooepidemicus*

SHI: synergistic hemolysis inhibition

TLR: toll-like receptors

TNF: tumor necrosis factor

TPP: three-phase partitioning

Chapter 1: Review of the Literature

Introduction and history of pigeon fever

Corynebacterium pseudotuberculosis is a Gram-positive, soil-borne bacterium with a worldwide distribution, and it is the causative agent of various clinical manifestations in goats, sheep, cattle, and horses. This bacterium was first identified and described as a cause of renal abscesses in sheep in 1891.[1] Since then, two different biotypes have been described, biovar *equi* and biovar *ovis.*[2] *Corynebacterium pseudotuberculosis* biovar *ovis* is the etiological agent of caseous lymphadenitis (CLA), a chronic contagious disease of small ruminants.[3] *Corynebacterium pseudotuberculosis* infection in horses, also known as pigeon fever, is caused by the biovar *equi*.[4] The disease in horses is a generally self-limiting, suppurative inflammatory process most commonly affecting the pectoral, axillary, ventral abdominal, and inguinal regions.[5,6] *Corynebacterium pseudotuberculosis* biovar *equi* infection occurs in Africa, the Americas, the Middle East, and India.[4]

Deep intramuscular abscesses caused by *C. pseudotuberculosis* were first reported in horses in 1915 in San Mateo County, California.[7] Since then, disease associated with this organism has been frequently reported in the western United States (California, Utah, Colorado and Texas) and Brazil.[8,9] It is currently considered to be one of the most common and economically important infectious diseases of horses in California.[4,10]

A recent increase in the number of cases in horses and disease spread to regions not previously considered endemic has been reported.[8,9] In 2002 and 2003, reports of outbreaks in Kentucky, Wyoming, Utah, and Colorado were described.[8] In 2005 and 2007, outbreaks were reported in Oregon and Idaho.[11] In 2012, more than 60 cases were reported from the northwestern panhandle region of Florida,[12] which led to cancellation of equestrian events in

surrounding counties. Therefore, the disease should no longer be considered to be restricted to the western United States, and it could be expected that climate change may result in further extension to new geographic locations. In order to control and limit the spread of the disease, further efforts to limit the bacterial transmission, develop accurate diagnostic tests, and investigate effective vaccines should be performed.

Clinical Manifestations of *C. pseudotuberculosis* infections in horses

Corynebacterium pseudotuberculosis disease in horses presents typically in one of three forms, including ulcerative lymphangitis, cutaneous abscesses, or internal abscesses. In a study of *C. pseudotuberculosis* infection in 538 horses in California; external abscesses were diagnosed in 91%, internal abscesses in 8%, and ulcerative lymphangitis in 1% of the cases.[4] The most common form, deep intramuscular external abscessation, has been given the names such as chest abscess, pigeon fever, pigeon breast, dryland distemper, false distemper, and Wyoming strangles.[6]

External abscess

External abscesses are located primarily in the pectoral and ventral abdominal region. The colloquial term of "pigeon fever" is due to the markedly swollen pectoral region, which resembles the breast of a pigeon. In a retrospective study of 117 cases, 50% of the cases involved the typical pectoral and ventral abdominal regions.[13] Other anatomic locations include prepuce, mammary gland, axilla, inguinal region, limbs and head. Other less common areas are the thorax, neck, parotid gland, guttural pouches, larynx, umbilicus, tail, and rectum. These atypical sites often make diagnosis more difficult.[13]

Some of the more consistent signs associated with developing abscesses are superficial or deep swellings, ventral pitting edema, ventral midline dermatitis, lameness, depression, and fever.[13] About 20-25% of horses develop fever up to 40ºC (104ºF).[13] In one study, twenty six percent of horses had multiple abscesses. The internal capacity of the abscess pocket can vary from 5 to 400ml of odorless, thick, creamy and, often caseous exudate.[13] Of 538 horses, 91.4%

had a single episode of infection, without recurrence in subsequent years, and 8.6% had recurrent infections in subsequent years.[4]

Internal organ infection

Internal *C. pseudotuberculosis* infections occur seasonally but more commonly between November and January, two months after the typical external abscesses presentation.[9] This difference may represent a true delay in the development of internal infections, delayed owner recognition of illness, or owner delay in seeking veterinary care after development of clinical signs.

The most common clinical signs are anorexia, lethargy, fever (up to 41.1ºC), and weight loss.[9] Other clinical signs depend on the localization of the abscesses, and include colic, ventral or limb edema, ventral dermatitis, ataxia, hematuria, and nasal discharge.[13]

The most frequently affected anatomic location is the liver, followed by mesentery, mediastinum, lungs, kidneys, diaphragm, spleen, pericardium, and uterus.[4,9] Bacteremia can also occur. Both single-organ and multiple-organ involvement have been documented; however, involvement of a single organ is most common.[9] In two different studies, between 50 to 63% of horses that had internal abscesses also had concurrent or historical external abscesses.[4,9]

Respiratory disease caused by *C. pseudotuberculosis* occurs in approximately 40% of horses that develop internal *C. pseudotuberculosis* infection.[14] The lower respiratory tract may be the only organ system affected and infection can manifest as pneumonia or pleuropneumonia. Approximately half of the horses that develop *C. pseudotuberculosis* lower respiratory tract infection have concurrent abdominal organ involvement.[14]

The pathophysiological development of internal abscesses in horses is unclear. Experimentally induced infections in small ruminants reveal that once *C. pseudotuberculosis* gains access via wounds or abrasions in the skin or mucous membranes, macrophages migrate to the invasion site and engulf the organism.[4] Infection can spread via the lymphatic system resulting in development of abscesses in subcutaneous tissues, peripheral lymph nodes, or internal organs.[15]

Ulcerative lymphangitis

This form of the disease has a worldwide distribution. In a recent study, it was found that the prevalence of ulcerative lymphangitis in the United States was much higher during 2011 through 2012, and the majority of the cases (96%) were reported in Texas.[11] Interestingly, in this study, significantly more females (71%) than males (29%) had ulcerative lymphangitis; and the cases appeared to occur more during March, April, and May, in contrast to the other forms of the disease which tend to occur in fall months.

The onset of lymphangitis is slow and the condition usually becomes chronic.[16] The affected lymphatic vessels are swollen and firm and nodules form along their length. Severe cellulitis and edema develop in the affected limbs with ulcerated nodules that exude a thick, odorless, greenish, blood-tinged pus.[16] The most remarkable clinical sign is non-weight-bearing lameness, associated with fever, lethargy, and anorexia.

Musculoskeletal disease

Recently, *Corynebacterium pseudotuberculosis* has been recognized as the causal agent of severe lameness in a retrospective study of 35 horses.[17] Deep abscesses located in the axillar

or triceps region was the most common finding. Lymphangitis as well as osteomyelitis and septic arthritis were also described, which have a poorer prognosis.[17] In another retrospective study, in 32 of 117 cases, lameness was noted and attributed to stiffness and discomfort from edema and abscesses adjacent to the limb, with the most severe lameness associated with abscess development in the axillary region.[13]

Other manifestations

Other clinical manifestations have been described in the literature such as guttural pouch empyema, maxillary sinusitis, and uterine infection.[4] The abortigenic potential of *C. pseudotuberculosis* has been demonstrated by descriptions of mares that were pregnant when internal infection with *C. pseudotuberculosis* was diagnosed and they aborted due to in utero infection.[9,13] The majority of fetuses had well-developed abscesses of internal organs. One mare was reported to abort without previous systemic infection, and *C. pseudotuberculosis* was postulated to access the fetus and allantochorion by invading through the cervical opening.[18]

A case of septic fibrinoeffusive pericarditis and pleuritis was associated with *C. pseudotuberculosis* infection and was successfully treated medically through a combination of drainage and lavage of the pericardial sac.[19]

Suppurative facial cellulitis and panniculitis caused by *C. pseudotuberculosis* was reported in two horses in Colorado.[20] One horse presented with a facial wound and multiple small abscesses located around the commissure of the lips, lateral aspect of the neck, intermandibular space, and ventral aspect of the abdomen. The wound was suspected to be the result of a kick injury. The second horse presented with a facial lesion caused by a gun shot and

extensive facial edema. *C. pseudotuberculosis* was isolated from the lesions of both horses. Both horses responded positively to trimethoprim-sulfamethoxazole therapy and local debridement.

A case of unilateral bacterial orchitis caused by *C. pseudotuberculosis* has been described in a stallion with a history of a resolved chest abscess, likely due to hematogenous spread.[21] Excision of the affected testis resulted in complete clinical recovery of the stallion and anatomical and functional preservation of the contralateral testis.

Recently a case of otitis media-interna with secondary meningitis was associated with *C. pseudotuberculosis* in a one-year old colt.[22] The colt presented with fever, ataxia, and facial and vestibulocochlear nerve deficits. Right-sided otitis media-interna with osteomyelitis of the temporal bone was made based on computed tomography findings. *Corynebacterium pseudotuberculosis* pure culture was grown from the fluid taken from the middle ear and cerebrospinal fluid collected from the atlanto-occipital site. The clinical signs improved with medical therapy but were not completely resolved 8 months after discharge.

Treatment and prognosis

Treatment of external abscesses typically consists of conservative management until the abscess is mature and can be surgically lanced, drained, and lavaged.[4] One hundred and forty six out of 496 cases with external abscesses in a retrospective study resolved without systemic antimicrobial treatment.[4] The use of antimicrobial therapy for external abscesses is controversial, but it is the only option for internal abscesses and ulcerative lymphangitis. It has been used after external abscess drainage in order to decrease cellulitis. On the other hand, antimicrobial use prior to surgical drainage of external abscesses was reported to prolong the course of the disease.[4]

Identification and treatment of internal abscesses is more challenging, considering the nonspecific clinical signs and clinico-pathologic findings in horses with thoracic or abdominal cavity infections.[4] Horses that develop internal infection require long-term antimicrobial treatment for resolution, between 4-6 weeks, and some cases can take longer than 6 months to resolve.[13]

The susceptibility pattern of *C. pseudotuberculosis* to antimicrobial agents varies among isolates obtained from various sources; however, *in vitro* studies have demonstrated susceptibility to the majority of antimicrobials.[3] The minimal inhibitory concentrations (MIC) of 39 antimicrobial agents for 54 isolates of *C. pseudotuberculosis* from different animals was determined *in vitro*.[23] The most active agents were penicillins, macrolides, tetracyclines, cephalosporins, lincomycin, chloramphenicol, and rifampicin. Most isolates were resistant to aminoglycosides, nitrofurans, polymyxins, nalidixic acid, and cycloheximide. In a recent study, the trend of *in vitro* antimicrobial MIC concentration of isolates from 196 horses with naturally occurring disease was evaluated.[24] No changes in the MIC trends over time were detected and no significant relationship was detected between the MIC patterns and abscess localization.

In vivo, antimicrobials are less effective due to the intracellular location of the bacteria, the thick encapsulation around the typical lesions, and the thick and caseous nature of the purulent material; therefore, these characteristics and the risk of complications must be considered when selecting an antimicrobial drug.[4,23] Antimicrobial-associated diarrhea was seen in 6% of horses being treated for *C. pseudotuberculosis* in a Californian study.[4] Based on MIC values for *C. pseudotuberculosis* in horses, pharmacokinetics and side effect of specific drugs in horses; doxycycline and enrofloxacin are probably the best antibiotic choices for long term treatment of abscesses.[24] However, other drugs such as penicillin, potentiated sulfonamides,

rifampin, and chloramphenicol are other options that can be considered in individual cases.[24] The use of gallium maloate showed no inhibition of bacterial growth in an *in vitro* study.[25]

Alternatives to antimicrobial therapy are being investigated. An integrated structural proteomics approach using 15 previously sequenced *C. pseudotuberculosis* strains from both biovar *equi* and biovar *ovis* was used to identify a set of 31 essential and non-host homologous potential drug-binding targets.[26] Out of 31 global targets, 9 targets have already been reported in other pathogenic microorganisms, and 3 of them (3-isopropylmalate dehydratase small subunit, 50S ribosomal protein L30, and chromosomal replication initiator protein DnaA) have been reported previously in *C. pseudotuberculosis*.[26,27] Another *in silico* study used a computational approach to evaluate protein-protein and host-pathogen interactions of *C. pseudotuberculosis* and other pathogens.[28] This study found a novel promising broad spectrum target, acetate kinase, whose activity was inhibited by penicillin, ceftiofur and two *Piper betel* derived natural compounds: piperdardine and dehydropipernonaline.

The overall case fatality rate is low (3.9%), and is considerably lower in horses with external abscesses (0.8%) than for horses with internal abscesses (40.5%).[4] In horses with internal abscesses that receive antimicrobials, case fatality rate is lower (29%), than untreated internal abscesses, which are uniformly fatal (100%).[4,9] In an epidemiological study performed in Colorado in 2001, participants reported a mean of 13 days (range 0-75) of lost use of affected horses and a mean treatment cost of $139 (range $0-850) per case.[5]

The prognosis for external abscesses is good. Most resolve in 3 weeks from the day of drainage. The prognosis for horses with internal abscesses and ulcerative lymphangitis is guarded.[4] The prognosis improves if infection is detected early and appropriate therapy is administered.

Clinical Manifestations of *C. pseudotuberculosis* infections in other animals

Despite equine disease has long been present in some regions, the equine biovar has not received much attention and the majority of the work regarding *C. pseudotuberculosis* has been performed with the ovine biovar. Infections in small ruminants have been further investigated due to its widespread and economic consequences. Information regarding *C. pseudotuberculosis* pathogenesis in horses have been extrapolated from investigations in small ruminants; however, some differences exist from clinical presentations in other species.

Small ruminants

Caseous lymphadenitis (CLA) is a chronic suppurative condition of sheep and goats. The two clinical presentations in small ruminants are abscessation and enlargement of superficial or internal lymph nodes, which may coexist in the same animal.[29] When infection becomes chronic, the affected lymph nodes exhibit characteristic encapsulated abscesses which have a concentric ring appearance in cross-section. This disease causes substantial economic losses due to sacrifice of infected animals, condemnation of carcasses, requirement for additional meat inspection and carcass trimming, devaluation of hides, and decreased milk and wool production.[29,30]

Caseous lymphadenitis in sheep is characterized by abscess formation in the superficial lymph nodes and internal organs such as the lungs and liver.[29] Prescapular lymph nodes are the most common site of superficial lesion development, and the lungs are the most commonly involved visceral organ which is a rare presentation in horses. Other organs involved include the liver, kidneys, udder, mediastinal, bronchial and lumbar lymph nodes; and more rarely heart, testis, scrotum, uterus, joints, brain or spinal cord.[29] In sheep experimentally infected with *C.*

pseudotuberculosis, the bacteria spreads to local lymph nodes by hematogenous and lymphatic routes which is suspected in horses, but has not been confirmed. Sheep with abscesses in the lungs may be a source of *C. pseudotuberculosis* infection to other sheep because of airborne transmission, which would be a rare mode of transmission in horses.[31]

On the other hand, similarly to horses, goats more frequently present with external abscess formation. In contrast, the lymph nodes of the head and neck are the most common lesion localization; however, any superficial lymph node of the body may be affected, depending on the original point of entry of the bacteria.[29]

It is generally believed that *C. pseudotuberculosis* infection in sheep and goats is initiated through superficial wounds to the skin or mucous membranes followed by extension to the regional lymph nodes with, perhaps, further dissemination; and this has been extrapolated to horses.[29] Evidence that shearing wounds are the primary route for infection in sheep has been provided by the demonstration of a specific serological response to *C. pseudotuberculosis* toxin in sheep 3 months after shearing.[32] A mathematical model developed to investigate transmission of *C. pseudotuberculosis* in sheep determined that the overt abscess form was the most frequent source of transmission, causing most disease initially in an epidemic; whereas respiratory abscesses were less frequent, but had an important role in establishment of endemic situations.[33] Infected animals are not the only reservoir of infection, as *C. pseudotuberculosis* survives well in the environment, which it is thought to be an important factor in horses.[34]

Cattle

Infection in cattle occurs as a herd problem with a sporadic incidence, but may sometimes occur in an epidemic form.[35] The most common clinical form affecting cattle is

cutaneous excoriated granuloma.[35] Infection in cattle is reported most frequently in Israel and manifests as lymphadenitis and lymphangitis with abscess formation and ulceration, which responds poorly to antibiotics.[35] This presentation resembles ulcerative lymphangitis in horses. Less commonly, mastitis and a clinical syndrome involving lesions of the coronary band with resulting lameness in affected dairy cattle have been reported.[35,36] Mastitis can be mild, with the only signs being the appearance of clots in the milk; or severe, with a greatly enlarged and tender mammary gland and complete cessation of milk production. In a rare visceral form of the disease, widespread abscessation occurs in lymph nodes associated with the upper and lower respiratory tract.[37] The organism has also been associated with necrotic and ulcerative dermatitis of the heel in heifers.[35] In an outbreak in a dairy cattle herd in Israel, most of the affected animals were mature cows and the organism was isolated from milk samples of 25% of the animals in the herd.[38] *Corynebacterium pseudotuberculosis* has been recorded as a cause of ulcerative lymphangitis and mastitis of cattle in countries other than Israel, although rarely.[37]

New World Camelids

Corynebacterium pseudotuberculosis has been recognized as the etiological agent of external and internal abscess and mastitis in alpacas in South America.[39] Abscesses in alpacas are mainly in renal lymph nodes in adult alpacas and in superficial lymph nodes in younger alpacas, which is a rare presentation of equine disease.[40] Pulmonary lesions as occur in sheep and goats, do not occur commonly in camelids. After experimental infection, abscessation of renal lymph nodes was most commonly observed, with pulmonary abscesses occasionally seen.[40] In a study involving postmortem examination of alpacas in Peru it was determined that the most frequently affected organ were superficial lymph nodes (38%), mammary gland (33%), and renal

lymph nodes (23%).[39] Less frequent localization included liver (4%) and lung (2%). The consequences of *C. pseudotuberculosis* disease in alpacas include debilitation and decreased fiber production, but the main economic loss is caused by condemnation of affected carcasses. [40]

In North America, several reports have described *C. pseudotuberculosis* disease in alpacas. Anderson *et al*. described 5 alpacas with superficial abscesses in the cervical and submandibular area.[41] A case report of a hepatic abscess was recently described in Texas in an alpaca that was evaluated for weight loss, anorexia, diarrhea, abdominal distension, and ventral edema, which resembled the equine internal abscess form.[42]

Other Camelids

Corynebacterium pseudotuberculosis can cause internal and external abscesses in dromedary camels.[43] Multiple muscle and subcutaneous abscesses at various sites of the body, particularly in the lungs were described in three adult camel herds in Jordan and confirmed to be caused by *C. pseudotuberculosis* biovar *ovis*.[43]

Other ungulates

Infections with *C. pseudotuberculosis* in the African continent are poorly documented, however in South Africa, the disease is known to be common in sheep from semi-arid farming areas and was first described in 1909.[44] A study performed in a South African game reserve revealed a 33% prevalence of *C. pseudotuberculosis* in antelopes causing tuberculosis-like lesions.[44]

Oedematous Skin Disease is an endemic disease in buffalos in Egypt and its causal agent is *Corynebacterium pseudotuberculosis* biovar *equi*.[45] The disease occurs during summer

months, is transmitted by the arthropod *Hippobosca equina*, and consists of ulcerative dermatitis characterized by diffuse swelling and thickening of the skin of the limbs, abdomen, and dewlap. This disease resembles equine ulcerative lymphangitis and the association with insect vectors further supports the hypothesis that vector transmission can play an important role in equine disease.

One recent case report demonstrated the presence of abscesses in the head of a captive elk in proximity of horses in Utah caused by *C. pseudotuberculosis* biovar *equi*.[46] An outbreak of CLA in an Iberian ibex (*Capra pyrenaica hispanica*) stock reservoir, that was established for conservation purposes in north-eastern Spain, was described.[47] A recent report described for the first time the isolation of *C. pseudotuberculosis* biovar *ovis* from caseous lymphadenitis lesions in swine affecting two Portuguese farms of Black Alentejano pig breed (*Sus scrofa domesticus*).[48] These reports alert for the importance of the establishment of suitable control and sanitary management practices to control the infection and avoid further dissemination of this important pathogen to other animal hosts.

Corynebacterium pseudotuberculosis zoonotic potential

Human infection, although rare, is considered an occupational zoonosis causing lymphadenitis.[3] The first human case was described in 1966.[49] Most of the cases of human lymphadenitis due to *C. pseudotuberculosis* have been reported from Australia; usually in those who have been occupationally exposed to sheep, such as sheep shearers, farmers, abattoir workers, or butchers. Other cases have been described in New Zealand, USA, Panama, France, Switzerland, Spain, and China.[50] Of the 32 cases that were reported from 1966-2009 worldwide, 85% were considered professional zoonoses.[51] Some exceptions with respect to sheep contact have been reported, including a man in USA that regularly drank unpasteurized cow's and goat's milk, who presented with cervical lymphadenitits.[52]

Corynebacterium pseudotuberculosis human infection usually causes ulcerative lesions and associated necrotizing and suppurative granulomatous lymphadenitis, without concomitant immunosuppression or known underlying disease.[51] It is likely that sheep are the source of *C. pseudotuberculosis* infection and that the hands and arms are the primary sites of infection in patients with axillary lymphadenitis, which is the predominant clinical presentation. In contrast, environmental contamination can be the source in patients with inguinal lymphadenitis, with the feet and legs as the primary sites of infection.[51] Other uncommon presentations reported in humans include arthritis and ocular infection.[51] Dissemination of infection to visceral lymph nodes and internal organs, as may occur in animals, has not been reported in human infections.

One case of human eosinophilic pneumonia was reported in a 28-year-old veterinary student in the USA exposed to *C. pseudotuberculosis* in a microbiology laboratory.[53] A second case *of C. pseudotuberculosis* pneumonia was recently reported in a veterinary student after working with the bacteria in a laboratory in Norway.[54]

Human illness caused by *C. pseudotuberculosis* is characterized by its chronicity rather than its severity, and prolonged courses with sinus formation and relapsing abscesses are common, especially if the diagnosis is delayed.[51] Surgical excision of affected lymph nodes in combination with a prolonged course of intracellularly active antibiotics (such as newer macrolides) is usually necessary for successful recovery.[51,55]

Because of its zoonotic potential, *C. pseudotuberculosis* infection of animals can contaminate meat and milk, putting consumers at risk.[56] The ability of *C. pseudotuberculosis* to infect both animals and humans makes studies on prevention and diagnosis of this pathogen important. A multiplex PCR was developed to identify and determine the toxigenicity of *C. pseudotuberculosis, C. ulcerans* and *C. diphtheria*, which could potentially be applied for the diagnosis of human disease caused by these bacteria.[57] It has been speculated that as vaccination against caseous lymphadenitis becomes more widely used in Australian sheep flocks, the incidence of the human form of this disease in those occupationally exposed to sheep or their environment would also decline.[51]

Epidemiology of *C. pseudotuberculosis* in horses

The majority of information regarding the epidemiology of *C. pseudotuberculosis* infection in horses comes from large retrospective studies performed in endemic areas such as California. However, no experimental studies have been performed to determine the course of the disease or the effect of risk factors.

No breed predisposition for *C. pseudotuberculosis* infection has been detected in large retrospective studies or epidemiological investigations.[4,6,13,58] Thoroughbred horses were associated with decreased risk for the disease in one study, however this association was not statistically significant and was most likely related to different management conditions in this breed and referral bias.[6]

The median age of horses with external abscesses was 5 years in a large retrospective study, with a range of 3 months to 28 years.[4] In a retrospective study of 30 horses, the mean age of horses affected by internal infection was 8 years.[9] Aleman *et al.* suggested that young horses may be at higher risk of infection, because 52% of horses in that study were 5 years of age or younger.[4] Horses aged between 1 and 2 years and between 3 and 5 years had significantly increased odds of being diagnosed with *C. pseudotuberculosis* infection in another study.[6] A low incidence of disease was observed in horses < 6 months (2.4%), suggesting that foals born to mares in endemic areas may be protected for several months by colostral transfer of immunoglobulin during the first few months after birth.[4,11] However, no effect of age has been found in investigation of risk factors in outbreaks involving naïve populations. [5,58]

Both sexes can be affected by *C. pseudotuberculosis* and gender was not considered a risk factor for the disease in some studies.[6,58] However, in a recent study, male horses compared to female horses were more likely to have external abscesses than internal abscesses or ulcerative

lymphangitis.[11] In a study of internal form cases, a female predilection (70%) was apparent; however, confounding variables such as the time spent at pasture by the mares, could have influenced this predilection.[9] In another recent cross-sectional study, 60% of the horses with an internal abscess were female; however, because of the low number of horses with internal abscesses in the study this association should be interpreted with caution.[11]

Management factors play an important role in the occurrence of the disease.[6] In a study performed in California, horses in contact with other horses or horses on summer pasture had significantly increased risk of disease, supporting a horse-to-horse route of transmission.[6]

The disease has a strong seasonal incidence.[6] Cases can be detected throughout the year; however, the disease is most frequently recognized during the fall and early winter, with the highest incidence in September, October, and November; which are the most arid months of the year in endemic areas.[4] Incidence also varies markedly among years, with a high annual incidence seemingly following winters with above-average rainfall and elevated temperatures.[13] Periods marked with drought conditions have been associated with unusual outbreak situations in Texas and Kansas.[58,59] This increased incidence has been hypothesized to be a result of optimal breeding, hatching, and survival conditions for many types of insects that are suspected to transmit the infectious agent mechanically.[4,13,58]

A recent increase in the number of cases in horses, and disease spread to regions not previously considered endemic (Kentucky, Wyoming, Utah, Colorado, Oregon, Idaho, and Florida) has been reported.[8,9,11] A recent cross-sectional study identified an increasing occurrence of *C. pseudotuberculosis* in horses during the 10-year period of the study.[11] A greater proportion of *C. pseudotuberculosis* culture-positive samples were submitted to state veterinary diagnostic laboratories in United States during 2011 through 2012 compared to the period from 2003 to

2010. Furthermore, *C. pseudotuberculosis* was confirmed from horses in states where the disease had not been previously recognized as endemic (Louisiana, Michigan, North Carolina, South Carolina, South Dakota, Vermont, and Wisconsin).

In Texas, pigeon fever cases increased 10-fold between 2005 and 2011, supporting the theory that the disease is re-emerging in the Texas horse population.[59] Two seasonal peaks in the number of cases were observed during the last 3 years of the study, with the first peak in June-July, followed by a second peak in November-December. One critical region was identified in central Texas, which did not appear to be a high-risk area for pigeon fever prior to 2008.[59] The moderate to severe drought conditions might explain the high-risk areas identified and the presence of 2 seasonal peaks.

Numerous factors can contribute to the recent increase in the number of cases including reporting bias;[11] environmental and climatic conditions facilitating infection, such as changing insect populations;[60] or conditions that may promote persistent survival of the organism in the soil.[4] Increased susceptibility of the equine population in regions where the disease is not endemic may also contribute to increased numbers of observed cases.[8,61] A recent Bayesian geostatistical study determined key environmental and climatic risk factors, which could be associated with a recent *C. pseudotuberculosis* outbreak in Kansas.[58] Higher soil moisture content was considered a protective factor, whereas higher land surface temperature and habitat fragmentation had higher risk of *C. pseudotuberculosis* infection. These physical environment and climatological factors could have influenced the infection among horses involved in the outbreak.

Nine genotype groups were identified using random amplified polymorphic DNA polymerase chain reaction (PCR) assay on isolates of *C. pseudotuberculosis*.[8] This study found

that while horses in Utah were likely infected by a single expanding clone, infections from the other states were from multiple strains not of a single source, concluding that pathogen factors are not the only factors driving emergence in these areas, and environmental and host factors still require further investigation.

Transmission of *C. pseudotuberculosis* in horses

The route of infection of C. *pseudotuberculosis* infection in horses remains undetermined, but transmission through horse-to-horse contact, contaminated soil, or vectors is suspected.[3,9] In a study from California, a statistically significant spatial and temporal clustering of horses with pigeon fever was detected, confirming that animal-to-animal transmission either by direct contact, insect mechanical vectors, or contaminated environment, could be plausible.[61] Similar to other infectious diseases that require close proximity of space and time for transmission, a certain population density of susceptible animals and a sufficient number of successful contacts between infected and susceptible animals are required for an outbreak to occur.[61] The variability of incidence over the years could be explained by the association between age and disease risk. If there is a period of several years in which only sporadic cases are reported; then a younger, susceptible horse population develops which can become infected. Given a sufficient rate of successful contacts, an outbreak can develop. In subsequent years, acquired immunity prevents new cases of disease until the density of susceptible horses again becomes high.[61] A challenge transmission model of *C. pseudotuberculosis* in horses had not been developed and little information is available concerning the transmission mechanisms of this disease. Experimental *C. pseudotuberculosis* infection by subcutaneous or intradermal inoculation of bacteria in sheep and goats results in 100% rate of transmission based on culture isolation and serological response.[15,62]

The geographic distribution of cases does not always reflect the distribution of the general equine population, and some particular areas or farms have a large number of cases.[13] The seasonal patterns of occurrence of ventral midline dermatitis and *C. pseudotuberculosis*

abscesses in California are similar.[13] This could be due either to transmission of the bacterium by biting flies or to contamination of ventral midline dermatitis lesions from other sources.

The portal of entry for this soil-borne bacteria is thought to be through abrasions or wounds in the skin and mucous membranes.[4] Transmission by insect vectors is suspected because of the seasonal pattern of occurrence, being most common during fall and early winter in the western US, which might be related to optimal breeding conditions for some arthropods.[4,13,58] Landscape fragmentation, which indicates breaking the habitat into several patches, was significantly associated with *C. pseudotuberculosis* infection among horses in one study, further supporting possible arthropod-born disease transmission.[58] This is explained because of a loss of "dilution effect". In other words, with a fragmented habitat there is higher risk of competent vectors of the disease to get infected by feeding in their host, while in a wider habitat the presence of other hosts (incompetent reservoirs) with a low capacity to infect feeding vectors dilute the effect of highly competent reservoirs, thus reducing disease risk.

Flies and other mechanical vectors may play an important role in the transmission of pigeon fever between horses and may explain the emergence of infections in previously non-endemic states. Three fly species, including *Haematobia irritans* (horn flies), *Stomoxys calcitrans* (stable flies), and *Musca domestica* (house flies), have been confirmed as potential vectors by PCR detection of *C. pseudotuberculosis* biovar *equi* phospholipase D (PLD) exotoxin gene.[60] In that study, evidence of bacterial DNA was detected in higher frequency in October, when most clinically affected horses were observed. *Corynebacterium pseudotuberculosis* was identified in up to 20% of the house flies examined in the vicinity of diseased horses.[60] Detection of the PLD gene in flies does not provide direct evidence that viable *C. pseudotuberculosis* organisms are present; however, another study demonstrated that from house flies caught with

fly nets from *C. pseudotuberculosis* infected horses, the viable organism was isolated from the body surface of 44% of flies and from the internal organs in 31%.[63] In house flies contaminated with *C. pseudotuberculosis* from cattle, bacteria were isolated from the flies' intestine and feces for 1-4h and after 1-3h in saliva after experimental inoculation with contaminated milk or sugar cubes.[35,64]

The role of *Hippobosca equina* in the transmission of Oedematous Skin Disease in Egyptian buffalos was confirmed based on the seasonal pattern of the disease, feeding habitat of the flies, intradermal introduction of the bacteria as the only route of infection, and isolation of the bacteria from the flies.[45] Other arthropod vectors have been linked to *C. pseudotuberculosis*. Both larval and adult ticks (*Dermacentor albipictus*) were shown to harbor the *C. pseudotuberculosis* when recovered from clinically affected deer.[65]

The number of viable bacteria in purulent discharge from caseous lymphadenitis lesions in small ruminants have been estimated at between 10^6 and $5x10^7$/g, but this number is unknown for pigeon fever lesions in horses.[29] The rupture of superficial abscesses therefore releases huge numbers of viable bacteria that contaminate the environment. Other animals can be exposed by direct physical contact with the affected animal or indirectly via contaminated fomites. The role of soil as a reservoir for *C. pseudotuberculosis* biovar *equi* is supported by a study in which the bacteria survived for eight months in different soil types.[66] Growth was better supported in sandy, rocky soil than in silty soil. Moist or slurry conditions yielded higher bacterial counts over dry samples, although *C. pseudotuberculosis* persisted in all conditions tested. *C. pseudotuberculosis* was recovered from moist silty samples held at 37°C during the entire eight-month period of evaluation. The addition of sterile feces enhanced survival and multiplication of bacteria, most likely by providing essential fatty acids and presumably other micronutrients that

aid in growth and survival. The role of soil as a reservoir of *C. pseudotuberculosis* is further supported by a recent study that found that areas with soil that hold higher moisture levels had a protective effect from infection for horses in Kansas; because soil types that retain higher levels of moisture typically have higher clay content, and are perhaps covered by vegetation that permits less run-off.[58]

Although natural cross-species infection does not appear to occur, conflicting epidemiologic evidence remains regarding disease potential and exposure to small ruminants. A survey of owners in western Colorado with horses infected by *C. pseudotuberculosis* indicated that most had sheep, goats, or both on or adjacent to their premises, indicating a potential risk factor.[5] Sixty-five percent of participants reported that sheep or goats were housed on, or adjacent to, their premises but only 19% reported that affected horses had exposure to sheep or goats prior to or during the onset of disease. A history of sheep or goats being housed on the premises was reported by 59% of participants, however the significance of this association was not discussed.[5] In contrast, a report from California concluded that close contact with livestock (sheep, goat, cattle) that potentially carried *C. pseudotuberculosis* did not increase the risk of disease.[6] Differences between these reports could be associated with geographic differences in ruminant populations.

A challenge transmission model of *C. pseudotuberculosis* in horses would provide information concerning the disease course and different mechanisms of transmission could be investigated. Further investigation regarding the role of insect vectors, soil, direct horse contact, and small ruminant contact in transmission of this bacteria to horses is needed.

Microbiological considerations of *Corynebacterium pseudotuberculosis*

The genus *Corynebacterium* belongs to the Actinomycetaceae, a family that also contains the *Mycobacterium, Rhodococcus,* and *Nocardia* genera.[29] *Corynebacterium* spp. are small, pleomorphic, Gram-positive bacteria which occur in coccoid, club and rod forms (pleomorphic coryneform morphology), and groups of the bacteria tend to show a characteristic palisade or "Chinese letter" arrangement in smears.[29,30] It is a non-sporulating, non-capsulated, and non-motile bacterium that possesses fimbria.[3] *Corynebacterium* spp. are catalase-positive, oxidase-negative, facultative anaerobes that require enriched media for growth such as blood agar, selective blood agar, or MacConkey agar.[30] Corynebacteria are characterized by small whitish colonies surrounded by a narrow zone of complete haemolysis when cultured in blood agar.[30] After several days, colonies become dry, crumbly and cream colored.

Corynebacterium pseudotuberculosis is host specific.[2] Two different biotypes are described, biovar *equi* and biovar *ovis*, with horse strains differing from those affecting small ruminants in genetic characteristics, including restriction fragment length polymorphisms, and the ability of cultured organisms to reduce nitrate to nitrite.[2,67,68] The non-nitrate-reducing biotype affects sheep and goats, and occasionally cattle and is the etiological agent of CLA. This biotype can be found in the environment or as a commensal on the skin and mucous membranes of affected species. The nitrate-reducing biotype affects horses and occasionally cattle, and is mainly found in the environment. Cross-infection by biotypes is thought to be minimal,[8] although non-nitrate-reducing strains have been isolated from clinical infections in horses.[69] Nitrate reduction may not absolutely distinguish between the isolates of *C. pseudotuberculosis*.[70] A study of 37 isolates of *C. pseudotuberculosis* on the basis of ribotyping demonstrated that sheep and goats isolates throughout the world appear to be distinct from equine isolates.[70] In this

study, one nitrate-negative isolate of equine origin resembled that of the nitrate-positive isolates from horses, rather than the nitrate-negative isolates from sheep and goats. Furthermore, there appeared to be two distinct groups of isolates from horses and cattle identified through ribotyping, depending on their geographical location: one from USA, and the other from South Africa and Kenya.[70]

Corynebacterium pseudotuberculosis is closely related to two other species of *Corynebacterium*, *C. diphteriae* and *C. ulcerans*, and shares two distinctive characteristics with these species: negative pyrazinamidase and positive cystinase activity.[51,71–73] The highly similar proteins produced by these bacteria could cross-react in serological tests designed for *C. pseudotuberculosis* antibody detection.[74,75]

The complete genome sequence of *C. pseudotuberculosis* has been reported from strains isolated from horses,[76–81] sheep,[82–84] goats,[85,86] cattle,[87] camels,[88] llamas,[89] buffalos,[90] antelopes,[91] and humans.[86,92] Sequencing of different isolates can allow comparison of genomes derived from the two distinct biovars, should offer insight into the organism's host specificity,[77] and should help investigators better understand the molecular and genetic bases of virulence of this bacterium.[76] Interestingly, no major differences between the structural characteristics of biovars *equi* and *ovis* have been observed, such as the numbers of coding sequences, genes, or proteins; which are very similar between strains of both biovars.[93] The differential pathogenicities of the biovars might be due to the presence of genes that are strain-specific, as each pathogen appears to preferentially infect particular hosts, therefore causing different disease symptoms.

A more clonal-like behavior has been observed in *C. pseudotuberculosis* biovar *ovis*, indicating acquisition of variable genes in a block through horizontal gene transfer and then

conserved, than the biovar *equi*, which contain great variability.[94] Particularly great variability of the pilus genes was observed in the biovar equi compared to ovis, which could be linked to the greater ability of the ovine biovar to adhere and internalize in cells, facilitating visceral organ abscess formation. More studies are needed to assess whether the *C. pseudotuberculosis* biovars *equi* and *ovis* truly present different patterns of pilin formation and, thus, variable degrees of host tissue adhesion, spreading, and cell internalization.[94]

Recently, a highly denaturing high-performance liquid chromatography depletion method linked with next-generation sequencing technology was developed to map the transcripts of *C. pseudotuberculosis* biovar *equi*.[95] This approach showed promise and encouraged the use of this method in future transcriptional evaluation of this bacteria for further elucidation of pathogenesis differences among strains.

Pathogenesis of *C. pseudotuberculosis*

Corynebacterium pseudotuberculosis is a facultative intracellular pathogen capable of surviving and replicating in phagocytes. The majority of investigation of virulence factors have been performed using in the ovine biovar, which genome includes seven putative pathogenicity islands, which contain several classical virulence factors, including genes for fimbrial subunits, adhesion factors, iron uptake, and secreted toxins.[96] Virulence is multifactorial and variations in the invasive potential of *C. pseudotuberculosis* strains exists.[97]

The phospholipase D (PLD) is considered the main virulence factor of *C. pseudotuberculosis*. The function of PLD is to hydrolyze lysophosphatidylcholine and sphingomyelin in mammalian cell membranes, such as the endothelial cells of blood vessels; thereby increasing vascular permeability and facilitating the establishment and spread of these bacteria in the host.[4,51,98] Furthermore, PLD inhibits neutrophil chemotaxis and degranulation of phagocytic cells and activates complement by the alternate pathway, thus evading the immune system.[99] The expression of PLD by intracellular *C. pseudotuberculosis* was shown to play a small but significant role in the reduction of macrophage viability following infection.[100] Although PLD seems to play a significant role in the virulence of *C. pseudotuberculosis,* it is not the only factor. Another exotoxin, the corynebacterial protease 40 (CP40) has been suggested to have an important role in virulence and pathogenesis due to its proteolytic activity.[101]

The second well characterized *C. pseudotuberculosis* virulence factor is the high content of lipid in its cell wall. There is a direct relationship between the percentage of lipid content in the cell wall and the induction of chronic abscessation in sheep.[102] It has been reported that the amount of corynomycolic acid contained in the lipid is more important than the total lipid content in determining pathogenicity.[102,103]

Another important *C. pseudotuberculosis* virulence factor is its capability for facultative intracellular survival for long periods. It has been suggested that the surface lipid of the bacteria is an effective barrier to phagosomal digestion by cellular enzymes, which may facilitate survival of the organism in macrophages.[15] This ability enables the bacteria to be carried within these cells to other locations. Similar to *Mycobacterium tuberculosis* and *Rhodococcus equi, C. pseudotuberculosis* infects and persists inside macrophages.[93] As an intracellular pathogen, this bacterium is subjected to different stresses in the phagolysosome and adaptation to oxidative stress is an important factor for survival. [104] Different proteins are involved in this function such as sigma factor E and adenine/guanine-specific DNA glycosylase (MutY), among 102 proteins identified in nitrosative stress resistance.[104–107]

The findings of an *in vitro* study using a cell line derived from ovine embryonic kidney cells raised the possibility that *C. pseudotuberculosis* can persist and disseminate *in vivo* through non-phagocytic cells, facilitating diffusion of the infection and/or maintenance of a carrier state.[108]

Other virulence factors recently investigated include the oligopeptide permease (Opp) transporter, which in addition to having an important role in cell nutrition, could have a role in the ability to adhere and infect macrophages and intercellular signaling.[109] Pili may play an important role in virulence as they enable the bacteria to bind to molecules in the host, and presence of two pilus gene clusters has been reported in some *C. pseudotuberculosis* strains.[92,94] Iron acquisition is essential for bacterial growth *in vivo* and proteins involved in the transport of iron are considered important virulence factors for *C. pseudotuberculosis.*[92,110] Recently, four genes within an operon that is involved in iron acquisition, designated as fag A, B, C and D, were shown to have an important role in the virulence of *C. pseudotuberculosis.*[111] Other

exotoxins produced by *C. pseudotuberculosis* include sphingomyelinase, hemolysis factor, dermonecrotoxins, and mouse lethality toxins, however their role in virulence has not been determined.[20,29]

Adaptation to environmental changes such as temperature induced stress is an essential property of all living organisms. The three most strongly downregulated genes by heat shock at 43ºC were PLD, fag C, and a fatty acid synthase (fas).[112] The importance of this regulation for the pathogenic processes involved with lesion formation by *C. pseudotuberculosis* warrants further study. Only the *C. pseudotuberculosis* cold shock protein A (Cp-CspA) has been studied, and the role in preserving cell viability during cold shock in the environment needs further investigation.[113]

In small ruminants, experimental subcutaneous or intradermal inoculation of *C. pseudotuberculosis* has enabled study of the pathogenesis of this pathogen. It is thought that the bacteria are carried intracellularly in macrophages via lymphatic drainage to the local lymph node.[15] After the lymph node has been colonized, pyogranulomatous inflammation occurs, which eventually leads to abscessation of the draining lymph nodes.[15,29] The subsequent development of visceral abscesses was related to the enlargement of regional lymph nodes draining the inoculation site.[15] An increase in acute phase proteins has been demonstrated in several experimental studies in small ruminants. In lambs, plasma levels of copper and haptoglobin increased rapidly following inoculation whereas zinc levels decreased[15]. The peaks were reached from 1 to 5 days post-inoculation, and thereafter the values came back slowly to the baseline. In another study; haptoglobin, serum amyloid A (SAA), and α1 acid glycoprotein (AGP) were shown to increase significantly in challenged sheep.[114] By day 7 post infection, haptoglobin and SAA concentrations reached mean values of 1.65 ± 0.21 g/L and 18.1 ± 5.2 mg/L respectively.

Thereafter, their concentrations decreased compared to those of the control sheep by day 18 post-infection. In contrast, the serum AGP concentration in infected sheep continued to rise to a peak of 0.38 ± 0.05 g/L on day 13 post-infection, after which a slow decline occurred. In a different study evaluating biologic markers of CLA in sheep, 38 clinically healthy ewes selected and segregated from a commercial flock of 2500 sheep in an area endemic for *C. pseudotuberculosis* were sampled every 30 days for 6 months.[115] Haptoglobin and fibrinogen concentrations and leukocyte counts failed to distinguish seronegative and seropositive sheep that were in the acute (IgM+/IgG±) or chronic (IgM-/IgG+) phases of infection.[115]

Immunity development after experimental infection has been well-studied in small ruminants. Specific IgG *C. pseudotuberculosis* PLD became detectable at 11 days post-infection in a study in sheep and continued to rise throughout the experiment.[114] One study assessed the kinetics of IgG in goats and showed variation between animals, but the maximum titers were detected between days 11 and 21 post infection.[116] The levels further declined until 140 days post-infection, but not all individuals remained positive throughout the 20 weeks of follow-up, indicating that humoral immunity might not be long lasting. However, other studies observed that antibody titers remained high in some goats even after complete healing of lesions, suggesting that recovered animals may continue to harbor the organism and thus retain seropositivity.[117,118]

Data analysis of an epidemiological study indicated that *C. pseudotuberculosis* had an incubation period of 3 to 4 weeks in horses;[61] however, no experimental inoculation studies have been performed in horses. In an experimental study performed with mice inoculated with *C. pseudotuberculosis* biovar *equi*, the intradermal route of inoculation was consistent with lymphadenomegaly in the draining nodes of the inoculation site, which was compatible with

lymphatic dissemination of the bacteria.[119] Internal abscessation developed with consistent involvement of the liver in all mice; however, the mice used were deficient in macrophage activation, T-helper-1 to T-helper-2 switching, and B cell maturation.[119] A recent study in horses found no significant relationship between strain and lesion location or extent of lesions, suggesting that phenotypic differences during *in vitro* culture do not account for external versus internal disease [120] Further work should be performed to identify determinants for bacterial virulence in horses; including genotypic differences among bacteria and the role of dose, environment, and host immune responses in determining the extent and severity of disease.[120] Further studies including experimental models are required to better understand the course of the disease in horses, the effective immune response of the host, and specific responses to *C. pseudotuberculosis* virulence factors.

Diagnosis of *C. pseudotuberculosis* infections

Diagnosis of *C. pseudotuberculosis* infections in horses

A presumptive diagnosis of pigeon fever in horses is based on presence of characteristic clinical signs such as pectoral or ventral abscesses in horses in endemic regions.[4] Culture of *C. pseudotuberculosis* from aspirated discharge provides a definitive diagnosis.[5] Other potential tests include a polymerase chain reaction (PCR) method to identify bacteria isolated from abscesses.[121]

Anemia of chronic disease, leukocytosis with neutrophilia, hyperfibrinogenemia, and hyperproteinemia are common clinicopathological findings in horses infected with *C. pseudotuberculosis;* however, they are not always present.[4] Acute phase protein concentrations and peripheral blood leukocyte counts are biologic markers of infection in animals.[122] Serum haptoglobin, α1-acid glycoprotein, and monocyte counts may have a role as markers for caseous lymphadenitis in sheep.[114,115] Information concerning the relative usefulness of markers of infection in *C. pseudotuberculosis* in horses is lacking.

Abdominocentesis analysis findings were abnormal in 27 out of 29 peritoneal samples obtained from confirmed internal abscess cases in a retrospective study.[4] These findings included elevated total protein, elevated fibrinogen concentrations, and an increased total nucleated cell count in the peritoneal fluid. Microbiological culture of peritoneal fluid samples yielded *C. pseudotuberculosis* in 7 out of 22 samples cultured. Ultrasonographic imaging is an important technique for identifying specific organs affected by the internal form of the disease, as an aid in obtaining samples for a definitive diagnosis, and monitoring response to treatment.[9]

The SHI test was developed in 1978 for detection of *C. pseudotuberculosis* phospholipase D exotoxin antibody detection in horses.[123] SHI measures IgG response to the

exotoxin in the patient´s serum by detecting the highest dilution that will prevent hemolysis of *Rhodococcus equi* exotoxin-sensitized bovine red blood cells when mixed with *C. pseudotuberculosis* exotoxin of a known concentration.[123] IgG response to the exotoxin depends on the chronicity and severity of the infection and antibody availability. A sensitivity of 100% and specificity of 80% was calculated from data of the first study evaluating the use of SHI in horses when a cut-off of 1:10 was used to determine infection.[123] If the cut-off was optimized and age was considered as a confounding factor, Knight et al. proposed an approximate cut-off of 1:80 and 1:160 to orientate diagnosis in young and old animals respectively, which lead to 26-27% of false negatives (sensitivity of 74-73%) and 14-15% of false positives (specificity of 86-85%).[123] However, the accuracy of the SHI test has not been further evaluated since 1978, and a bigger sample size, as well as modern statistical methods are needed to determine an optimal cut-off and the accuracy for the SHI test. The laboratory that performs the SHI test provides only guidance information regarding the interpretation of the test, without a determined cut-off. Specifically, the laboratory states that titers <1:8 are not considered significant and probably represent cross-reactions with common environmental organisms, that titers ≥1:256 are consistent with active infection, and that in the absence of external abscess, titers >1:256 have a high association with internal abscess formation.

The SHI test was developed initially to aid in detection of internal infections, such as cases of abdominal abscesses in which peritoneal fluid samples cannot be obtained or give negative results on microbial culture.[4,123] However, the interpretation of the test has been debated. In a retrospective study, the SHI titer of 174 horses with external abscesses ranged from negative (1:2) to positive (1:1,024), and 63.8% of the horses had titers ≥ 1:256.[4] In this study, all horses with internal abscesses in which a SHI test was performed had titers ≥ 1:512, with a range

from 1:512 to 1:1,024. Therefore, based on these findings, a SHI titer > 1:512 was used historically as evidence for internal abscesses; however, there is overlap between the lower titers which occur in horses with external abscesses, recovered horses in endemic regions, and horses with the internal form.

In a recent study, higher SHI titers were typically more indicative of active, external or internal, *C. pseudotuberculosis* infection rather than internal disease, specifically.[124] The SHI test was not a useful predictor of internal *C. pseudotuberculosis* infection in horses with external abscesses, but it was useful in the absence of external disease to detect internal abscesses. Excluding cases of horses with external abscesses, the likelihood ratio for detection of internal infection was 10.75 for horses with SHI titer ≥ 1:512. When only cases involving external abscesses were evaluated, the results indicated that SHI test results had limited usefulness in identifying the presence of internal infection. Overall, a titer ≤ 1:160 was associated with a low likelihood of active *C. pseudotuberculosis* infection (internal, external or both); a titer ≥ 10,240 most likely indicated the presence of an internal abscess; and titers between 160 and 10,240 were uncertain. The study concluded that practitioners should be cautious in making clinical decisions involving diagnosis of pigeon fever solely on the basis of antibody titers determined via SHI testing.[124]

The time for decline in serological titers detected by SHI test after resolution of infection in horses is unknown. Serum antibody titers remained high in some horses and goats in one study, even after complete healing of lesions, suggesting that recovered animals may continue to harbor the organism and thus retain seropositivity.[4,117]

The internal form of the disease may not be detectable ante-mortem, therefore a reliable serologic test is required to determine and treat the cause of illness in individual animals.[30] An

ELISA test for detection of cell wall antigens in sheep was reported to be not very accurate in horses;[16] however, further studies are needed to investigate the use of cell wall antigens in serological testing in horses.

Diagnosis of *C. pseudotuberculosis* infection in small ruminants

Similarly to horses, the diagnosis of CLA in small ruminants is mainly based on the characteristic clinical symptoms and on isolation of the agent from discharging abscesses, followed by identification of the cultured organisms as *C. pseudotuberculosis* by biochemical tests or PCR.[121,125]Cross-reaction with *C. ulcerans* has been observed with the available PCR tests. [121,126] However, since serological testing is of significant importance in the diagnosis of this disease because subclinically infected animals play a major role in introducing CLA into a healthy flock, further emphasis in developing accurate serological assays for small ruminants has occurred.[56]

Serological detection of CLA targets either IgG as a marker for humoral response or gamma-interferon (γ-IFN) as a marker for cell-mediated response.[127] These serological tests are associated with a wide spread scientific debate about advantages and disadvantages of each type and most of the serologic tests have been reported to lack either sensitivity or specificity.[3,128]

In small ruminants, many serological tests have been developed with the objective of detecting subclinically infected animals, but enzyme linked immunosorbent assay (ELISA) is the most commonly used because of its advantages; which include cost, effectiveness, ease of applicability, and acceptable sensitivity and specificity.[128,129] Other developed tests include agar gel immunodiffusion test (AGID)[130,131], microaglutination assay,[132], tube agglutination assay[133], synergistic hemolysis inhibition test (SHI),[123,134] hemolysis inhibition test,[135] dot-blot,[136] Western

blotting,[137], complement fixation assay,[138] indirect hemoagglutination test,[138] and a variety of ELISA procedures.

Specificity of AGID is claimed to be higher than that of ELISA[131], but some ELISA techniques have shown very high specificity (99 ± 1%).[75] Furthermore, a comparative study between AGID and one ELISA showed that the ELISA was more sensitive and more specific.[139] In addition, AGID has a lower testing capacity and takes 3 days compared to ELISA, which may be performed within hours.[131]

Different bacterial components have been used as the solid phase antigen in ELISAs, including whole cell sonicate (somatic antigen),[140] formalin-inactivated whole bacterial cells,[141–143] exotoxin (phospholipase D) extracted from culture supernatants,[137] and recombinant exotoxin.[144] Some studies have demonstrated that the exotoxin is better than the somatic antigen to detect both humoral and cellular responses against *C. pseudotuberculosis* based on higher sensitivity and higher production of γ-IFN *in vitro*.[145,146] On the other hand, other studies have shown higher sensitivity, but lower specificity, of cell wall antigen than using crude exotoxin as a solid phase ELISA antigen.[147] Recently, it has been shown that depending on a single antigen, even PLD, is not sufficient to detect all CLA cases.[127] Therefore, a combination of two or three *C. pseudotuberculosis* immunodominant antigens is necessary.[129]

Indirect PLD-based ELISA is considered the most sensitive type with a high specificity.[144] Furthermore, the indirect PLD-based ELISA is reported to be more specific than the indirect double antibody sandwich ELISA.[75,137,148] This indirect PLD-based ELISA has a specificity of 98±1% for goats and 99±1% for sheep and its sensitivity was 94±3% for goats and 79±5% for sheep.[75]

An ELISA was developed to detect whole blood γ-IFN as a marker of cell-mediated immunity against *C. pseudotuberculosis*,[116] but the indirect PLD ELISA was reported to be more predictive than γ-IFN assay for detecting goats with postmortem lesions.[128]

Specificity of an IgG-detecting ELISA through the use of PLD as a solid phase antigen is higher than that of total antibody-detecting ELISA because the latter also measures the IgM, which has a greater cross reactivity with many antigenically related bacteria.[140] Although testing for IgM may detect infection earlier than testing for IgG, this is of little value as CLA is a chronic disease.

In small ruminants, results of some studies indicate that the SHI test has low specificity for identification of animals with CLA, which could lead to inaccurate determination of the prevalence of CLA and culling of genetically valuable seropositive animals that would not have developed active CLA lesions.[117,149] The false positives from this test can be a reflection of current subclinical infection or positive titers in recovered animals, or cross reaction with other bacterial pathogens.[117,149]

Specificity and sensitivity of serological tests for *C. pseudotuberculosis*

Serological tests, particularly those detecting humoral response, have specificity and/or sensitivity insufficiencies.[128] Sensitivity of such tests varies according to immune status of the tested animals, route and extent of exposure to *C. pseudotuberculosis*, antigen type used and the infection stage as the interval between infection and sampling affects seropositivity.[139,140,150] Consequently, repetitive serological monitoring is better than single testing policy for diagnosis and control of CLA in sheep and goat flocks. False negatives can result from acute onset of infection and rapid maturation of the abscess before developing immunoglobulin response, presence of a thick capsule isolating the organisms and preventing a serologic response, and consumption of antibody during active infection. As well, some sera from positive animals were found to have a negative immune response against PLD in immunoblot analyses.[129]

Specificity problems of serological tests to identify *C. pseudotuberculosis* infected animals are mainly due to antigenically related bacteria, vaccination, or effect of maternal antibodies.[117,139,140] The CMNR group is a suprageneric group of the Actinomycetales family that are antigenically related to *C. pseudotuberculosis* and may cross-react with serological tests in small ruminants.[75] This group includes several genera that impact on animal and human health, including Corynebacterium (C), Mycobacterium (M), Nocardia (N) and Rhodococcus (R). Bacteria in the CMNR group share common features, including a high genomic G/C content and a specific cell wall structure composed of mycolic acid, peptidoglycan and arabinogalactan.[3] Cross-reaction caused by *Mycobacterium avium* subsp. *paratuberculosis* (Johne's disease) may occur since it is known that an infection with *C. pseudotuberculosis* in goats interferes with serological test for *M. avium* subsp. p*aratuberculosis.* This interference is presumably due to the presence of surface antigens used in the Johne's disease assays that are shared between the

genetically related Corynebacteria sp. and Mycobacteria sp.[151] There was no evidence that infection with *C. pseudotuberculosis* interferes with diagnostic testing for tuberculosis.[152] To avoid the effect of maternal antibodies on serological testing for CLA, it is recommended not to test animals under 6 months age[127]. Moreover, serological tests are unable to distinguish between exposed but recovered animals and those that were exposed and still harboring the infection, which necessitates retesting after 4 weeks to compare antibody titers.[127,139]

Similar to small ruminants, in horses possible cross-reactions may be observed with the SHI test due to exposure to closely related bacteria producing similar phospholipases [74,75,153]. *Corynebacterium ulcerans* is a closely related bacterium to *C. pseudotuberculosis*, that is known to circulate among wild life and dogs, and causes mastitis in cows and goats.[154,155] *Corynebacterium ulcerans* PLD shares a 87% amino acid homology with *C. pseudotuberculosis* PLD (Table 1.1).[74] Likewise, *Arcanobacterium haemolyticum* phospholipase shares a 64% homology with *C. pseudotuberculosis* exotoxin (Table 1.1).[74,156] A novel *Corynebacterium* spp. has been recently isolated from urogenital samples of mares, which was named *Corynebacterium uterequi.*[157] This bacterium produces a PLD, which shares 30% of amino acid homology with the PLD sequence of *C. pseudotuberculosis* at the N-terminal portion (Table 1.1). Despite the evidence on amino acid homology, further investigations are needed to determine if these proteins cross-react with the antibody response against the *C. pseudotuberculosis* PLD and are responsible for false positive results, lowering the specificity of this test.

An important factor that influences sensitivity and specificity of serological tests is the cut-off value, which is dependent on a reference gold standard test.[75,144] Selection of a gold standard test to judge serological tests of CLA is not simple.[127] Bacteriological identification of

Table 1.1: Amino acid sequence alignment between phospholipase D protein from different related bacteria and *C. pseudotuberculosis* phospholipase D protein. Identity indicates the number of amino acid exact matches (homology). Positives indicates the number of matches with similar amino acids base on side chain properties. E-value represents the number of alignments expected from chance and is is calculated with the formula E = (query length) * (length of database) * 2^-(S). A biologically significant e-value is less than or equal to 0.05.

Bacterium spp.	**Protein description**	**Amino-acid length**	**Identity (%)**	**Positives (%)**	**E-value**	**GenBank Accession number**
C. pseudotuberculosis	Phospholipase D	307	307/307 100%	307/307 (100%)	0.0	AAA64910.1
C. ulcerans	Phospholipase	307	266/307 (87%)	283/307 (92%)	0.0	KPJ25302.1
A. haemolyticum	Phospholipase D	318	196/306 (64%)	233/306 (76%)	$7e^{-153}$	AAA21882.1
C. uterequi	Phospholipase D-nuclease N-terminal	80	12/40 (30%)	18/40 (45%)	0.037	AKK11121.1

C. pseudotuberculosis as a reference gold standard method has some limitations such as: bacteriological culture negative status certainly does not equate to a non-infected animal, inaccessibility of internal lesion samples, seropositivity of some *C. pseudotuberculosis* culture negative animals, and seronegativity of some *C. pseudotuberculosis* culture positive animals.[127,140] Similarly, PCR as a reference gold standard has some limitations which include the inaccessibility of visceral lesions for sampling and false negative results when performed on blood samples due to the need of a lower detection limit.[126] Clinical signs or postmortem examination without bacteriological confirmation should not be used as a gold reference method to judge serological tests, because small non-progressive CLA lesions exist and other pyogenic bacteria may induce abscesses in sheep and goats.[127]

Recently, it has been shown that the same ELISA test was not equally suitable for both sheep and goats. This is due to the differences in serological responses of each species to different antigens of *C. pseudotuberculosis,* which vary according to the origin of the isolates from sheep or goats.[129] This ELISA based on PLD as a sole antigen was unable to identify all infected animals, particularly sheep,[129] which may explain the previous success of certain ELISA techniques to diagnose and eradicate CLA from goat rather than sheep flocks.[75,141] The species-specificity of serological diagnostic methods currently available require further evaluation.[127]

Development of new diagnostic methods for *C. pseudotuberculosis*

Efficient serological diagnostic tests for *C. pseudotuberculosis* infections, both for horses and small ruminants, are not available, due in part to a lack of sufficient information concerning *C. pseudotuberculosis* virulence determinants.[93,129] Identification of proteins that can induce an immune response with diagnostic value is essential for development of accurate serological diagnostic tests. The economic importance of CLA in small ruminants provides momentum for research in this area, and advances in equine diagnostics and preventative measures could benefit from this work.

Immunodominant antigens of *C. pseudotuberculosis* biovar *ovis*

Species-specific differences in the anti-*C.pseudotuberculosis* humoral immune response between sheep and goats are responsible for the differences in suitability of the available diagnostic tests.[129] Many ELISAs typically perform adequately in goats, but with reduced sensitivity in sheep, especially in subclinically individuals with only internal abscesses as discussed above.[29,75] In one study, the most commonly used diagnostic antigen (PLD) was recognized by 100% of the *C. pseudotuberculosis* positive goats but only by 70% of the positive sheep; therefore, serological assays based on the PLD as single diagnostic antigen are not able to identify all infected animals, especially sheep, and the ideal serological assay should consist of a combination of 2 to 3 select immune dominant recombinant *C. pseudotuberculosis* proteins.[129] One study used the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting approach to characterize the whole cell fraction of *C. pseudotuberculosis*, and identified a total of 18 different antigens that reacted with ovine and caprine sera, with species variability in recognition.

Initially, with the aim of identifying new targets for the development of immunodiagnostics and vaccine targets to combat CLA, various research groups conducted proteomic studies using one-dimensional SDS-PAGE and immunoblotting to characterize the whole cell fraction and extracellular proteins of *C. pseudotuberculosis*. Antibodies to as many as 10 antigens in the whole cell lysate, ranging from 22 to 120 kDa were present in the sera of infected goats in one study.[102] Three antigens of about 120, 68, and 31.5 kDa were consistently recognized. Twenty out of the 40 infected animals also exhibited antibodies to antigens with approximate molecular mass of 64, 43, and 22 kDa. Only one of the antigens was further identified, the purified 31.5-kDa protein, as the phospholipase D (PLD).[102] Several similar studies identified multiple SDS-soluble proteins from whole cells and culture supernatant ranging in size from 20 to 119kDa.[158,159] The bacteria in these studies were grown in complex media containing exogenous proteins that could contaminate extractions of extracellular proteins.[93]

A chemically defined medium (CDM) for *C. pseudotuberculosis* growth in macromolecule-free conditions was developed as an effective strategy to identify bacterial components and exclude other exogenous proteins, which was used in successive studies.[160] Paule and colleagues developed an efficient protocol for extracting the extracellular proteins of *C. pseudotuberculosis* based on the three-phase partitioning (TPP) technique.[146] With this technique, 6 proteins from 26-66 kDa were recognized predominantly via immunoblotting in experimentally infected goats, however individual variability was present.[146] All infected animal sera recognized two proteins of approximately 30 and 41 kDa, which were suspected to correspond with PLD and CP40 antigens respectively, but this was not confirmed by mass

spectrometry (MS). This finding confirmed the results from a previous study indicating that a 40 kDa protein was an early immunoreactive antigen.[161] Another study identified two other antigens of 16 and 125 kDa not previously identified.[162] The differences in the number of proteins recognized among these studies could be associated to different methods of preparation of antigens, including sonication, ether-extraction, detergent emulsification, and three-phase partitioning with ammonium sulfate and buthanol.[162]

All of these results only indicated the molecular weights of the proteins or reactivity of the proteins against the sera of infected animals without protein characterization by mass spectrometry (MS), with the exception of one study identifying PLD by different methods.[102] A set of more exhaustive studies of the immunodominant proteins in *C. pseudotuberculosis* biovar *ovis* has been carried out using more advanced proteomic analyses. One study combined the techniques of TPP and gel-free separation using liquid chromatography coupled with mass spectrometry and identified 93 extracellular proteins of *C. pseudotuberculosis* that were associated with the physiology and virulence of this pathogen.[163] Another study used two dimensional gel electrophoresis to resolve the extracellular proteins of *C. pseudotuberculosis*, which were then digested with trypsin, and sequenced with matrix-assisted laser desorption/ionization spectrophotometry.[164] Combining the results from these studies, a total of 104 extracellular proteins ranging from 5 to 200kDa in size were identified in *C. pseudotuberculosis*.[93,163] The results obtained from these investigations showed quantitative and qualitative changes between the exoproteomes of two strains, one from caprine origin and the other from ovine.[165] Out of the 104, 29 proteins were common to the two strains studied, but some proteins were unique to only one of the strains. However, these investigations did not link these antigens with the immune response.

In silico predicted pan-exoproteome studies are a recently developed approach to identify vaccine, diagnosis and drug targets. An initial study identified a set of 150 genes classified as secreted proteins and 227 as potentially surface exposed proteins from *C. pseudotuberculosis* biovar *ovis.*[166]

Another approach that has been employed to analyze the exoproteome of *C. pseudotuberculosis* is serological proteome analysis (SERPA), which involves two dimensional immunoblotting and identification of antigenic spots by a MS technique.[167] In a preliminary study, 23 immunoreactive proteins were identified from the bacterial secretome.[167] Six out of the 23 proteins (Table 1.2) were further studied and they were sequenced by MS. In a more extensive study, the same group identified 16 immune reactive exoproteins (Table 1.2), of which 11 were common to the 2 strains used, one caprine and one ovine.[168] Interestingly, none of the immune-reactive proteins was identified as PLD or CP40, which have been considered the major immunodominant proteins of this bacteria. Unfortunately, the authors of this work did not comment on this issue. Two proteins of molecular size around 40 kDa were identified, the surface layer prot A (SplA, 38.67 kDa) and resuscitation-promoting factor (RpfB, 40.31 kDa), which could be the proteins identified as a 40kDa antigen in previous studies and not CP40.[146,161,167,168] However, other studies using a recombinant CP40 vaccine showed strong immune response by immunoblot analysis and protection of challenged mice.[169,170] This emphasizes the necessity of further studies to identify the truly immunodominant proteins of this pathogen.

Nine out of the 13 immune-reactive exoproteins identified by SERPA approach, which had not been previously described as antigenic in *C. pseudotuberculosis,* present little or even no similarity with proteins from other bacterial species.[168] These antigens might be promising

Table 1.2: Immuno-reactive proteins from *C. pseudotuberculosis* biovar *ovis*.

Protein identity	**Molecular Weight (kDa)**	**Isoelectric point**	**Function**	**Reference**
Phospholipase D (PLD)	32.45	8.77	Sphingomyelin phosphodiesterase	Muckle et al. 1992
Corynebacterial protease 40 (CP40)	43.00	6.48	Serine protease precursor	Silva et al. 2014 Droppa-Almeida et al. 2016
Surface layer prot A (SplA)	38.67	5.90	Transferase activity	Seyffert et al. 2011 Seyffert et al. 2014
Resuscitation-promoting factor (RpfB)	40.31	5.06	Hydrolase	Seyffert et al. 2011 Seyffert et al. 2014
Putative secretive Metalloendopeptidase-like	24.83	7.23	Metalloendopeptidase	Seyffert et al. 2011
Putative secreted prot	24.39	5.34	Unknown	Seyffert et al. 2011
Putative efflux system prot	59.55	5.6	Protein transporter	Seyffert et al. 2011
Cell-surface hemin receptor (HtA)	63.96	5.82	Unknown	Seyffert et al. 2014
Trehalose corynomycolyc transferase (CmtB)	36.55	6.91	Transferase	Seyffert et al. 2014
Trehalose corynomycolyc transferase C (CmtC)	70.23	5.61	Transferase	Seyffert et al. 2014
Nlp C/P60	36.63	5.62	Hydrolase	Seyffert et al. 2011 Seyffert et al. 2014
Neuranimidase (NanH)	74.63	5.05	Neuranimidase	Seyffert et al. 2014
9 Hypotetical prots (Cphp1-9)	29.59 – 84.94	4.94 – 8.58	Unknown	Seyffert et al. 2014

targets for developing an effective diagnostic test because they might not cross-react with other pathogens.

Immunodominant antigens of *C. pseudotuberculosis* biovar *equi*

Limited information regarding immunodominant proteins exist in horses. Soares et al. predicted 49 putative Atg targets that were shared by three strains from the two biovars using a reverse vaccinology approach.[171] At least two of these antigens, a resuscitation-promoting factor and a hypothetical protein with cell-surface hemin receptor (HtA) family domain, were found immunogenic in a previous study involving the ovine biovar.[168] The products of the PLD genes of the three *C. pseudotuberculosis* strains investigated by Soares et al. revealed variable results and, therefore, were not included in the data set. The only PLD gene product predicted to be secreted and antigenic was that of *C. pseudotuberculosis* biovar *ovis* investigated. The PLD from *C. pseudotuberculosis* biovar *equi* CIP 52.97 was predicted to be a cytoplasmic protein and the PLD from *C. pseudotuberculosis* biovar *equi* 258, although apparently secreted, presented a low adhesion probability. These variations in the prediction of protein features may be related to small differences in the sequence of signal peptides (CIP 52.97) and epitope sites (258). This observation requires further investigations *in vitro*, as PLD is considered the major virulence factor of both biovars of *C. pseudotuberculosis* and currently used for standard vaccine production and diagnostic testing.

No peer-reviewed studies could be found that confirmed the possible immune-reactive antigens of *C. pseduotuberuclosis* biovar *equi* using immunoblotting with equine sera; however, some abstracts from scientific conferences address this issue. One study developed an ELISA that measured the antibody response to PLD and screened 50 infected and uninfected horses.[172]

They reported that while most horses with internal abscesses showed high antibody responses to PLD, many horses with external abscesses and some uninfected horses also had high titers to PLD. In another study, sera from positive and negative *C. pseudotuberculosis* horses were subjected to three immunoassays.[173] Serum samples from 120 horses were tested for the intensity of their immune responses to the *C. pseudotuberculosis* antigens PLD and cell lysate by ELISA, IgG antibody responsiveness to PLD, and the IgG and IgM responses against bacterial cell lysate. It was found that horses mounted an immune response to one or more proteins in the cell lysate. Horses with the internal form had statistically significant higher antibody levels to both cell lysate and PLD than those with the nonfatal external abscess. Lastly, another study investigated whether infected or uninfected horse sera had IgG antibodies that cross-reacted or had some specificity for different PLD antigens.[174] Noticeable cross reactivity against the peanut and cabbage PLD was confirmed by ELISA using the Horse Radish Peroxidase system. In addition, bacterial cultures of *C. pseudotuberculosis* biovar *equi*, *C. pseudotuberculosis* biovar *ovis*, and *C. ulcerans* were grown, and their purified and concentrated supernatants, as well as their whole cell lysates, were tested against different horse sera by western blot to determine whether infected horse sera reacted with different bacterial strains with high PLD sequence homology. However, the results of this experiment were not reported in the abstract.

Putative antigens have been predicted for *C. pseudotuberculosis* biovar *equi* focusing towards finding a common vaccine antigen shared by both biovars or more than one pathogen,[81] but no prediction of a specific antigen for biovar *equi* not shared with biovar *ovis* or other similar bacteria has been made. Both *C. pseudotuberculosis* biovars are genetically very similar, and the immunoreactive antigens confirmed for biovar *ovis* have a high amino acid sequence identity to biovar *equi* (Table 1.3), which can lead to false positive results in serological tests. Further

investigation of the immunodominant proteins in horses is required to improve the diagnosis of pigeon fever and control the disease extension to non-endemic regions.

Table 1.3: Amino acid sequence alignment between confirmed immune-reactive proteins from *C. pseudotuberculosis* biovar *ovis* (Cp1002)[167,168] and the equivalent proteins of two biovar *equi* (Cp1/06-A and Cp316) strains.

Protein identity	**Cp1002**	**Cp1/06-A**	**Cp 316**
Phospholipase D (PLD)	100% (307/307)	98% (300/307)	98% (300/307)
Corynebacterial protease 40 (CP40)	100% (374/374)	-	91% (340/374)
Surface layer prot A (SplA)	100% (359/359)	99% (356/359)	99% (356/359)
Resucitation-promoting factor (RpfB)	100% (383/383)	99% (382/383)	99% (382/383)
Putative secretive Metalloendopeptidase-like	100% (233/233)	100% (233/233)	100% (237/237)
Putative secreted prot	100% (236/236)	100% (236/236)	100% (236/236)
Putative efflux system prot	100% (563/563)	99% (557/563)	-
Cell-surface hemin receptor (HtA)	100% (654/654)	99% (650/654)	99% (650/654)
Trehalose corynomycolyc transferase (CmtB)	100% (341/341)	100% (341/341)	100% (341/341)
Trehalose corynomycolyc transferase C (CmtC)	100% (644/644)	99% (642/644)	99% (642/644)
Nlp C/P60	100% (334/334)	99% (332/334)	99% (332/334)

Prevention of *C. pseudotuberculosis* infection

Since existing serological tests are not reliable, disease prevention in horses involves early identification and isolation of clinical cases and the implementation of management changes such as improvement of stable hygiene, insect control and change of pasture practices.[6] Such measures would include frequent removal of discharge from draining abscesses, environmental and local fly control, wound protection in cases of ventral midline dermatitis, and isolation of infected horses.[13]

In small ruminants, most of the currently-available commercial vaccines for CLA are combined with vaccines against other pathogens.[3] These vaccines are based on inactivated PLD and are called toxoid vaccines. In sheep, vaccines using whole cells, cell walls, toxoids, and bacterin-toxoid combinations have shown to provide high degree of protection, decreasing the number of infected sheep and the number of abscesses per sheep.[51,62] Immunization with Cp40 antigen provoked a strong immune response in sheep.[161] Investigations of sheep immunized with live recombinant vaccine with inactivated PLD gene and challenged with the wild-type *C. pseudotuberculosis* demonstrated protection and strong humoral and cellular immune responses.[98,150] Other attempts to create live vaccine vectors against CLA have been less successful.[125,175] DNA vaccines have been less successful in the immunization of livestock than conventional vaccines against CLA, with weak and short-lived immune responses after challenge and antibody concentrations not significantly higher than those in non-vaccinated animals.[176] In general, the available vaccines confer variable levels of protection, but their safety profiles remain questionable mainly because of their side effects (lesions at injection site, fever, malaise, and reduced milk production), which are more intense in goats.[110,177]

In a mathematical model developed to evaluate control strategies in sheep flocks, a combination of vaccination and clinical examination reduced the prevalence of infection at a faster rate than using clinical examination or vaccination alone.[178] Lancing abscesses reduced the prevalence of infection when the initial prevalence was < 0.60, but elimination was unlikely. A vaccine efficacy of 0.79 or more led to elimination of infection from the flock, provided that the endemic prevalence of infection was < 0.60. Serological testing led to elimination of infection after five tests, but was highly dependent upon the diagnostic test sensitivity and specificity and management options used. Further investigations to obtain a diagnostic test with at least 0.90 specificity and sensitivity under field conditions are required before any methods of control can be recommended with confidence.[178]

In horses, an autogenous bacterin-toxoid and a toxoid vaccine were tested in horses in 1992, but despite no observed adverse reactions, the difference between the incidence of clinical infection between the vaccinated and control group was not significant.[179] An equine autogenous bacterin-toxoid vaccine against *C. pseudotuberculosis* infection was tested in a pilot study in mice.[180] Vaccinated mice showed significant protection from challenge infection, as evidenced by a higher survival rate, fewer gross and histopathological lesions, and lower bacterial levels on culture. Recently, the U.S. Department of Agriculture (USDA) granted a conditionally licensed vaccine commercialized by Boehringer Ingelheim Vetmedica (BIVI).[181] However, the vaccine was removed from the market after a few months due to side effects, and further efficacy and safety test studies are currently in progress.

Further investigation is needed to find an effective and safe vaccine for horses. Since the organism can exist as a facultative intracellular parasite, the key to immunoprophylaxis may be found in inducing effective cell-mediated immunity with attenuated living agents.[13] One study

using a subtractive genomic approach identified several drug and vaccine targets that were common to four *C. pseudotuberculosis* strains from different species. [27] In another study applying reverse vaccinology strategy found 49 putative antigenic proteins that could be used as candidate vaccine targets in *C. pseudotuberculosis* biovar *equi* [81]

Novel vaccination approaches have been tested in mice recently.[110,169,170] The use of an iron-acquisition-deficient mutant strain of *C. pseudotuberculosis* elicited both humoral and cellular responses in mice.[110] Another promising target is the serine protease CP40, which induced high protection after immunization with recombinant CP40 in mice.[161,169,170] These vaccines need to be tested *in vivo* in horses, therefore an equine experimental model able to recreate the disease consistently is needed in order to test the protection conferred by vaccination.

Despite use of vaccination in small ruminants, eradication has not been successful; rather, a combination of management changes (farm hygiene, shearing practices, etc.) and identification and culling of infected adult animals has proven to be necessary to eradicate the disease at the herd level.[3] The use of serology as a tool in disease eradication and control has been used in small ruminants in different countries such as the Netherlands and Norway, and the use of bulk tank milk ELISA has been investigated for herd-level surveillance.[182] Complete eradication of infection was demonstrated in a recent study in United Kingdom, where vaccination is not available, using clinical examination and regular ELISA testing in a test-and-cull program in two sheep flocks.[183] Such radical measures, however, might not be necessary or difficult to implement for a population of companion animals such horses that have considerable financial as well as emotional value. For horses, change of management including improved stable hygiene and insect-control measures, and early identification and isolation of infected horses will

continue to be the most logical approach for disease prevention.[6] In order to early detect affected horses, an accurate serological assay is needed, and further investigations should center in this important aspect in order to advance in prevention of *C. pseudotuberculosis* infection in horses.

Chapter 2: Statement of objectives

The general purpose of our studies was to investigate the mode of transmission and diagnosis of *C. pseudotuberculosis* infection in horses in order to institute measures to control further disease spread. Our specific objectives were to determine the role of house flies as a potential vector of this bacterium, and to provide further information to support the development of a more accurate diagnostic serological assay.

Specific aim 1: To find a suitable experimental model to inoculate house flies with *C. pseudotuberculosis* biovar *equi,* which could be used in future investigations. This bacteria has been isolated by culture and PCR in house flies in the surroundings of diseased horses.[60,63] House flies have been demonstrated to be experimentally inoculated with *C. pseudotuberculosis* biovar *ovis* contaminated milk or sugar cubes.[35,64] We hypothesized that house flies would harbor *C. pseudotuberculosis* biovar *equi* following inoculation in our laboratory conditions.

Specific aim 2: To investigate how long house flies would harbor live bacteria after experimental inoculation with *C. pseudotuberculosis* biovar *equi.* House flies inoculated with *C. pseudotuberculosis* biovar ovis harbored the bacteria for up to 4h after experimental inoculation.[35,64] We hypothesized that house flies inoculated with the equine biovar harbor the bacteria long enough to be considered plausible vectors in horses.

Specific aim 3: To investigate if house flies inoculated with *C. pseudotuberculosis* biovar *equi* would transmit the bacteria to ponies. Despite the suspicion of transmission by insect vectors based on seasonal occurrence of cases and finding the bacteria in fly species such as house flies,

clear evidence for transmission of the bacteria to horses by flies is lacking.[4,60] We hypothesized that inoculated house flies would be able to transmit the bacteria to naïve ponies.

Specific aim 4: To develop an experimental model of *C. pseudotuberculosis* biovar *equi* infection in ponies. In contrast to small ruminants, no experimental infection model has been developed to study pathogenesis, epidemiology, and prevention of the disease.[15,56,62,118] We hypothesized that our model will provide insights regarding the course of the disease in horses.

Specific aim 5: To find the prevalence of detectable SHI titers in the non-endemic state of Alabama. Horses without history or clinical signs of the disease were found to have detectable SHI titers in Alabama when screening for naïve ponies. Low SHI titers (≤ 1:128) have been linked both with external abscessation and with low likelihood of active *C. pseudotuberculosis* infection, which makes difficult the interpretation of this test.[13,124] We hypothesized that a large number of horses in Alabama have low detectable SHI titers.

Specific aim 6: To find risk factors associated with detectable SHI titers in a non-endemic population. The duration of SHI titers after disease has not been determined in horses. Cross-reactions with the SHI test due to exposure to closely related bacteria producing similar phospholipases is possible.[74,75,153] We hypothesized that previous travel to endemic areas and small ruminant exposure are associated with an increased SHI titer.

Specific aim 7: To compare the agreement of the SHI test and a caprine specific ELISA based on *C. pseudotuberculosis* biovar *ovis* exotoxin and cell wall antigens. In small ruminants, indirect

PLD-based and cell-wall based ELISA are considered to have high accuracy, and combination of two antigens has been recommended.[129,144,147] We hypothesized that the caprine ELISA successfully detects antibodies against *C. pseudotuberculosis* biovar *equi* in horses.

Specific aim 8: To analyze the immune-dominant proteins from *C. pseudotuberculosis* in horses. The analysis of immuno-reactive proteins has helped to evaluate and develop more accurate diagnostic tests in small ruminants, [127,129,147,167] but limited information is found regarding these proteins in horses. We hypothesized that proteins from the equine biovar induce an immune reaction not present in horses prior to infection and that is different from the reaction against proteins from the ovine biovar.

Chapter 3:

Experimental inoculation of house flies, *Musca domestica* L., with *Corynebacterium pseudotuberculosis* serovar *equi*

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Abstract

Corynebacterium pseudotuberculosis (Actinomycetales: Corynebacteriaceae) infection in horses causes three different disease syndromes: external abscesses, infection of internal organs and ulcerative lymphangitis. The exact mechanism of infection in horses remains undetermined, but transmission by insect vectors is suspected. The present study first determined the optimal culture media for inoculation of house flies (*Musca domestica* L.) (Diptera: Muscidae), with *C. pseudotuberculosis* biovar *equi* and the time required for fly inoculation. A second experiment determined the duration of bacterial survival on flies. Exposure of house flies to 3 different preparations of blood agar supplemented with dextrose and colonized with *C. pseudotuberculosis* determined that a 10 minute exposure to the bacteria was enough to inoculate the flies. *C. pseudotuberculosis* could be recovered for up to 24 hours after house flies were exposed for 30 minutes to a blood agar plate colonized with the bacteria and moistened with 10% dextrose. These findings support the hypothesis that the house fly is a potential vector of pigeon fever and aid in establishing a protocol for a future experimental model to demonstrate the role of house flies as mechanical vectors in *C. pseudotuberculosis* infection.

Introduction

Corynebacterium pseudotuberculosis (Actinomycetales: Corynebacteriaceae) is a pleomorphic, facultative intracellular, Gram-positive rod with a worldwide distribution.[30] Two biotypes of *C. pseudotuberculosis* are described and are distinguished by genetic characteristics, including restriction fragment length polymorphisms and the ability of cultured organisms to reduce nitrate to nitrite. Nitrate-positive biovar *equi* is commonly isolated from horses and cattle but nitrate-negative biovar *ovis* is typically recovered from small ruminants and cattle.[2,67] In ruminants, *C. pseudotuberculosis* causes caseous lymphadenitis, abscesses, and occasionally mastitis.[35] In horses, *C. pseudotuberculosis* biovar *equi* causes three clinical forms of disease.[4] The most common form, known as "pigeon fever" or "dryland distemper", is characterized by subcutaneous abscessation of the pectoral region; the second form causes abscesses of internal organs; and the third causes ulcerative lymphangitis.[4] Natural cross-species infection by specific biotypes is not known to occur.[8] Human infection, although rare, is considered an occupational zoonosis.[3]

C. pseudotuberculosis biovar *equi* infection is prevalent in the western United States (California, Utah, Colorado and Texas) and Brazil, but a recent increase in case numbers and spread to regions previously considered non-endemic have been reported.[8,9,11]

The routes of *C. pseudotuberculosis* infection in horses remain undetermined.[3,9] Transmission by insects is suspected because of the seasonal occurrence patterns during fall and early winter in the western United States.[13] The highest annual incidences in horses have been observed during dry months of the year following winters with above average rainfall, which provides optimal breeding conditions for insects in the subsequent summer and fall.[4,11,13,59] Three fly species, including the horn fly, *Haematobia irritans* L., the stable fly, *Stomoxys calcitrans* L.

and the house fly, *M. domestica* L, were reported as potential vectors by PCR detection of the *C. pseudotuberculosis* biovar *equi* phospholipase D (PLD) exotoxin gene in field samples of fly homogenates.[60] In house flies contaminated with *C. pseudotuberculosis* biovar *ovis* from cattle, bacteria were isolated from the flies' intestine and feces, and saliva for 1-4 and 1-3 h, respectively, post exposure.[35,64]

The objectives of the present study were to determine the optimal inoculation time of *C. pseudotuberculosis* biovar *equi*, the most appropriate inoculation media, the duration of bacterial retention in house flies after exposure and the survival of flies after inoculation. The overall goal of this project was to develop a house fly inoculation system which could be used subsequently in a controlled experimental model to evaluate the role of house flies as mechanical vectors of *C. pseudotuberculosis* in horses.

Materials and Methods

1. Bacterial identification

A field strain of *C. pseudotuberculosis* biovar *equi* isolated from an abscess on a horse from California was grown aerobically on 5% bovine blood agar for 24 h at 37°C in 10% carbon dioxide and confirmed as *C. pseudotuberculosis* biovar *equi* by morphology, culture characterization, and conventional biochemical testing: the bacterium was beta-hemolytic, catalase and nitrate-positive, and fermented glucose without gas production.

2. Fly rearing

Naïve laboratory-bred *Musca domestica* pupae (*n* = 500) from the USDA (Gainesville, FL) *Musca domestica* colony were placed in cages of nylon netting 1.50 x 1.50 mm mesh (24.5 x 24.5 x 24.5 cm) (BugDorm, MegaView Science Co., Ltd., Taiwan), and were maintained at a temperature = 37°C and relative humidity = 30% until emerging. Adult flies emerged in 1 to 3 days, were supplied with a mix of 2 ml of water, 5 g of powdered milk and 5 g of sugar in a small petri dish, and were maintained under laboratory conditions (temperature = 27°C, relative humidity = 40%). Flies were starved for 24 h before being used in experiments.

3. a. Experiment One: Optimization of bacterial inoculation of flies (*n* = 180)

Bacterial preparations

Three different media were used to propagate *C. pseudotuberculosis* to determine if different media preparations would affect feeding and thus recovery of the bacterium. Preparation A consisted of overnight cultures incubated at 37° C of *C. pseudotuberculosis* grown to confluence on 5% bovine blood agar. Preparation B consisted of overnight cultures of *C.*

pseudotuberculosis grown to confluence on 5% bovine blood agar containing 10% dextrose. The Preparation A and B plates were inoculated with a standardized suspension of *C. pseudotuberculosis* in saline at a high concentration of 3×10^8 CFU/ml. The saline suspension was prepared from a culture of *C. pseudotuberculosis* grown in 5% CO_2 for 18-24 h on 10% bovine blood agar. Prior to exposing the flies to Preparations A and B, the entire agar surface of each plate was moistened with a sterile swab impregnated with sterile 10% dextrose solution. Preparation C consisted of 5% bovine blood agar in which the surface was swabbed with a 10% dextrose solution containing a suspension of *C. pseudotuberculosis* (3×10^8 organisms per mL) immediately before exposure to flies. There were 10 petri dishes (60 mm x 15 mm height) for each of the three preparations.

Fly feeding on the bacterial preparations

To determine the exposure times necessary for inoculation of the flies with the bacterium, an inverted plastic cup (1 oz: 45 mm x 40 mm height) (Bio-Serv, Flemington, NJ) containing 6 naïve flies (3-5 days old, mixed sexes) was placed over each of 10 agar plates of the three preparations (n = 30 plates) (Fig. 3.1). The flies were allowed to feed for 10 or 30 min or 1, 2, 4, 6, 8, 12, 24 or 48 h. The flies were observed for 1 min 3 times during the first 10 min of the experiment, and the number of individuals feeding at every time point until termination was recorded to assess how often they fed on the preparation. Following the period of exposure, the number of surviving flies was recorded. Then, the plastic cup containing the flies over the agar plate was inverted and placed in the freezer at -20^o C for 3 min. The cold immobilized flies fell into the cup, were removed using forceps, and placed in a 2-ml sterile microcentrifuge tube. Four flies out of each group of six flies were manually homogenized in the microcentrifuge tubes with

Figure 3.1: Clear plastic cups were used to contain house flies over the preparations.

a sterile stick and 100 µl of phosphate buffered saline (PBS). An aliquot of the homogenate was inoculated onto a 5% bovine blood agar plate for culture. After 36 h of incubation at 37^{o} C, the density of bacterial growth was semi-quantitatively scored using standard methods. Bacterial growth was recorded based on each quadrant to delineate numbers since the method of plating dilutes the specimen from quadrant to quadrant.[185] Heavy growth corresponded to the presence of *C. pseudotuberculosis* colonies in the fourth quadrant of the agar plate, moderate growth corresponded to isolated colonies in the third and/or second quadrant, whereas light growth was equivalent to colony formation limited to the first quadrant and no growth was the total absence of *C. pseudotuberculosis* growth.

Culture Controls:

Bacterial viability in each of the three preparations was evaluated by sampling *C. pseudotuberculosis* from each culture at 24 and 48 h and spreading on 5% bovine blood agar plates as described above.

3. b. Experiment two: Bacterial survival in/on flies (*n* = 60)

Six naïve flies (9-11 days of age, mixed sexes) were contained in inverted plastic cups (described above) over each of 10 agar plates containing Preparation A (5% bovine blood agar with *C. pseudotuberculosis* colonies moistened with sterile 10% dextrose solution). Preparation A was selected for use in this experiment, based on the result from experiment one. After 30 min, the number of surviving flies was recorded and then the flies were cold immobilized at -20^{o}C for 3 min and the agar plate was removed. The flies were transferred to a sterile petri dish at room temperature without addition of water and food. Shortened fly survival was expected due to

starvation, but this method decreased the risk of escape of flies carrying *C. pseudotuberculosis* and minimized the addition of confounding factors due to fly interaction and contamination with other bacteria. The retention of *C. pseudotuberculosis* in flies was examined at T = 10 and 30 min and 1, 2, 4, 6, 8, 12, 24 and 48 h using the same protocol as experiment one.

Negative Fly Controls (*n* = 48)

Prior to the start of experiments one and two, six additional naïve flies were collected and homogenized as described above for bacterial culture at T = 0 to verify the absence of contamination with *C. pseudotuberculosis*. In addition, six naïve flies were contained by an inverted plastic cup over a Petri dish with food (milk, sugar and water) for the duration of the experiment (48 h). The number of surviving flies was recorded and then the flies were cold immobilized and homogenized for culture to confirm absence of contamination with *C. pseudotuberculosis* due to physical manipulation.

3. c. Statistical Analysis

Descriptive statistics was used to summarize the bacterial growth on the different media, fly survival and fly feeding time. Fly survival was analyzed using logistic regression techniques as implemented in SAS® PROC GLIMMIX. Time after treatment was used as a covariate and fly survival modeled as Growth Medium + Time (Growth Medium). Estimates for intercept and slope were compared using linear contrasts. Least squares means were calculated for the two experiments at time points 0 and 48 h; the means for the media were then compared to the negative control using Dunnett's test.

Results

Experiment One: Optimization of bacterial inoculation of flies

Corynebacterium pseudotuberculosis was readily transmitted to house flies by feeding (Table 3.1). Heavy growth of *C. pseudotuberculosis* recovered from fly homogenates indicated that house flies could become highly inoculated after an exposure of 10 min to 24 h to preparations A and B (Fig. 3.2). After 48 h of exposure, heavy growth of *C. pseudotuberculosis* was observed from preparation A, while moderate growth was observed from preparation B. Preparation C showed more variable bacterial growths, with moderate growth observed after 6 h and 24 h of exposure and no growth after 48h of exposure. These results indicated that exposure for 10 min to the bacterium was sufficient to inoculate house flies.

The flies were assessed by subjective observation to feed more often on preparations A and C than on preparation B, in which they expended more time resting on the wall of the cup. The negative controls evaluated at T = 0 and T = 48 h were culture-negative for *C. pseudotuberculosis*. The positive culture controls yielded heavy growth of *C. pseudotuberculosis* confirming bacterial viability.

Preparation A was selected as the ideal medium for the remainder of experiments because of its simplicity of preparation, the consistency of bacterial growths, and the subjective observation of higher feeding frequency of the flies. Overnight bacterial culture on 5% blood agar is also a standard culture method.

One or two out of the six flies exposed to the preparations died during the experiment in some of the groups due to manipulation or contact of the wings with the preparation. These flies were not included in the four flies sampled per group. The mortality of flies during the experiment is shown in Table 3.1. The survival of flies was not statistically different among

groups whether exposed or non-exposed to the bacteria at the beginning or end of the experiment, indicating that exposure to the bacteria did not increase fly mortality (Figure 3.3).

Experiment two: Bacterial survival on/in flies

Inoculated flies continued to harbor viable *C. pseudotuberculosis* for at least 24 h following exposure (Table 3.2). *Corynebacterium pseudotuberculosis* was not detected in samples from negative controls. The growth scores of *C. pseudotuberculosis* recovered from homogenates of exposed flies were heavy growths from T = 0 to T = 6 h and moderate growths from T = 8 to 24 h. No growth of *C. pseudotuberculosis* was obtained from flies sampled at T = 48 h.

The mortality of flies during the experiment is shown in table 2. Fly survival was not statistically different between group A and control at the beginning or end of the experiment (Figure 3.4).

Table 3.1: Number of flies alive at the end of each experiment out of 6 initial flies and culture scores of *C. pseudotuberculosis* recovered from homogenates of flies after 10 different exposure times to three experimental preparations. Negative controls were evaluated before (T0) and after the experiment (T48h). Viability cultures were evaluated after 24 and 48 h.

	Bacterial preparations *					
Exposure times	A	Flies alive	B	Flies alive	C	Flies alive
10 min	Heavy growth	6	Heavy growth	6	Heavy growth	5
30 min	Heavy growth	6	Heavy growth	6	Heavy growth	5
1 h	Heavy growth	6	Heavy growth	6	Heavy growth	4
2h	Heavy growth	6	Heavy growth	6	Heavy growth	5
4 h	Heavy growth	6	Heavy growth	6	Heavy growth	5
6 h	Heavy growth	5	Heavy growth	6	Moderate growth	6
8 h	Heavy growth	6	Heavy growth	6	Heavy growth	4
12 h	Heavy growth	6	Heavy growth	6	Heavy growth	6
24 h	Heavy growth	5	Heavy growth	4	Moderate growth	6
48 h	Heavy growth	4	Moderate growth	6	No growth	4
Neg control T0	No growth	6	No growth	6	No growth	6
Neg control T48h	No growth	6	No growth	6	No growth	4
Viability culture 24h	Heavy growth	6	Heavy growth	6	Moderate growth	6
Viability culture 48h	Heavy growth	6	Heavy growth	6	No growth	4

*A: overnight cultures of *C. pseudotuberculosis* grown to confluence on 5% bovine blood agar. B: overnight cultures of *C. pseudotuberculosis* grown to confluence on 5% bovine blood agar containing 10% dextrose. C: 5% bovine blood agar in which the surface was swabbed with a 10% dextrose solution containing a suspension of *C. pseudotuberculosis.*

Table 3.2: Number of flies alive at the end of each experiment out of 6 initial flies and culture scores of *C. pseudotuberculosis* growth at 10 post-exposure intervals from homogenates of flies exposed for 30 minutes to Preparation A* and from a negative control.

Post-exposure times	Culture scores	Number of flies alive
0 min	Heavy growth	6
10 min	Heavy growth	5
30 min	Heavy growth	6
1 h	Heavy growth	6
2h	Heavy growth	5
4 h	Heavy growth	4
6 h	Heavy growth	4
8 h	Moderate growth	5
12 h	Moderate growth	4
24 h	Moderate growth	4
48 h	No growth	2
Negative control T0	No growth	6
Negative control T48h	No growth	2

* Preparation A: overnight cultures of *C. pseudotuberculosis* grown to confluence on 5% bovine blood agar.

Figure 3.2: Heavy growth of *C. pseudotuberculosis* pure culture observed in a blood-agar plate recovered from fly homogenates after a 10-min exposure to preparation A.

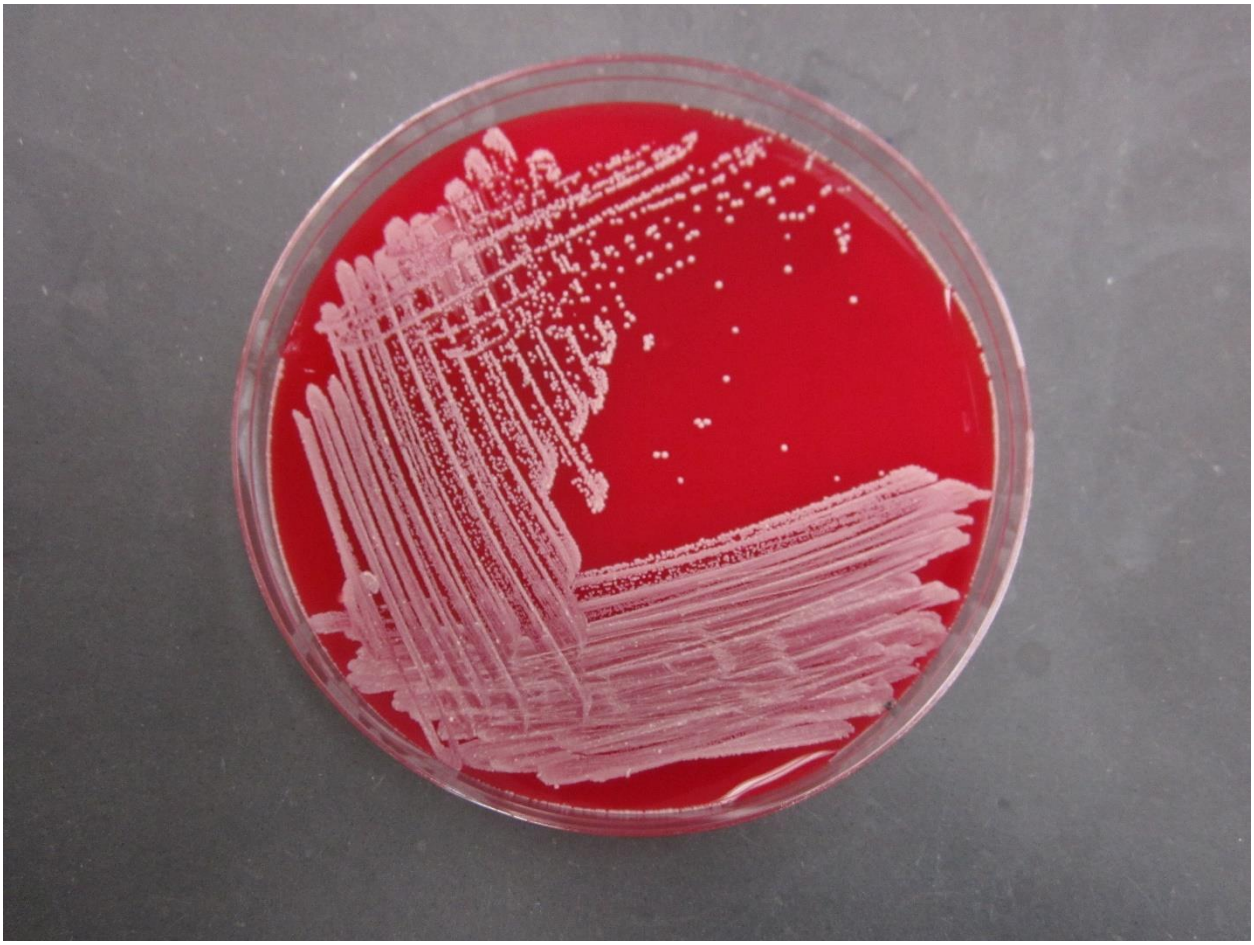


Figure 3.3: Fly survival estimates, 95% CI(p), and contrast *P*-values reflect the contrast of each *C. pseudotuberculosis* medium versus the negative control at T = 0 and time T = 48h in experiment 1.

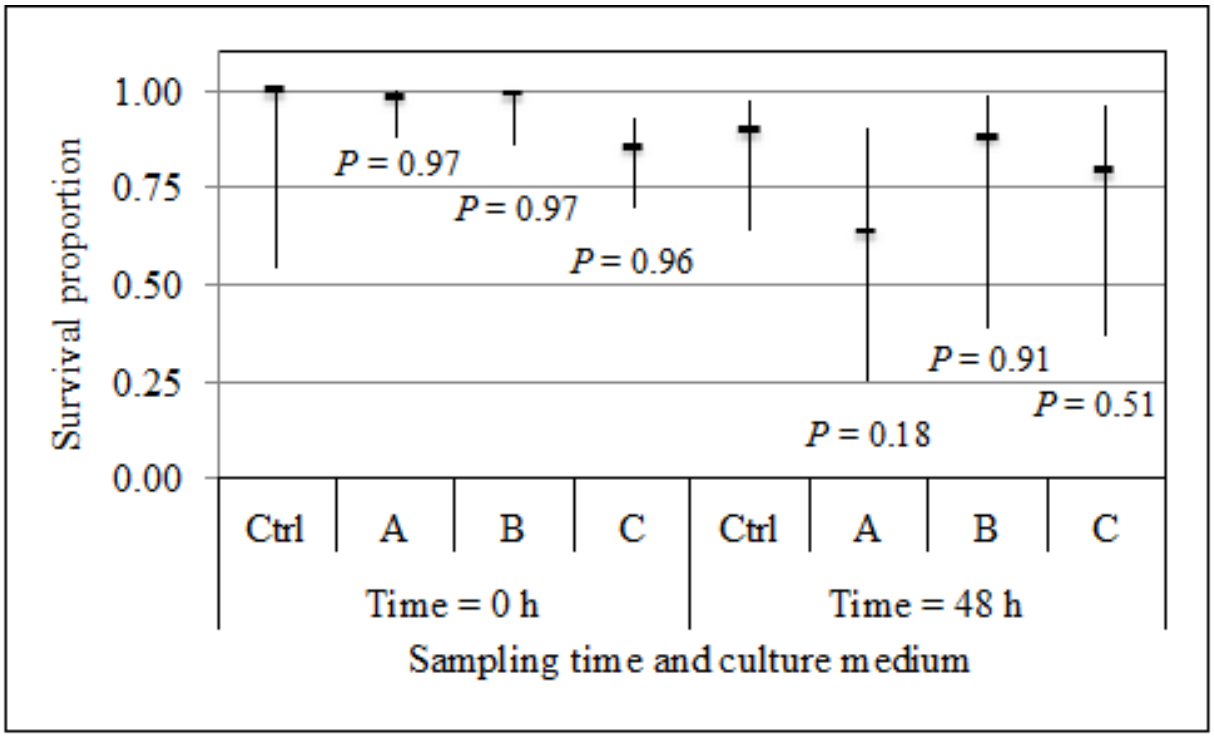
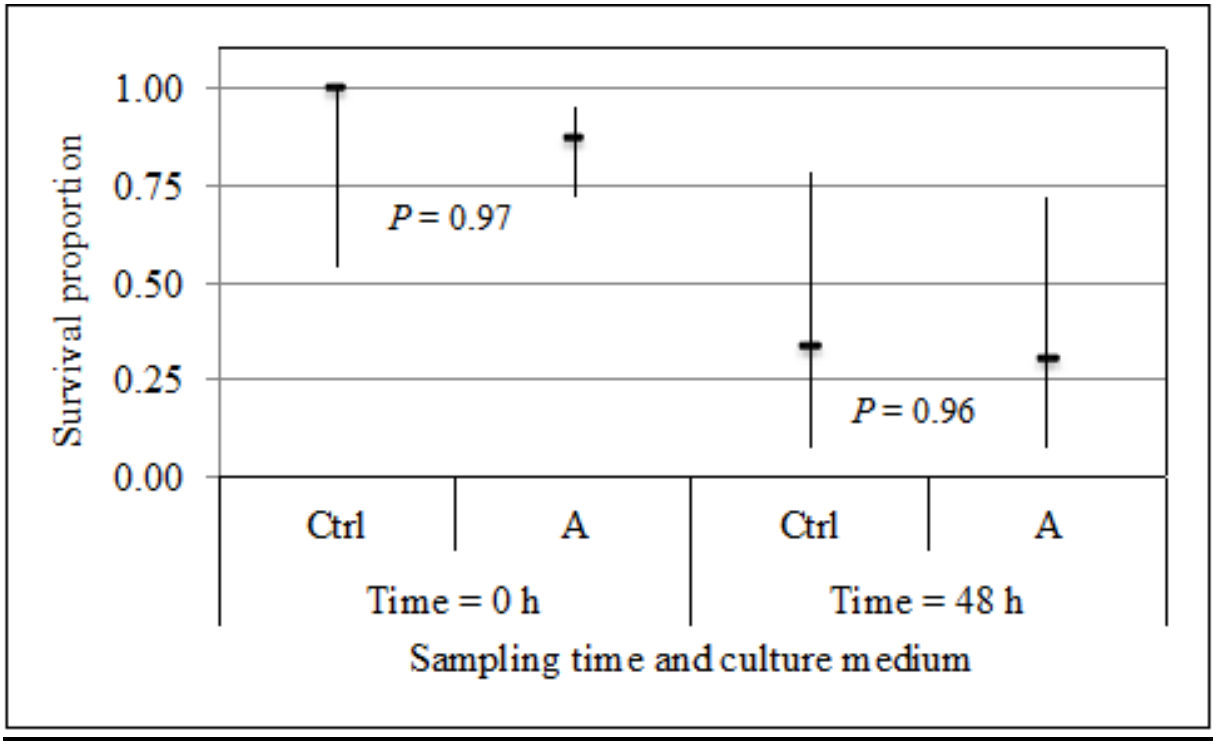


Figure 3.4: Fly survival estimates, 95% CI(p), and contrast *P*-values reflect the contrast of the *C. pseudotuberculosis* medium A versus the negative control at time T = 0 and T = 48h in experiment 2. No difference in survival was detected between the exposed and the control groups. However a decrease in survival was observed at the end of the experiment, which was attributed to the long period of starvation.



Discussion

The results of this study show that adults of *Musca domestica* can be experimentally inoculated with *C. pseudotuberculosis* in an exposure as short as 10 min and under the conditions of this experiment the flies harbored the bacteria for up to 24 h post-exposure. Of the three preparations used to investigate the optimal technique for inoculation of adult house flies, two preparations (A and B), were overnight incubated cultures of *C. pseudotuberculosis* and one (preparation C), was freshly swabbed with a suspension of *C. pseudotuberculosis*. Blood agar was used in all three preparations and a 10% dextrose solution was applied to the surface of all three prior to their exposure to the flies to increase the attractiveness for the flies and to moisten the surface of the agar thus providing a water source to the flies during the experiment. All 3 preparations were successful in allowing inoculation of the flies, and therefore any of these preparations could be used in future experiments. However greater growth was observed when flies were exposed to the overnight incubated cultures of *C. pseudotuberculosis*, and flies appeared to feed more readily on preparation A despite the fact that the blood agar in preparation B also contained dextrose. Because of the consistent heavy growth on preparation A at all exposure times, the maintenance of a heavy growth in the viability controls performed from the media, the simplicity of preparation and the subjective feeding time of the flies, preparation A was selected for use in experiment 2.

In the present study, *C. pseudotuberculosis* was recovered from the flies for up to 24 h following 30 min of exposure to the bacteria. Thirty minutes of exposure was chosen as the feeding time for this second experiment due to operational convenience. These results contrast with previous studies. Braverman *et al.* (1999) recovered *C. pseudotuberculosis* from the intestine of house flies only between 1 - 4 h after feeding for 2 – 4 h on contaminated milk.

Similar results were obtained in another study, in which *C. pseudotuberculosis* was recovered from house flies for up to 4 h after they fed on contaminated milk.[35] The strain used by Braverman *et al.* (1999) and Yeruham *et al.* (1996) was nitrate-negative, which is commonly isolated from ruminants.[2] In contrast, a *C. pseudotuberculosis* nitrate-positive strain was investigated in this study, which has been previously isolated from house flies recovered from horses with ulcerative lymphangitis;[63] this strain may have characteristics different from the ruminant strain that allow it to survive longer in mechanical vectors or our experimental methods may have been more successful at fly inoculation and /or microbiological culture. In addition, the inoculation method of these studies included contaminated milk or sugar cubes, which differed from the method used in the present study. Our results showed that *M. domestica* could be more easily inoculated with *C. pseudotuberculosis* and that the length of bacterial survival was 6 times as long as previously reported.[35,64]

Exposure to *C. pseudotuberculosis* did not affect mortality of the flies, however a higher mortality of flies was observed at the end of experiment 2 in both the exposed and the control groups. This could be attributed to the starvation time of 48h at the end of that experiment.

There are several important limitations to this study. Firstly, ten minutes of exposure to *C. pseudotuberculosis* was chosen as the shortest feeding time due to operational management reasons. The homogenates of flies exposed for only 10 min to the inoculated blood agar preparations produced heavy growth scores when plated on sterile blood agar. The shortest time of exposure to the *C. pseudotuberculosis* investigated in previous experiments was 1 hour.[64] In our experiments the house flies were confined in plastic cups over the inoculated preparations, and it is unlikely that flies fed or walked continuously on the blood agar during the entire exposure time, however due to the previous starvation the flies fed on the blood agar to some

degree. Thus inoculation was likely achieved in less than the specified time intervals. House flies in nature will rarely have the opportunity to feed undisturbed on infected abscesses for 10 min, thus additional research is needed to determine the minimum exposure period required to enable a house fly to become contaminated. Our results indicated how easily house flies became inoculated in the laboratory with *C. pseudotuberculosis*, however replication of the experiments would have been beneficial to demonstrate the reproducibility of the inoculation protocol.

Second, the variability of the individual flies feeding behavior was not evaluated in this study. Each fly could have ingested a variable quantity of *C. pseudotuberculosis*. The ability or need to feed can be related to several factors such as the age, sex of the fly, or the egg development stage for female flies. Another confounding factor would be the potential cross-contamination among the 6 flies kept in the same cup. Differentiation of individual fly feeding behaviors was beyond the scope of this study. Additional research would be required to determine how much *C. pseudotuberculosis* growth could be attributable from an individual fly of a particular sex or life stage. By homogenizing 4 flies some of these individual variations were averaged out, which is more reflective of a field situation where a large number of flies are likely to feed on an abscess or wound on a horse.

Third, the experimental conditions of this study differ substantially from the natural conditions that are present during the potential transmission of *C. pseudotuberculosis* between horses. Our results show *Musca domestica* was inoculated under the laboratory conditions discussed above, however further research is needed to investigate inoculation under natural conditions from a horse abscess.

A fourth limitation of this study is that the mechanism of transmission was not investigated. Yeruham *et al.* (1996) demonstrated excretion of *C. pseudotuberculosis* biovar *ovis*

in *Musca domestica* feces for up to 4 h and in saliva for up to 3 h post infection, but the bacteria survived on the external organs of house flies for no longer than 10 min post exposure.[35] Several factors including the time of exposure, environmental conditions, competition with other bacteria in the fly digestive tract flora or the immune response of the house fly may affect the survival time of pathogenic bacteria.[186] Further research is needed to determine the potential mechanism of transmission of *C. pseudotuberculosis* and the survival of the bacterium within the fly.

Flies and other mechanical vectors may play an important role in the transmission of *C. pseudotuberculosis* among horses, as indicated by the recent emergence and spread of the disease in previously non-endemic areas. Although three main muscoid fly pests have been suspected as mechanical vectors of *C. pseudotuberculosis,* the house fly likely has the highest potential because of its tendency to readily move between locations and feed on numerous materials.[187] House flies can fly 8 km/h[188] and disperse 20 km or more in one day.[189] Our experiments indicate that *C. pseudotuberculosis* can survive in/on house flies for at least 24 h. Therefore, flies could potentially spread the disease over a wide geographic area, making the house fly a plausible mechanical vector for dissemination of *C. pseudotuberculosis* and transmission among horses. More research is needed to better define the role of *Musca domestica* in *C. pseudotuberculosis* transmission to emphasize the need for effective fly management as a means of limiting host-to-host *C. pseudotuberculosis* transmission.

Conclusions

The results obtained from this study will be essential in developing a house fly inoculation system for further investigating the role of *M. domestica* as a mechanical vector of *C. pseudotuberculosis* biovar *equi* in horses. Results show that house flies can become contaminated within 10 minutes and continue to harbor live bacteria up to 24 h following a 30 min exposure. Although not demonstrated experimentally, this is indicative of the possible duration of the mechanical transmission following a single exposure. Flies have been implicated as vectors of *C. pseudotuberculosis* biovar *equi* (Spier *et al*. 2002), however, this is the first experiment to demonstrate actual contamination of *M. domestica*. Transmission studies are needed to demonstrate a causative relationship between *M. domestica* inoculated with *C. pseudotuberculosis* biovar *equi* and abscess development in horses.

Chapter 4:

Experimental transmission of *Corynebacterium pseudotuberculosis* biovar *equi* in horses by house flies

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Abstract

Background: The route of *Corynebacterium pseudotuberculosis* infection in horses remains undetermined, but transmission by insects is suspected.

Objectives: To investigate house flies (*Musca domestica* L.) as vectors of *C. pseudotuberculosis* in horses.

Animals: Eight healthy, adult ponies.

Methods: Randomized, controlled, blinded prospective study. Ten wounds were created in the pectoral region where cages for flies were attached. Three ponies were directly inoculated with *C. pseudotuberculosis.* Four ponies were exposed for 24 hours to 20 *C. pseudotuberculosis*-inoculated flies. One negative control pony was exposed to non-inoculated flies. Ponies were examined daily for swelling, heat, pain, and drainage at the inoculation site. Blood was collected weekly for complete blood cell count and biochemical analysis, and twice weekly for synergistic hemolysis inhibition titers.

Results: Clinical signs of local infection and positive cultures were observed in 7/7 exposed ponies and were absent in the negative control. In exposed ponies, peak serologic titers (1:512 to 1:2048) were obtained between days 17 and 21. Seroconversion was not observed in the negative control. Neutrophil counts were higher in the positive and fly-exposed groups than in the negative control (P=.002 and P=.005) on day 3 post-inoculation. Serum amyloid A concentrations were higher in the positive control than in the negative control and fly-exposed ponies on days 3 (P<.0001) and day 7 (P=.0004 and P=.0001). No differences were detected for other biochemical indices.

Conclusions and clinical importance: House flies can serve as mechanical vectors of *C. pseudotuberculosis* and can transmit the bacterium to ponies.

Introduction

Corynebacterium pseudotuberculosis (Actinomycetales: Corynebacteriaceae) is a pleomorphic, facultatively intracellular, Gram-positive rod, with a worldwide distribution.[30] The two described biotypes of *C. pseudotuberculosis* are distinguished by genetic characteristics, including restriction fragment length polymorphisms and the ability of cultured organisms to reduce nitrate to nitrite.[2] The nitrate-positive biovar *equi* is commonly isolated from horses and cattle but the nitrate-negative biovar *ovis* is typically recovered from small ruminants and cattle.[3] In ruminants, *C. pseudotuberculosis* causes caseous lymphadenitis, abscesses, and occasionally mastitis.[35] In horses, *C. pseudotuberculosis* infection manifests itself in three clinical forms of disease.[4] The most common form, known as "pigeon fever" or "dryland distemper", causes external subcutaneous abscessation, especially in the pectoral region; a second form causes abscesses of internal organs including liver, lung, and kidneys; and a third form causes ulcerative lymphangitis in the limbs.[4] Natural infection in horses caused by the ovine biovar and vice-versa is not known to occur.[8] Human infection, although rare, is considered an occupational zoonosis.[3]

Very little is known about *C. pseudotuberculosis* biovar *equi* infection, except that it is unpredictable and contagious. Previously confined to the arid, western United States (California, Utah, Colorado and Texas) and Brazil, numbers of *C. pseudotuberculosis* cases have been recently increasing at alarming rates in regions where the disease was previously unknown or considered non-endemic (Wyoming, Kentucky, Tennessee, Oklahoma, Louisiana, Florida, and South Carolina).[8,9,191]

The routes of *C. pseudotuberculosis* transmission in horses remain undetermined.[3,14] Speculated routes include horse-to-horse contact, contact with pathogen-infested soil, and via insect vectors. *Corynebacterium pseudotuberculosis* is able to penetrate the skin through

abrasions. Transmission by insects is suspected because of the seasonal occurrence of clinical cases during fall and early winter in the western United States.[13] The highest annual incidences in horses have been documented during dry months of the year following winters with above average rainfall, which provides optimal breeding conditions for insects in the subsequent summer and fall.[4,11,13,59] Three fly species, including the horn fly (*Haematobia irritans* L.), the stable fly (*Stomoxys calcitrans* L.), and the house fly (*Musca domestica* L.) were reported as potential vectors following PCR detection of *C. pseudotuberculosis* biovar *equi* phospholipase D (PLD) exotoxin gene in field samples of fly homogenates.[60] In house flies inoculated with *C. pseudotuberculosis* biovar *ovis* from cattle, bacteria were isolated from the flies' intestine and feces for 1–4 h and from saliva for 1-3 h.[35,64]

In a previous study [a] a fly inoculation system developed with *C. pseudotuberculosis* was used to demonstrate that house flies can become contaminated within 10 minutes and continue to harbor live bacteria for up to 24 h following 30 minutes of exposure. This suggests that mechanical transmission following a single exposure is possible; however, to investigate the capability of house flies as vectors, experimental transmission studies with horses are needed. Using a challenge model in ponies, the overall goal of the present study was to evaluate the role of house flies as mechanical vectors of *C. pseudotuberculosis* in horses. We hypothesized that ponies directly inoculated with *C. pseudotuberculosis* would be confirmed to be infected by presence of clinical signs, bacterial isolation from external or internal abscesses, and seroconversion as indicated by reciprocal serum synergistic hemolysin inhibition (SHI) titers. The second hypothesis was that ponies inoculated with *C. pseudotuberculosis* using house flies as mechanical vectors would become infected, resulting in clinicopathological, bacteriological, and serological responses similar to those of the directly inoculated ponies.

Materials and Methods

Bacterial identification

A field strain of *C. pseudotuberculosis* biovar *equi* isolated from an abscess on a horse from Salinas, CA was cultivated aerobically on 5% bovine blood agar for 24 h at 37° C in 10% carbon dioxide and confirmed as *C. pseudotuberculosis* by cellular and colony morphology, and conventional biochemical testing. The bacterium was beta-hemolytic, catalase and nitrate positive, and fermented glucose without gas production.

Fly rearing

Naïve laboratory-reared house fly pupae (*n* = 500) from the USDA house fly colony[b] were placed in cages (24.5 x 24.5 x 24.5 cm) of nylon netting (1.50 x 1.50 mm mesh)[c] supplied with a mix of 2 ml of water, 5 g of powdered milk and 5 g of sugar in a small petri dish under laboratory conditions (temperature = 37°C, relative humidity = 30%). Adult flies emerged in 1 to 3 days and were maintained according to established protocols.[192] Flies were starved for 24 h before being used in experiments.

Bacterial inoculation of flies

Ninety naïve adult flies (2-4 days of age) were inoculated for 30 min while contained in inverted plastic cups (6 flies per cup; 1oz: 45 mm x 40 mm height) over 15 agar plates (1 cup per agar plate; 60 mm x 15 mm height) containing 5% bovine blood agar with *C. pseudotuberculosis* colonies moistened with sterile 10% dextrose solution for 30 minutes as previously described.[a] Following inoculation, each plastic cup/agar plate combination was inverted and placed in a freezer at -20°C for 3 min. The cold-immobilized flies dropped into the cups, were removed

using forceps and then transferred to cylindrical fly cages (9cm diameter x 1.5cm height, covered with nylon screen with 64 holes per cm^2). Twenty flies were placed inside each cage at the start of an experiment. Flies recovered mobility 5 minutes after the immobilization.

To confirm successful inoculation, 6 additional inoculated flies were homogenized in 100µl of phosphate buffered saline (PBS) and an aliquot was inoculated onto a 5% bovine blood agar plate for culture. To verify the absence of *C. pseudotuberculosis*.in the naïve flies, six flies were selected and homogenized as described above for bacterial culture.

Animals

This research was performed under approval of the Institutional Animal Care and Use Committee of Auburn University. Eight healthy, adult ponies owned by Auburn University were used in a randomized, controlled, blinded prospective study. Before the study began, each animal was subjected to a complete physical examination, thoracic and abdominal ultrasound examinations, and SHI titers, complete blood count (CBC), biochemical profile, and fibrinogen concentration were determined. Inclusion criteria included SHI titers ≤1:32.

Experimental design

The ponies were randomly assigned to fly-exposed (n = 4), positive control (n = 3), or negative control (n = 1) groups via random draw of numbers from a hat and were housed individually in isolation stalls for 5 weeks after inoculation. Following aseptic preparation and local anesthesia with 2% lidocaine of the left pectoral region of each pony, 10 wounds (each with a diameter of 6mm) were created over an area of approximately 63 cm^2 using a skin biopsy punch. Wounds were clustered so they would all fit underneath a fly cage. In fly-exposed ponies,

fly cages containing 20 *C. pseudotuberculosis*-inoculated house flies were sutured over the wounds using non-absorbable suture material. The nylon screen, which was in contact with the wounds, allowed flies to access and feed on the wounds, but prevented the flies from escaping (Figure 4.1). The flies and cages were removed after 24 h, and fly homogenates were tested for *C. pseudotuberculosis* by culture as described above.

The negative control pony was exposed to 20 non-inoculated flies contained in cages sutured over the wounds and attached to the pony as previously described. Positive control ponies were inoculated by swabbing the skin wounds with a solution containing 6 x 10^8 viable *C. pseudotuberculosis* bacteria suspended in 2 ml saline. An empty fly cage was also sutured over the wounds as described above. The fly cages were removed after 24h. The investigators handling the ponies (MB, AS, TP, AW, MC) were blinded as to which cage contained the non-inoculated flies, therefore they remained blinded to which ponies were the fly-exposed ponies and which was the negative control pony. A physical examination was performed daily on all ponies, and attitude, appetite, body temperature, heart rate, respiratory rate, mucous membrane color, and capillary refill time were recorded. Presence of heat, pain, swelling, and discharge over the inoculation site was recorded as present or absent. Heat was subjectively assessed by palpation of the skin surrounding the inoculation site. Pain was subjectively assessed by applying digital pressure around the inoculation site. Blood was collected on days 0, 3, 7, 14, 28, and 35 to analyze complete blood cell counts and serum concentrations of fibrinogen, albumin, globulin, iron, and serum amyloid A. Ultrasound examination of the inoculation site and thoracic and abdominal cavities was performed weekly, and findings were recorded. Swabs from the inoculation site were submitted for culture before exposure. Samples of wound discharge and aspirates of any detectable abscesses were collected and submitted daily for the first 3 days and

Figure 4.1A: Image of the pectoral region of one of the ponies after creation of the skin wounds to allow the non-bloodsucking flies to feed.

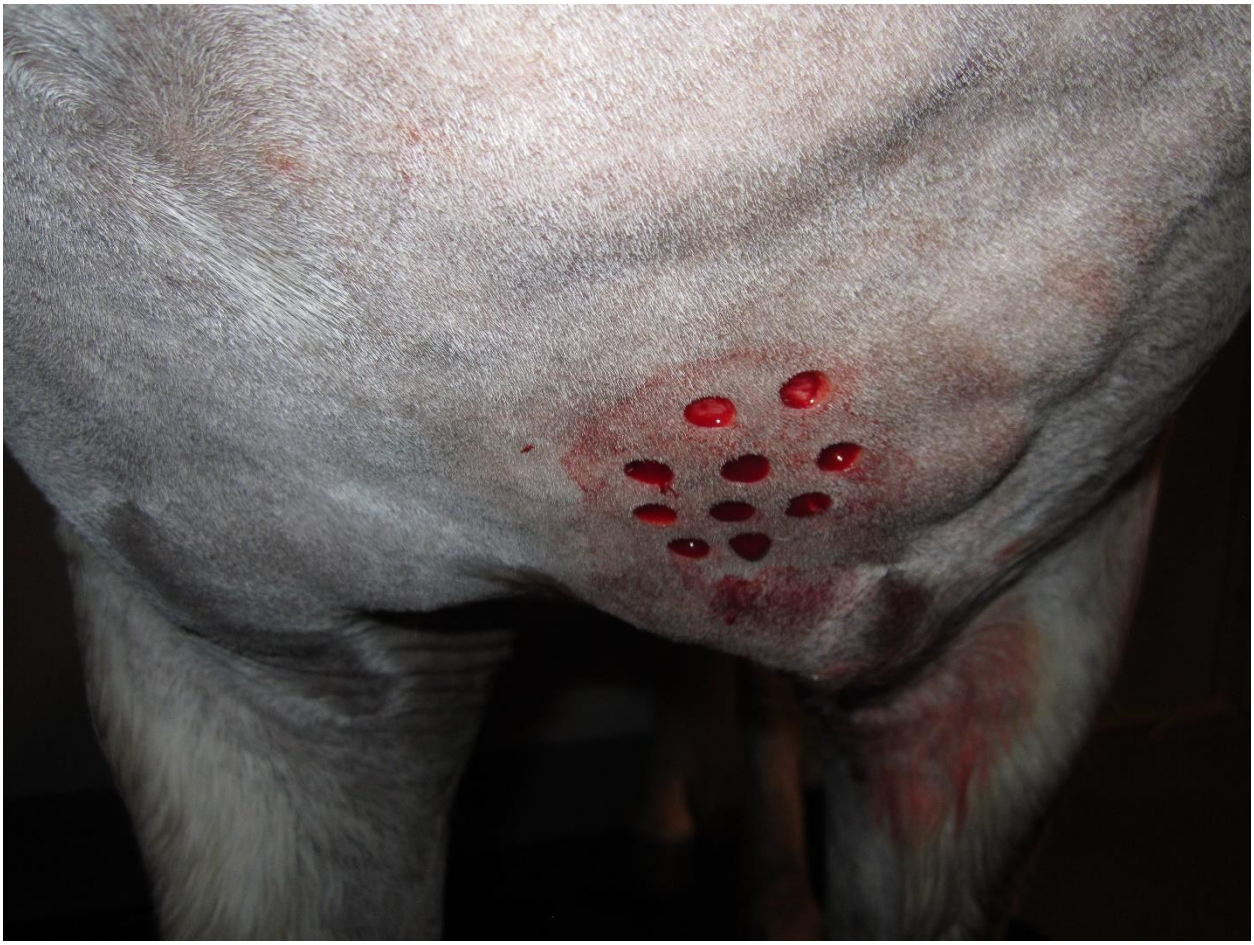
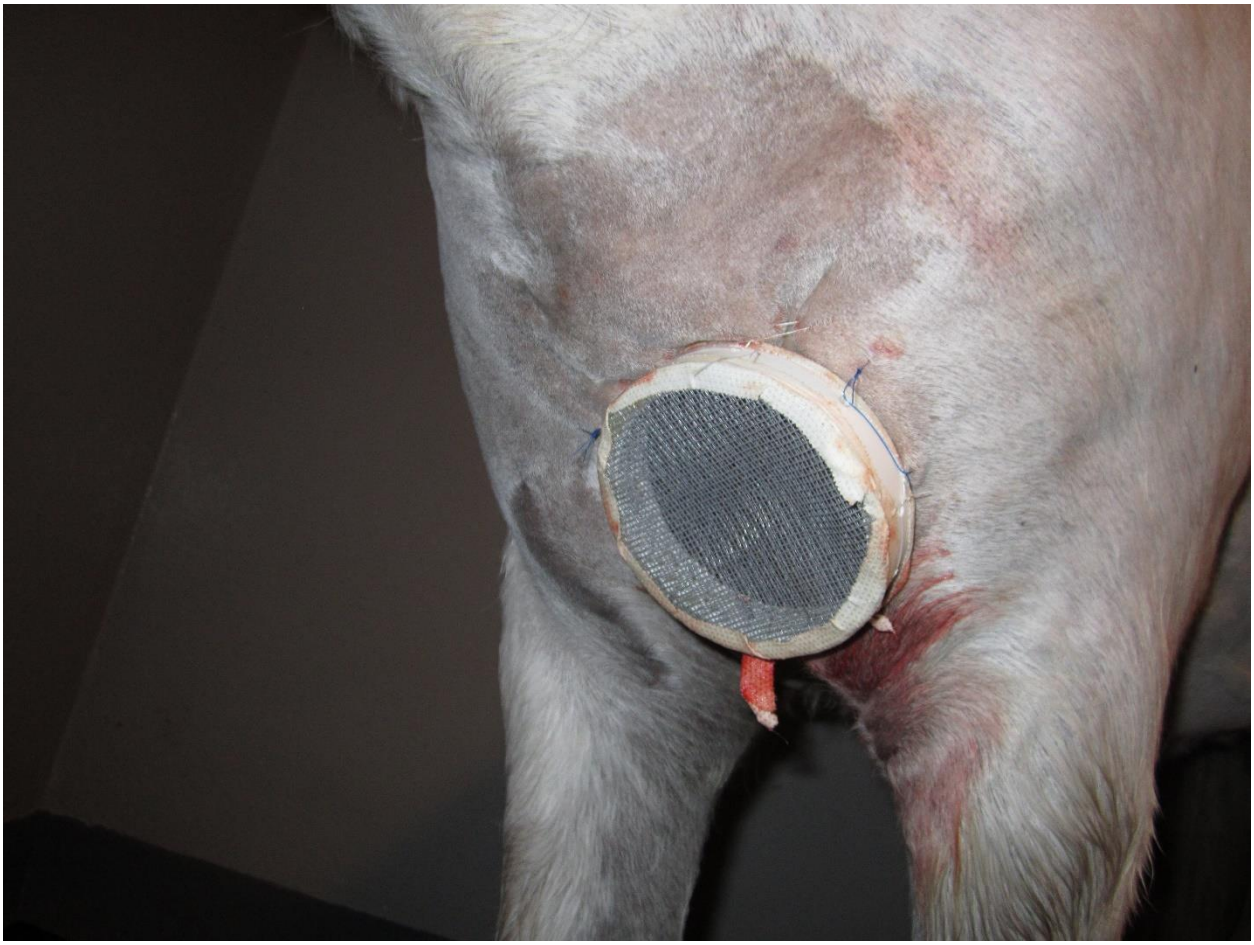


Figure 4.1B: Fly cage attached to the pectoral region.



then twice weekly until the discharge abated. Serum samples were collected at 0, 7, 10, 14, 18, 21, 24, 28, 31, 35 and 38 days after inoculation and frozen for batched *C. pseudotuberculosis* SHI antibody titers.[d]

Five weeks after inoculation, all ponies were humanely euthanized. A full necropsy examination was performed. Samples from any abscess and aspirates from the inoculation site were submitted for microbiological culture. Histopathology was performed on tissue samples from the inoculation sites.

Statistical analysis:

Normality of the data was assessed using analysis of residuals (Plots = StudentPanel' option in the procedure call[e]). All responses, except SHI titers, fulfilled the normality assumption and were therefore analyzed using a generalized linear mixed model (PROC GLIMMIX[f]), and factors included in the model were pony, treatment, time, and interactions of those variables. Based on the limitations of study design and availability of isolation facilities, the negative control was not replicated. The analysis thus assumed homogeneity of variances among treatment groups, but there was no variance estimate for the negative control. The residual variances for the control treatment were therefore estimated from the other treatments; which was possible since the ponies were assigned at random to the treatment. A parametric analysis with PROC GLIMMIX was chosen based on 2 parameters: the higher power of parametric tests (versus non-parametric), and the ability of this procedure to analyze non-Gaussian distributed data and the correlated data typical of repeated measures experiments.[e] Differences in rectal temperature, heart rate, respiratory rate, duration in days of clinical signs (heat, pain, swelling, and discharge), and clinicopathological parameters over time within groups

or between groups at each evaluation time were analyzed. Random effects due to repeated measures were accounted for in the GLIMMIX procedure using R-side modeling. The SLICEDIFF option was utilized to calculate pairwise comparisons among treatment means for each time point at a standard Type I error rate of $\alpha = 0.05$.

Because serum SHI titers did not fulfill the normality assumption, they were subjected to $\log^2$ transformation and were analyzed with SAS® PROC NLMIXED using a 3-variable logistic growth model. Non-detectable samples were set to $\log^2 = 1$. Pairwise contrasts for each time point were constructed from the regression equation. Synergistic hemolysis inhibition data is shown as median and range, which were calculated in Microsoft Excel 2010.[g] Because the fly-exposed group consisted of 4 ponies, the median was calculated as the average of the second and third highest values. Values of $P < .05$ were considered significant.

Results

Clinical signs

Inoculation-site reactions, including moderate swelling, increased heat, sensitivity and purulent discharge was observed in all 7 *C. pseudotuberculosis*-exposed ponies (Figure 4.2). A mild reaction was seen in the negative control which lasted two days and did not develop purulent discharge. The mean duration of local heat, pain, swelling, and discharge in the exposed groups was 13, 12, 16 and 12 days, respectively. Differences between exposed groups and the negative control were detected for the duration of pain ($P = .0139$), heat ($P = .0085$), swelling ($P = .0045$) and discharge ($P = .00001$) (Figure 4.3). There were no statistically significant differences between the fly exposed and positive control groups.

Signs of local inflammation resolved without treatment in all ponies. Pyrexia (rectal temperature > 38.6ºC) was not observed in any pony at any time point. However, mean rectal temperatures in the positive control and fly exposed groups were significantly higher ($P = .034$ and $P = .048$, respectively) on day 1 post-inoculation than that in the negative control. Mean rectal temperature was significantly higher in the positive control group compared with the negative control pony on day 19 ($P = .049$). No statistically significant differences were detected for respiratory and heart rate.

Figure 4.2A: Images from ponies 36 h after exposure to *C. pseudotuberculosis* biovar *equi*.

A: Positive control pony, lateral view.

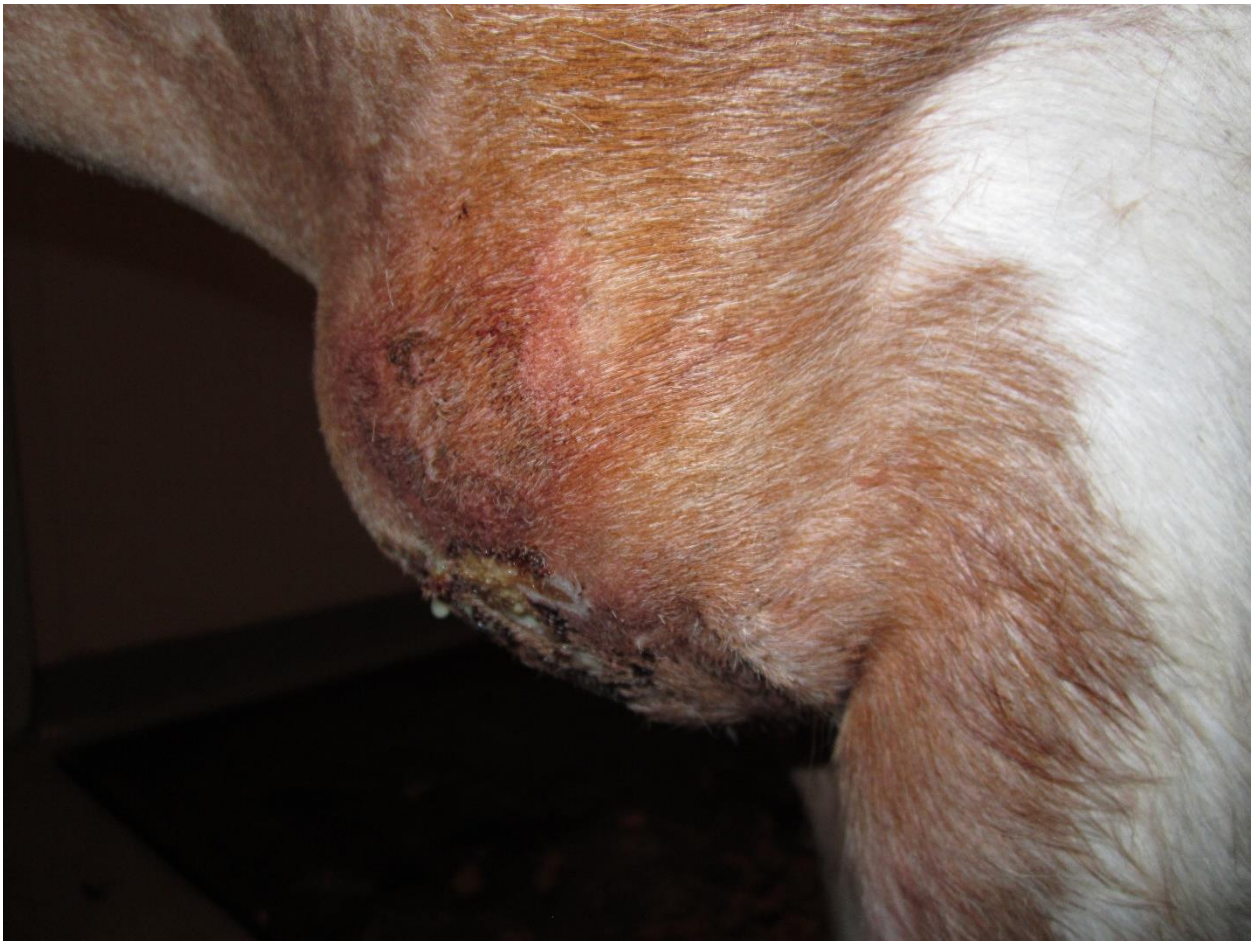


Figure 4.2B: Images from ponies 36 h after exposure to *C. pseudotuberculosis* biovar *equi*.

B: Positive control pony, frontal view.

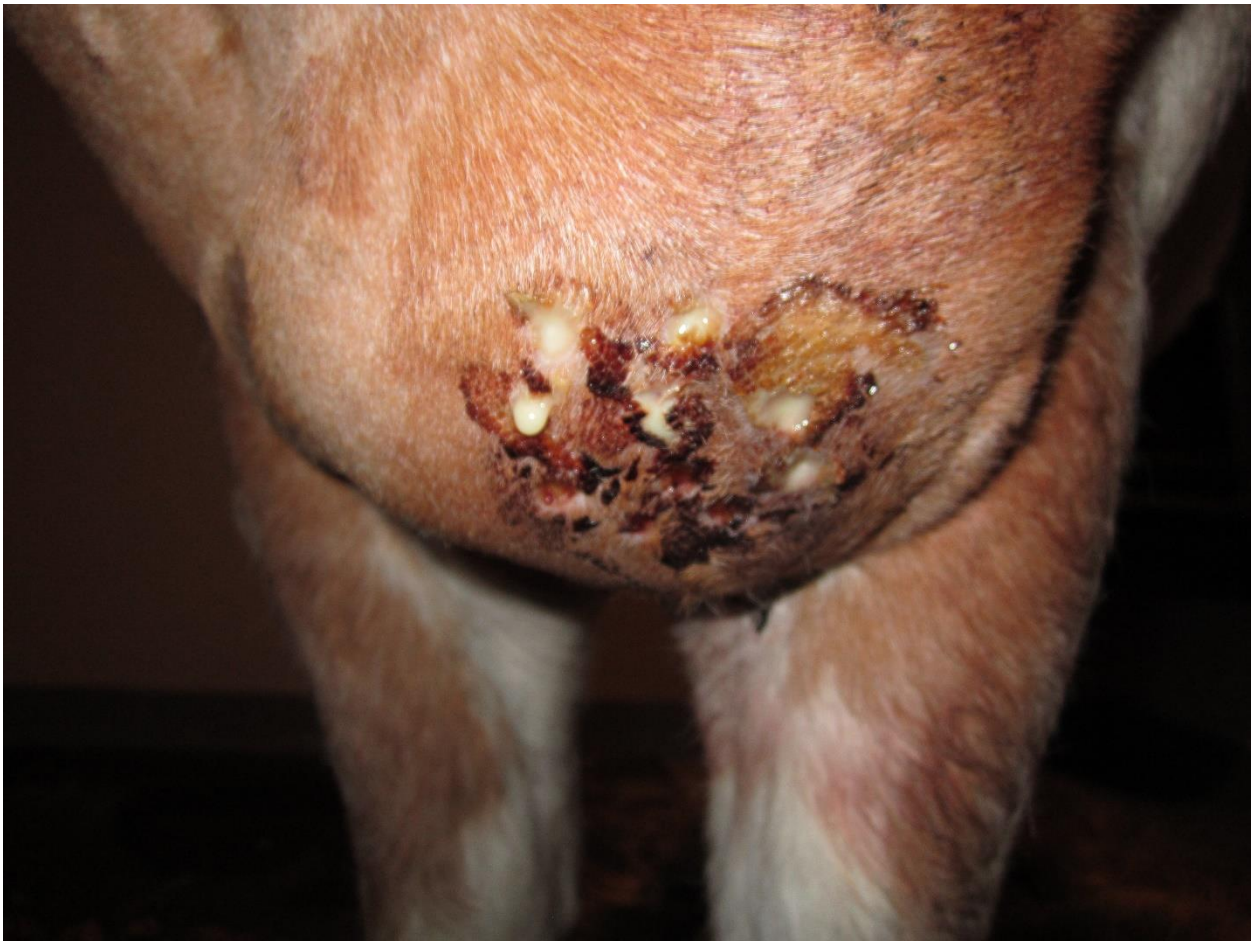


Figure 4.2C: Images from ponies 36 h after exposure to *C. pseudotuberculosis* biovar *equi*

C: Fly-exposed pony, lateral view.

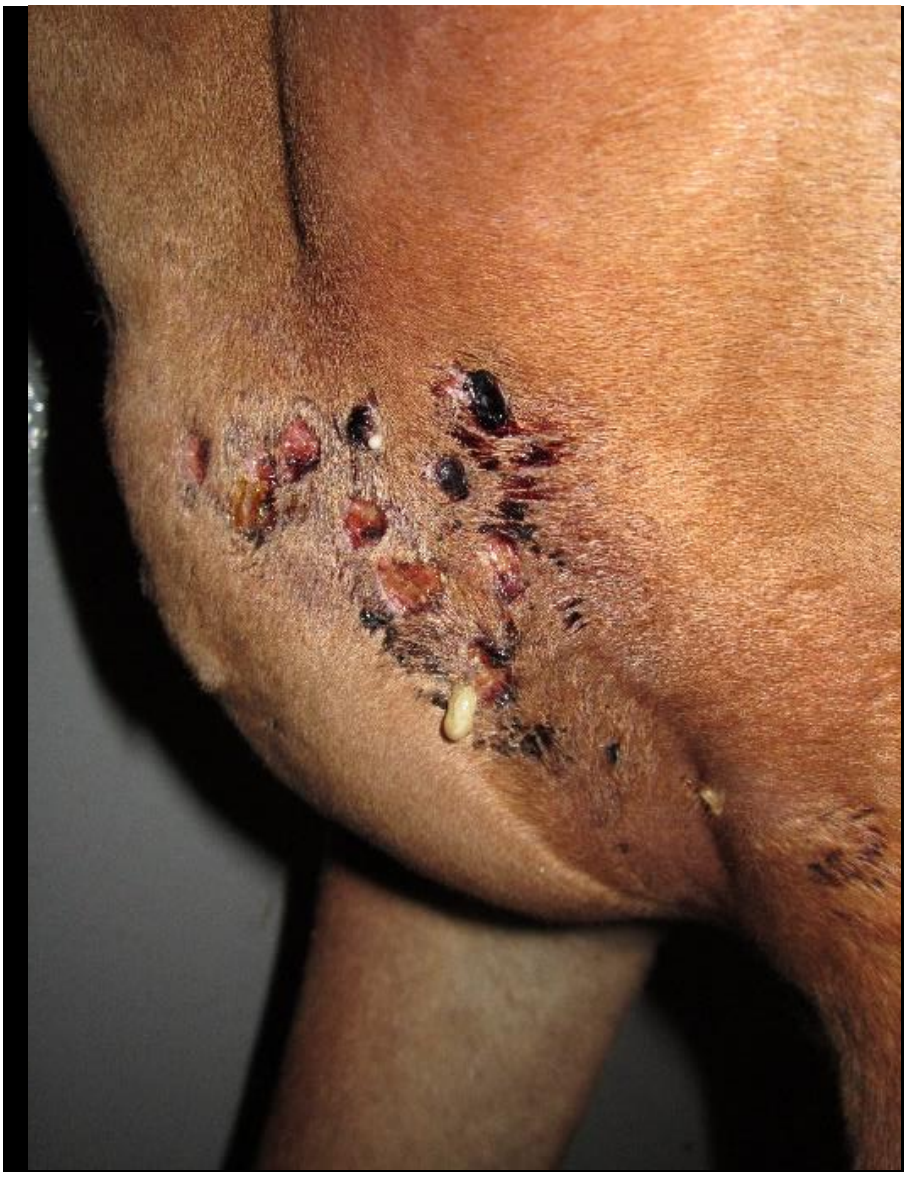


Figure 4.2D: Images from ponies 36 h after exposure to *C. pseudotuberculosis* biovar *equi*.

D: Fly-exposed pony, frontal view.

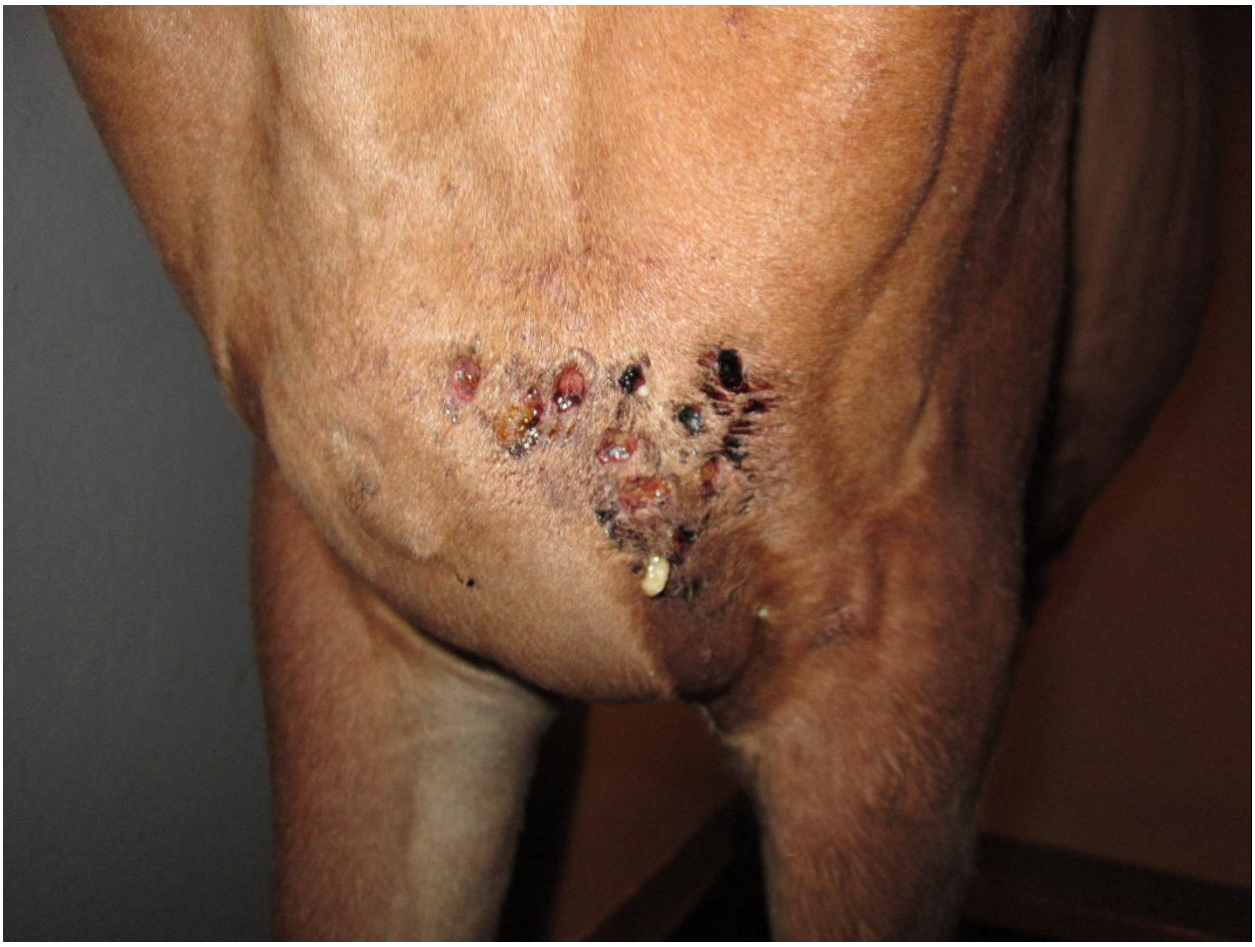


Figure 4.2E: Images from ponies 36 h after exposure to *C. pseudotuberculosis* biovar *equi*.

E: Negative control pony, lateral view.

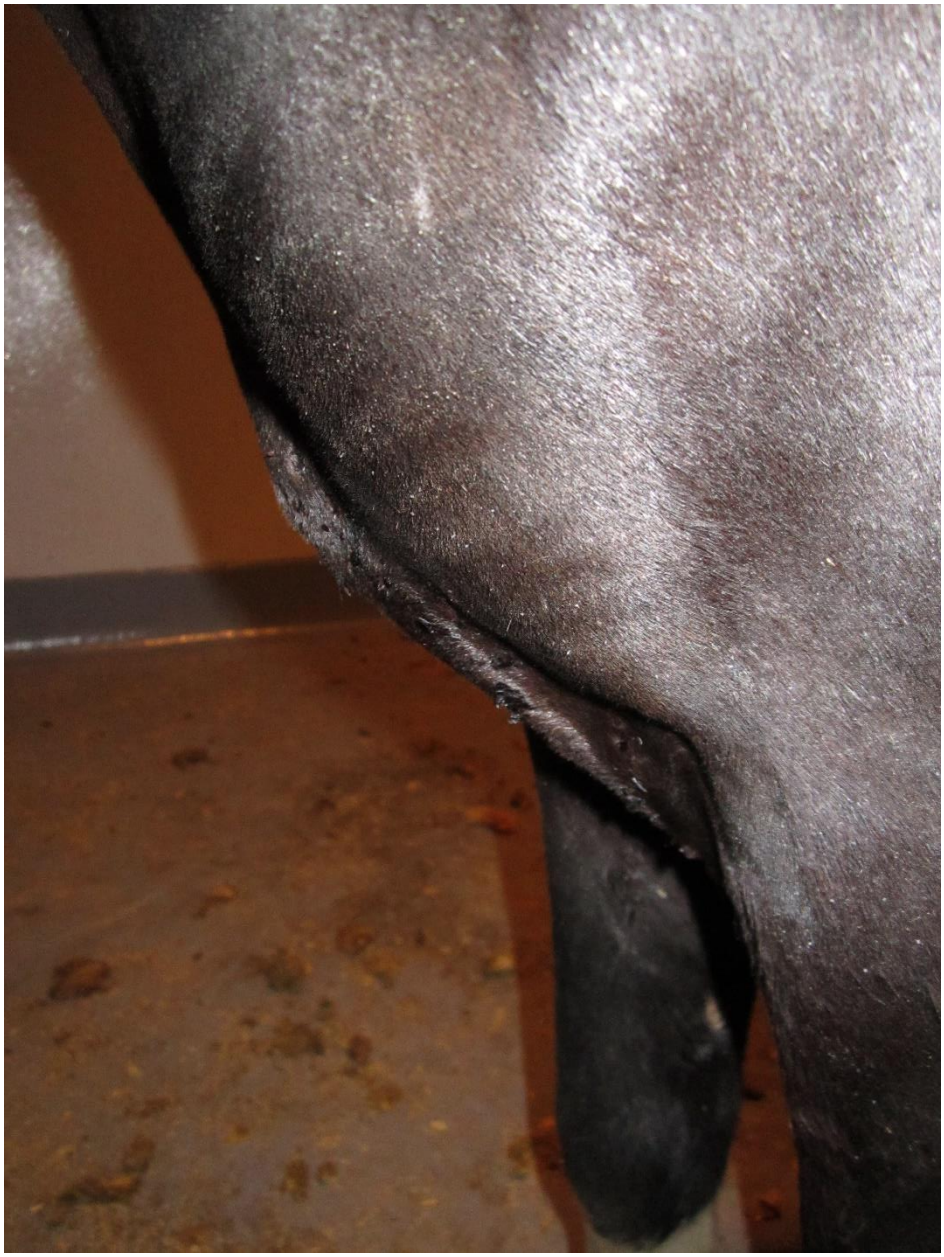


Figure 4.2F: Images from ponies 36 h after exposure to *C. pseudotuberculosis* biovar *equi*.

F: Negative control pony, frontal view.

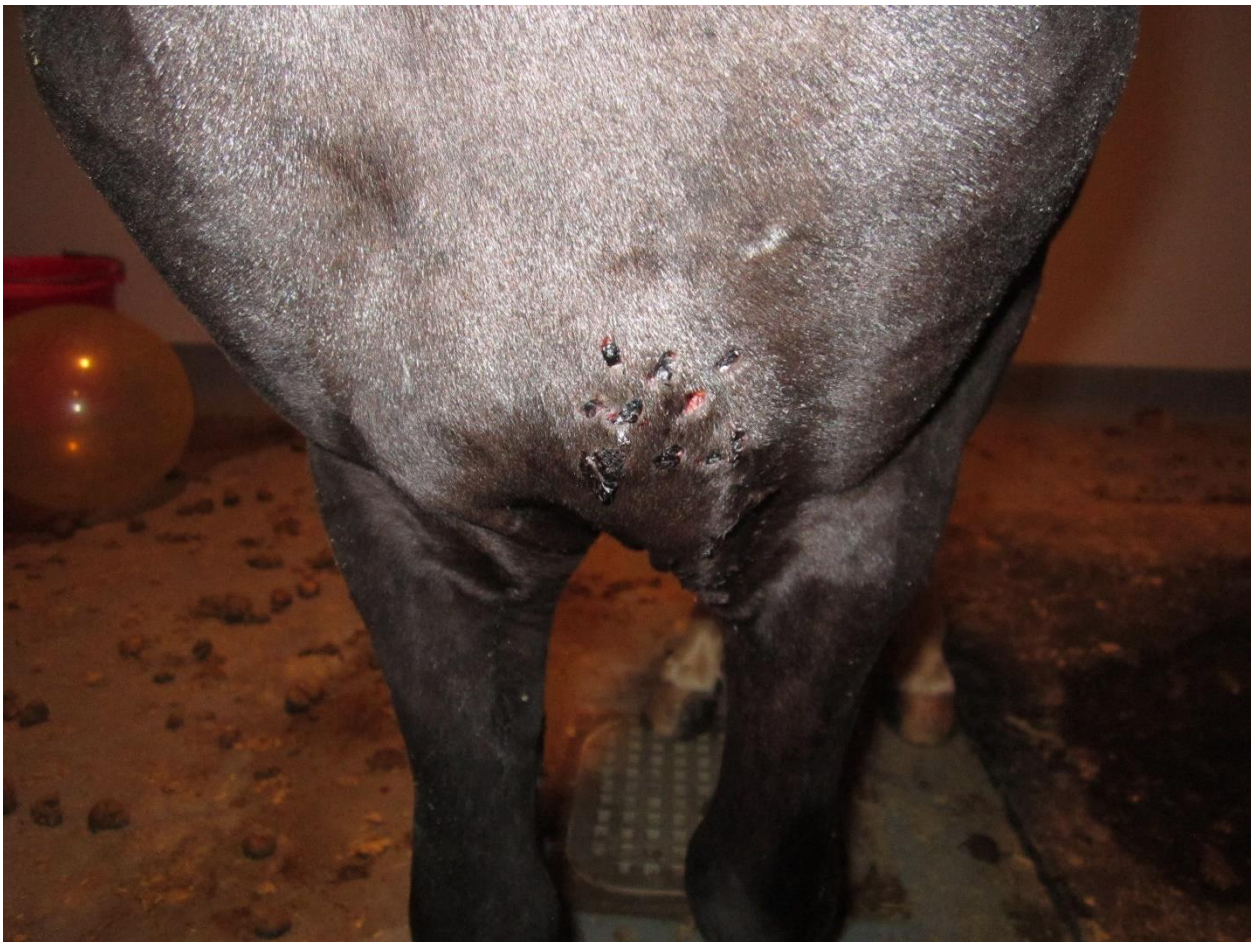
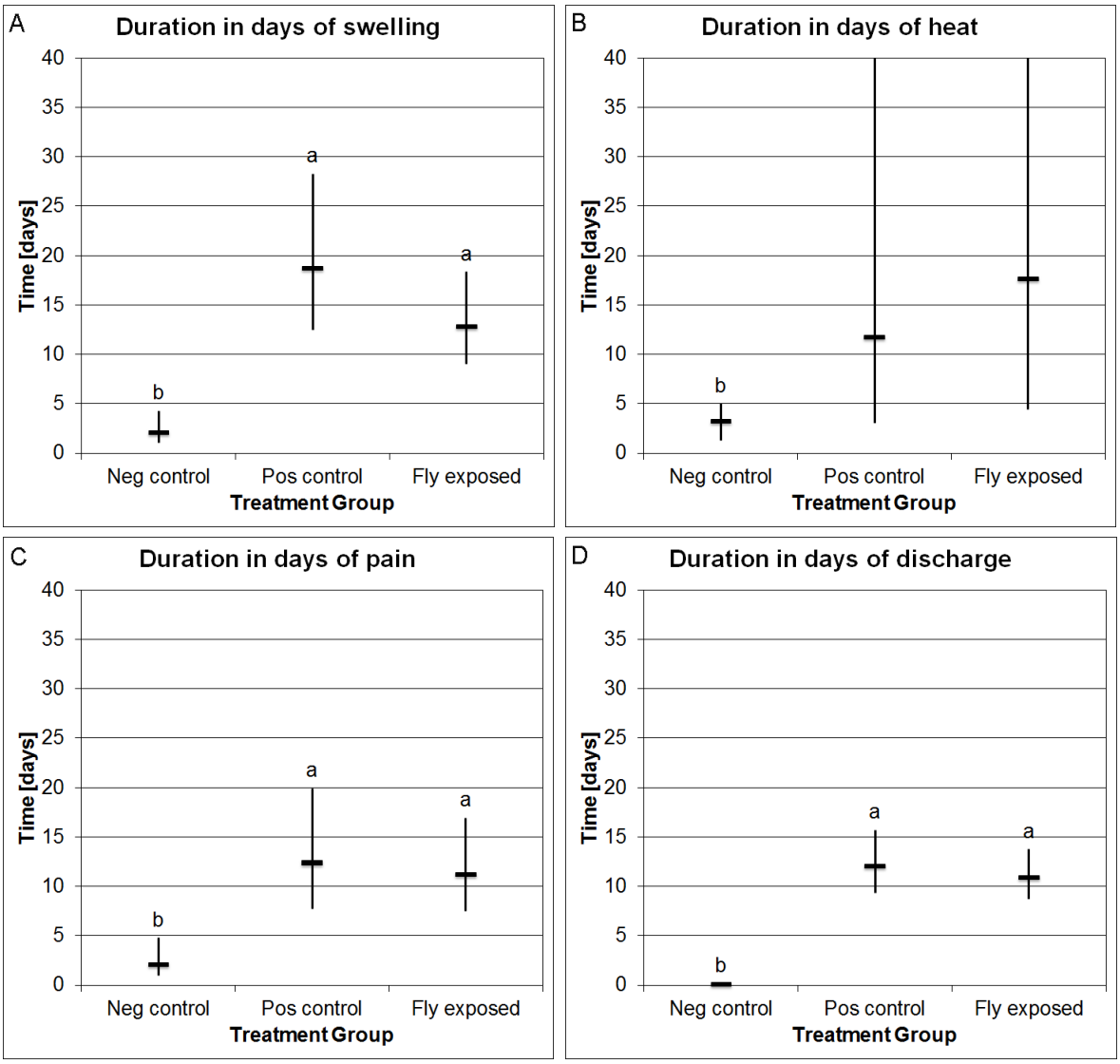


Figure 4.3: Mean duration (in days) and 95% confidence interval of local swelling (A), heat (B), pain (C) and discharge (D) from the inoculation site for the three groups of ponies after exposure to *C. pseudotuberculosis* biovar *equi*. Groups with different letter superscripts are significantly different ($P < .05$).



Clinical pathology

Neutrophil counts were significantly higher in the positive and fly exposed group than in the negative control (P = .002 and P =.005, respectively) on day 3 post-inoculation. Serum amyloid A concentrations were significantly higher in the positive control group than in the negative control on days 3 (P < .0001) and 7 (P = .0004), and significantly higher in the positive control group than in the fly exposed group on days 3 (P < .0001) and 7 (P = .0001; Figure 3.4). No statistically significant differences were detected among groups for the rest of the clinical pathology indices (Figure 3.4).

Ultrasound examination

Although superficial abscesses were detected externally in all exposed ponies for the time that purulent discharge was present, no deep abscesses were detected by ultrasound examination. Marked swelling associated with hypoechogenic areas within the subcutaneous tissue was observed compatible with cellulitis. Thoracic and abdominal ultrasound examinations remained unremarkable in all ponies during the experiment.

Post-mortem examination

Several epidermal crusts with minimal superficial dermal thickening were observed in all ponies at the inoculation site. No gross abnormalities of the regional lymph nodes were observed. In one fly-exposed pony, a 2 cm pustular epidermal-dermal wound was observed at the inoculation site, and the axillary left lymph node was enlarged. Heavy growth of *C. pseudotuberculosis* was obtained from the purulent material but the lymph node was negative.

Multiple hepatic white parenchymal and/or capsular foci (2-4 mm in diameter) were observed in all ponies, which were considered incidental findings and not related to *C. pseudotuberculosis* exposure. Cultures of these samples were negative. The remainder of the necropsy was unremarkable. Histopathology of the inoculation site tissue revealed minimal to mild intra-epidermal edema, infiltration of leukocytes and orthokeratosis. Mononuclear cell infiltrate was present perivascularly in the superficial and deep dermis in addition to focal scarring characterized by fibrovascular tissue formation and replacement of adnexal structures.

Bacteriological cultures from ponies

All samples collected from the pectoral region before inoculation yielded negative growth for *C. pseudotuberculosis*. Twenty-four hours after removal of the fly cage, heavy growth of *C. pseudotuberculosis* biovar *equi* was obtained from the samples taken at the inoculation site from all the 7 exposed ponies; a sample from the negative control produced no growth. Positive cultures were obtained from the purulent discharges collected from the 7 exposed ponies, daily for the first 3 days and then twice weekly until the discharge abated (between 7 and 13 days post exposure). Daily negative cultures for the first 3 days post-inoculation were obtained in the negative control from the inoculation site. After this time, absence of discharge and presence of normal crusting as a result of normal wound healing precluded further collection of culture samples from the negative control. Almost all samples collected during postmortem examinations, including inoculation site, regional lymph nodes, lung, spleen and liver, were culture-negative. The exception was the fly-exposed pony with the small superficial closed abscess at the pectoral region, which yielded heavy growth of *C. pseudotuberculosis* biovar *equi*.

Figure 4.4A: Mean ± SD SAA concentration compared over time after exposure to *C. pseudotuberculosis* biovar *equi* for the three groups of ponies. Days with asterisk (*) are significantly different between positive control and negative control ($P < .05$).

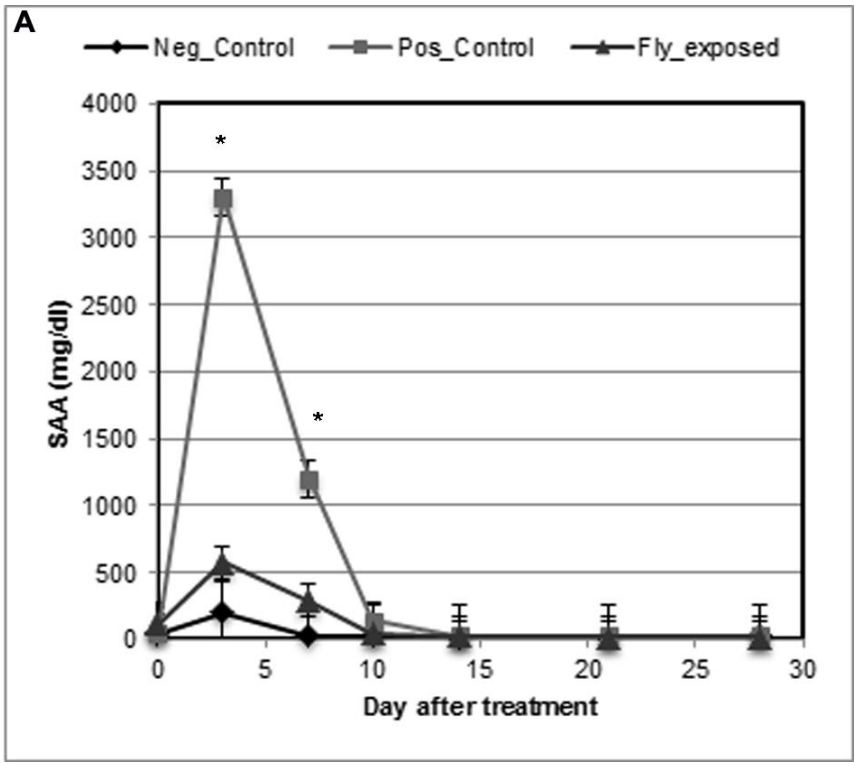


Figure 4.4B: Mean ± SD neutrophil count concentrations compared over time after exposure to *C. pseudotuberculosis* biovar *equi* for the three groups of ponies. Days with asterisk (*) are significantly different between positive control and negative control ($P < .05$).

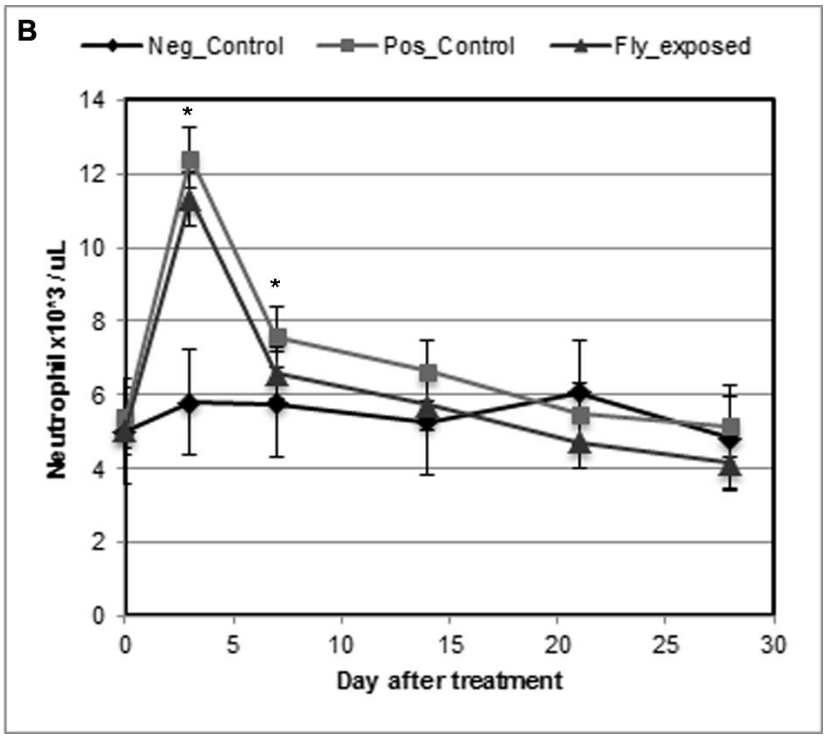


Figure 4.4C: Mean ± SD Iron concentrations compared over time after exposure to *C. pseudotuberculosis* biovar *equi* for the three groups of ponies.

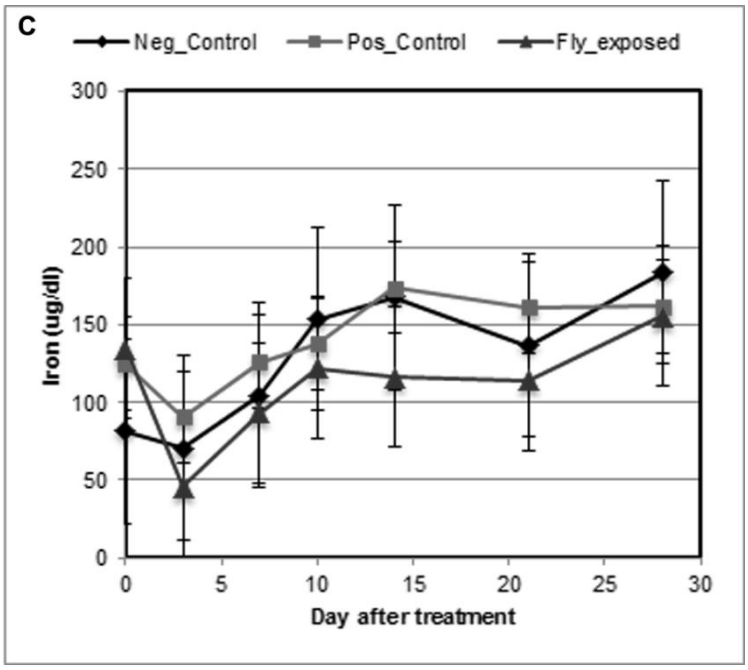
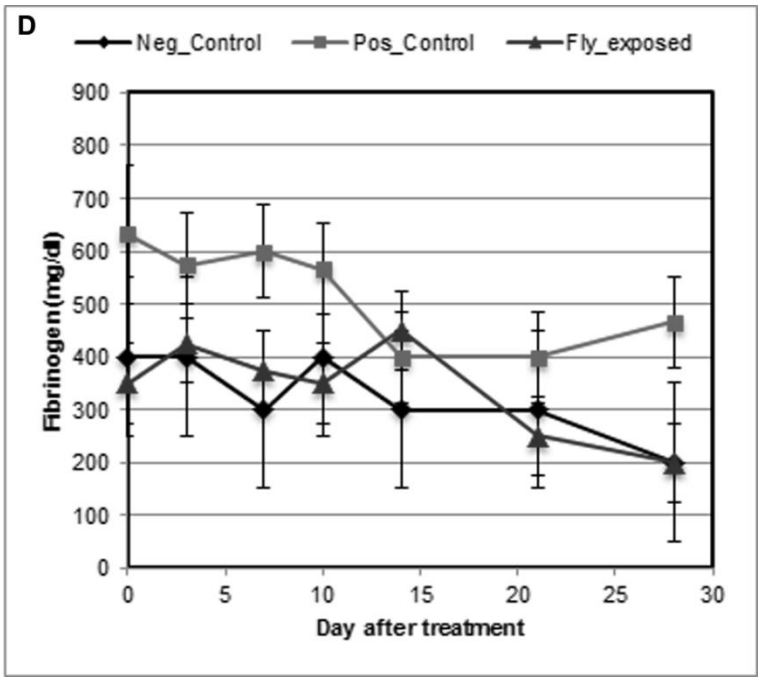


Figure 4.4D. Mean ± SD Fibrinogen concentrations compared over time after exposure to *C. pseudotuberculosis* biovar *equi* for the three groups of ponies.



Serology

Initial serologic titers prior to exposure were all ≤1:32. The peak serologic titer was obtained between 17 and 21 days after infection in the directly inoculated and fly exposed groups respectively (Figure 3.5). The maximum peak titer in the directly inoculated group was 1:2048 and in the contaminated fly-exposed group was 1:512 (Table 3.1). No seroconversion was observed in the negative control. A statistical difference was detected between negative and positive control and between fly-exposed group and negative control for the increase in titers, respectively ($P = .05$).

Fly survival and culture

Subjective evaluation 12h after the fly-cage placement identified that the majority of flies were alive in all the cages. All flies were dead by the time of cage removal 24h after exposure to the ponies, with the exception of one cage, where 2 out of 20 flies were still alive. Cultures from homogenates of non-inoculated flies were negative. Culture from flies after experimental exposure to the bacterium and after removal of the fly cages 24h post-inoculation of the ponies yielded heavy *C. pseudotuberculosis* growth in all samples.

Figure 4.5: Median and range of synergistic hemolysis inhibition (SHI) titers compared over time for the three groups of ponies exposed to *C pseudotuberculosis* biovar *equi*.

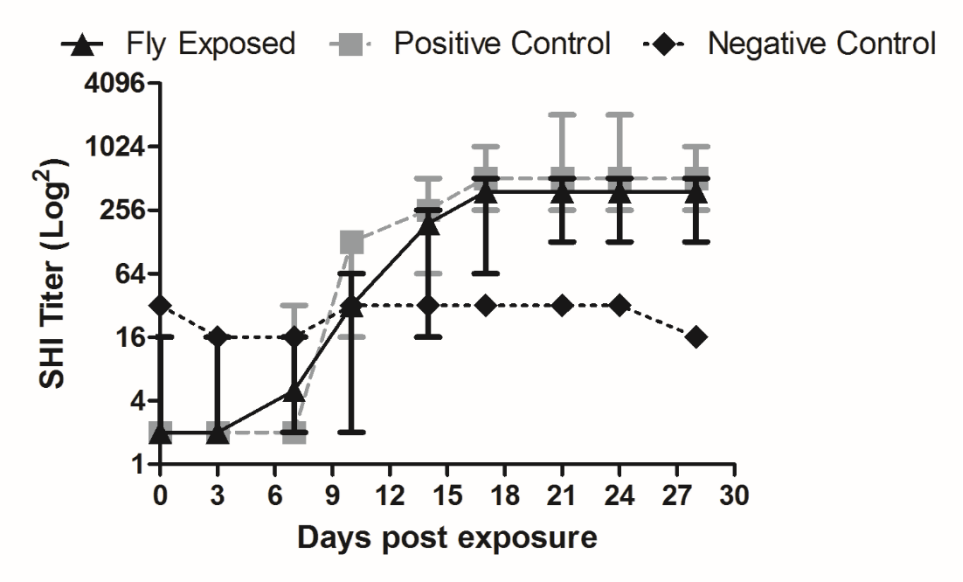


Table 4.1: Summary of the SHI titer of each individual pony over time.

Day post exposure	**Positive control group**			**Fly-exposed group**				**Negative control group**
	Pony 1	Pony 2	Pony 3	Pony 5	Pony 6	Pony 7	Pony 8	Pony 4
0	0	0	16	0	0	0	16	32
3	0	0	16	0	0	0	16	16
7	0	0	32	0	8	0	16	16
10	16	128	128	32	64	0	32	32
14	64	512	256	256	256	16	128	32
17	256	1024	512	512	512	64	256	32
21	256	2048	512	512	512	128	256	32
24	256	2048	512	512	512	128	256	32
28	256	1024	512	512	512	128	256	16

Discussion

This study has shown that *C. pseudotuberculosis* can be transmitted to superficial skin wounds in horses by direct inoculation and by using a mechanical insect vector. Clinical signs were consistent with those expected from *C. pseudotuberculosis* infection with all exposed ponies developing pectoral edema, heat, pain and swelling and superficial abscess development with positive *C. pseudotuberculosis* culture. Laboratory findings were also consistent with *C. pseudotuberculosis* infection with increased SHI titers, neutrophil counts, and SAA concentrations.

Clinical signs of infection in ponies in the present study were characterized by marked local swelling, mild heat and pain, and moderate purulent discharge from the inoculation site for 7-22 days. A more marked response was observed in the positive control group, which is likely due to the greater bacterial dose used in this group by the direct swabbing of the inoculation sites with a highly concentrated bacterial solution. Variability introduced during inoculation of flies and differences in feeding behavior of individual flies while maintained on the wounds precluded determination of the precise inoculation dose in the fly-exposed group. Although the bacterial dose transmitted by flies was suspected to be lower than in positive controls, the dose was sufficient for disease transmission to all fly-exposed ponies. Using this transmission model, the classic pectoral swelling (pigeon breast) was induced, but only superficial abscesses were detected by ultrasound examination. The marked local swelling that resulted from exposure to *C. pseudotuberculosis* was a result of severe cellulitis and not large deep tissue abscesses. The severe inflammation was likely induced by bacterial exotoxins, including phospholipase D.[4] Phospholipase D damages the vascular endothelium increasing its permeability and resulting in edema.[4,99] The results of this study explain why field veterinarians reportadly have difficulty

identifying abscesses by ultrasound examination in the swollen brisket area of acutely infected horses. The percentage of field cases that develop the initial pectoral cellulitis and go on to develop the classic large draining abscesses is unknown. A proportion of field cases also may have complete resolution of initial cellulitis without abscess development. Such lesions may occur, but are not usually differentiated from trauma. Although an exact clinical representation of naturally occurring disease was not induced, we propose that the model developed in this study will be particularly important for evaluation of early stages of *C. pseudotuberculosis* infection.

Compared to the frequency of the development of external abscesses, internal infection is thought to represent < 10% of *C. pseudotuberculosis* biovar *equi* infections in the field.[4] No cases of internal abscesses were detected in the 7 exposed ponies in our study. An approximately 2 month delay in the detection of internal infections was observed in a large retrospective study.[9] The present study was terminated after 5 weeks, but as clinicopathological signs of external inflammation had resolved, the risk of internal abscesses was considered to be low. Other factors including varying virulence among bacterial strains and varying immunological responses of the animal likely play a role in development of internal abscessation.[119,120]

Neutrophil counts and SAA concentrations were significantly increased in both exposed groups. The same findings were observed in experimental infection of sheep with caseous lymphadenitis, where serum concentrations of SAA and haptoglobin peaked 7 days post-infection, and then declined to concentrations equal to those of control sheep by day 18.[114] Based on results of this study, neutrophil count and SAA are the best indicators of acute infection compared to fibrinogen, iron, or globulin concentrations. This observation may be useful for

veterinarians monitoring for transmission of *C. pseudotuberculosis* within a herd or in a horse with suspected exposure.

Obtaining seronegative animals for the study was difficult even if ponies came from non-endemic regions, and one pony included in the study had a titer of 1:32. Rapid increases in serological titers was observed in both exposed groups, with an increase of titers between 5 to 10 fold in the positive control and 4 to 9 fold in the fly-exposed groups in 17-22 days. A higher serologic response was observed in the positive control group which could be attributed to the higher bacterial inoculation dose. Results of serologic testing in this study indicated that the clinical signs observed in the exposed ponies were attributable to the bacteria. The variability of the titers can be related to individual response or to the infectious dose. A one-fold decrease in titer was detected in one pony after 29 days, but further continuation of the study would have been necessary to determine the length of time the ponies would remain seropositive after exposure. The SHI titers in the positive control (median 1:512; maximum 1:2048) and inoculation group (median 1:384; maximum 1:512) were comparable to titers obtained from horses with naturally occurring external abscesses which can be between <1:8 and 1:10,240.[4] Horses with naturally occurring internal abscesses can have titers between 1:256 and 1:10,240,[4,124] but no internal abscesses were found at necropsy. Higher titers and more severe disease have been reported in many naturally occurring cases than that induced in our experimental model.[4,9]

Natural transmission might occur via insect vectors, and biting flies such as the horn fly (*Haematobia irritans* L.), and the stable fly (*Stomoxys calcitrans* L.), and the non-biting house fly (*Musca domestica* L.), have been implicated. [60] Because house flies are not bloodsucking flies, but may transmit the bacterium through excoriated skin via mouthparts and tarsal

exoskeleton, superficial skin wounds were created on the pectoral region of each pony. This method provided consistent and reproducible experimental induction of infection in both exposed groups; this was advantageous, particularly considering the small sample size. The fact that the majority of flies were dead 24h after exposure was attributed to the lack of water consumption since the blood and discharge from the wounds had dried at that time. Further investigation is warranted to determine if house flies act as mechanical vectors or amplify the bacteria, and to determine if bacteria are carried on the external or in the internal organs. House flies were chosen for this study, but further experiments could be performed using biting flies and the established fly cage system without creation of skin wounds. Similar studies were performed using the same fly cage system using biting horn flies to evaluate transmission of Bovine Viral Diarrhea virus in cattle.[193]

Limitations of this study included the small sample size and the presence of only one pony in the negative control group. However, it was decided to terminate the study after successful inoculation of 7/7 ponies because proof of concept of induction of disease by house flies had been achieved. Additional animals would have made statistical analysis easier, and additional inflammatory parameter data may have been useful but sacrifice of additional animals, was not considered warranted. The differences between the positive and fly-inoculated groups compared to the negative control were significant but quantitative aspects of the results could be influenced by biological variability of the individual horses and differences in the inoculum between these two groups.

The methods utilized in this study did not allow for quantification of the bacterial load transmitted by flies, which may have been different from that used for inoculation of positive controls. All flies were exposed to bacterial culture plates in which *C. pseudotuberculosis* had

been grown to confluence, but the bacterial load carried by individual flies could have been affected by time of feeding on the culture, survival of bacteria on individual flies, location of bacteria on flies, and bacterial growth on the flies. Furthermore, the amount of bacteria transmitted to each horse could be affected by the mechanism of transmission, time spent on the wounds, and survival of flies. Therefore, future research should evaluate the mode of transmission by flies (e.g. contact with mouth parts or other external parts, by feces, saliva, or regurgitate) and number of bacteria that individual flies can transmit.

In conclusion, we have demonstrated that house flies can serve as mechanical vectors of *C. pseudotuberculosis* biovar *equi* in horses demonstrated by development of clinical signs of local infection with positive culture and increase in neutrophil count, serum amyloid A concentration and SHI titers. To our knowledge, this is the first time that *C. pseudotuberculosis* biovar *equi* has been experimentally transmitted to horses. The disease induced experimentally resolved spontaneously without the formation of large abscesses that required draining, which is different from most reported cases of naturally occurring disease. This model can potentially be used in future epidemiological investigations or to test the effectiveness of vaccination or other treatment.

Footnotes:

[a] Barba M, Xing PH, Hathcock T, Hogsette JA, Wooldridge AA, Passler T, Chamorro MF, Cattley R and Stewart AJ. Experimental inoculation of house flies, *Musca domestica* L., with *Corynebacterium pseudotuberculosis* biovar *equi*. Abstract presented at the Entomological Society of America annual meeting November 2013, Austin, TX

[b] USDA/ARS/CMAVE, Gainesville, FL

[c] MegaView Science Co, Ltd. Taichung 40762,Taiwan

[d] California Animal Health and Food Safety (CAHFS-Davis), University of California, Davis, W. Health Sciences Drive, Davis, CA 95616, USA

[e] http://support.sas.com/documentation/cdl/en/statug/63347/ HTML/default/viewer.htm#glimmix_toc.htm

[f] SAS 9.2, SAS Institute Inc., Cary, NC

[g] Microsoft Excel 2010, Redmond, WA

Chapter 5:

Seroprevalence and risk factors associated with *Corynebacterium pseudotuberculosis* detectable antibodies in equids in Alabama

Journal article submitted to Journal of Equine Veterinary Science.

Abstract

A cross-sectional serological survey was carried out to screen the equine population of the non-endemic state of Alabama for presence of detectable antibody titers against *Corynebacterium pseudotuberculosis*. A second objective was to determine the association of detectable titers with risk factors such as exposure to small ruminants or previous travel to endemic states.

A total of 342 equine serum samples from 40 Alabama counties were analyzed using the synergistic hemolysis inhibition test (SHI). The prevalence of detectable antibody titers (≥ 1:8) was 52.5% (95% CI, 47-57.9%). Titers ≥1:128 were detected in 2.63% (95% CI, 1.2-4.9%) and titers ≥1:512 were detected in 0.3% (95% CI, 0-1.6%) of the sampled population. In the final generalized linear model, age ($P < 0.001$), breed ($P = 0.023$), and contact with cattle ($P = 0.05$) were associated with increasing SHI titers. Contact with goats was associated in the initial but not in the final analysis ($P = 0.19$). Previous travel was not associated with increasing SHI titer ($P = 0.97$). The results demonstrated a high prevalence of detectable titers in a non-endemic population and therefore call into question the validity of low positive SHI titers for the diagnosis of pigeon fever. Further evaluation of SHI cut-off titers and accuracy is warranted to reduce the risk of a false positive diagnosis. Possible false positive results may be caused by cross-reaction with antibodies against phospholipases from *C. pseudotuberculosis* biovar *ovis* or soil-borne *Corynebacterium* spp. and warrant further investigation.

Introduction

Corynebacterium pseudotuberculosis is a pleomorphic, facultatively intracellular, Gram-positive rod, with a worldwide distribution.[30] There are two described biotypes of *C. pseudotuberculosis;* the nitrate-positive biovar *equi* is commonly isolated from horses and cattle and the nitrate-negative biovar *ovis* is typically recovered from small ruminants and cattle.[2,67] In horses, *C. pseudotuberculosis* infection manifests itself in three clinical forms of disease: external subcutaneous abscessation, known as "pigeon fever"; abscesses of internal organs, and ulcerative lymphangitis in the limbs.[4] Natural infection in horses caused by the ovine biovar and vice-versa is not known to occur.[8]

Pigeon fever is considered endemic in the western United States (California, Utah, Colorado, and Texas) and Brazil, causing important economic losses.[4,8,9] A recent increase in the number of cases in horses, and disease spread to regions not previously considered endemic such as Kentucky, Louisiana, Florida, and North and South Carolina has been reported; therefore, the disease should no longer be considered to be restricted to the western United States.[8,9,11] Despite the increasing spread, control measures to limit dissemination from endemic to non-endemic areas have not been established.

A presumptive diagnosis of pigeon fever is based on presence of characteristic clinical signs in horses in endemic regions; however, only culture of *C. pseudotuberculosis* from purulent material provides a definitive diagnosis.[5] Serology using a synergistic hemolysis inhibition (SHI) test is used to help diagnose this disease, especially the internal form; however, its accuracy and ideal cut-off values for diagnosis have not been rigorously evaluated. Historically, cut-off values of ≥ 1:128 and ≥1:512 have been used in endemic areas to indicate exposure and internal *C. pseudotuberculosis* infection, respectively.[4,9] To our knowledge, the

SHI test has not been rigorously evaluated in a disease-free population. We detected low positive titers (1:8 to 1:256) in several ill horses in a non-endemic area (Alabama) in which *C. pseudotuberculosis* infection was initially a differential diagnosis, but was subsequently definitively ruled out. Similar low positive results were then detected in normal horses with no history of disease, which raised concerns about the likelihood of false positive results (data not shown). Although the SHI test is commercially available and has been used for nearly 40 years,[123] the sensitivity and specificity of the test has not been rigorously evaluated. Positive serological results, even if titers are considered relatively low, in horses with equivocal clinical signs may lead to a false positive diagnosis with instigation of unnecessary treatment and possible quarantine of animals or premises.

The objective of this study was to screen the equine population of the non-endemic state of Alabama [11] for presence of detectable SHI titers against *C. pseudotuberculosis*. A second objective was to determine the risk factors associated with detectable titers. We hypothesized that previous travel to endemic areas and exposure to small ruminants would be associated with an increased SHI titer.

Materials and Methods

Study design

A population-based cross-sectional survey was designed to estimate the seroprevalence of detectable antibody titers against *C. pseudotuberculosis* in the equid population of Alabama. Based on the number of horses in the last USDA published census (87,111 horses; USDA 2009), an estimated prevalence of elevated titers of 3% (from unpublished preliminary screening data), an assumed sensitivity and specificity of the SHI test of 85% and 85%,[123] respectively, and a desired confidence level of 95%; the aim was to sample 443 horses. An online epidemiological calculator[a] was used for sample size determination. Equine veterinarians practicing in Alabama were contacted by letter and/or by phone and requested to participate in the study. Horse owners were contacted by their local veterinarian, and a maximum of 5 horses were sampled per farm. For horses that had a titer ≥1:128, the owners were contacted to obtain a second serum sample through their local veterinarian to repeat SHI testing. This research was performed under approval of the Clinical Research Review Committee and Institutional Animal Care and Use Committee of Auburn University.

Questionnaire development

A one-page questionnaire requesting information on geographical information (location of the horse), horse signalment, use of the horse, travel history, animal health history, contact with other animals, and insect control was developed. Animal health history questions included previous medical conditions, history of external abscess, internal abscess, or ulcerative lymphangitis, and history of or vaccination against strangles. The questionnaire was completed by the owner at the time of blood collection.

Sample collection and serological examination

Jugular blood samples were collected into plastic tubes between August 2013 and May 2015 by the owners' local veterinarians. Blood was centrifuged and serum was separated and stored at -20°C until transportation within 3 months of collection to the laboratory for analysis. Serum samples were analyzed using the SHI test for detection of *C. pseudotuberculosis* IgG antibody titers at the California Animal Health and Food Safety Laboratory[b] using a 2-fold serum dilution series of 10 steps, starting with 1:8.

Statistical analysis

Standard statistical software[c] was used to determine descriptive statistics of SHI titers grouped by each variable and to represent the geographical mapping of the data. The prevalence of antibodies against *C. pseudotuberculosis* was estimated with the exact binomial confidence intervals of 95% using an online epidemiological calculator.[d]

The association of SHI titers and risk factors was analyzed using a generalized linear model approach in two steps. First, the interaction between SHI titer and the covariates was tested. Second, the non-significant interactions ($P < 0.2$) were dropped and a revised model was fitted to the data in the final test of the SHI effect. A lognormal distribution function,[195] as implemented in SAS PROC GLIMMIX[e], was used to analyze SHI titers. First, a complete model was evaluated with age as a covariate and class variables; breed, sex and use of the horse as variables related to horses; insect control (yes, no, unknown), previous medical conditions (yes, no, unknown), previous identification of abscess (yes, no, unknown), history of strangles (yes, no, unknown), and strangles vaccination (yes, no, unknown) as variables related to

medical condition; and cattle, goats, sheep, wildlife, cats, and dogs as variables related to contact. The compound variable State Score reflected the information that an animal originated in a state other than Alabama or traveled to another state. States were classified as history of endemic pigeon fever (CA, NV, NM, TX), sporadic pigeon fever (KY, WY, UT, CO, AZ, OR, ID, FL, LA, MI, NC, SC, SD, VT, WI, TN, OK) or no history of pigeon fever (WA, MT, NH, ND, NE, NJ, CT, KS, MN, IA, MO, AR, IL, IN, OH, MS, AL, GA, ME, MA, NY, PA, MD, DE, VA, WV, RI) based on previous reports.[4,8,11] The State Score was created in the following manner; score 0: unknown travel history; score 1: horse did not travel/move to/from another state; score 2: horse traveled/moved to/from another state with no history of pigeon fever; score 3: horse traveled/moved to/from another state with history of sporadic pigeon fever; score 4: horse traveled/moved to/from another state with history of endemic pigeon fever. This variable was then used as a classification variable in the full main effects model. Data from the remaining variables (localization of abscess, history of lymphangitis, years lived in Alabama, and history of caseous lymphadenitis if history of contact with small ruminants) were not amenable to analysis due to high dispersion of the answers and non-completed answers in the questionnaire. Forty-two out of 342 questionnaires had incomplete answers regarding the contact with cattle and goats, therefore these observations (horses) had to be removed to create a dataset that was amenable to analysis in the final model. An additional observation had to be removed because no age information was reported in one questionnaire.

The reduced model, containing only the significant effects of the initial analysis was used to calculate least squares means and associated 95% CI(μ), which were then back-transformed to the original data scale. Since this study can be characterized as an initial survey type experiment no adjustment was made for multiple comparison, as recommended by Milliken et al. (2009).[196]

Results

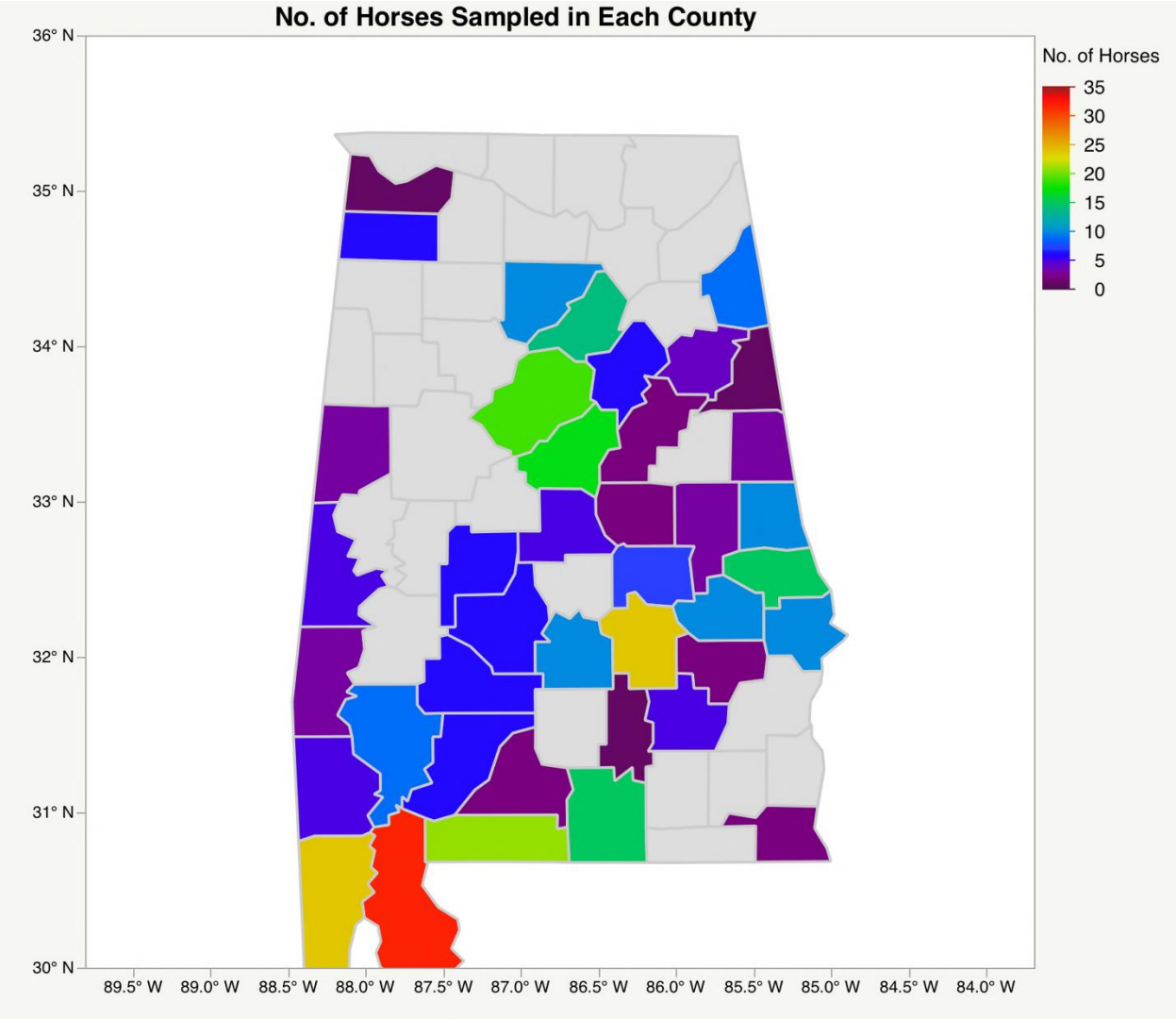
Survey response

Of the 61 invited veterinarians, 43 (70.5%) agreed to participate in the study and 17 (27.8%) returned serum samples. From August 2013 to May 2015, 345 serum samples from horses in 40 Alabama counties were obtained, but 3 horses did not have a complete questionnaire and were not included in the study. The maximum number of horses sampled per county was 32 and the minimum was 1. The geographical distribution of the sampled horses and number of horses sampled in each of the counties that constitute the state of Alabama are shown in Fig 5.1, with the counties with highest number of horses sampled being Baldwin (32), Mobile (24), Montgomery (24), and Escambia (21).

Characteristics of horse population

A total of 342 horses were included in the study. There were 187 geldings, 133 mares, and 16 stallions; sex was not reported for 6 horses. Breeds or breed types were identified as Quarter Horse, Paint or Appaloosa (182), Tennessee Walking horse, Saddlebred, and other gaited horses (73), Thoroughbred (21), Warmblood (11), Arabian or Arabian cross (10), Draft or Draft cross (3), Mustang (3), donkey or mule (3), pony or miniature horse (17), and mixed breed (19). Sixty-one horses were 1 to 5 years old, 251 were 6 to 20 years old, and 29 were > 20 years old; age was not recorded for 1 horse. One hundred and seventy-two horses were used for pleasure, 56 for western, 19 for jumping, 8 for dressage, and 87 for other disciplines.

Figure 5.1: Map of Alabama counties showing the geographical distribution of the number of horses sampled for SHI testing. The counties with highest number of horses sampled are Baldwin (n=32), Mobile (n=24), Montgomery (n=24), and Escambia (n=21).



Seroprevalence of *C. pseudotuberculosis* in horses in Alabama:

One hundred and sixty-two horses (47.4%) had a negative SHI titer of < 1:8. Of the 180 horses (52.6%) that had a SHI titer ≥ 1:8; 59 horses (17.2%) had a titer of 1:8, 72 horses (21.0%) had a titer of 1:16, 31 horses (9.1%) had a titer of 1:32, 9 horses (2.6%) had a titer of 1:64, 5 horses (1.5%) had a titer of 1:128, 3 horses (0.9%) had a titer of 1:256, and 1 horse (0.3%) had a titer of 1:512 (Table 5.1).

The seroprevalence of horses with a detectable titer (titer ≥ 1:8) was 52.5% (95% CI, 47-57.9%). The seroprevalence of horses with a titer ≥ 1:128 or ≥ 1:512 was 2.63% (95% CI, 1.2-4.9%) and 0.3% (95% CI, 0-1.6%), respectively.

The median anti-*C. pseudotuberculosis* toxin antibody titer determined by serum SHI testing for all horses in the study was 1:8. The maximum and median titer by county are represented in figure 5.2 and 5.3 respectively. The counties with higher maximum titers were Lowndes (1:512), Chambers (1:256), and Montgomery (1:256); followed by Talladega (1:128), Wilcox (1:128), and Covington (1:128). The county with highest median titer was Talladega (1:96), followed by Conecuh (1:32) and Wilcox (1:32).

Risk factors associated with SHI titer

Sex was not associated with increasing SHI titers ($P = 0.78$) in the initial model and was not included in the final model. An association with increasing SHI titers was found for age ($P = 0.002$), which remained significant in the final model ($P < 0.001$) (Figure 5.4). Breed was associated with increasing SHI titers in the initial ($P = 0.016$) and final model ($P = 0.023$) (Table 5.2).

Figure 5.2: Map of Alabama counties showing the geographical distribution of the maximum SHI titer obtained for each county. The counties with the highest maximum titers were Lowndes (1:512), Chambers (1:256), and Montgomery (1:256); followed by Talladega (1:128), Wilcox (1:128), and Covington (1:128).

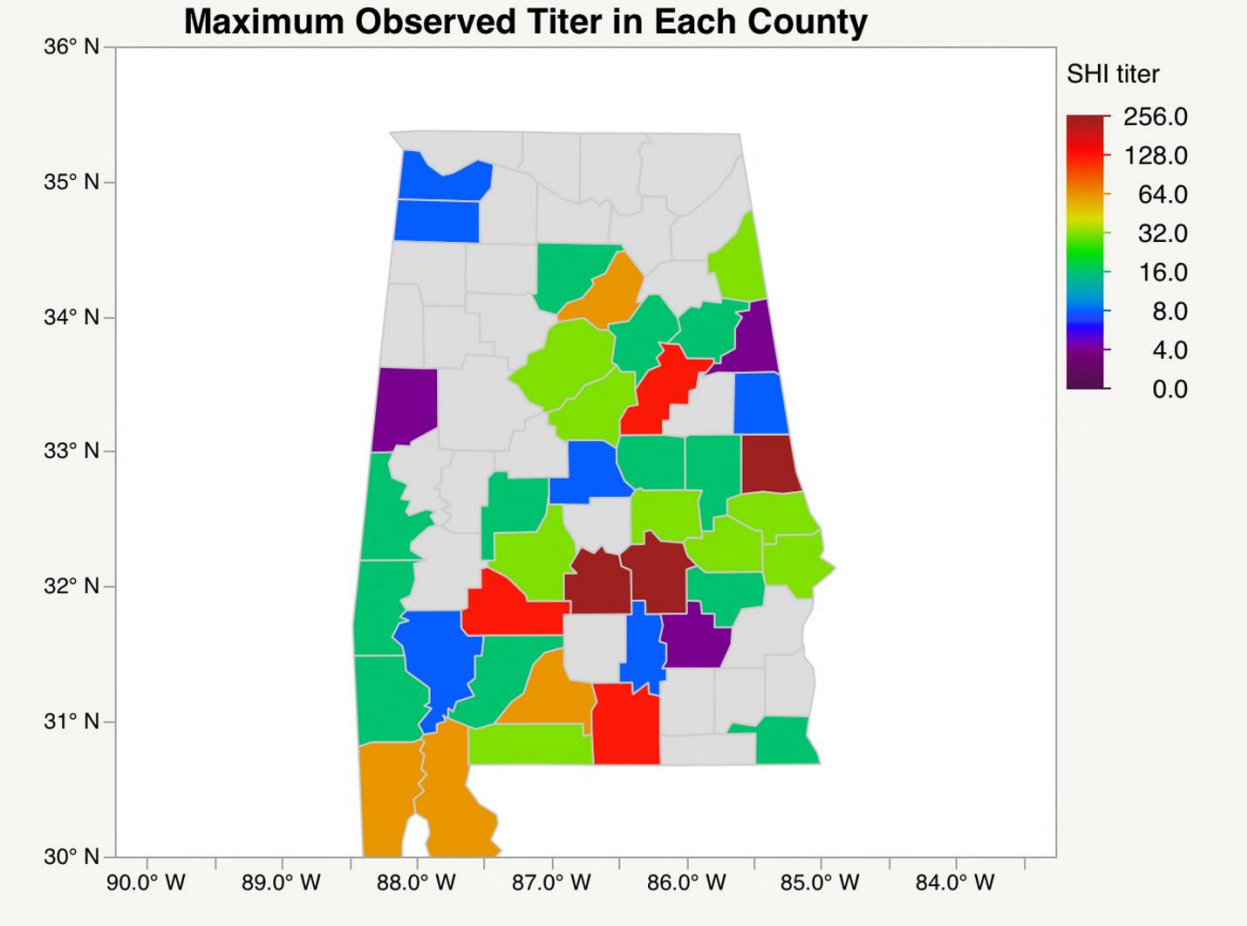


Figure 5.3: Map of Alabama counties showing the median SHI titer obtained for each county. The county with highest median titer was Talladega (1:96), followed by Conecuh (1:34) and Wilcox (1:32).

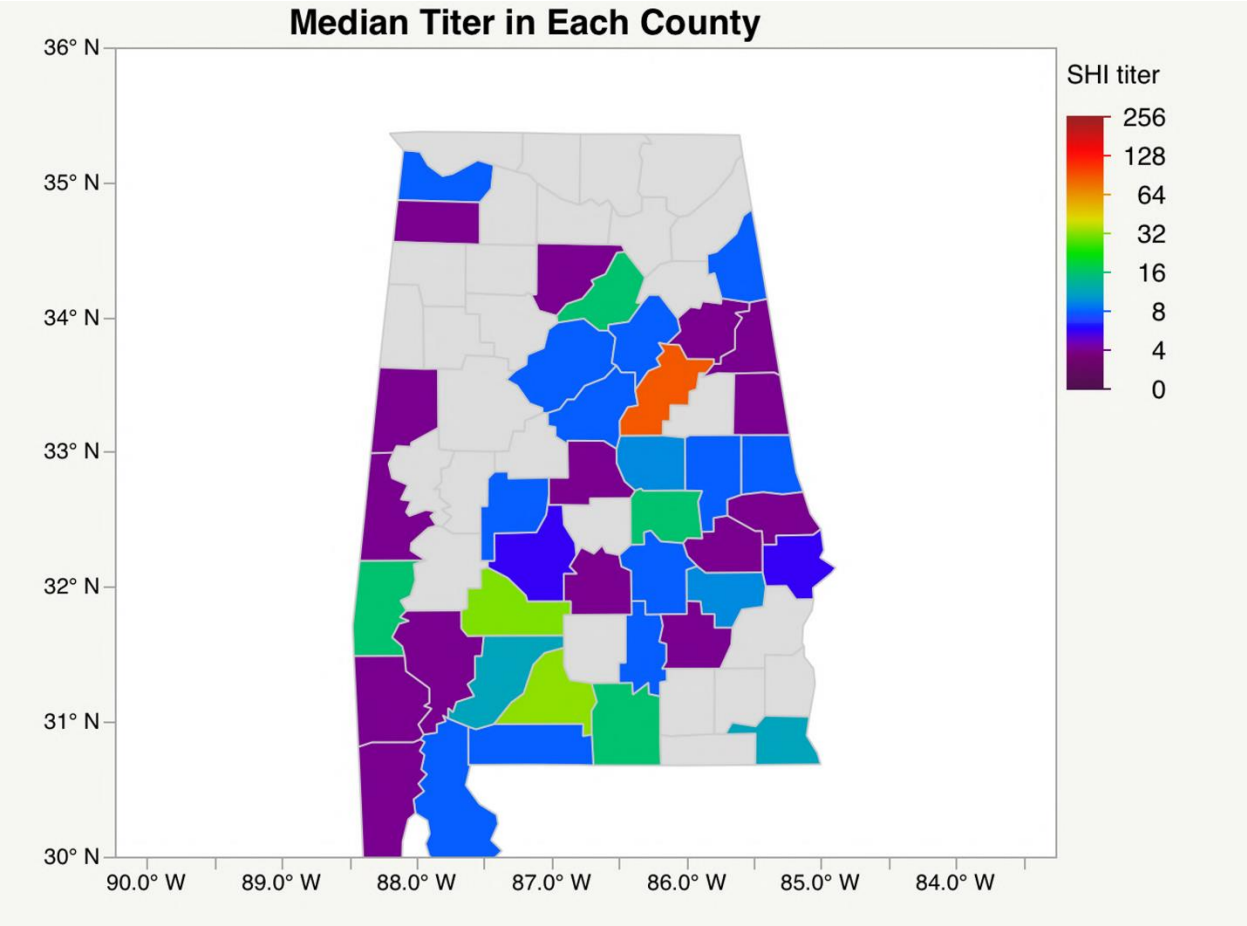


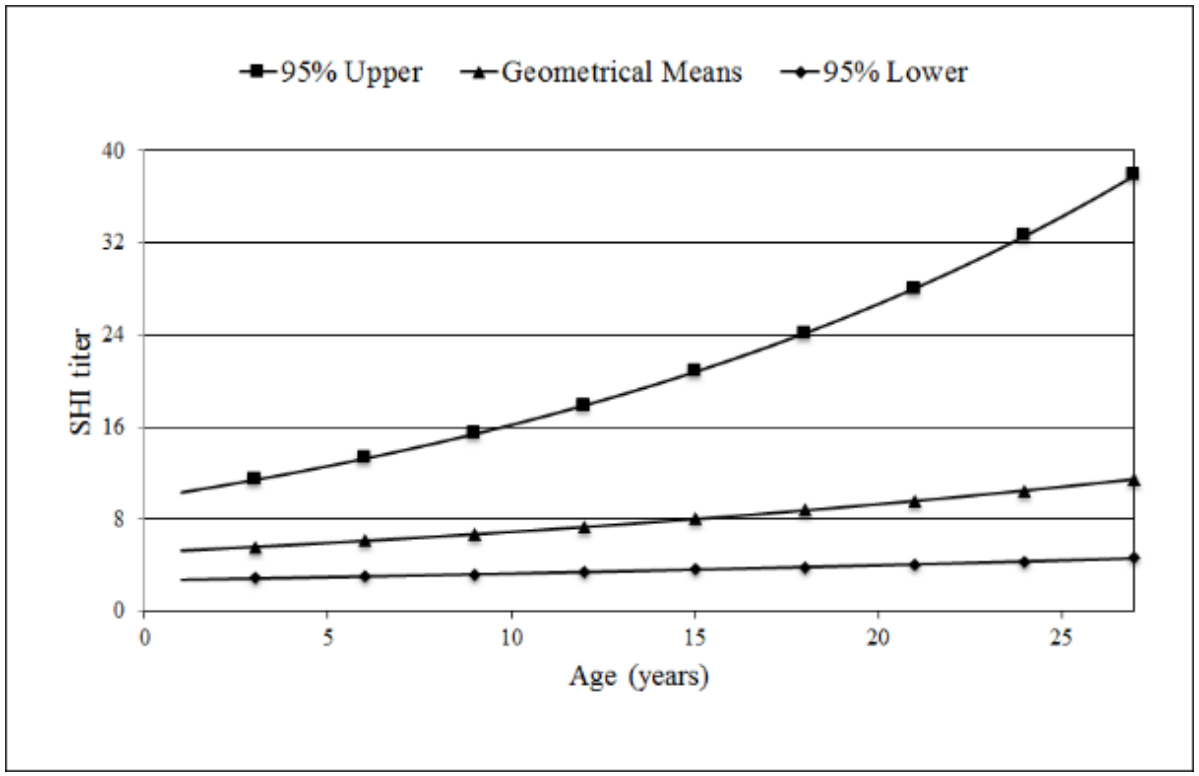
Table 5.1: Frequency of horses and seroprevalence ratios (95% CI) for *C. pseudotuberculosis* in Alabama grouped by SHI titer.

Titer	Number of horses	Seroprevalence (%)	95% Confident Interval
<1:8	162	47.37	42-52.8
1:8	59	17.25	13.4-21.7
1:16	72	21.05	16.9-25.8
1:32	31	9.06	6.2-12.6
1:64	9	2.63	1.2-4.9
1:128	5	1.46	0.5-3.4
1:256	3	0.87	0.2-2.5
1:512	1	0.29	0-1.6

Table 5.2: Distribution of the variables: breed, contact with cattle and contact with goats identified as significant (P < 0.2) in the initial generalized linear model and included in the final model to determinate the risk factors associated with *C. pseudotuberculosis* titer in Alabama. The titers are reported as the geometric mean and 95% CI after antilog transformation of the log titers.

Risk factor variable	Geometric mean SHI titer	95% CI	Number of animals	P value
Breed				0.023
Donkey and Mule	41	111-15	3	
Thoroughbred	15	23-9	14	
Arabian and crosses	11	21-5	7	
Gaited	11	14-8	61	
Mixed breed	10	16-6	17	
Quarter horse	9	11-8	164	
Draft and crosses	7	21-3	3	
Mustang	7	19-3	3	
Pony and Miniature	7	11-4	17	
Warmblood	6	10-3	10	
Cattle				0.05
No	9	12	187	
Yes	11	15	112	
Goat				0.19
No	9	12	253	
Yes	11	15	46	

Figure 5.4: Geometrical mean (95% CI) for the SHI titers grouped by age calculated by antilog transformation of the log titers. An association with high SHI titers was found for age in the final generalized linear model ($P < 0.001$).



Three hundred and three owners reported the use of insect control methods but neither the use nor the type of insect control method used had an association with increasing SHI titer (P = 0.72).

Two hundred and eighty horses had no previous reported medical conditions. There was no association between the different types of medical conditions (including colic, lameness, ocular problems, neurological signs, etc.) reported and increasing SHI titers (P = 0.85). History of external or internal abscesses or *Streptococcus equi* subsp. *equi* (strangles) infection or vaccination did not have an association with increasing SHI titers.

Three horses had a history of lymphangitis in the past; two of them had a negative SHI titer at ≤ 1:8, but one horse had a SHI titer of 1:256. The owner of this horse reported that the horse had recurrent dermatologic problems in the lower limbs after the horse was diagnosed with ulcerative lymphangitis by culture of *C. pseudotuberculosis* 2 years prior. The horse had lived in Texas before the owner purchased the horse and had traveled to Florida, Mississippi, and Oklahoma multiple times.

There was no association of SHI titers and reported contact with sheep (P = 0.29), dogs (P = 0.63), cats (P = 0.71), or wildlife (P = 0.59). Contact with cattle and goats was associated with increasing SHI titer (P = 0.048 and P = 0.068, respectively) in the initial model. In the final model cattle contact remained significant (P = 0.05), however contact with goats was not significant (P = 0.19). Of the 46 horses that had contact with goats, only 3 had a confirmed history of caseous lymphadenitis in the goats and the titers of these horses were 1:256, 1:128, and 1:16. The owners of ten of 46 horses did not know if their goats had had caseous lymphadenitis in the past and 33 had not observed the disease in their goats.

Travel to other states was not associated with increasing SHI titers, with the State Score not significant in the initial model ($P = 0.97$).

Repeated SHI testing

Of the 9 horses with a titer ≥ 1:128, serum samples for repeated SHI testing were only obtained from 6 horses (Table 5.3). Two horses from the same farm initially had titers of 1:128 and 1:256, and repeated SHI testing one year later resulted in titers of 1:64 for both of the horses. The horses on this farm were in contact with goats with history of caseous lymphadenitis and had previously traveled to Georgia, Kentucky (state with sporadic outbreaks), and Ohio. Another horse with a titer of 1:128 maintained the same serologic titer 2 months later. This horse had travelled to Kentucky (state with sporadic outbreaks) and Tennessee. Two horses from the same farm initially had titers of 1:128 and 1:256, and repeated SHI testing one year later resulted in identical titers; no travel history or contact with small ruminants was reported, however the horses had contact with cattle. The remaining horse, had an initial titer of 1:256 and, one month later, the titer of this horse had risen to 1:1,024. This was the horse that had been previously been diagnosed with *C. pseudotuberculosis* ulcerative lymphangitis after residing in Texas (endemic state). Bacteriological culture of a skin biopsy taken at the time of the 1:1024 titer could not confirm *C. pseudotuberculosis* recurrence. A second sample for repeated testing of the horse with a titer of 1:512 could not be obtained. This horse had no history of previous travel outside Alabama but had contact with goats.

Table 5.3: Summary of the SHI titer results and questionnaire responses for the horses that had a titer >1:128 on initial testing.

First titer	Second titer	Time between titers (months)	Travel Endemic State	Travel Sporadic State	Contact	Previous diagnosis *C. pseudo*
128	128	12	No	No	Cattle	No
256	256	12	No	No	Cattle	No
128	128	2	No	TN, KY	No	No
256	1024	1	TX	FL, MS	No	Yes
128	64	12	No	GA, KY, OH	Goats	No
256	64	12	No	GA, KY, OH	Goats	No
128	-	-	No	LA	No	No
512	-	-	No	No	Goats	No
128	-	-	No	No	Unknown	No

Discussion

In the present study, the seroprevalence of detectable SHI titer (>1:8) in a non-endemic population was 52%. Two point six percent of the horses in this study had a titer ≥1:128, and 0.3% had ≥ 1:512. This is the first time the prevalence of detectable titers has been evaluated in a non-endemic area. The results are consistent with data from a study from an endemic area where horses not affected by *C. pseudotuberculosis* had a median titer of 1:8 and numerous others had titers between 1:128 and 1:1,024.[124]

From the first study that evaluated the use of the SHI in horses in 1978, a sensitivity of 100% and specificity of 80% could be calculated if a cut-off of 1:10 was used.[123] If age was considered as a confounding factor, Knight et al. proposed an approximate cut-off of 1:80 and 1:160 to suggest positive diagnosis in young and old animals respectively, which lead to 26-27% false negatives (sensitivity of 74-73%) and 14-15% false positives (specificity of 86-85%).[123] However, a rigorous evaluation of the accuracy of this test using a large sample size involving horses from endemic and non-endemic states has not been performed. Historically, cut-off titers of ≥ 1:128 and ≥1:512 have been used to indicate exposure and internal *C. pseudotuberculosis* infection, respectively.[4,9] In a retrospective study, of 174 horses with external abscesses SHI titers ranged from negative (1:2) to positive (1:1,024).[13] Although 63.8% had titers ≥ 1:256, 36.2% of horses with external abscesses had relatively low titers of ≤ 1:128. In contrast, Jeske et al. (2013) demonstrated that a titer ≤ 1:160 was associated with a low likelihood of active *C. pseudotuberculosis* infection and a titer ≥ 1:10,240 most likely indicated the presence of an internal abscess.[124] One laboratory that performs the SHI states that titers <1:8 are not considered significant and probably represent cross-reactions with common environmental organisms, that titers ≥1:256 are consistent with active infection, and that in the absence of external abscess,

titers >1:256 have a high association with internal abscess formation. Rigorous identification of an appropriate cut-off value for diagnosis of pigeon fever and evaluation of the diagnostic accuracy of the SHI test have not been performed and the interpretation of titers between 1:128 and 1:1,024 remains questionable. This is a limitation for diagnostic evaluation in sick animals, as well as for control of disease spread to non-affected areas. Our results suggest that a cut-off titer greater than 1:512 should be used for diagnosis of the disease in non-endemic areas; however, evaluation of the accuracy of the SHI test was not the purpose of our study and this needs to be further evaluated with a greater sample size including non-affected and naturally infected horses from multiple regions.

This study identified several risk factors associated with increasing SHI titers. It was hypothesized that horses exposed to small ruminants or cattle could have been exposed to *C. pseudotuberculosis* biovar *ovis* and develop antibody titers. The SHI test detects *C. pseudotuberculosis* phospholipase D exotoxin (PLD) antibodies and does not differentiate between biovar *equi* and biovar *ovis*.[123] Goats inoculated with an equine-origin strain of *C. pseudotuberculosis* developed SHI antibody titers within one month of inoculation with minimal clinical signs compared to the goats inoculated with *C. pseudotuberculosis* biovar *ovis* inoculation.[118] The effect of infection or exposure of *C. pseudotuberculosis* biovar *ovis* to horses has never been investigated, but development of antibody titers without appreciable clinical disease is plausible. Interestingly, in our study, there was a significant association of increasing SHI titers in horses that had been in contact with cattle but not with small ruminants. Despite no significant effect on the final analysis, contact with goats was significant in the initial analysis, and this effect should be investigated using a greater sample size. As numbers of horses that had been exposed to small ruminants with caseous lymphadenits (CLA) was very low, this could not

be assessed in the statistical modeling. Of the three horses that had been exposed to small ruminants with confirmed CLA, two had titers ≥1:128. Experimental inoculation of horses with *C. pseudotuberculosis* biovar *ovis* would be required to determine the nature of the mounted immune response. The role of cattle in transmission of *C. pseudotuberculosis* and potential cross-reactivity in serological titers needs to be further investigated. Other genetically related bacteria such as *C. ulcerans* or *C. haemolyticum* are known to produce a similar phospholipases,[74] but the effect of antibody titers in horses exposed to these bacteria has never been investigated and cross-reactivity is possible.

Our results indicate that there is a significant association between increasing SHI titer and increasing age. One study stated that aged horses with clinical signs of infection had higher SHI titers that younger affected horses.[123] Some reports indicated that horses between 1 and 2 years of age are at higher risk of *C. pseudotuberculosis* infection,[6] whereas other studies reported that this age range represented the lowest percentage of cases (9%) compared to horses 2 to 10 years of age (60%) or those over 10 years old (31%).[5] This difference may arise from different regional management practices in endemic areas. The increasing SHI titer in older animals in our study could reflect their greater likelihood of exposure and persistence of antibodies, as occurs with other infectious diseases.[197] Further investigation is needed to determine if age should be taken into account to stablish an appropriate cut-off for the SHI test.

Breed, specifically donkeys, was significantly associated with increasing SHI titer. This association should be interpreted with caution because only 3 animals were included in that group. Limited information concerning the susceptibility of donkeys to *C. pseudotuberculosis* infection exists, and few reports mention the involvement of donkeys.[24] No breed predisposition was detected in large retrospective studies or epidemiological investigations of *C.*

pseudotuberculosis infection in horses.[4,6,13] Thoroughbred horses were associated with decreased risk for the disease in one study; however, this association was not statistically significant and was most likely related to different management conditions in this breed and referral bias.[6]

One possible cause of detectable serological titers without clinical disease is persistence of antibodies from previous exposure or recovery from *C. pseudotuberculosis*. Since the state of Alabama is free from the disease, it was hypothesized that horses could have been exposed to the bacteria during previous travel to endemic states. However, in our study, no statistically significant association between increasing SHI titer and travel to states with presence of endemic or sporadic outbreaks was detected. The time for decline in titers after resolution of infection in horses is unknown. In this study, only one horse had a historical diagnosis of *C. pseudotuberculosis* infection. Of the 9 horses that had titers > 1:128, we were able to obtain follow-up titers from 6 horses. For the 4 horses that had titers repeated after 1 year, two had declined and two remained the same. Further investigation of follow-up titers in horses that have recovered from *C. pseudotuberculosis* is warranted. Serum antibody titers remained high in goats in one study, even after complete healing of the lesion, suggesting that recovered animals may retain seropositivity.[117]

Strangles history or vaccination was included as a potential factor causing increasing titers based on a clinical case seen by the authors (MB, AJS); that involved a pony with clinical infection with *Streptococcus equi* subs. *equi* and no signs of *C. pseudotuberculosis* infection ante and post-mortem that had a titer of 1:256. However, our study did not detect an association between strangles history or vaccination and increasing SHI titers.

One of the tested horses living in Alabama was suspected to have had *C. pseudotuberculosis* infection in the past. This horse was healthy at the time of initial testing, but

had a small non-healing wound on the right hind limb pastern. This horse had been diagnosed with *C. pseudotuberculosis* ulcerative lymphangitis after residing in Texas two years prior. Since then, the horse had the small chronic wound and episodes of dermatitis that partially responded to systemic antimicrobial therapy. Two weeks after initial serological testing, the wound reactivated and local signs of dermatitis appeared on the limb. *Corynebacterium pseudotuberculosis* was not confirmed after bacteriological culture of a biopsy and skin swab. Recurrence of *C. pseudotuberculosis* was suspected based on a two-fold-increase in the SHI titer over a 1-month-period. Although this was an isolated case in which a horse infected with *C. pseudotuberculosis* had been imported into Alabama, there had been no known spread of the infection to any other horses on the farm or in the surrounding area. However, this case is an example of the risk of spread of the disease into non-endemic areas.

One limitation of this study is that the sampled horses were a sample of convenience and not a random sample of the equine population of Alabama. Horses were selected by their local veterinarians, therefore selection bias towards animals that had higher risk of being exposed to the bacteria could have inadvertently occurred. Only 27.8% of the contacted veterinarians participated in the study, therefore a volunteer bias could have occurred if veterinarians were aware of recent pigeon fever outbreaks nearby, as could had happened in the counties of Mobile, Baldwin or Escambia, that border with Florida or Louisiana. In addition, independent-sampling could not be guaranteed, therefore to limit this problem, a maximum of 5 horses per farm was defined as a restriction for sample collection.

Conclusions

The results of this study indicate that the prevalence of detectable SHI titers for *C. pseudotuberculosis* is high in the non-endemic population of Alabama. The association between increasing SHI titers and contact with ruminants warrants further evaluation of possible false positives caused by cross-reaction with antibodies against phospholipases from *C. pseudotuberculosis* biovar *ovis.* Additional research about the diagnosis of *C. pseudotuberculosis* infection is needed to control disease extension to non-endemic regions and to accurately diagnose disease in individual horses, including determining an appropriate cut-off and the accuracy of the SHI test. Making decisions involving *C. pseudotuberculosis* infection in horses only based on antibody titers determined via SHI testing should be avoided even in non-endemic areas.

Conflict of interest statement

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper**.**

Footnotes

a. http://epitools.ausvet.com.au

b. CAHFS, Davis; University of California, Davis;620 W. Health Sciences Dr.; Davis, CA 95616

c. JMP, SAS Institute, Cary, NC

d. EpiTools epidemiological calculators http://epitools.ausvet.com.au

e. SAS 9.3, SAS Institute, Cary, NC

Chapter 6:

Corynebacterium pseudotuberculosis antibody detection in horses: comparison between the synergistic hemolysis inhibition test and a caprine specific ELISA; and analysis of immunoreactive proteins.

Abstract

False positive results may occur when testing horses for antibodies against *Corynebacterium pseudotuberculosis* by synergistic hemolysis inhibition (SHI). In small ruminants, ELISA tests based on exotoxin and cell wall antigens have greater accuracy than SHI. Our first objective was to compare the detection of *C. pseudotuberculosis* antibodies in equine serum by SHI and a caprine ELISA test that uses exotoxin and cell wall antigens. Sera from 7 ponies experimentally infected with *C. pseudotuberculosis* were analyzed by both tests. Correlation and agreement were calculated by Spearman's and Kappa coefficients, respectively. Receiver operating characteristic analysis was used to obtain the optimal cut-off value for the calculated ELISA score. Reactivity to antigens in the ELISA to the equine sera was evaluated by immunoblotting. When SHI titers ≥1:128 were considered a positive result, the optimal ELISA score cut-off to determine positive status was 106%, with 73% sensitivity and 72% specificity with respect to SHI. Positive correlation was found between both tests (r_s=0.692; P=0.085). Agreement in determining positive status was poor (Kappa=0.439; (95%CI 0.226-0.652). Correlation and agreement were strong in 3/7 ponies, but very weak in 4/7. Immunoblot analysis showed a band 13.4 times more intense in infected compared to pre-infected sera corresponding with the exotoxin antigen, but only non-specific reactivity with the cell wall antigen. The use of this ELISA test in horses is not recommended. Development of an ELISA test with specific antigens from *C. pseudotuberculosis* biovar *equi* is needed.

The second objective was to identify immune-dominant antigens of *C. pseudotuberculosis* that evoke a humoral response in horses compared to small ruminants. Immunoblot analysis of the proteins present in bacterial whole cell lysates from the two biovars was performed using sera from infected ponies and goats. The results revealed multiple antigenic

differences between both *C. pseudotuberculosis* strains and differences in caprine and equine responses. The results of the present study offer promise for the establishment of an accurate serological test for the diagnosis of *C. pseudotuberculosis* in horses, however, further investigation of the possible role of these antigens in pathogenesis and immunity is warranted.

Introduction

Corynebacterium pseudotuberculosis is a Gram positive, intracellular, facultative anaerobic bacterium that causes disease in horses (biovar *equi*) and in small ruminants (biovar *ovis*).[2] Definitive diagnosis of *C. pseudotuberculosis* infection in horses is based on bacteriological culture.[5] The internal form is difficult to detect and diagnostic samples for culture are usually not available; therefore, a reliable serologic test is required to determine and treat the cause of illness in individual sick and subclinical animals.[4,14] Serological diagnosis is important for control and eradication programs in small ruminants; a strategy that could also be considered in horses to limit the spread of the disease.[125,127]

Serology using the synergistic hemolysis inhibition (SHI) test, which detects antibodies against *C. pseudotuberculosis* phospholipase D (PLD) exotoxin, has been used as a diagnostic tool for both horses and small ruminants.[118,123,124] In small ruminants, results of some studies indicate that the SHI test has low specificity to distinguish healthy animals from DLA affected animals, which resulted in the development of multiple serological tests including ELISAs with high sensitivity and specificity.[75,117,149] Indirect PLD-based ELISA is considered the most sensitive of the ELISA tests, with a high specificity of 98-99%.[75,144] In horses, the SHI is the only commercially available test, but its accuracy has not been rigorously evaluated. Historically, a titer > 1:512 has been used to diagnose internal infection based on large retrospective studies; however, an appropriate cut-off has not been determined.[4,124] There is overlap between the lower titers that occur in horses with external abscesses, recovered horses in endemic regions, and horses with the internal form; and even with non-exposed clinically normal horses as we previously demonstrated in chapter 5.[4,13] One possibility for the high number of false positives present in this test is cross-reaction with antibodies against *C. pseudotuberculosis* biovar *ovis*

PLD or other similar bacteria. We demonstrated that ruminant exposure was a risk factor for increasing SHI titers in a non-endemic equine population, however further evaluation of the immune-dominant proteins of this bacteria in horses is needed to determine if cross-reaction exists.

Identification of *C. pseudotuberculosis* biovar *equi* proteins that can induce an immune response, and therefore have the potential to be used in the development of an accurate serological diagnostic tests would be advantageous.[93,167] In small ruminants, many studies have investigated the immune-reactive proteins of *C. pseudotuberculosis* biovar *ovis* using multiple approaches.[102,129,168] On the other hand, limited information is available regarding immune-reactive proteins of *C. pseudotuberculosis* biovar *equi* in horses.

The purpose of this study was to compare the detection of *C. pseudotuberculosis* antibodies in equine serum by SHI and a small ruminant ELISA test that uses both an exotoxin and cell wall components as solid phase antigens. Furthermore, a second objective was to investigate immune-dominant antigens of *C. pseudotuberculosis* that evoke a humoral response in horses compared to small ruminants by immunoblot analysis of the two biovars using sera from infected ponies and goats.

Material and Methods

Bacterial strains

Field strains of *C. pseudotuberculosis* biovar *equi* and biovar *ovis*, were cultivated aerobically on 5% bovine blood agar at 37° C in 10% carbon dioxide and confirmed as *C. pseudotuberculosis* by cellular and colony morphology, and conventional biochemical testing. The bacteria were beta-hemolytic, catalase positive, and fermented glucose without gas production. The biovar *equi* strain originated from an external abscess of a horse from Salinas, CA and was nitrate positive. The biovar *ovis* strain was originated from an external abscess of a goat in Auburn, AL and was nitrate negative. After the first isolation, the isolates were sub-cultured twice.

Serum samples

Sera from 7 ponies experimentally infected with *C. pseudotuberculosis* biovar *equi* was collected and frozen at -80ºC until used for the ELISA and immunoblotting. The ponies developed local abscesses at the site of inoculation and *C. pseudotuberculosis* was cultured from purulent secretions.[190] Infected ponies also showed a systemic inflammatory response. Sera from the ponies on the day prior to infection were considered negative and sera from the ponies on day 21 post-infection (peak) were considered positive.

Since the sera from the experimentally infected ponies was considered the unknown in this study, in order to calculate the ELISA score, a negative standard OD was obtained from sera from 17 horses without a history of exposure or clinical signs of *C. pseudotuberculosis* that were sent to the collaborating laboratory[a] for reasons other than this study and was used for score calculations.

Goat sera were collected and frozen at -80ºC until use for ELISA and immunoblotting. Sera were collected from 4 goats naturally infected with *C. pseudotuberculosis* biovar *ovis* were considered as positive controls. Two of the goats had external abscesses in the parotid region and *C. pseudotuberculosis* was cultured from aspirated purulent material. The other two goats from the same flock had systemic signs and lymph node enlargement, but bacteriological culture confirmation was not possible due to absence of mature external abscesses. Sera collected from 4 goats from a herd without history of clinical disease of *C. pseudotuberculosis* were considered negative controls. None of the goats had CLA clinical signs upon physical examination.

Synergistic hemolysis inhibition test

The SHI test for detection of *C. pseudotuberculosis* IgG antibody titers was performed at the California Animal Health and Food Safety Laboratory (CAHFS)[b] as previously described.[123] Sensitized blood agar plates were made by dissolving agar base with *Rhodococcus equi* toxin in distilled water and bovine red blood cells, and sterilized. A 2-fold serum dilution series of 10 steps, starting with 1:8, was incubated at room temperature with *C. pseudotuberculosis* toxin in a titration plate and then individually absorbed onto a filter paper disc. The wetted discs were placed serially on the sensitized blood agar plates and incubated for 18-24h at 37ºC. The results were expressed as the highest serum dilution for which the disk had no surrounding hemolysis, i.e. the presence of antibody against *C. pseudotuberculosis* toxin at that dilution inhibited the hemolytic activity in sensitized plates. [123]

Enzyme-linked immunosorbent assay (ELISA)

A commercial indirect ELISA[a] developed for goat samples was used to analyze horse sera. This test was chosen because it was commercially available and because it used the combination of two antigens. The ELISA was performed on the horse sera samples at the collaborating laboratory[a]. Briefly, 96-well plates were coated with two antigens obtained from crude preparations from *C. pseudotuberculosis* biovar *ovis* culture. The exotoxin antigen preparation was obtained by collection of culture supernatant, salt precipitation, and purification by column chromatography. The cell wall antigen preparation was obtained by bacterial whole cell pellet washing, enzymatic digestion, and extraction of DNA in an organic solvent. The cell wall antigen was sonicated to expose hidden epitopes in the membrane proteins, and then used to coat the wells. Diluted serum (1:50) was added to the coated wells and incubated for 60 min. After washing, the specifically bound antibodies were detected with horse-radish-peroxidase (HRP) - labelled rabbit anti-horse IgG instead of the anti-goat IgG used in the commercial assay. After incubation for 60 min, the wells were washed and then o-phenylenediamine dihydrochloride (OPD) and hydrogen peroxide were added as chromogen and substrate, respectively. After a 20 min incubation period, the stop solution was added (0.1M H_2SO_4) and the optical density was read at 495 nm wavelength. The results were expressed as a score equal to the percent value of the optical density (OD) value of negative control serum.

Score = (Patient OD/Negative control OD) * 100

The OD of the negative control serum was calculated by averaging the OD values from serum samples of 17 asymptomatic horses that fell within 2 standard deviation of the average OD.

To estimate the ELISA repeatability, three well-defined samples from ponies determined by the SHI titer [high (1:2,048), low (1:128), and negative (<1:8)]; were selected to evaluate intra

and inter-assay variation. For intra-assay variation, duplicates of the 3 well-defined samples were run in the same assay and this procedure was repeated 3 times. Inter-assay variability was evaluated by repeating the ELISA 3 times for the 3 well-defined samples. The coefficient of variation (% CV) from the replicates of each sample and between plates was calculated.[c] Linearity of the assay for equine samples was assessed by recovery calculation after serial dilution of the 3 well-defined samples.

Whole cell lysate preparation

Field strains of *C. pseudotuberculosis* biovar *equi* and biovar *ovis*, characterized by biochemical and molecular methods, were grown in a chemically defined medium (Table 6.1) as previously described[160,168] at 37ºC until reaching the exponential growth phase: OD 600nm = 0.4. Bacterial cells were separated from the supernatant by centrifugation at 2,900g (4,300 rpm, rotor radius 14cm) for 10min. Bacterial cells were washed twice with phosphate buffered saline (PBS) and centrifuged. The pellet was resuspended in PBS and a protease inhibitor cocktail[d] was added. The suspension was sonicated[e] on ice with 20-second pulses for 2 min (100% amplitude, 20kHz, 600W) to obtain a whole cell lysate as previously described.[102,129,198] The sonicated solution was centrifuged at 8000g for 20min at 4ºC and the supernatant was collected. Protein concentrations were determined by the Bradford method with a protein assay[f]. The whole cell lysate was frozen at -80ºC until needed.

Immunoblot analysis

Corynebacterium pseudotuberculosis antigen (exotoxin and cell wall) and whole cell lysate reactivity with the serum samples from infected and non-infected ponies was evaluated by

immunoblot analysis. The cell wall antigen was sonicated with 20-second pulses for 2 min (100% amplitude, 20kHz, 600W) to expose hidden epitopes on the membrane proteins. Protein concentrations were determined by the Bradford method with a DC Protein Assay[f], and the maximum amount of protein was loaded into the gel[g].[199] Antigens or lysates were thawed on ice.

Table 6.1. Constituents of the chemically defined medium

Reagent	**Concentration**
Na_2HPO_4 anhydrous	6.85g/L
KH_2PO_4	2.55g/L
NH_4Cl	1g/L
$MgSO_4$ anhydrous	0.098g/L
$CaCl_2$.$2H_2O$	0.026g/L
Tween 80 [d]	0.05% (v/v)
100X minimal essential medium (MEM) vitamin solution	4% (v/v)
50X MEM amino acids solution	1% (v/v)
100X MEM non-essential amino acids solution	1% (v/v)
Filter-sterilized glucose	1.2% (w/v)

and boiled for 10 min in 5x sample buffer (0.5M Tris-HCl pH=6.8, sodium dodecyl sulfate, glycerol, 0.25% bromophenol blue and β-mercaptoethanol). Antigens (26.25μg of exotoxin antigen and 7μg of cell wall antigen) or whole cell lysate (12.75μg) were loaded on 10% polyacrylamide gels[g] and separated with SDS-PAGE. Bovine serum albumin (12.75 μg, BSA) was added as a positive control for protein transfer. Electrophoresis was performed for 35 min under a constant voltage of 200V.

Separated proteins were transferred onto a polyvinyldene fluoride transfer membrane (PVDF, 0.45μm pore)[h] for 1.5 hours at 100V. Membranes were stained with Ponceau S to confirm transfer. Membranes were blocked in 5% nonfat milk for 1 hour and then incubated with equine or caprine serum as the primary antibody (1:100 in 5% nonfat milk) for 12-16 hours at 4°C. Membranes were incubated for 1 h at room temperature with either rabbit anti-horse IgG horseradish peroxidase–conjugated secondary antibody[d] (1:2000 in 5% nonfat milk) or rabbit anti-goat IgG horseradish peroxidase–conjugated secondary antibody[i] (1:2000 in 5% nonfat milk). Signal was detected using enhanced chemiluminescence. A digital imager[j] was used to image the membranes, and band density was analyzed with imaging software.[k]

To optimize the concentration of secondary antibody, two different dilutions of secondary antibody, 1:2,000 and 1:10,000, were used to blot a membrane with different concentrations of serum as a positive control and a lysate of equine endothelial progenitor cells as a negative control. The 1:2,000 dilution showed an intense band without increasing non-specific binding and was chosen as the dilution for the rest of immunoblot assays.

Statistical analysis

Synergistic hemolysis inhibition test results were expressed as the reciprocal of highest serum dilution that prevents hemolysis, and ELISA results were expressed as a score as described above. Therefore, in order to be able to compare the agreement of both tests, the data was transformed to categorical results: positive or negative. Since an established cut-off for SHI is not available, a cut-off for SHI that reflected the seroconversion (at least 2-fold dilution increase in titers) of experimentally infected horses was determined. Since the maximum SHI titer observed in the ponies prior to inoculation was 1:32, 1:128 was chosen as the cut-off for positivity; because it is a 2-fold dilution increase from 1:32. A receiver operating characteristic (ROC) analysis was performed to estimate the optimal ELISA cut-off for the various values of sensitivity and specificity compared to SHI using statistical software.[l]

Normality of the data was assessed using analysis of histograms and Quartile-Quartile plots. The data did not fulfill the normality assumption and therefore, the correlation between SHI and ELISA numerical results was determined using non-parametric Spearman's coefficient test. Since the sera was collected from each pony at multiple timepoints, the 68 data points could not be considered independent to test for correlation. Therefore, the median titer for the SHI test and median OD for ELISA of the 7 inoculated ponies obtained at each time point were calculated. Overall correlation over time was then determined using Spearman's coefficient. Correlation over time was also calculated for each individual pony. Agreement between SHI (1:128 cut-off) and ELISA (106.3% cut-off) categorical results was calculated using Kappa coefficient. The analysis was performed using a statistical software package.[m]

Results

Repeatability and linearity of the caprine ELISA for analysis of equine samples

The overall intra-assay coefficient of variation (CV) for the ELISA for equine samples was 16.16%. The intra-assay CV was 11.11% for the negative samples, 10.31% for the low-positive samples, and 24.15% for the high-positive samples. The overall inter-assay CV was 18.2%. The inter-assay CV was 13.85% for the negative samples, 19.21% for the low-positive samples, and 24.53% for the high-positive samples. The serum dilution used in the assay (1:50), was in the range of linearity (approximately a 2-fold serum dilution produces a drop in OD of 0.2 units) in the three well-defined samples (Fig 6.1).

Determination of ELISA cut-off value with reference to SHI test

Receiver operating characteristic (ROC) analysis for the ELISA test compared to SHI using a cut-off $\geq$ 1:128 for positivity showed that the optimal cut-off for the ELISA score was 106% (Figure 6.2). Using this cut-off, sensitivity and specificity for ELISA compared to SHI was 73% and 72%, respectively (Table 6.2).

Comparison of SHI and ELISA test results

The SHI and ELISA test results are summarized in table 5.4 and figure 5.3. A positive correlation between numerical results from both tests was found overall (Spearman r_s=0.692; P=0.085). Correlation was strong and positive in 3/7 ponies, with Spearman correlation coefficients between 0.833 and 0.805; however, very weak correlation was present in 4/7, which ranged between 0.122 and -0.451 (Figure 6.3). A poor agreement in determining positive status between both tests was found (Kappa=0.439; 95%CI 0.226-0.652).

Table 6.2. Sensitivity and specificity of the ELISA in relation to SHI for different cut-off using 68 serum samples from 7 experimentally infected ponies.

ELISA Score cut-off	**SHI + ELISA + (A)**	**SHI – ELISA + (B)**	**SHI + ELISA – (C)**	**SHI – ELISA – (D)**	**Sensitivity A/(A+C)**	**Specificity D/(D+B)**	**1-Specificity**
66.13	30	38	0	0	1.000	0	1.0
85.20	27	24	3	14	0.900	0.359	0.641
106.26	22	11	8	27	0.733	0.718	0.282
133.63	9	4	21	34	0.300	0.897	0.103
210.36	0	0	30	38	0	1.0	0

A: number of True Positives, B: number of False Positives, C: number of False Negatives, D: number of True Negatives. Sensitivity = number of True Positives / (number of True Positives + number of False Negatives). Specificity = number of True Negatives / (number of True Negatives + number of False Positives).

Table 6.3. Summary of the results of sera from the 7 inoculated ponies tested with both serological tests at each time point after infection. IQR: Interquartile range.

Time	**Median SHI titer**	**IQR SHI titer**	**Median ELISA OD**	**IQR ELISA OD**	**Median ELISA score**	**IQR ELISA score**
t0	4	12	0.326	0.136	114.269	47.719
t3	4	12	0.305	0.059	106.901	20.702
t7	4	12	0.244	0.064	85.497	22.339
t10	32	112	0.251	0.030	88.187	10.526
t14	256	192	0.306	0.077	107.485	27.135
t21	512	256	0.352	0.174	123.626	61.170
t28	512	256	0.368	0.167	129.006	58.480

Figure 6.1. Linearity of ELISA test based on *C. pseudotuberculosis* biovar *ovis* exotoxin and cell wall as solid phase antigens assessed by representation of optical density (OD) values versus increasing dilution factors of equine well-defined serum samples. Negative sample: SHI titer <1:8. Low-positive sample: SHI titer 1:128. High-positive sample: SHI titer 1:2,048.

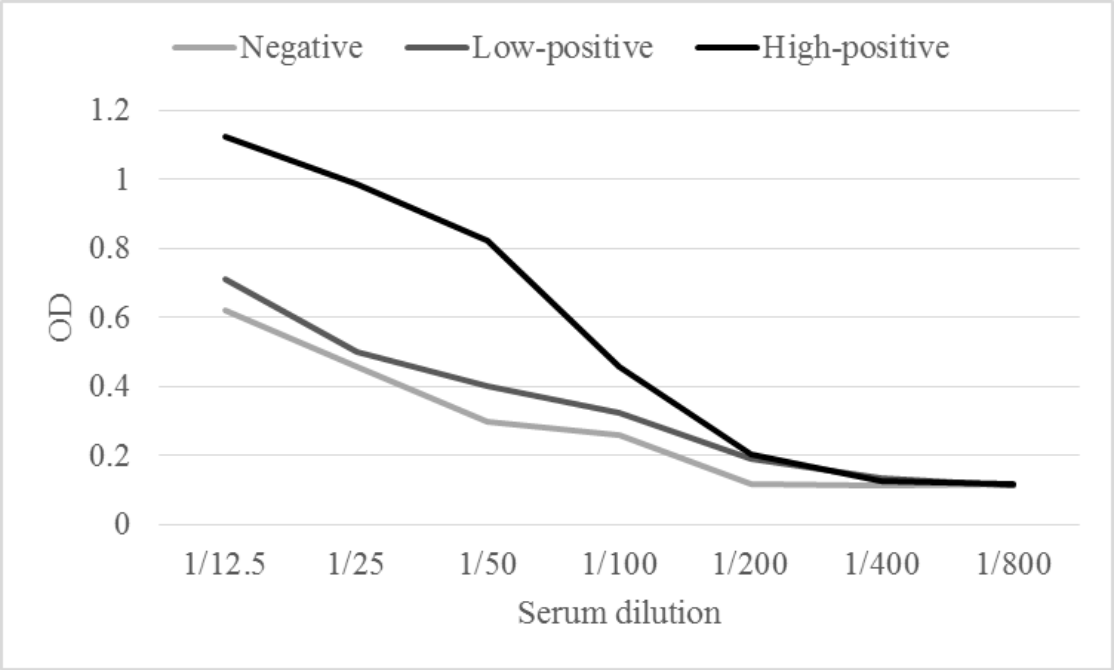


Figure 6.2. Receiver operating characteristic (ROC) curve for the ELISA test. The data are based on a comparison of a total of 68 serum samples from 7 experimentally infected ponies tested both with the ELISA and SHI assays.

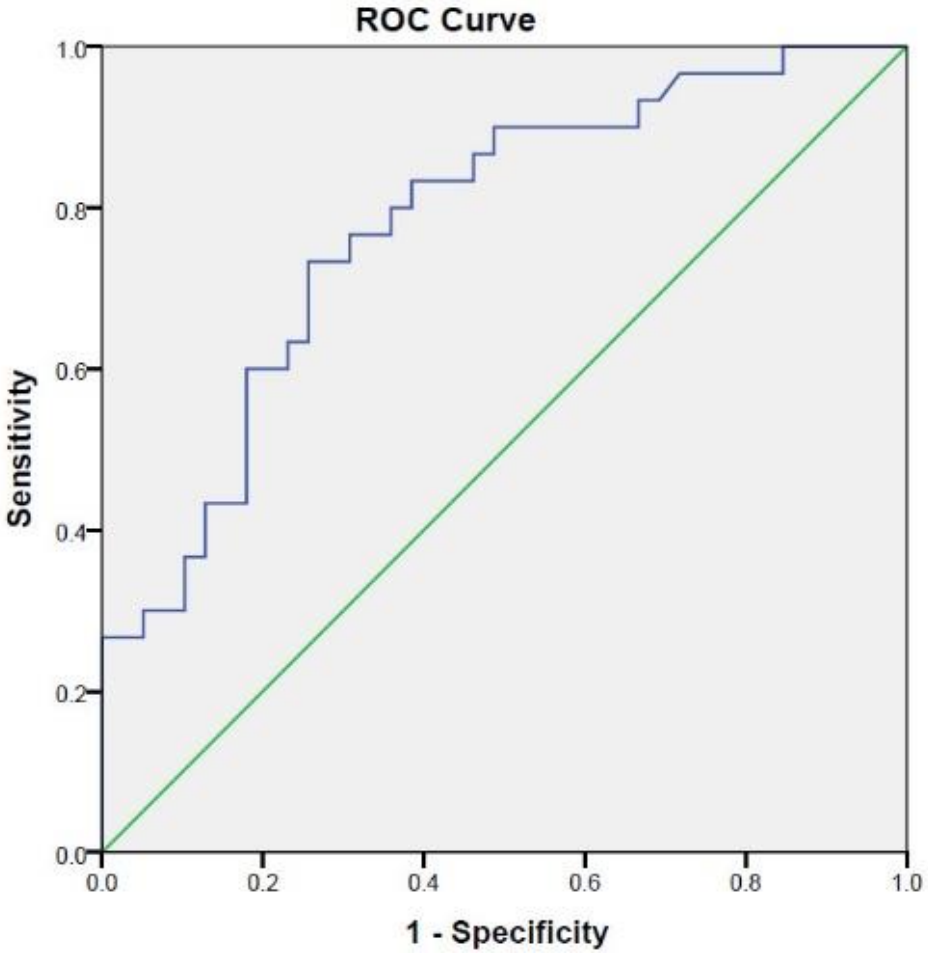


Figure 6.3. Scatter plot representing the median ELISA optical density (OD) versus the median SHI titer of the 7 experimentally infected ponies at each of the 7 time points.

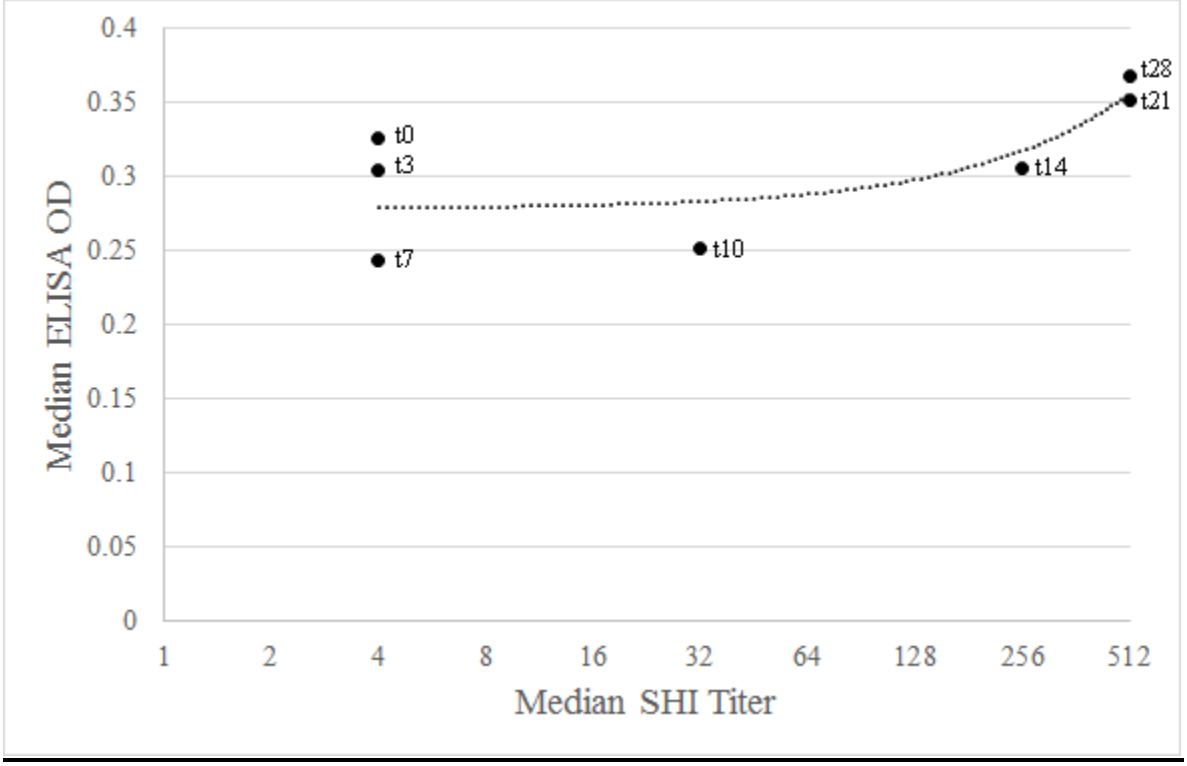
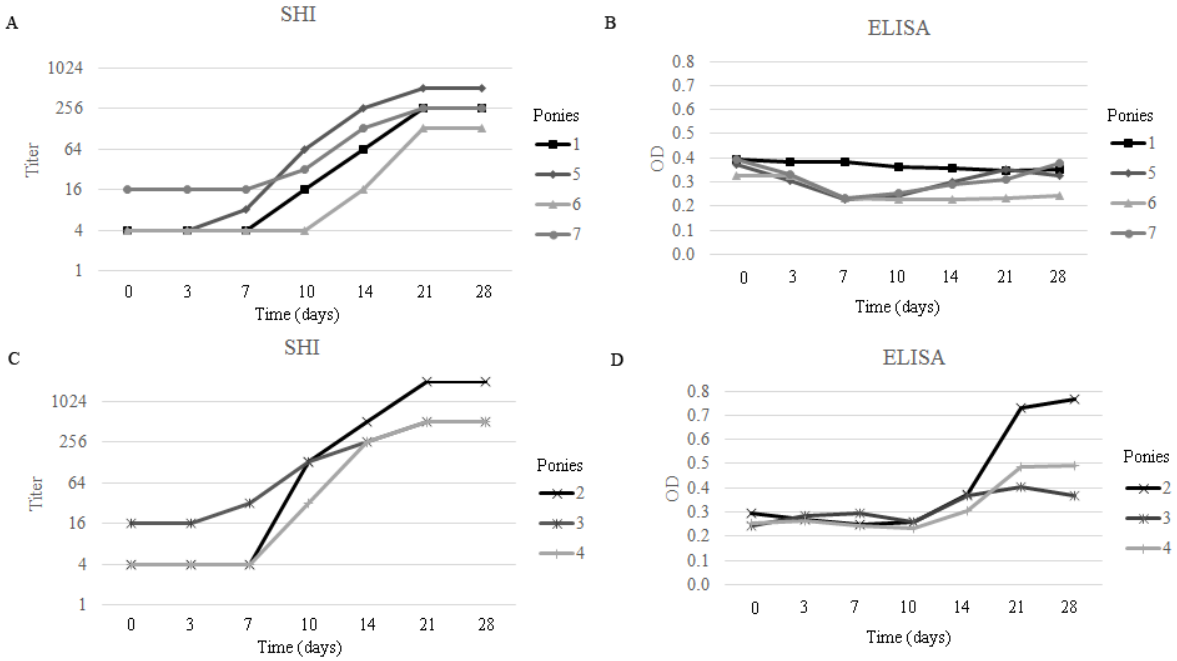


Figure 6.4. Graphic representation of serological results tested with SHI or ELISA over time (in days) after inoculation with *C. pseudotuberculosis* biovar *equi* at t0 in ponies. A and B shows the results for the 4 ponies (1, 5, 6, and 7) which the two tests had weak correlation. C and D shows the results for the 3 ponies (2, 3, and 4) for which the two tests had strong correlation.



Optimization of immunoblot assay

To confirm specificity of the horse and goat polyclonal IgG secondary antibody used in the immunoblot, two membranes with the ELISA antigens, whole bacterial lysates, equine serum, caprine serum and BSA were prepared and incubated with each of the secondary antibodies directly without serum as primary antibody for 1 hour. The membrane blotted with horse polyclonal secondary antibody showed a signal only to 2 equine serum proteins of ~50kDa and ~25kDa (Figure 5.5). These two proteins presumably correspond to the IgG light chains (26-27 kDa) and heavy chains (51-52 kDa) that in normal conditions are connected by disulfide bonds, which are disrupted by treatment with β-mercaptoethanol during antigen preparation for electrophoresis.[200] These findings confirmed the absence of non-specific binding with proteins present in bacterial antigens (Figure 6.5). The membrane blotted with the goat secondary antibody showed a signal only to caprine serum proteins, of similar size as the light and heavy chains of IgG proteins, confirming as well analytical specificity (Figure 6.5).

Immunoreactivity to *C. pseudotuberculosis* antigens from a commercial caprine ELISA in *C. pseudotuberculosis* infected ponies and goats

Immunoblot analysis revealed differences in the reactivity of equine serum compared to caprine serum to the cell wall ELISA antigen (Fig. 6.6, lane 7). A stained band of moderate intensity (~30kDa) was observed in positive caprine sera, whereas non-specific reactivity was observed in post-infection equine sera. Immunoblots incubated with negative caprine sera and pre-infection equine sera showed no antigenic bands or bands with low intensity, respectively.

Similar reactivity to the ELISA exotoxin antigen was observed with caprine and equine serum (Fig 6.6, lanes 5 and 6). Immunoblots incubated with positive caprine or post-infection

equine sera showed a strongly stained band (~70kDa). A few low-intensity bands were also observed with both caprine and equine sera. Immunoblots incubated with negative caprine or pre-infection equine sera showed no antigenic bands or a weak band of the same size, respectively. Analysis showed that the antigenic immune-reactive 70kDa band was 13.4 times more intense in infected equine sera compared to pre-infected sera (Fig 6.7).

Immunoreactivity to whole cell lysate from *C. pseudotuberculosis* biovars in C. *pseudotuberculosis* infected ponies and goats

Immunoblot analysis revealed antigenic differences between both *C. pseudotuberculosis* biovars and differences in caprine and equine responses (Fig 6.6, lines 1-4). Immunoblots incubated with equine sera showed multiple strong antigenic bands that were common for both bacterial strains (~150, 90 and 45kDa). A strong antigen band (~115kDa) was observed in biovar *equi* lysate (Fig 6.6, lines 1 and 2) that was not present in biovar *ovis* lysate (Fig 6.6, lines 3 and 4) after incubation with post-infection equine sera as the primary antibody. Non-specific binding obscured the analysis of smaller antigens (37-20kDa), and separate bands could not be distinguished. Immunoblots incubated with pre-inoculation equine serum showed multiple weakly stained bands following a similar pattern as post-inoculation sera blots but of lower intensity.

Immunoblots incubated with positive caprine sera showed a similar pattern as equine sera blots with a few differences (Fig 6.6, lines 1-4). A 130kDa band was only observed in the biovar *ovis* blots and no band of 115kDa was observed either biovar blot. Similarly, non-specific binding obscured the analysis of smaller antigens. Immunoblots incubated with negative caprine sera showed a few weak bands of low intensity.

Figure 6.5. Immunoblot showing specificity of the secondary IgG polyclonal secondary antibodies. A. Membrane blotted with equine secondary antibody. B. Membrane blotted with caprine secondary antibody. 1. Equine serum. 2. Caprine serum. 3. BSA. 4 and 5. *C. pseudotuberculosis* biovar *equi* whole cell lysate. 6 and 7. *C. pseudotuberculosis* biovar *ovis* whole cell lysate. 8. ELISA toxin antigen. 9. ELISA cell wall antigen.

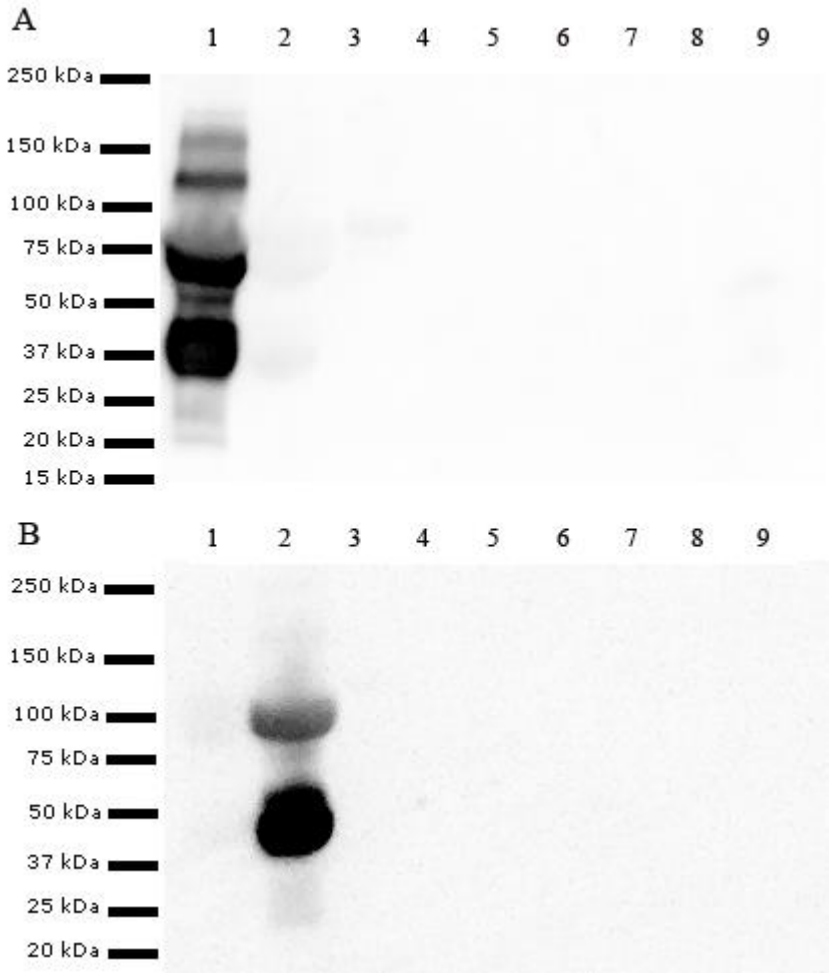


Figure 6.6. Immunoblot showing the immunoreactivity of the different antigens with pre-inoculation equine serum (A), post-inoculation equine serum (B), negative caprine serum (C), and positive caprine serum (D). Lanes 1 and 2. *Cp. equi* whole cell lysate; lanes 3 and 4. *Cp. ovis* whole cell lysate; lanes 5 and 6: ELISA exotoxin antigen; lane 7: ELISA cell wall antigen; lane 8: BSA negative control. The green rectangle indicates the cell wall ELISA antigen band at 30kDa. The red rectangle indicates the exotoxin ELISA antigen at 70kDa. The blue rectangle indicates the 115kDa band observed in *Cp. equi* lysate in equine serum. The yellow rectangle indicates the 130kDa band observed in *Cp. ovis* lysate in caprine serum.

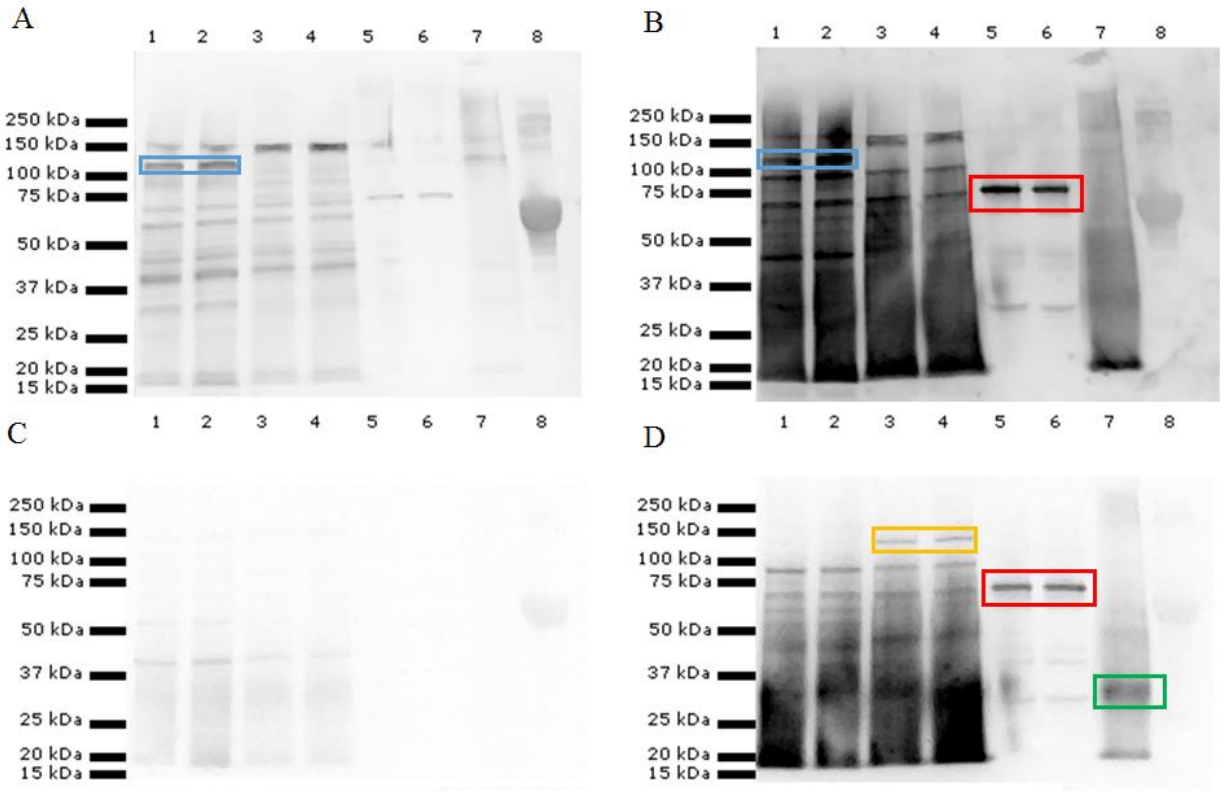
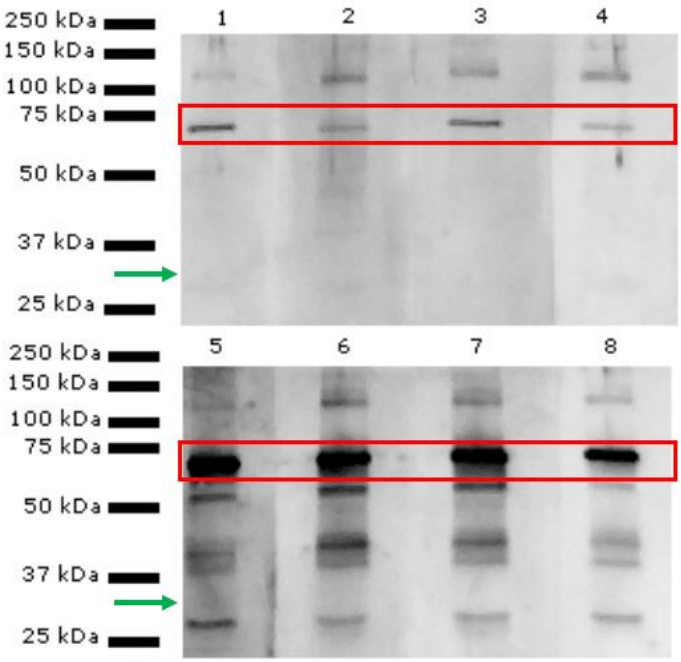


Figure 6.7. Immunoblot showing immune-reactivity of the ELISA exotoxin antigen blotted with sera from 4 ponies, 1-4 pre-inoculation and 5-8 post-inoculation with *C. pseudotuberculosis* biovar *equi.* The red rectangle indicates the 70kDa band. The green arrow indicates the position where phospholipase D should show reactivity (~30kDa).



Discussion

Serological diagnostic testing is essential for diagnosis of horses infected with *C. pseudotuberculosis* internal form and subclinical cases.[4,124] Synergistic hemolysis inhibition test is the only serological test available for horses but is not very sensitive or specific. The results of the present study did not support recommending the use of an ELISA test that uses a combination of *C. pseudotuberculosis* biovar *ovis* exotoxin and cell wall as solid phase antigens based on comparison of the detection of *C. pseudotuberculosis* antibodies in equine serum to SHI test. Secondly, this study provided evidence that the *C. pseudotuberculosis* biovar *equi* antigens recognized by antibodies from sera of experimentally infected ponies differed from sera before inoculation and from the biovar *ovis*. These studies offer promise for establishment of an accurate serological test applicable for horses if the specific antigenic proteins can be identified and then developed into an equine-specific ELISA. Two-dimensional electrophoresis and sequencing of immune-dominant proteins by MS would be the next step in investigation.

A positive correlation between numerical results from the SHI and the ELISA tests was found overall; however, agreement was poor. Results from sera of 4 out of the 7 experimentally infected ponies showed a very weak correlation; furthermore, 2 animals had negative correlation having a higher pre-infection OD, and subsequently ELISA score, than after infection (Fig. 5.6). This affected the agreement between the tests because there were several samples classified as negative by SHI test that were considered positive by the ELISA, therefore false positives. Despite the use of ROC analysis to stablish the optimal cut-off of the ELISA with respect to the SHI, the optimal combination of sensitivity and specificity for the ELISA compared to SHI was only 73% and 72% respectively; which reflected the poor agreement between both tests. Based on these results, the use of this specific caprine ELISA in horses cannot be recommended.

Immunoblot of the solid phase antigens from the caprine ELISA showed that the cell wall antigen led to non-specific signal when blotted with equine serum, whereas when blotted with caprine serum, led to a sharp band with strong signal of approximately 30kDa. The fact that the band observed in caprine blots was the same size of the major *C. pseudotuberculosis* exoprotein, PLD, could indicate that the detected immune reaction was against PLD and not a cell wall antigen. In a study in small ruminants, animals vaccinated with a cell wall antigen developed antibodies against PLD.[102] One could argue that the cell wall antigen could be the source of non-specific signal leading to false positive results in equine serum with this caprine ELISA, which supports the anecdotal report that an ELISA test for detection of cell wall antigens in sheep was not very accurate in horses.[16] One study found that horses mounted an immune response to one or more proteins in cell lysate, which was higher in horses affected with the internal form of the disease.[173] Further studies are needed to investigate the use of cell wall antigens in serological testing in horses to evaluate if the addition of biovar *equi* cell wall antigens will improve the performance of any serological testing.

Immunoblot of the exotoxin antigen showed a strong signal band of approximately 70kDa. The major *C. pseudotuberculosis* exotoxin has been identified as PLD, which has a size of 31kDa, therefore the results of this study show that the immune reaction detected by the antigen solution used in the solid phase of the ELISA may not be to PLD and is against another exoprotein. Proteins can run on SDS-PAGE at higher molecular weights if they are not completely unfolded, if they possess abundant hydrophobic residues, or if they are modified (i.e. glycosylation or phosphorylation), which could also explain the size discrepancy.[201] This exotoxin antigen was isolated using column chromatography by the laboratory that developed the ELISA from whole bacterial preparations and was never sequenced by MS to our knowledge.

Other weaker bands were observed against antigens from the exotoxin preparation, and one of them was approximately of 30kDa with could correspond to PLD. Only non-published studies have reported detection of antibodies against PLD in equine serum by using a PLD ELISA and the tests are not commercially available.[172,173] One conference abstract reported that while most horses with internal abscesses showed high antibody responses to PLD, many horses with external abscesses and some uninfected horses also had high titers to PLD.[172] The other abstract reported that horses with the internal abscess form of the disease had higher antibody levels to both cell lysate and PLD than those with external abscesses.[173] Lastly, another abstract reported noticeable cross reactivity against peanut and cabbage PLD antigens using an ELISA.[174] Further investigation to evaluate if an ELISA with the exotoxin antigen alone as solid phase antigen would perform better than the 2 antigens combined, or if substitution by equivalent biovar *equi* antigens will improve performance of the test is needed.

The SHI test was used in this study to compare against the caprine ELISA serological test because the SHI is the only serological test commercially available to detect antibodies against *C. pseudotuberculosis* in horses and multiple retrospective studies have found it useful for diagnosis of internal infection in horses.[4,9] In order to be able to compare the agreement between the SHI and ELISA tests, the results needed to be converted to categorical data: positive and negative results. Since an appropriate cut-off for the SHI has not been rigorously stablished, 1:128 was chosen as the cut-off for positivity because it is a 2-fold dilution increase from 1:32, which was the maximum titer observed in the ponies prior to infection. However, we have found that a 2.33% of clinically normal horses from a non-endemic population have titers ≥1:128 (false positives) (Unpublished results from chapter 5). The arbitrary choice of 1:128 as the cut-off for

positivity is a limitation of this study and could have introduced errors in the latter analysis. Further evaluation is needed to stablish an accurate cut-off for the SHI test in horses.

The SHI test is based on ability of *C. pseudotuberculosis* phospholipase D (PLD) exotoxin to produce a zone of hemolysis when applied to a blood agar plate containing RBC sensitized with *R. equi* culture broth. If anti-*C. pseudotuberculosis* PLD toxin antibodies are present, this reaction is neutralized. The SHI test was developed in 1978, and from the original data it could be calculated to have an approximate sensitivity of 75% and a specificity of 85%;[123] however, the accuracy of the SHI test has not been further evaluated since then and an optimal cut-off for the SHI test has not been determined with modern statistical methods, such as ROC analysis.[123] Since a rigorous evaluation of its diagnostic accuracy is lacking, this could have affected the results of this study since the ELISA results were compared to SHI test results; however, the equine sera was obtained from ponies that were experimentally infected with *C. pseudotuberculosis* and confirmed by bacteriological culture that is considered to be the gold standard. The SHI results in the experimental ponies correlated with bacteriological results, however, the peak SHI titers varied from 1:128 to 1:2,048 between the 7 ponies.[190]

Diagnostic tests based on anti-PLD antibody detection can lead to false positives and false negatives. False positives can be caused by possible cross-reactions due to exposure to closely related bacteria producing similar phospholipases.[74,75,153] *Corynebacterium ulcerans*, present in wild-life and recognized caused of ruminant mastitis, produces a phospholipase D that has a 87% amino acid identity with *C. pseudotuberculosis* PLD (Table 1.2).[74,154,155] Likewise, *Arcanobacterium haemolyticum* and *Corynebacterium uterequi* phospholipases shares 64% and 30% of their amino acid sequence with *C. pseudotuberculosis* exotoxin, respectively (Table 1.2).[74,156,157] In addition, false negatives can arise from animals infected with strains that do not

produce PLD.[129] Further evaluation is needed to stablish the diagnostic accuracy of SHI for it to be used clinically with diagnostic certainty and even before it can be used to compare with newly designed serological tests.

The development of a more accurate serological diagnostic test is necessary for diagnosis of clinical cases, detection of subclinical cases, limiting the spread of the disease, and epidemiological investigations. Performance and applicability of serological assays depend on antigenic selection because antigen candidates have to be immunodominant and therefore recognized by the vast majority of a population infected with *C. pseudotuberculosis.* Little is known about the immunodominant antigens of *C. pseudotuberculosis* evoking a humoral response in horses, however extensive work has been published in small ruminants.

The electrophoretic profile of the *C. pseudotuberculosis* biovar *ovis* whole cell lysate has been reported as very complex, containing proteins ranging in molecular mass from approximately 5 to 200 kDa.[102,146,158,159,162] Different antigen preparation methodologies have been used to disrupt *C. pseudotuberculosis* cells including sonication, ether-extraction, detergent emulsification, and three-phase partitioning with ammonium sulfate and buthanol. In this study, sonication was chosen to extract the proteins from the whole cell lysate. Ellis et al. compared different methodologies including sonication, ether extraction, physical disruption, lysozyme digestion, and octyl glucoside digestion; which all resulted in solubilization of virtually the same constellation of antigens by visual assessment of the molecular masses of separated bands in PAGE gels.[159] However, the three-phase partitioning method was claimed to be more efficient for extraction and concentration of immune-reactive excreted proteins of *C. pseudotuberculosis* biovar *ovis*, and it is possible that a higher number of immune reactive proteins could have been detected in the present study using this technique.[162] Other factors that can affect the recovery of

expressed proteins is the bacterial growth phase, because the expression of different proteins change in early compared to late exponential growth phase.[202] The number of passages might alter expression of proteins, as it was demonstrated in a study where reference ATCC strains showed less immune reactive proteins than field strains.[129] Lastly, a CDM was developed to allow the growth of the bacterium and the production of secreted proteins in macromolecule-free conditions that could interfere with immunological studies.[160] Despite following these published protocols for extraction of proteins from bacterial lysates, such as the use of CDM and the use of field bacterial strains with low number of passages at the mid-exponential growth phase; other factors could have affected the expression of the proteins or its extraction in this study which may differ from *in vivo* situations.[202] In a study using a membrane shaving technique to study host-pathogen interactions *in vivo* of *C. pseudotuberculosis*, 13 proteins were identified that were not identified in culture media, suggesting that a different surface protein repertoire is expressed *in vivo*.[203] Since *C. pseudotuberculosis* protein expression can differ between *in vitro* and *in vivo* conditions, there is no absolute certainty that the proteins expressed in the *in vitro* conditions are indeed the same as those produced during infection. Thirty-one bacterial surface proteins were identified in a study using a membrane shaving technique to study natural host−pathogen interaction *in vivo* on *C. pseudotuberculosis* harvested directly from naturally infected sheep lymph nodes.[203] Thirteen of the 30 proteins were not identified in culture media, suggesting that a different surface protein repertoire is expressed in this hostile environment. Further evaluation of the differences between both biovars is warranted including the evaluation of secreted proteins *in vivo* and in culture supernatant in addition to whole cell lysates.

Once the immune-dominant antigens are recognized and identified, the use of standardized recombinant *C. pseudotuberculosis* antigens in serological assays, as used in some

small ruminant ELISAs based on recombinant PLD,[144] would be a promising approach to overcome the problem of differences in the antigen structure of isolates used to obtain the antigens in the laboratory.[129]

The study of the immune-reactive proteins in *C. pseudotuberculosis* biovar *ovis* has been performed using different methodologies from SDS-PAGE followed by immunoblot to the more complex SERPA; which includes 2 dimensional electrophoresis, immunoblotting, and identification of antigenic proteins by a MS technique.[102,129,168] Multiple proteins have been shown to react with small ruminant sera, and at least 21 have been identified (9 as hypothetical proteins) among different studies (Table 1.1).[102,167–170] Differences in the number of proteins among these studies could be associated to different methods of preparation of antigens or posterior evaluation. The present study evaluated the immune-reactive proteins of *C. pseudotuberculosis* biovar *equi* through SDS-PAGE followed by immunoblotting, which limited the differentiation of antigens, especially of smaller size due to the strong reactivity in infected animals leading to high intensity signal. Further evaluation using another approach such as 2-DE would allow better differentiation of the antigens, which can separate proteins of similar molecular weight based on isoelectric point. Another possibility to separate proteins further, would be to immune-precipitate the lysate proteins first via a pull-down assay. This process binds immune-reactive proteins to serum antibodies that have been immobilized on a solid-phase substrate. Then these proteins are washed out and examined by electrophoresis and immunoblotting, without the interference of proteins that are not immune-reactive. In addition, in order to absolutely identify the proteins, a MS technique needs to be used, which will help to further characterize the immune-reactive proteins from this bacterium in horses.

Reaction to antigens in the whole cell lysate of 150 and 115kDa was observed in immunoblots incubated with pre-infection equine sera. One study reported that sera from goats with no detectable infection consistently reacted with an antigen of approximate molecular mass of 64 kDa.[158] Identification of these proteins by MS would help to understand the role of these antigens and to confirm the hypothesis that cross-reaction with antibodies that may have been induced by closely related bacteria exists, leading to confusion between *C. pseudotuberculosis* specific antigens or exposure to other organisms in the environment.[159,204]

Putative antigens have been predicted for *C. pseudotuberculosis* biovar *equi* focusing towards finding a common vaccine antigen shared by both biovars or more than one pathogen.[81] At least two of these antigens, a resucitation-promoting factor and a hypothetical protein with cell-surface hemin receptor (HtA) family domain, were found to be immunogenic in a study involving the ovine biovar.[168] However, no prediction of a specific antigen for biovar *equi* not shared with biovar *ovis* or other similar bacteria has been published. Both *C. pseudotuberculosis* biovars are genetically very similar, and the immune-reactive antigens confirmed for biovar *ovis* have a high amino acid sequence identity to biovar *equi* (Table 1.3), which can lead to false positive results in serological tests. Further studies involving reaction of sera from naturally infected and disease free horses with various isolates of *C. pseudotuberculosis* will determine which of these proteins have epitopes exposed at the surface that can be useful for diagnostic purposes. Mapping of immunodominant proteins would help to identify specific epitopes which could be the basis of newly developed serological assays based solely on antigenic oligopeptides, as developed for other pathogens. [129,205]

Conclusions

The use of a caprine ELISA test based on the combination of *C. pseudotuberculosis* biovar *ovis* exotoxin and cell wall antigens as solid phase for detection of antibodies in horses is not recommended. Development of an ELISA test with specific antigens from *C. pseudotuberculosis* biovar *equi* is needed. We provided evidence that bacterial antigens recognized by antibodies from sera of experimentally infected horses differed from sera before inoculation and from biovar *ovis*. Further investigation of the possible role of these antigens in pathogenesis and immunity is warranted in order to find the most appropriate immune-dominant antigens that can be used to develop an accurate serological test for horses that can help to improve the diagnosis of pigeon fever and control the disease extension to non-endemic regions.

Footnotes

a. Pan American Veterinary Laboratories, Hutto, TX

b. California Animal Health and Food Safety (CAHFS-Davis), University of California, Davis, CA

c. Excel 2010, Microsoft, Redmond, WA

d. Sigma-Aldrich, St. Louis, MO

e. Sonic Dismembrator, Model 500, Fisher Scientific

f. DC Protein Assay, Bio-Rad Laboratories, Hercules, CA

g. Mini-Protean TGX Precast Gels, Bio-Rad Laboratories, Hercules, CA

h. BioTrace, Life Sciences, Pall Corporation, Port Washington, NY

i. Santa Cruz Biotechnology, Inc., Dallas, TX

j. ChemiDoc-It^{TS2} Imager, UVP, LLC. Upland, CA

k. ImageJ, Open Source, Public Domain

l. SPSS, IBM, Armonk, NY

m. JMP, SAS 9.2, SAS Institute Inc., Cary, NC

Chapter 6: Summary and Conclusions

Corynebacterium pseudotuberculosis infection in horses is a re-emerging disease in the United States. The disease can no longer be considered limited to the western United States due to the recent increase in the number of cases and spread to regions not previously considered endemic. This disease is considered as one of the most economically important equine infectious diseases of horses in some endemic areas such as California and has caused important economic losses to the equine industry of historically non-endemic regions with recent outbreaks in Florida leading to cancellation of equestrian events.[4,10,12] In order to limit the spread of the disease, several aspects of the epidemiology of *C. pseudotuberculosis* infection in horses need to be elucidated, including investigation of vector transmission and the development of accurate serological assays.

The overall purpose of the investigations reported in this dissertation was to study different aspects of the epidemiology of *C. pseudotuberculosis* infection in horses that could provide practical information towards limiting further spread and controlling this disease in horses. Our specific objectives were first, to determine the role of house flies (*Musca domestica* L.) in the transmission of *C. pseudotuberculosis;* and second, to provide further information to aid in the development of a more accurate equine serological diagnostic assay.

Results from our studies support the hypothesis that the house fly is a potential vector of pigeon fever. Our first study investigated the optimal laboratory protocol to inoculate house flies with *C. pseudotuberculosis* biovar *equi*; including the most appropriate inoculation time and media, that could be used for future studies. Three different preparations of blood agar supplemented with dextrose and colonized with *C. pseudotuberculosis* were investigated for

inoculation at different time-points. It was determined that a 10-minute exposure to the bacteria was enough to inoculate the flies and that a protocol consisting of a 30-minute exposure to a blood agar plate colonized with the bacteria and moistened with 10% dextrose was the most convenient protocol. In addition, we evaluated if the duration of bacterial retention in house flies after exposure was long enough to have biological significance. We demonstrated that house flies harbored live bacteria for up to 24 h following a 30-minute exposure in the laboratory; therefore, flies could potentially spread the disease over a wide geographic area, making the house fly a plausible mechanical vector for dissemination of this bacteria and transmission among horses. Future research would be necessary to evaluate for how long naturally inoculated flies harbor the bacteria and where the flies carry the bacteria, on external parts or internal organs.

Our second study evaluated the role of house flies as mechanical vectors of *C. pseudotuberculosis* biovar *equi* in ponies experimentally exposed to house flies inoculated with the bacteria using the protocol we had previously stablished. We demonstrated that the ponies exposed to inoculated house flies developed local clinical signs of infection, had an increase in blood inflammatory markers, and in serological titers. *Corynebacterium pseudotuberculosis* was cultured from the purulent material collected from the site of infection. All these findings supported that house flies are mechanical vectors for *C. pseudotuberculosis* infection in horses. Furthermore, the experimental equine model we developed in these studies could potentially be used in future epidemiological investigations regarding other insects, such as blood sucking flies, or to test the effectiveness of vaccination or novel therapies.

In order to control the spread of the disease, accurate diagnosis of the disease is essential to stablish measures to limit bacterial transmission. Definitive diagnosis of *C.*

pseudotuberculosis infection in horses is based on bacteriological culture. Serological diagnostic tests are primarily used to help diagnose the internal form of the disease because samples for culture are usually not available and clinic-pathological signs are non-specific. Serology using the synergistic hemolysis inhibition (SHI) test, which detects antibodies against *C. pseudotuberculosis* phospholipase D (PLD) exotoxin, is the only commercially available test in horses; however, its accuracy has not been rigorously evaluated.

Our third study investigated the seroprevalence and risk factors for detectable antibodies against *C. pseudotuberculosis* using the SHI assay in the non-endemic equine population of Alabama. During the process of selection of ponies for our experimental study, multiple animals that had not lived outside Alabama (a non-endemic state) tested positive using SHI serological titers, despite no known history or clinical signs of the disease. This was further confirmed by our study of 342 equine serum samples from 40 Alabama counties, which revealed that the prevalence of detectable SHI titers (≥ 1:8) was 52.5%. In addition, higher titers were detected in a small number of horses; with titers ≥1:128 detected in 2.63% and titers ≥1:512 detected in 0.3% of the sampled population. The analysis of risk factors associated with increasing SHI titers revealed association with age, breed, and ruminant exposure. Despite our hypothesis, previous travel was not associated with increasing SHI titer. The combination of a high prevalence of detectable titers in a non-endemic population and the possible false positive results caused by cross-reaction with antibodies against phospholipases from *C. pseudotuberculosis* biovar *ovis*, calls into question the validity of low detectable SHI titers for the diagnosis of pigeon fever in horses. Further evaluation of SHI cut-off titers and accuracy is warranted to reduce the risk of a false positive diagnosis.

The development of a more accurate serological diagnostic test for horses is necessary for diagnosis of clinical cases, detection of subclinical cases, reduction of the spread of the disease, and epidemiological investigations. Therefore, in order to further investigate the serological diagnosis of this disease in horses, our last study compared the performance of the SHI test with an ELISA test based on exotoxin and cell wall *C. pseudotuberculosis* biovar *ovis* antigens. We chose this type of ELISA because it was commercially available for goats and it has been shown to have greater accuracy than SHI in diagnosis of caseous lymphadenitis in small ruminants. Since both biovars have high genetic homology, we hypothesized that antibody detection could be performed with this test in horses. However, our results showed that despite good correlation between the results obtained with both tests in serum samples from the ponies experimentally infected from previous studies, the agreement between both tests was poor. Therefore, it was concluded that this specific ELISA test cannot be recommended for diagnosis in horses. Analysis of the immunological response induced by each of the antigens used in this ELISA by immunoblot, showed that the cell wall antigen did not induce an appropriate signal. Further research is needed to stablish if addition of a cell wall antigen from *C. pseudotuberculosis* biovar *equi*, in addition to a PLD antigen, would provide greater results in an ELISA test for horses.

Since the immune-dominant antigens that evoke a detectable humoral response have not been determined in horses, we analyzed the immune-reactive proteins from both *C. pseudotuberculosis* biovars in horses. The study of the signal from bacterial whole cell lysate proteins separated by SDS-PAGE and incubated with equine serum provided evidence that *C. pseudotuberculosis* biovar *equi* antigens recognized by antibodies from sera of experimentally infected horses differed from sera before inoculation and differed from the antigens recognized from biovar *ovis*. Further investigation of the identity of these antigens by using more advanced

techniques such as 2D-PAGE separation and MS analysis, is necessary in order to find the most appropriate immune-dominant antigens that can be used to develop an accurate serological test for horses. Further work in warranted to understand the possible role of these antigens in pathogenesis and immunity in horses.

In conclusion, different aspects of the epidemiology of *C. pseudotuberculosis* infection in horses were studied during the progress of this dissertation. Evidence was provided to support the role of house flies as mechanical vectors of this bacteria in horses, which justifies emphasizing insect control measures on equine farms where the disease is present to limit its spread. In addition, further work is needed to improve the diagnosis of pigeon fever in horses, both for individual cases and for future epidemiological studies, as our results showed that the current serological test, the SHI test, could lead to false positive results which potentially can be attributed to cross-reaction from *C. pseudotuberculosis* biovar *ovis* antigens. Furthermore, the experimental equine model developed could be used to test the efficacy of vaccination in horses. We believe that the results of these studies can help to improve the diagnosis and control measures of this disease in order to prevent further extension to non-endemic regions or decrease its prevalence in endemic areas.

Appendix: Research project at the University of Nottingham:

In vitro* evaluation of cytokine production in response to co-infection of equine influenza virus and *Streptococcus equi* subsp. *zooepidemicus.

Scope of this project

In order to further develop my molecular biology skillset and expand my ability to investigate infectious diseases in horses, a collaboration with Dr. Janet Daly from the University of Nottingham was arranged, which allowed me to participate in a research project related with equine influenza virus.

Despite the change of research topic from *C. pseudotuberculosis* towards equine influenza virus and secondary bacterial infection with *Streptococcus* spp.; this represents a continuation of my investigation of equine infectious pathogens, which is my main research interest. The shift in research topic will allow me to continue with this line of investigation and use my skillset during my transition to a faculty position in Europe. Viral respiratory infection and bacterial pneumonia are common diseases encountered worldwide in equine internal medicine practice.

Understanding the role of the innate immune response in cases of co-pathogenesis is essential in order to develop new strategies to prevent and treat these diseases in horses. Furthermore, the development and investigation of new *in vitro* models to study respiratory pathogens and their interactions with the host are needed in equine infectious disease research, and results from these investigations can have a human public health impact since zoonosis from these pathogens can occur.

During this research experience, I developed skills in techniques including cell culture, DNA sequencing, conventional PCR, quantitative PCR, ELISA, and other bacteriological and virological culture techniques. The results obtained during this period will be submitted as a conference abstract and will constitute preliminary results that will be used when I apply for research funding in the future.

Abstract

Equine influenza virus (EIV) is considered one of the leading causes of infectious respiratory diseases in equids worldwide. Often, the worst disease outcomes are associated with secondary bacterial pneumonia caused primarily by *Streptococcus equi* subsp. *zooepidemicus* (SEZ). The mechanisms of synergism between respiratory viruses and opportunistic pathogenic bacteria remain unknown and effective *in vitro* models may help provide answers to these questions. The objective of this study was to investigate the innate immune response induced by different EIV strains and co-infection with SEZ *in vitro* in DH82α cells, a canine macrophage-like cell line. Cells were infected with Sussex/89 (full length non-structural protein 1 (NS1)) or Kentucky/5/02 (truncated NS1) strains of EIV, and the mRNA cytokine expression was measured by quantitative (qRT)-PCR. Secondly, the effect on SEZ growth and cellular cytokine responses was investigated in cells co-infected with EIV Kentucky/5/02 and SEZ 1h or 24h apart and it was compared to Kentucky/5/02- and SEZ-mono-infected cells. Data were analyzed by one-way analysis of variance and Tukey's multiple-comparison test. A higher expression of TNFα and IL6 was observed in Sussex/89 infected cells compared to Kentucky/5/02 at 48h post-infection (P=0.046 and P=0.015 respectively). Cells infected with Kentucky/5/02 had higher IFNβ expression at 48h (P=0.013) and IFNα expression at 24h post-infection (P=0.02) compared to Sussex/89. Cells co-infected with Kentucky/5/02 and SEZ showed a higher expression of TNFα 48h post-bacterial infection, especially if co-infected 24h apart. Lower bacterial growth in the supernatants of cells co-infected with Kentucky/5/02 and SEZ 24h apart was observed 48h post-infection compared to cells mono-infected with SEZ or cells co-infected with SEZ and Kentucky/5/02 only 1h apart. Differences in the cytokine response was observed when DH82α cells were infected with different EIV strains or were co-infected with EIV and SEZ, which

agree with previous results observed in other studies performed in other models. These preliminary results suggest that DH82α cells can be used as an *in vitro* model to investigate cytokine production after EIV infection; however further evaluation is needed to confirm the relevance of these results.

Introduction

Equine influenza virus (EIV), an enveloped virus with a segmented, single-stranded, negative-sense ribonucleic acid (RNA) genome from the Orthomyxoviridae family, is considered one of the leading causes of infectious respiratory diseases in equids worldwide.[206,207] The influenza viruses currently circulating among horses are of H3N8 subtype, with Florida sub-lineage clade 1 predominating in America and clade 2 predominating in Europe.[208] Despite intensive vaccination programs in some horse populations, equine influenza virus remains a serious health and economic problem throughout most parts of the world.[209] Since 2000, several widespread EIV outbreaks have occurred in different parts of the world [210–212] and the global transportation of horses has been responsible for numerous outbreaks by introducing the virus into previously unexposed horse populations.[213]

Often, the worst disease outcomes associated with EIV infection are related to secondary bacterial pneumonia, and one of the bacteria most commonly involved is *Streptococcus equi* subps. *zooepidemicus* (SEZ).[214,215] The co-pathogenesis between respiratory viruses and opportunistic pathogenic bacteria is characterized by complex interactions between the co-infecting pathogens and the host. Influenza virus enhances bacterial colonization in several ways including decreased airway function, epithelial damage, upregulation and exposure of receptors, and alteration of the innate immune response.[216]

Different factors of the innate immune response have been investigated in animal models. Both bacteria and viruses are recognized by toll-like receptors (TLRs), which lead to activation of signaling cascades and the generation of an inflammatory response.[216] *Streptococcus* spp. are recognized by TLR-2 and TLR-4, leading to NF-kB activation, which leads to production of pro-inflammatory cytokines and chemokines such as interleukin (IL) -1, IL-6, tumor necrosis factor

alpha (TNF-α), and gamma interferon (IFN-γ); as well as the anti-inflammatory cytokine IL-10. Influenza virus is recognized through TLR 3, TLR7, and TLR8, which activate similar pathways leading to the same cytokine expression.[216] The use of the same pathways, and the overlap in the inflammatory mediators produced, creates an opportunity for either interference with or augmentation of the immune response during dual or sequential infection. In mice, an unbalanced elevation of both pro-inflammatory and anti-inflammatory cytokines after influenza virus and pneumococcus co-infection leads to massive influx of neutrophils and macrophages that invade the lung, cause inflammatory damage, but do not effectively clear the bacteria.[217] Influenza-induced type I IFN has been shown to induce susceptibility to Gram-positive and Gram-negative bacterial pneumonia in mice,[218–220] which has been associated with attenuation of the production of neutrophil chemoattractants impairing neutrophil recruitment and inability to efficiently resolve pneumococcal superinfection.[221–223] TLR downregulation occurs after influenza infection which leads to decreased bacterial clearance.[224] In mice, reduced expression of TLR-4 coincided with increased lung pneumococcal burden, reduced production of neutrophil and macrophage chemoattractants and growth factors, and reduced neutrophil recruitment.[220] The association of post-influenza innate immune response with secondary bacterial infection has not been investigated in horses.

The predisposition to bacterial pneumonia after influenza infection appears also to be strain dependent.[225] In humans and animal models, several virulence factors that are expressed by the virus have viral strain-specific effects on the host that enable bacteria to cause disease, such as low glycosylation of hemagglutinin, high neuraminidase activity, or expression of proapoptotic proteins.[225] In addition, the virulence factor non-structural protein 1(NS1) has been associated with an important role in modulating host antiviral response, including TNF-α, IL6,

and type I IFN.[226,227] Ponies experimentally infected with EIV developed more pronounced clinical signs and higher cytokine response, particularly IFN and IL-6, with a more virulent strain (Sussex/89) than with Newmarket/2/93.[228] Equine influenza viruses that belong to the Florida clade 2 are characterized by an 11-amino acid C-terminal truncation of the NS1 protein; Kentucky/5/02 was the first strain that possessed this truncation.[229,230] There is little information regarding the effect of influenza virus strain virulence and NS1 truncation on the effect of host immune response and its association with secondary bacterial infection in horses. Further investigations are needed to elucidate the complex interactions between EIV and opportunistic bacteria.

In order to study the pathogenesis and interactions between these equine respiratory pathogens, a relevant in vitro equine model is necessary to respect the principle of the 3Rs (replacement, refinement, and reduction of animals in research). Limited continuous equine cell lines are available and these are not necessarily permissive to infection by EIV (Daly, unpublished data). The DH82α canine cell line is a macrophage-like cell line that has been used as a model for replication of other equine viruses[231] and can be infected by EIV. Therefore, the DH82α cell line was studied as a potential model for immune response investigation after EIV infection.

The overall purpose of this study was to investigate the innate immune response induced by different EIV strains and co-infection with SEZ *in vitro*. The first objective was to investigate the cytokine response against infection with two EIV strains with main differences in the C-terminal region of the NS1 protein. The second objective was to determine the effect of co-infection with EIV and SEZ compared to EIV- and SEZ-monoinfection on SEZ growth and cytokine responses *in vitro*.

Material and Methods

Cell culture

DH82α cells, a canine macrophage-like adherent cell line, were routinely grown at 37ºC in a 5% CO_2 atmosphere. Cell culture medium was Dulbecco's Modified Eagle Medium (DMEM)[a] supplemented with L-Glutamine (2mM)[f,] penicillin (100IU/ml)[b], streptomycin (100µg/ml)[b], and fetal bovine serum (FBS)[b] (10% v/v) unless otherwise stated. Confluent monolayer cultures of cells were prepared in 24-well tissue culture polystyrene plates 24-48h prior to each experiment by seeding 2 x 10^5 cells/well. Each experiment consisted of 3 replicates of each treatment group.

Virus source and techniques

Two equine influenza A virus strains isolated from outbreaks in horses, Kentucky/5/02 and Sussex/89, were obtained from the University of Nottingham repository. The viruses were grown in embryonated hens' eggs. Three different dilutions (1:10, 1:100 and 1:1000) of each virus strain were inoculated (0.1ml/egg) into the egg allantoic cavity 2-3mm below the line of the air sac. The allantoic fluid was harvested 72h after inoculation and centrifuged (2500rpm for 5min) to purify the virus. A haemagglutination assay was performed on the harvested allantoic fluid to confirm growth of virus at each dilution of inoculum. Briefly, two fold dilutions of the virus were incubated with 0.5% chicken red blood cells and the titer was calculated as the reciprocal of the highest dilution that induced haemagglutination of the red blood cells. Allantoic fluid with a titer <1:32 was discarded. Allantoic fluid with a titer > or equal to 1:32 for each viral strain was pooled and aliquots of the virus stock were stored at -80°C until use.

The 50% Tissue Culture Infective Dose ($TCID_{50}$) of the virus stock was calculated by $TCID_{50}$ titration method on DH82α cells. Briefly, for each sample, 10-fold serial dilutions were prepared, and of each dilution, 100ul per well was added to confluent DH82α cells in a 96-well plate; with 4 replicates per dilution. Plates were incubated for 5 days, and the wells were analyzed for virus-induced cytopathic effects and stained with crystal violet (2%) after ethanol fixation. The $TCID_{50}$ represents the endpoint dilution required to produce cytopathic effects in 50% of the wells. Calculation of the $TCID_{50}$ was performed following this formula:

$$LogTCID_{50} = L - d\ (S - 0.5)$$

Where L is initial log dilution of virus, d is the distance between successive log dilutions, and S is the sum of the proportion of wells testing positive.

The identity of the viruses was confirmed by sequencing of the HA and NS1 genes. Viral RNA was extracted from the 100µL of each strain of virus stock following the manufacturer's instructions of a commercial kit for RNA isolation.[c] A reverse transcription reaction was performed to obtain complementary DNA (cDNA) from the extracted RNA as described below. An end-point PCR with the cDNA was performed as described below. The PCR product was run in a 1% agarose gel to confirm the reaction (Figure A.1). PCR products generated were purified using a PCR purification kit [d] according to manufacturer's instructions. Nucleic acid concentration was calculated using a spectrophotometer[e]. Sanger sequencing [f] of the HA and NS1 genes was performed using a set of 3 primers for the HA gene and 2 primers for NS1 (Table A.1). Analysis of the sequence and alignment results were performed and compared to published reference sequences using standard software[g].

Figure A.1: PCR products of the individual influenza genes. Lane 1 represents PCR amplification of NS1 and lane 4, HA. Lanes 2 and 5 represent the respective reverse transcription negative controls, and lanes 3 and 6 represent the respective non-template PCR negative controls.

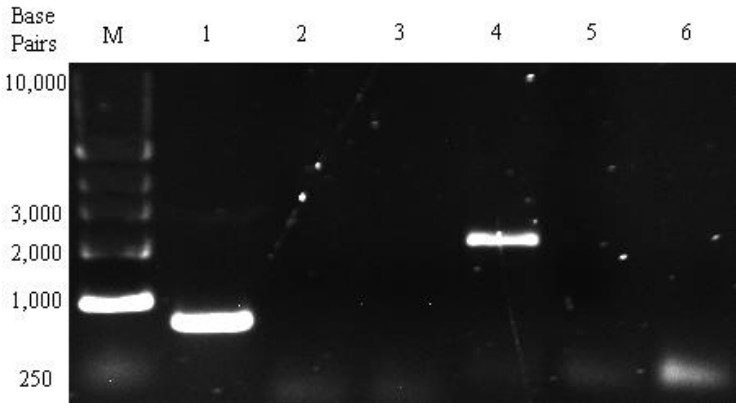


Table A.1: Primers used for PCR and sequencing of Influenza virus genes

Gene	Primer	Use	Sequence (5'→3')	Tm (°C)	Reference
HA from H3 Influenza A virus	H3HA1/1 (F)	End-point PCR Sanger Sequencing	AGCAAAAGCAGGGGATATTTCTG	61.5	Designed by Janet Daly
HA from H3 Influenza A virus	H3HA1/2 (R)	End-point PCR Sanger Sequencing	GCTATTGCTCCAAAGATTC	51	Designed by Janet Daly
HA from H3 Influenza A virus	HA F525	Sanger Sequencing	CTGAATTGGCTAACAAAATC	54	Designed by Janet Daly
NS1 from Influenza A virus	EQNS1F391	End-point PCR Sanger Sequencing	AAAGCAAACTTTAGTGTGATTTTCGAAAGG	68.8	Lee et al. 2016 [232]
NS1 from Influenza A virus	EQNS1R772	End-point PCR Sanger Sequencing	CATAAATGTTATTTGTTCAAAACTATTTTC	60.9	Lee et al. 2016 [232]

Bacterial sources and techniques

The D2a strain of *S. equi* subsp. *zooepidemicus* (SEZ) isolated from a horse with pneumonia was obtained from the Animal Health Trust repository. The identity of the bacteria was confirmed by sequencing the 16S ribosomal RNA gene (as described above).

The bacteria was grown in Columbia agar plates supplemented with 7% sheep blood under aerobic conditions at 37ºC overnight. Then, one colony was sub-cultured in Todd Hewitt Broth (THB) supplemented with 10% fetal bovine serum (FBS)[b] overnight until mid to late-log phase. An equal amount of 50% sterile glycerol was added to the bacterial solution (0.5ml of solution + 0.5ml of glycerol) and aliquoted in 1ml cryogenic screw-cap vials. The vials were stored at -80ºC until use.

A bacterial growth curve was calculated in order to correlate the optical density (OD) of bacterial solution and the approximate amount of colony forming units (CFU) present per ml of solution. Briefly, 100ml of pre-warmed (37ºC) THB supplemented with 10% FBS was inoculated with 2ml of an overnight culture of SEZ. Each hour, from time point 0 to 6 hours post-inoculation, the OD was measured and serial dilutions were performed in THB from 10-1 to 10-8. 50µl of each dilution was grown overnight on THA plates at 37ºC. The next morning, each individual colony was manually counted and the CFU/ml was calculated.

Infection with EIV

For viral infection, 500µl of 10^3 $TCID_{50}$/ml was inoculated per well of DH82 cells for each of the virus strains. The virus solution was a 100 fold dilution of the cryopreserved virus stock in FBS-free medium with penicillin/streptomycin and crystalline trypsin (1.25µg/ml). The plate was incubated for 1h, and then, the supernatant was discarded and replaced by fresh FBS-

free medium. For mock infection, the cells were inoculated with culture media without the pathogen.

Infection with SEZ

For infection experiments, cryopreserved bacteria were grown in THB broth overnight. The next morning, the bacteria were sub-cultured at a 1:50 dilution and grown for approximately 3h to early exponential growth phase (OD_{550} of 0.2, which should correspond to approximately 10^8CFU/ml) (Figure 3). Numbers of viable bacteria were determined by serial plating of serial dilutions (1:10) in THA agar plates and counting of colony forming units (CFU) the following day.

Firstly, an experiment to determine the best protocol for inoculation of DH82α cells with SEZ was performed. Three different bacterial inoculum doses were investigated: ~10^7 CFU/ml, ~10^6 CFU/ml, and ~10^5 CFU/ml. The cell culture plate was incubated for 1h and then the supernatants were discarded. The wells were washed with 500µl of PBS to remove non-attached bacteria and two types of medium replacement were investigated: FBS and antibiotic-free medium or FBS-free medium with penicillin and streptomycin. For mock infection, the cells were inoculated with culture media without the pathogen. Therefore, 7 treatment groups were investigated in duplicate. The supernatant was removed to measure bacterial counts as described above at 1, 2, 4, 6, 24, and 48h after washing.

Following identification of the optimum inoculation protocol, for bacterial infection of cells in later experiments, 1ml of ~10^5 CFU/ml SEZ was inoculated per well of DH82 cells. The bacterial solution was a 1000 fold dilution of the ~10^8 CFU/ml solution diluted in FBS and antibiotic-free medium. The plate was incubated for 1h and then the supernatant was discarded.

The well was washed with 500µl of PBS to remove non-attached bacteria and fresh FBS and antibiotic-free medium. Supernatants were removed and replaced by fresh FBS and antibiotic-free medium every 12 h.

Co-infection with EIV and SEZ

The cells that were co-infected were inoculated using the protocols described above for mono-infection. Cells were inoculated with EIV first as described, then incubated for 1h. After discarding the supernatant, the EIV infected cells were then infected with SEZ as described above, incubated for 1h, washed with PBS, and the supernatant replaced by fresh medium.

Treatment with LPS

Confluent cells were treated with 500ul/well of lipopolysaccharide (LPS) diluted in FBS-free medium at 100ng/ml solution. The LPS used originated from *Escherichia coli* 111:B4[b] and was used as a positive control to assure that DH82α cells produced cytokines.

Cell harvesting and supernatant collection for response testing

Prior to harvesting the cells, cell media were collected and stored at -20°C until used. Cells were harvested at different time-points post-infection with EIV and/or SEZ. Cells were trypsinized with 500µL of 1X trypsin/EDTA, centrifuged at 11,000g for 1 min, and re-suspended in 100µL of PBS. Then, 500µL of RNALater[b] was added. The cell solution was frozen at -20°C until used. For processing after thawing, 500µl of PBS was added per sample and centrifuged at 16,000 g for 1 min to remove the RNA preservative. The cell pellet was re-suspended in β-mercapthoethanol and lysis buffer from the RNA isolation kit[c]. Following the manufacturer's

instructions, the cells were lysed and RNA was extracted. Concentration of RNA was measured by a spectrophotometer[e] and RNA was stored at -20°C until used.

Reverse transcription

The same protocol was followed for viral and cell RNA samples. Samples and reaction mixes were prepared in DNAse/RNAse-free PCR tubes and filtered DNAse/RNAse-free tips[h], for all PCR techniques. Reactions with the omission of RNA and SuperScript IV reverse transcriptase (RTase)[a] were set up as negative control samples. Master mixes were prepared in the following way and scaled up as appropriate according to the number of reactions required:

Reverse transcription mix:

11 µL RNA

1 µL 10 mM 2'-deoynucleoside5'-triphosphate (dNTP mix)

1 µL 50 µM Oligo d(T)20

13 µL total volume

The reverse transcription mix was heated for 5 min at 65°C and then hold on ice for 1min. The following reagents[a] were then added to each sample tube:

4 µL 5X RT buffer

1 µL 0.1 M EDL-dithiothreitol (DTT)

1 µL RTase

Control Tubes:

1. Negative control: water instead of RNA
2. Genomic DNA contamination control: water instead of RTase

End-point polymerase chain reaction

Master mixes of the PCR reaction were prepared as appropriate according to how many samples were to be amplified. The negative controls from the RT reactions were included and further PCR negative controls were prepared such as a reaction with the omission of cDNA (NTC, non-template control). Primers used for all PCR applications are given in Table 1. Primer melting temperatures (Tm) were calculated by the manufacturer.[b]

PCR reaction mix (25 µL total volume)

5 µL cDNA

2.5 µL 10x Taq buffer [i]

1.5 µL 25mM $MgCl_2$

0.5 µL 10 mM dNTP mix

0.5 µL gene-specific 5' cRNA primer (10 µM)

0.5 µL gene-specific 5' vRNA primer (10 µM)

0.125 µL Taq DNA polymerase [i]

14.375 µL nuclease free water

The samples were placed in a thermo-cycler[j] and the following cycles were used:

- Initial denaturation 95°C for 30 seconds
- 30 cycles:
    - 95°C for 30 seconds
    - 56°C for 1 min
    - 68°C for 1 min
- Final extension at 68°C for 5 min and hold at 4°C

Agarose gel electrophoresis was performed to visualize PCR products in 1-2% agarose gels. DNA samples were prepared by diluting 2µl of loading buffer (0.04 % bromophenol blue, 0.015 % xylene cyanol FF, 10 % glycerol in 5 x TBE) per 5µl of sample, and loaded into the wells with DNA standards for determination of molecular size. The agarose gels were run at 80V for 45 min to 1 h and visualized using the long wave setting on a ultra-violet transilluminator.

Quantification of cell cytokine transcripts by qPCR

Real time quantitative polymerase chain reaction (qPCR) assay was carried out on a thermo-cycler[k] 480k using gene-specific primers to selected cytokines (IL-6, TNF-α, IFN-α, and IFN-β) reported in the literature (Table A.2). A panel of standard reference genes (18S ribosomal RNA, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), succinate dehydrogenase complex flavoprotein subunit A (SDHA), and hydroxymethylbilane synthase (HMBS)) were used as an internal controls (Table A.3).

The protocol was followed according to the manufacturer's instructions.[k] A 96-well-plate was set up using different master mixes for each set of primers for a 25µl reaction:

- 10µl SYBR Green Master mix[k]
- 7µl of water
- 1µl of forward primer
- 1µl of reverse primer
- 1µl of template: cDNA (sample), RNA (RTC, reverse transcriptase control for genomic DNA contamination) and water (NTC, non-template control)

Table A.2: Primers used for qPCR of canine target genes

Gene	Primer	Sequence (5'→3')	Tm (°C)	Reference
TNFα	F	ACCACACTCTTCTGCCTGCT	61.41	Designed by Janet Daly
	R	CTGGTTGTCTGTCAGCTCCA	59.61	Designed by Janet Daly
IL-6	F	TCTCCACAAGCGCCTTCTCC	62.17	Designed by Janet Daly
	R	TTCTTGTCAAGCAGGTCTCC	57.45	Designed by Janet Daly
IFNα	F	TGGGACAGATGAGGAGACTCTC	60.36	Jassies-van der Lee et al. 2014 [233]
	R	GAAGACCTTCTGGGTCATCACG	60.68	Jassies-van der Lee et al. 2014 [233]
IFNβ	F	CCAGTTCCAGAAGGAGGACA	58.65	Park et al. 2015 [234]
	R	TGTCCCAGGTGAAGTTTTCC	57.64	Park et al. 2015 [234]

Table A.3: Primers used for qPCR of canine reference genes

Gene	Primer	Sequence (5'→3')	Tm (°C)	Reference
GAPDH*	F	GAGAAAGCTGCCAAATATG	51.91	Designed by Janet Daly
	R	CCAGGAAATGAGCTTGACA	55.09	Designed by Janet Daly
18S rRNA[1]	F	TGCTCATGTGGTATTGAGGAA	57.0	Peters et al. 2007 [235]
	R	TCTTATACTGGCGTGGATTCTG	57.68	Peters et al. 2007 [235]
SDHA[1]	F	GCCTTGGATCTCTTGATGGA	56.98	Peters et al. 2007 [235]
	R	TTCTTGGCTCTTATGCGATG	55.92	Peters et al. 2007 [235]
HMBS[1]	F	TCACCATCGGAGCCATCT	57.62	Peters et al. 2007 [235]
	R	GTTCCCACCACGCTCTTCT	59.63	Peters et al. 2007 [235]

The plate was centrifuged at 2000rpm for 2min. A 3-step cycling protocol was followed. First, the plate was heated at 95°C for 2 minutes for polymerase activation. Then the mix was denatured at 95°C for 5 seconds, the primers were annealed at 60°C for 10 seconds, elongated at 72°C for 10 seconds, and fluorescence was read. This was repeated for 40 cycles. Melting curve analysis was performed from 65-95°C with plate read every 2.2°C increase in temperature.
A PCR reaction was run for each sample resulting in 3 crossing points (Cp) values for each treatment group. The Second Derivative Maximum method was followed to determine Cp. A mean Cp value was calculated for each group using these values. Relative expression of each gene was calculated using these Cp values.

The geNorm VBA applet for Microsoft Excel[l] was used to determine the most stable genes from the set of tested genes.[236] The geNorm program calculates stability and assumes that the two genes whose expression is most correlated are the most appropriate and a normalization factor can be calculated. The program defines a stability measure (M) as the average pairwise variation between a gene and all other control genes, genes are then ranked from best to worst on the basis of the M values.

Real-time PCR efficiencies (E) were calculated from the slopes given by LightCycler software according to the equation: E = 10[-1/slope] using an online calculator[m] (Table 4) The mean Cp value was converted to a relative copy number value using the EΔCt method (E stands for reaction efficiency) which compensate for differences in target and reference gene amplification efficiency.[237] ΔCp with values calculated relative to the reference genes GAPDH/HMBS with the following equation:

$$ratio = \frac{(E_{target})^{\Delta CP_{target}}}{(E_{ref})^{\Delta CP_{ref}}}$$

Quantification of cell cytokine protein production by ELISA

Direct quantification of cytokines was measured in stored supernatants with commercially available ELISA kits following manufacturer's instructions.[n] Briefly, 96-well plates were coated overnight with mouse anti-canine TNFα or IL-6 capture antibody. Wells were blocked with 1% BSA for 1h, then the samples were incubated for 2h and followed by incubation of biotinylated goat anti-canine TNFα or IL6 for 2h. Streptavidin conjugated to horseradish-peroxidase was incubated for 20 min and the substrate solution (H_2O_2 and tetramethylbenzidine mix) was added and incubated for another 20 min. Then the stop solution (2N H_2SO^4) was added and the optical density was measured using a microplate reader set at 450nm. Standard curves were calculated with the recombinant canine TNFα standard provided in the kit. The mean absorbance values from the standards prepared were used to calculate the concentration of cytokines in samples.

The ELISA kits used had already been used a few months prior, and some reagents were not conserved following manufacturer's instructions. It was decided to perform the tests, knowing that the performance of the test could be altered.

Bacterial growth in supernatants

Bacterial replication and growth kinetics was determined by duplicate plating of pooled supernatants freshly on THA agar plates at the different sample collection time points. Colony forming units were manually counted the following day and the CFU/ml were calculated.

Statistical analysis

Experiments were performed three times, and results were expressed as means and standard deviations (SD). Data were analyzed for fulfillment of the normal distribution assumption by graphical assessment of histograms and Q-Q plots. The cytokine mRNA expression of each group at each time-point was compared within treatment group over time and within a time-point among treatment groups by one-way analysis of variance (ANOVA) and Tukey's multiple-comparison test (normally distributed data) or Mann-Whitney-Wilcoxon test (non-normally distributed data) using a common statistical software package.[o] A P value of 0.05 or less was considered significant.

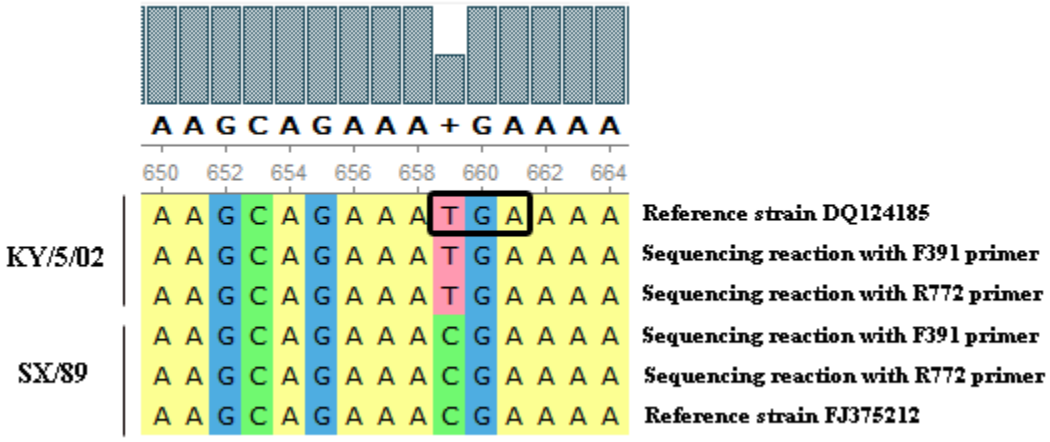
Results

Virus titer and sequencing

Allantoic fluid inoculated with 1:10 stock virus dilution had a hemagglutination assay titer of 1:16 for both viral strains, therefore it was discarded. Allantoic fluid inoculated with 1:100 and 1:1000 dilutions had a hemagglutination titer of 1:32, therefore it was pooled for virus stock formation for each strain. The $TCID_{50}$ obtained for the Kentucky/5/02 (K/5/02) strain was 105.25 $TCID_{50}$/ml and for the Sussex/89 (SX/89) strain was 105.75 $TCID_{50}$/ml.

The hemagglutinin (HA) gene of K/5/02 virus strain sequence had 99% identity when aligned with the sequence of the HA gene of the reference strain AY855341.1 (A/equine/Kentucky/5/2002 (H3N8)). The HA gene of SX/89 virus strain sequence had 99% identity with the HA gene of the reference strain KJ643906.1 (A/equine/Sussex/89(H3N8). The NS1 gene of K/5/02 virus strain had 100% identity with the NS1 sequence of DQ124185.1 (A/equine/Kentucky/5/2002(H3N8)). The NS1 gene of SX/89 had 99% identity with FJ375212.1 (A/equine/Sussex/89(H3N8)). The stop codon (TGA in cDNA, UGA in RNA) was observed due to a substitution at the position 659 with a Thymidine (T) (Uracil (U) in RNA); in the K/5/02 strain, leading to a truncated NS1 (Figure A.2). In contrast, the SX/89 had a Cytosine (C) at the position 659, leading to the codon CGA which encodes an arginine in the full length NS1 protein.

Figure A.2: Results of the sequence alignment of the NS1 gene of the reference strains and the virus strains grown for the experiments. A substitution at the position 659 is responsible for the appearance of a stop codon (framed bases in black) in the Kentucky/5/02 compared to Sussex/89, which is the cause of the truncation of the NS1 protein in the K/5/02 strain.



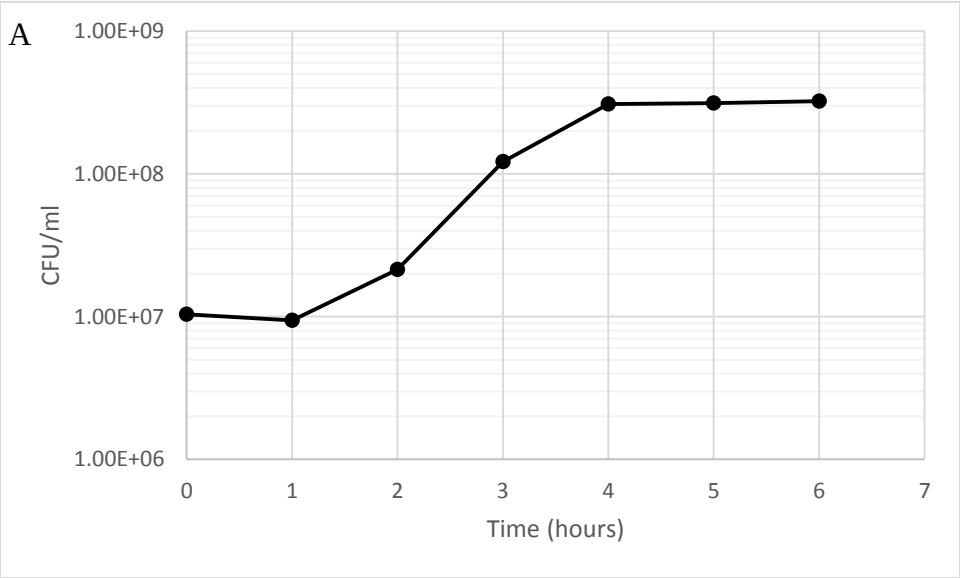
Bacterial growth kinetics

Streptococcus equi subsp. *zooepidemicus* was grown in THB at 37ºC and the CFU/ml and OD was measured every hour to produce a growth curve (Figure A.3). The bacteria grew logarithmically for 4 hours, after which they reached a stationary growth phase. After 3 hours of growth, the bacteria were actively growing, so the 3 hour time point and OD_{550} between 0.15 and 0.3 were used for preparation of bacterial solution for inoculation of cell culture, which approximately represented 10^8CFU/ml.

Optimization of bacterial inoculation dose

For the experiment to optimize the dose of bacterial inoculation, the OD_{550} at 3h of growth was 0.2; which corresponded to 6.6×10^8 CFU/ml measured by CFU counting the next day. Infection of DH82α cells with the two highest doses of bacterium ($6.6x10^7$ CFU/ml and $6.6x10^6$ CFU/ml) lead to complete cell death at 24h post-infection, whereas the lowest dose ($6.6x10^5$ CFU/ml), showed only partial cell death and lower bacterial counts (Figure A.4). However, at 48h post-infection the majority of cells were dead in the three groups. Replacement with fresh medium with antibiotics resulted in complete bacterial death, with subsequent negative cultures. Replacement with fresh medium without antibiotics allowed the growth of bacteria, but very high bacterial counts were obtained in the supernatant if the media was not replaced for 24h. Therefore, the best protocol to inoculate DH82α cells with SEZ was determined to be infection with the lowest dose of bacteria (~10^5 CFU/ml) and replacement of the supernatant with medium without antibiotics every 12h.

Figure A.3. A. Growth curve of *S. equi* subsp. *zooepidemicus* (SEZ) over time. B. Relationship between the OD read from bacterial solution at different times during the growth SEZ with the CFU/ml.



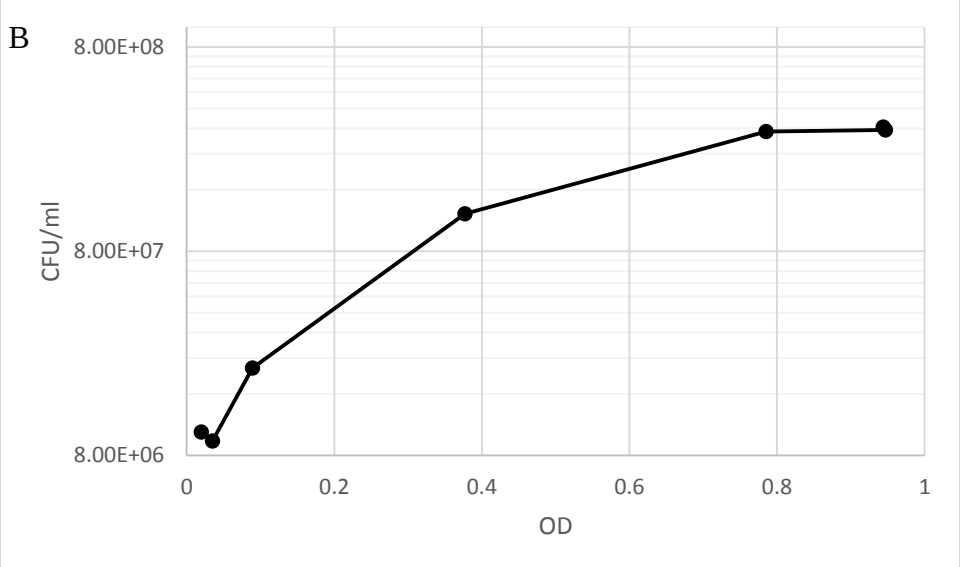
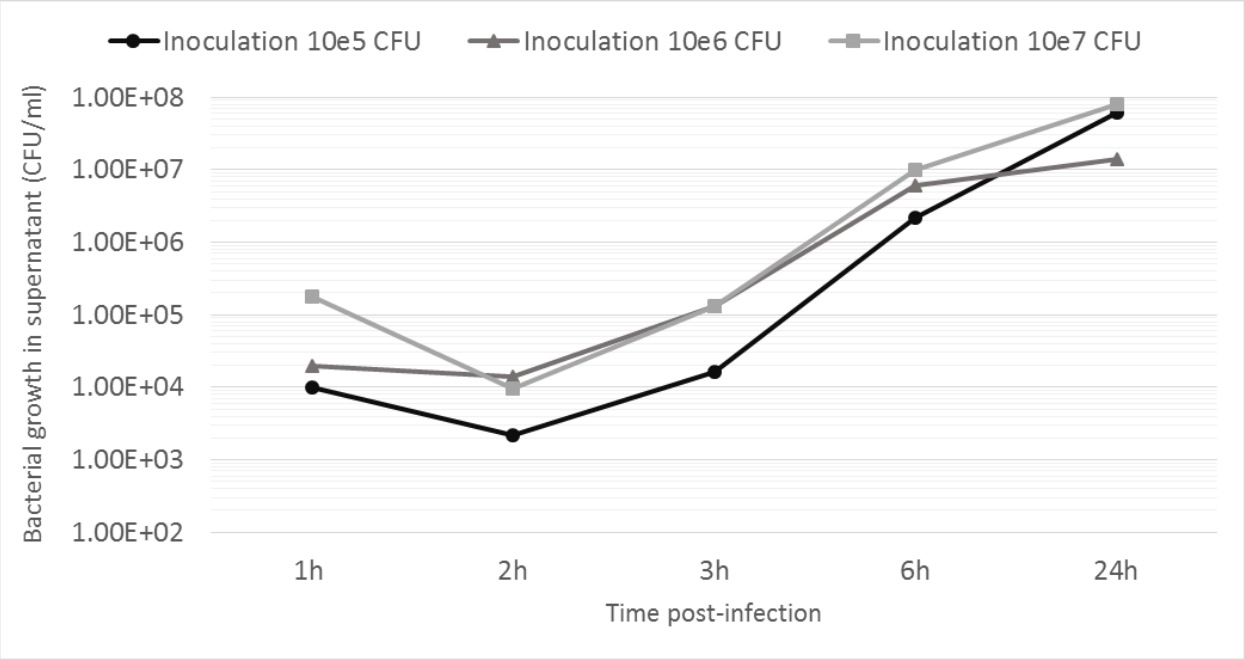


Figure A.4: Bacterial counts measured from supernatants of DH82α cells infected with different doses of *S. equi* subsp. *zooepidemicus* and had the supernatant replaced with medium without antibiotics after each time point.



Optimization of quantitative PCR

High amplification efficiency was obtained for the housekeeping genes and moderate for the target genes (Table A.4). Specificity of RT-PCR products was documented with 2% agarose gel electrophoresis and resulted in a single product with the desired length (Figure A.5 and A.6). In addition a melting curve analysis was performed which resulted in single product-specific melting temperatures. No primer-dimers were generated during the 40 real-time PCR amplification cycles in the target samples.

The stability of gene expression of the 4 reference (housekeeping) genes was evaluated over time and after LPS treatment (Figure A.7). The two reference genes most stably expressed were GAPDH and HMBS (Table A.5). The M value of the combination of these two genes was 0.032 and $M < 1.5$ is considered stable.[236] An average of the Cp values of these two genes was used to normalize the expression of the target genes for relative quantification in the following experiments. The stability of the 2 chosen reference genes was checked after virus infection, which remained stable, with an M value of 0.024.

Table A.4: Results from standard curve analysis by serial dilution of pooled cDNA samples for the different set of primers used in qPCR with the objective of calculating the amplification efficiency of each gene examined.

Gene	**Slope**	**Efficiency (%)**
GAPDH	-3.263	102.5
18S rRNA	-2.948	118.3
SDHA	-3.239	103.5
HMBS	-3.387	97.3
TNFa	-4.04	76.82
IL6	-4.319	70.43
IFNa	-3.755	84.63
IFNB	-3.575	90.42

Table A.5: Results of stability analysis of the reference genes.

Gene	Mean Cp	Cp Error	CV (%)	M value
GAPDH	20.628	0.394	1.911	0.099*
18S rRNA	21.978	3.698	16.830	0.240*
SDHA	26.875	1.054	3.922	0.100*
HMBS	26.367	0.847	3.214	0.100*
2 most stably expressed GAPDH/HMBS	23.497	0.588	2.502	0.032**

M values were calculated with GeNorm Excel app. *M value calculated utilizing the 4 housekeeping genes tested. **M value calculated considering only the 2 most stably expressed genes GAPDH and HMBS and eliminating 18S rRNA and SDHA from the algorithm.

Figure A.5. PCR products of the individual canine reference genes. Lane 1 represents PCR amplification of GAPDH; lane 3, 18S rRNA; lane 5, SDHA; lane 7, HMBS; and lanes 2, 4, 6, and 8 represent the respective non-template negative controls.

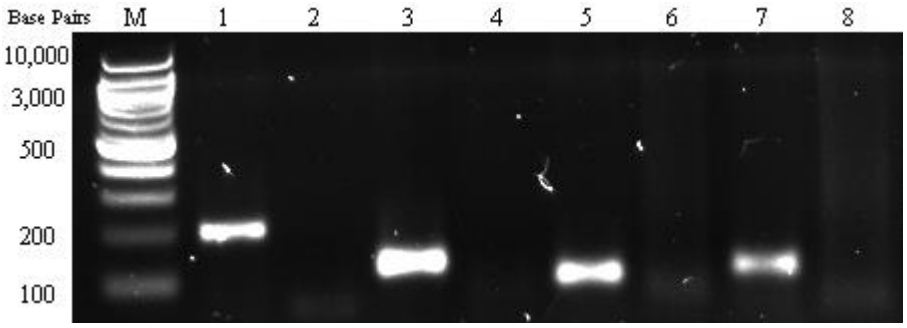
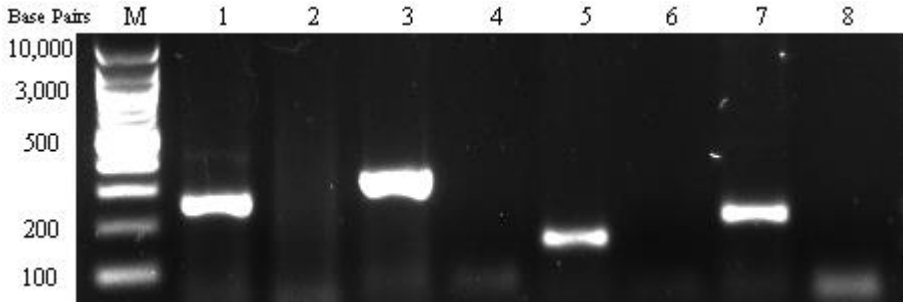


Figure A.6. Gel profile showing the PCR products of the individual canine reference genes. Lane 1 represents PCR amplification of TNFα; lane 3, IL6; lane 5, IFNα; lane 7, IFNβ; and lanes 2, 4, 6, and 8 represent the respective non-template negative controls.



Cytokine expression over time in DH82α cells infected with Sussex/89 and Kentucky/5/02

One initial experiment was performed to optimize the experimental protocol and cytokine expression was evaluated up to 6h post-infection. An increase of TNFα, IL6, and IFNβ was observed in the LPS-treated group after 1h post-wash compared to mock-infected cells, in which levels were already decreasing at 6h post-wash (Figure A.8). A delayed increase of IFNα was observed in LPS-treated cells, where the highest expression was detected at 6h post-wash. A significant increase of TNFα expression was observed in the cells infected with SX/89 6h post-infection compared to mock-infected ($P= 0.036$) or cells infected with K/5/02 6h post-infection ($P= 0.037$), however this increase was significantly lower than the increase observed in LPS-treated cells ($P< 0.001$)(Figure A.9). No significant increase of IL-6 expression was observed in virus infected cells compared to mock-infected cells (Figure A.10). No significant increase of IFNβ expression was observed in virus infected cells from 1h to 6h post-infection compared to mock-infected cells (Figure A.11). A significant decrease in IFNβ expression was observed in K/5/02 virus infected cells 4h post-infection compared to 1h post-infection or compared to mock-infected cells ($P= 0.029$ and $P= 0.034$, respectively). A significant decrease in IFNβ expression was observed in SX/89-infected cells 6h post-infection compared to mock-infected cells ($P= 0.499$). A significant decrease in IFNα expression was observed in K/5/02 and SX/89 virus infected cells 4h post-infection compared to mock infected ($P=0.034$ and $P=0.0499$ respectively) (Figure A.12). No significant difference in the IFNα was observed between the two viruses tested.

The same experiment was repeated, with evaluation of the expression of cytokines up to 48h post-infection. Regarding TNFα expression (Figure A.13), a mild increase in mRNA expression was observed in the mock-infected group over time, however this was not significant.

The LPS group showed an early significant increase in TNFα expression (P<0.0001). Both virus strains induced a significant increase in TNFα at 48h compared to 1h post-infection (K/5/02 P<0.0001 and SX/89 P=0.002), which was significantly higher for the SX/89 virus (P=0.046). A similar response was observed for IL-6 (Figure A.14). The LPS group showed an early increase in IL6 expression (P<0.0001). Both viral strains induced a late increase in IL6 compared to 1h (K/5/02 P=0.015 at 24h, SX/89 P<0.0001 at 48h), which was higher for the SX/89 virus at 48h (P=0.015). On the other hand, a different response was observed in the type I IFNs. A significantly higher late increase was observed in the K/5/02 group compared to SX/89 in IFNβ (P=0.013 at 48h) (Figure A.15) and IFNα (P=0.02 at 24h) (Figure A.16).

Protein concentrations over time in DH82a cells infected with Sussex/89 and Kentucky/5/02

TNFα protein was not detected in the cell supernatants of virus-infected cells; neither in the LPS treated cells. Protein concentration for IL-6 was detected in the LPS group treated group at 48h (1241.66pg/ml ± 643.8) but not at 24h. No IL-6 was detected in the Sussex/89 infected group but it was detected in the K/5/02 at 48h (583.78pg/ml ± 422.95), in the mock infected group at 48h (331.65pg/ml ± 351.08), and in the mono-SEZ infected group at 24h (6,040.2pg/ml ± 279.5) and 48h (4,785.5pg/ml ± 231.17).

Figure A.7. Reference gene expression stability over time in mock infected and LPS treated DH82 cells. The data is presented as mean and SD indicated by the error bars.

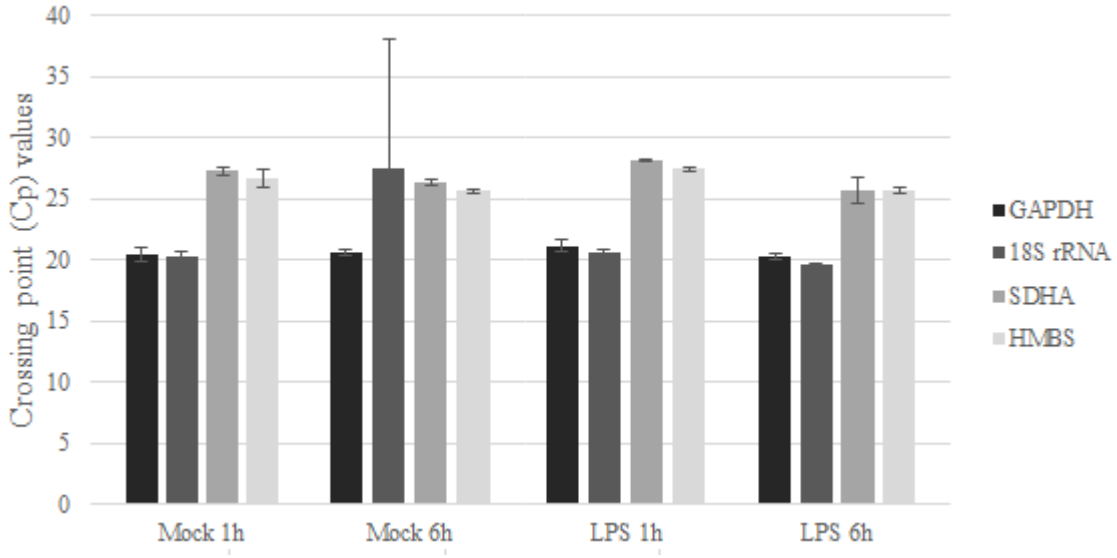


Figure A.8. Relative cytokine expression with respect to GAPDH/HMBS reference genes in mock-infected and LPS-treated DH82α cells over time. A. TNFα, B. IL6. C. IFNα D. IFNβ. The data is presented as mean and SD indicated by the error bars.

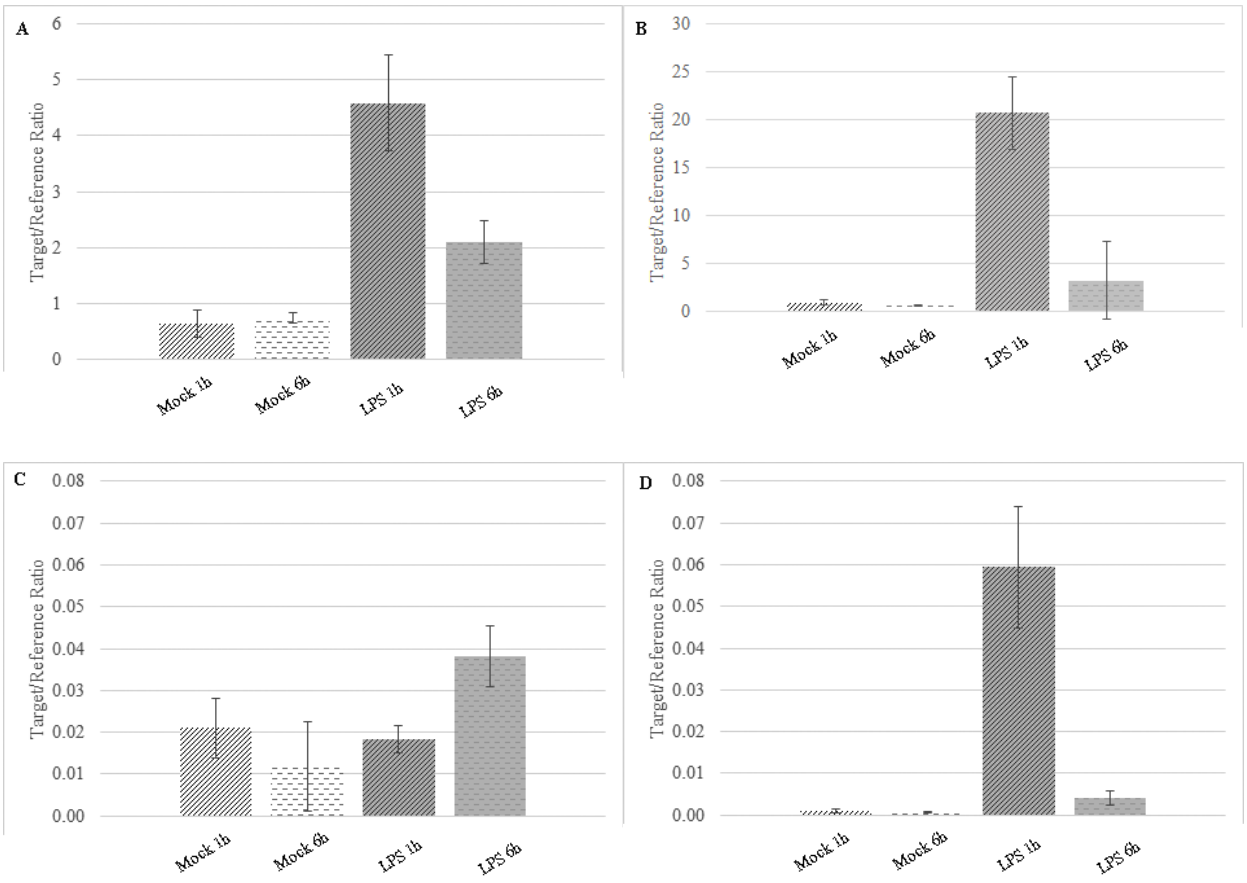


Figure A.9. Relative quantification of TNFα mRNA expression with respect to GAPDH/HMBS reference genes in virus infected cells over time. KY indicates cells infected with Kentucky/5/02 and SX indicate cells infected with Sussex/89. Significant results ($P \leq 0.05$) obtained from comparison with the mock infected group are indicated by *. The data is presented as mean and SD indicated by the error bars.

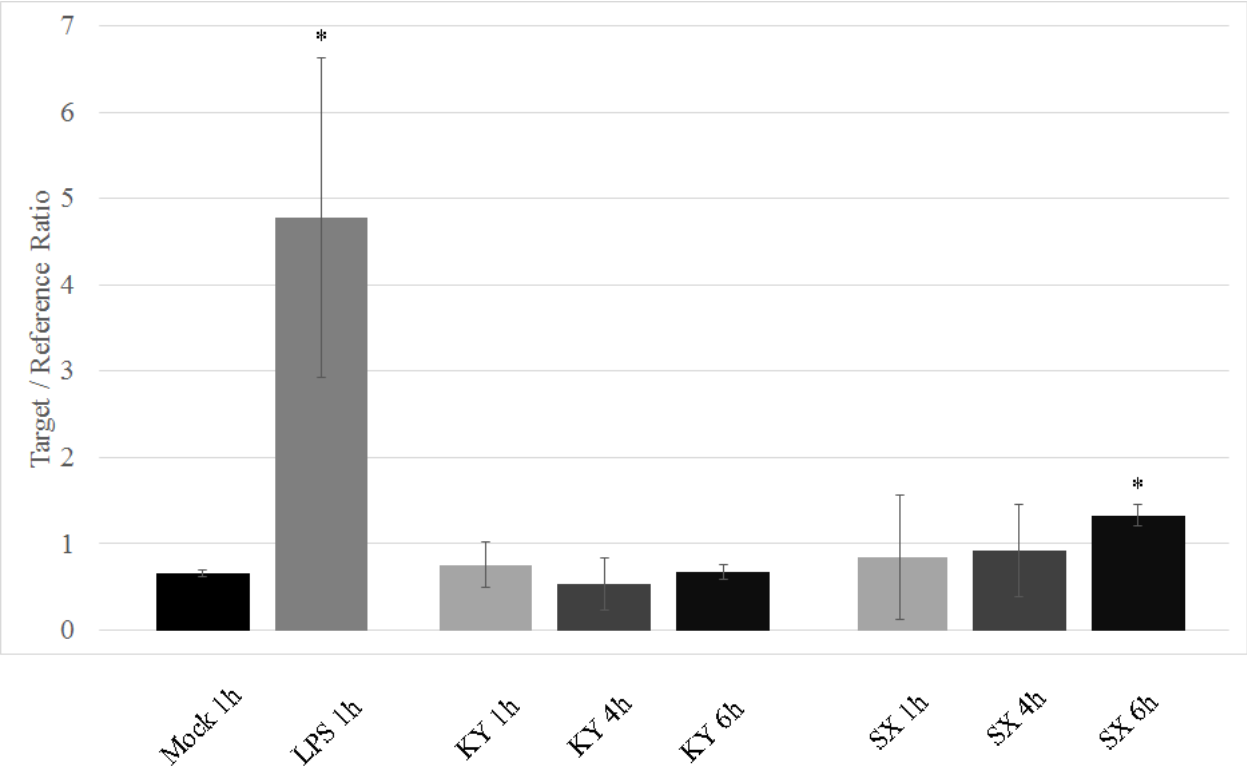


Figure A.10. Relative quantification of IL-6 mRNA expression with respect to GAPDH/HMBS reference genes in virus infected cells over time. KY indicates cells infected with Kentucky/5/02 and SX indicate cells infected with Sussex/89. Significant results ($P \leq 0.05$) obtained from comparison with the mock infected group are indicated by *. The data is presented as mean and SD indicated by the error bars.

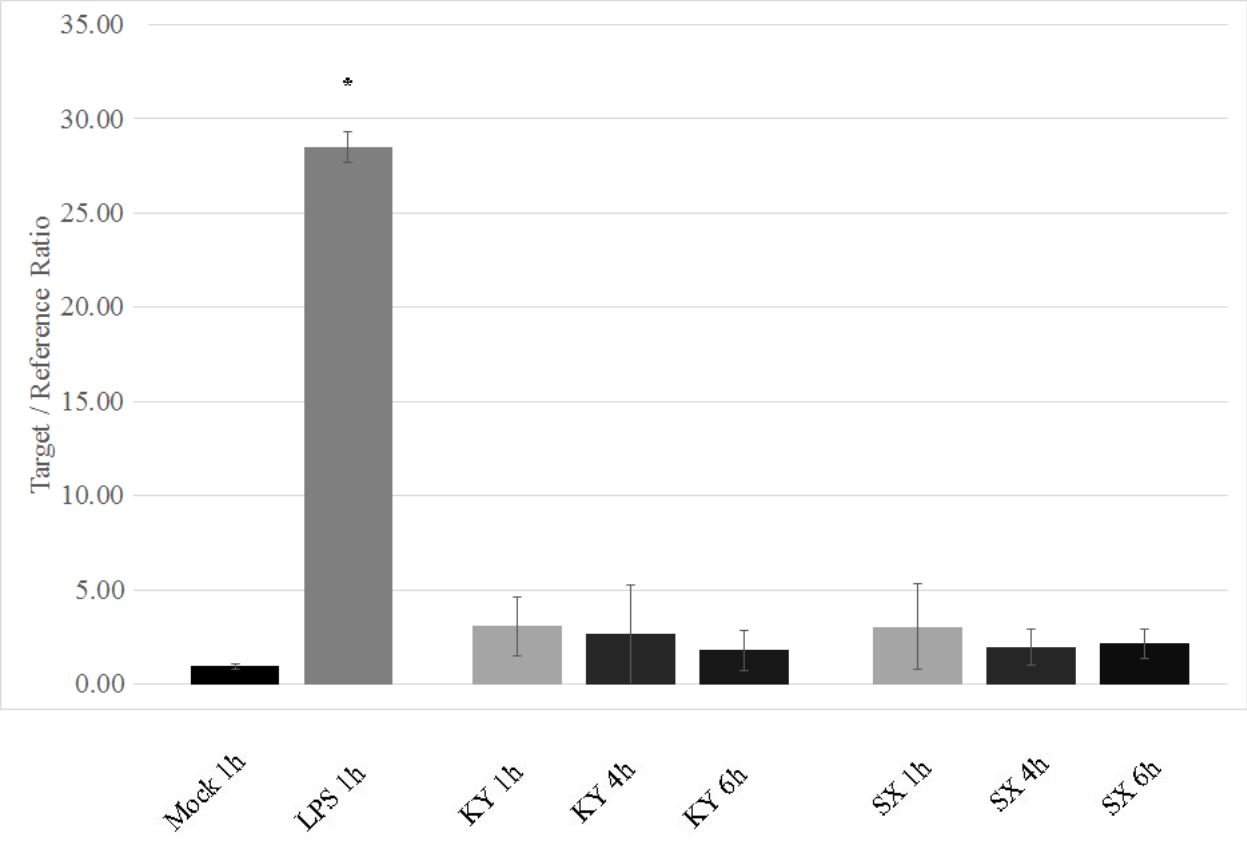


Figure A.11. Relative quantification of IFNβ mRNA expression with respect to GAPDH/HMBS reference genes in virus infected cells over time. KY indicates cells infected with Kentucky/5/02 and SX indicate cells infected with Sussex/89. Significant results ($P \leq 0.05$) obtained from comparison with the mock infected group are indicated by *. The data is presented as mean and SD indicated by the error bars.

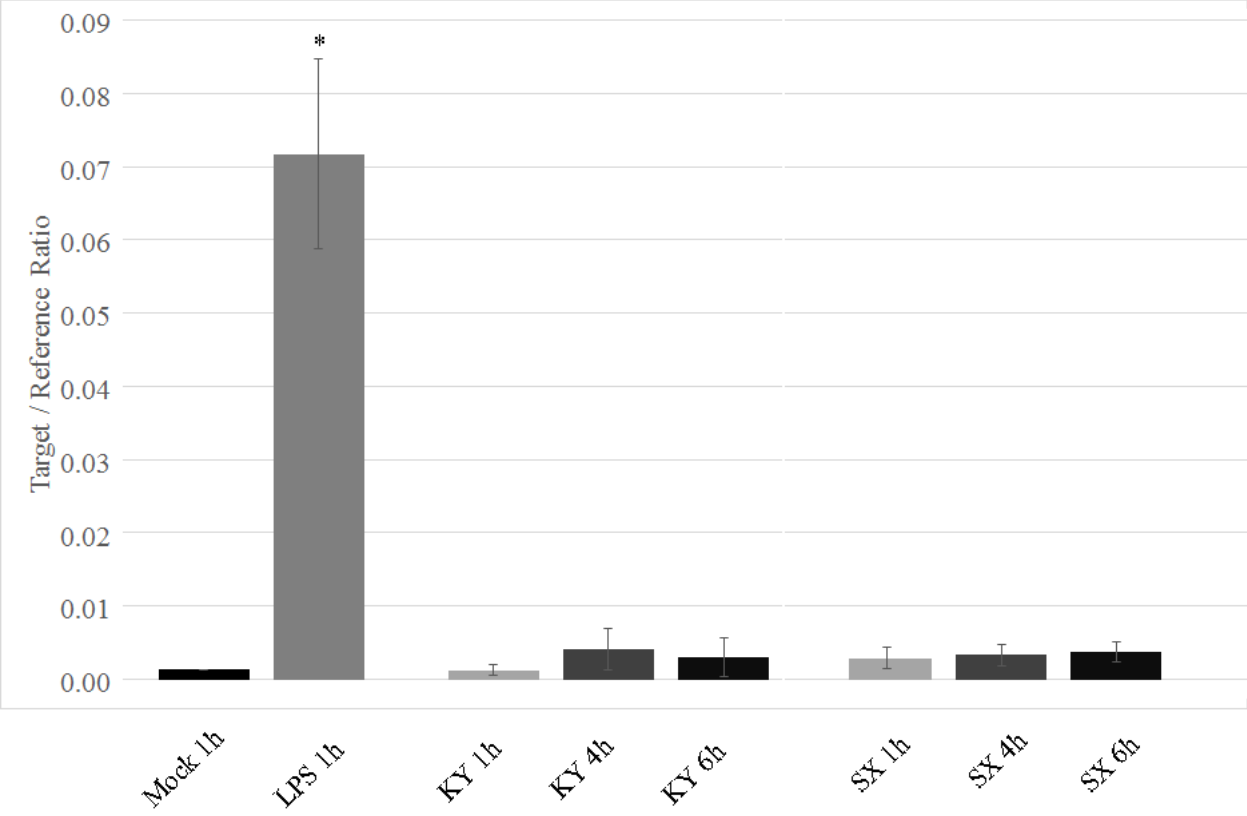


Figure A.12: Relative quantification of IFNα mRNA expression with respect to GAPDH/HMBS reference genes in virus infected cells over time. KY indicates cells infected with Kentucky/5/02 and SX indicate cells infected with Sussex/89. Significant results ($P \leq 0.05$) obtained from comparison with the mock infected group or within group over time are indicated by *. The data is presented as mean and SD indicated by the error bars.

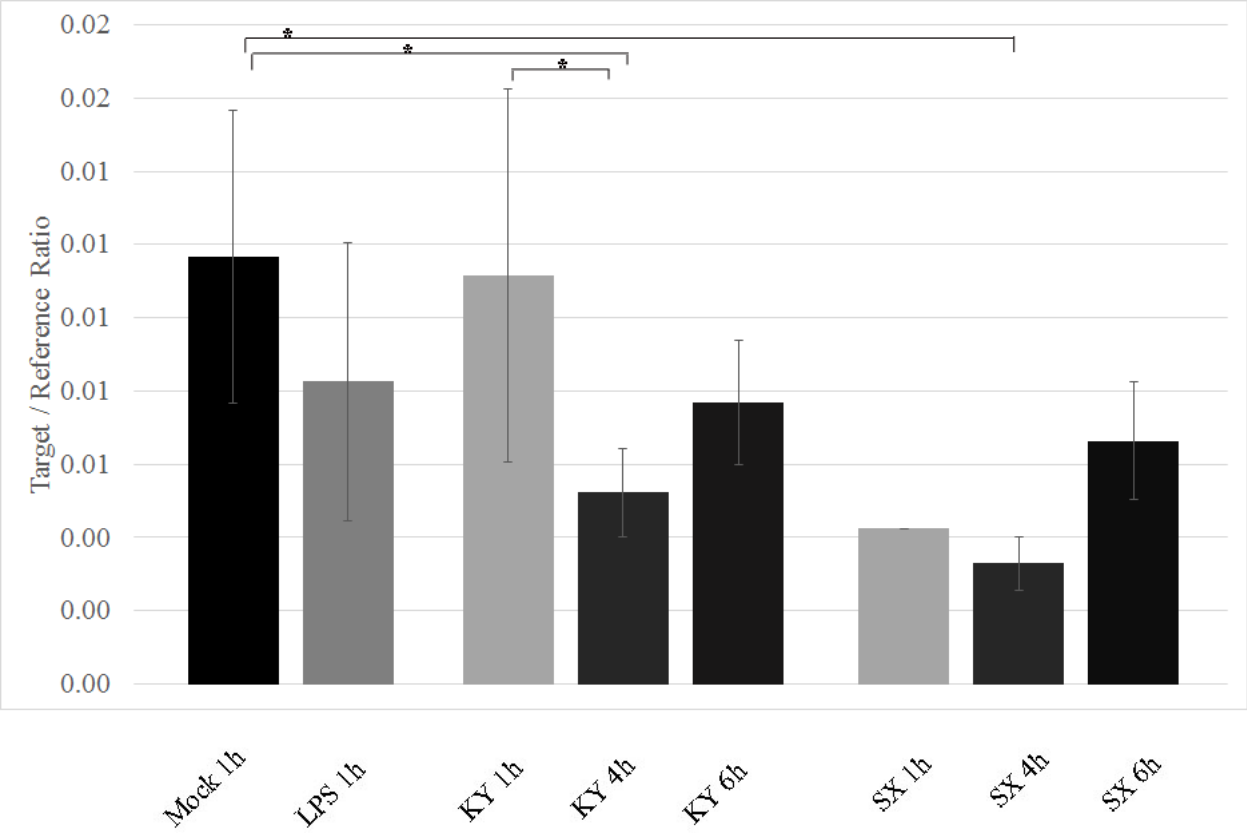


Figure A.13: Relative quantification of TNFα mRNA expression with respect to GAPDH/HMBS reference genes in virus infected cells over time. The data is presented as mean and SD indicated by the error bars. KY indicates cells infected with Kentucky/5/02 and SX indicate cells infected with Sussex/89. Significant results obtained by comparison within treatment group over time are represented with an asterisk (*) inside the graph over the bars and by comparison within a time-point among treatment groups below the legend. P values ≤ 0.05 are represented as *, P ≤ 0.01 as **, and P ≤ 0.001 as ***.

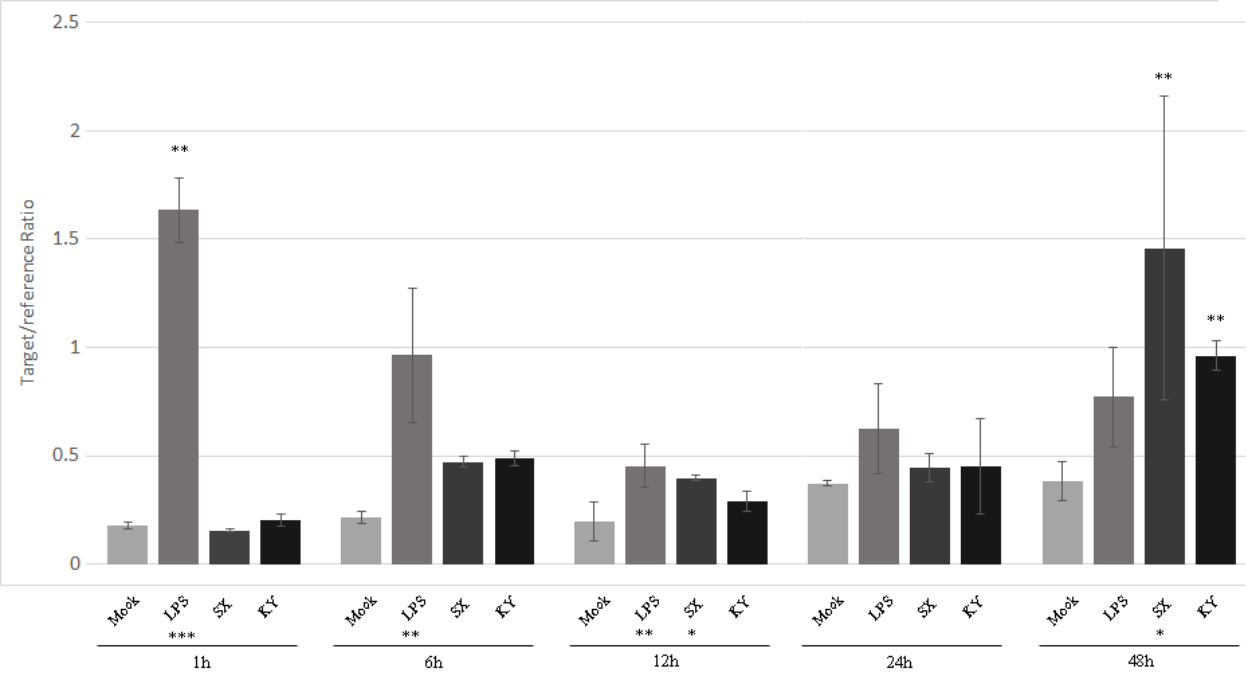


Figure A.14. Relative quantification of IL6 mRNA expression with respect to GAPDH/HMBS reference genes in virus infected cells over time. The data is presented as mean and SD indicated by the error bars. KY indicates cells infected with Kentucky/5/02 and SX indicate cells infected with Sussex/89. Significant results obtained by comparison within treatment group over time are represented with an asterisk (*) inside the graph over the bars and by comparison within a time-point among treatment groups below the legend. P values ≤ 0.05 are represented as *, P ≤ 0.01 as **, and P ≤ 0.001 as ***.

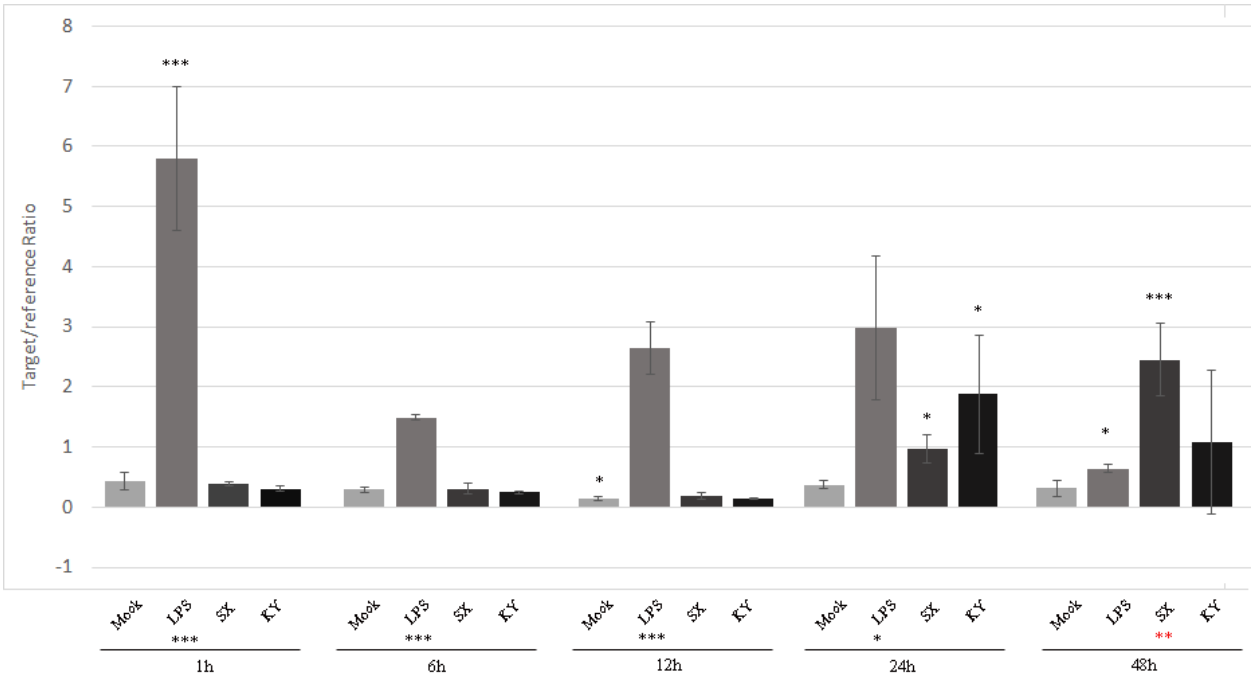


Figure A.15. Relative quantification of IFNβ mRNA expression with respect to GAPDH/HMBS reference genes in virus infected cells over time. The data is presented as mean and SD indicated by the error bars. KY indicates cells infected with Kentucky/5/02 and SX indicate cells infected with Sussex/89. Significant results obtained by comparison within treatment group over time are represented with an asterisk (*) inside the graph over the bars and by comparison within a time-point among treatment groups below the legend. P values ≤ 0.05 are represented as *, P ≤ 0.01 as **, and P ≤ 0.001 as ***.

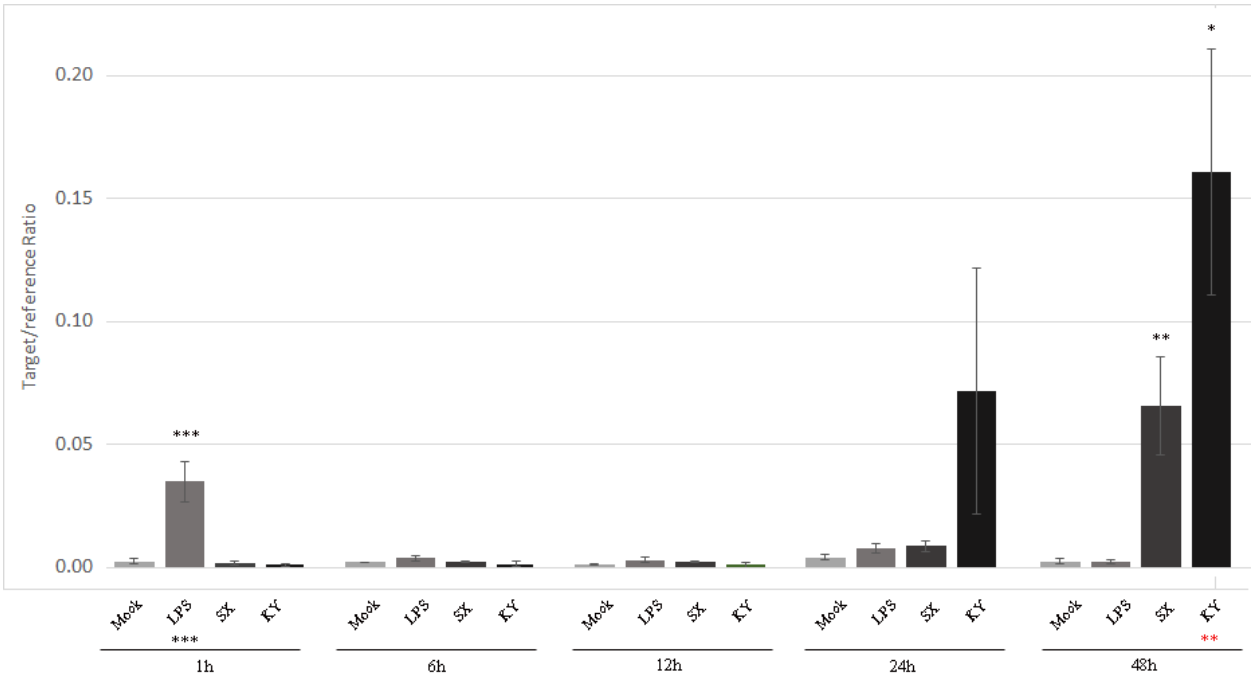
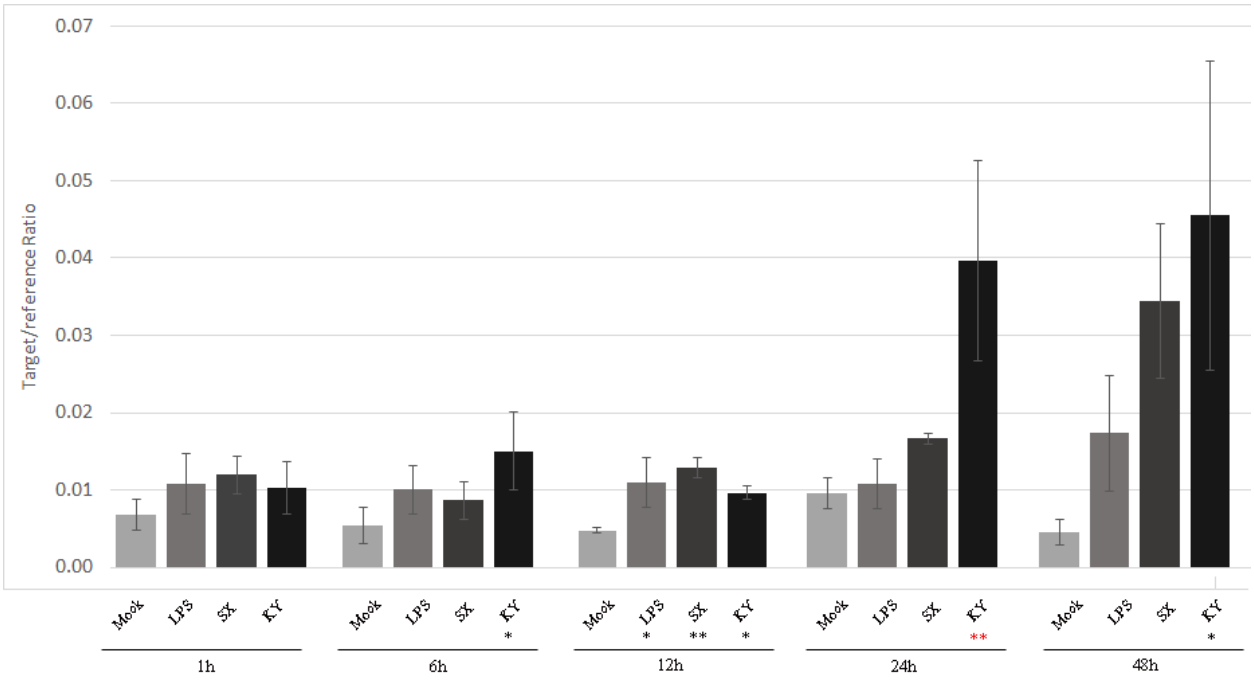


Figure A.16. Relative quantification of IFNα mRNA expression with respect to GAPDH/HMBS reference genes in virus infected cells over time. The data is presented as mean and SD indicated by the error bars. KY indicates cells infected with Kentucky/5/02 and SX indicate cells infected with Sussex/89. Significant results obtained by comparison within treatment group over time are represented with an asterisk (*) inside the graph over the bars and by comparison within a time-point among treatment groups below the legend. P values ≤ 0.05 are represented as *, P ≤ 0.01 as **, and P ≤ 0.001 as ***.

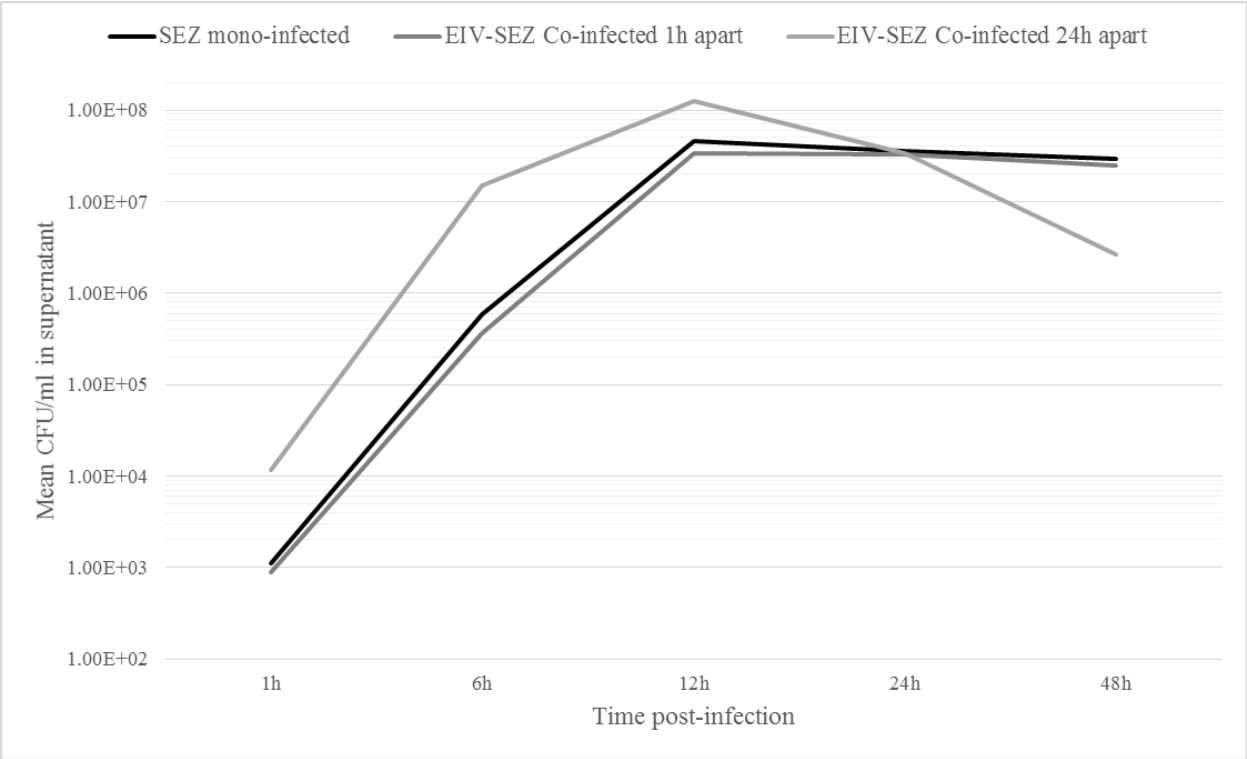


Bacterial growth in supernatants of cells co-infected with Kentucky/5/02 and SEZ

For the co-infection experiment, the OD_{550} at 3h of growth was 0.18 for mono-infection and co-infection 1h apart groups ($7.2x\ 10^7$ CFU/ml) and 0.19 for the co-infection 24h apart group ($8.2x\ 10^7$ CFU/ml). This lead to an actual infectious dose of 7.2×10^4 CFU/ml (1ml/well) and 8.2×10^4 CFU/ml (1ml/well), respectively.

Similar bacterial growth kinetics were observed in the group mono-infected with SEZ and the group co-infected with EIV and SEZ 1h apart (Figure 16). The group co-infected with EIV and SEZ 24h apart (Co-24h) had greater bacterial counts from 1h to 12h post-infection compared to the other 2 groups, but followed the same curve of growth. At 12h the Co-24h group started to have a drop in the bacterial counts whereas the other 2 groups maintained the same bacterial growth. At 48h the Co-24h group had lower bacterial counts than the other 2 groups, however it was not statistically significant (Figure 16). The mock-infected and virus mono-infected groups tested negative for SEZ throughout the duration of the study.

Figure A.17. Results of the bacterial count measurement in the supernatant of DH82α cells mono-infected with *S. equi* subsp. *zooepidemicus* (SEZ) compared to cells co-infected with equine influenza virus (EIV) 1h apart or 24h apart.



Cytokine expression over time in DH82a cells co-infected with Kentucky/5/02 and SEZ

The peak of TNFα expression in the SEZ mono-infected group was observed at 6h post-bacterial infection but it was not significantly higher than mock infection. A higher increase of TNFα expression was observed in the group of cells co-infected with K/5/02 and SEZ 24h apart at 6h ($P<0.001$) compared to 1h post-bacterial infection and compared to the mock-infected or mono-infected groups. A significant increase of TNFα expression was observed at 24h post-bacterial infection in the group of cells co-infected with K/5/02 and SEZ 1h apart compared to 1h post-infection ($P=0.031$) and compared to mock infection ($P=0.026$) but was not significantly higher than SEZ-mono infected cells.

Regarding IFNβ expression, SEZ mono-infected cells showed an increase in expression that peaked at 48h post-bacterial infection and was higher than K/5/02 mono-infected cells, which had a peak of expression at 24h, however the differences were not significant. Co-infected cells showed a similar increase in IFNβ expression than SEZ mono-infected, however the co-infected cells 1h apart showed a higher increase than mono-infected and 24h apart co-infected cells, although not significant.

Figure A.18: Relative quantification of TNFα mRNA expression with respect to GAPDH/HMBS reference genes in cells over time. The data is presented as mean and SD indicated by the error bars. KY indicates cells infected with Kentucky/5/02, SEZ indicates cells infected with SEZ, Co1 indicates cells co-infected with K/5/02 and SEZ 1h apart, and Co24 indicates cells co-infected with K/5/02 and SEZ 24h apart. Significant results obtained by comparison within treatment group over time are represented with an asterisk (*) inside the graph over the bars and by comparison within a time-point among treatment groups below the legend. P values ≤ 0.05 are represented as *, P ≤ 0.01 as **, and P ≤ 0.001 as ***.

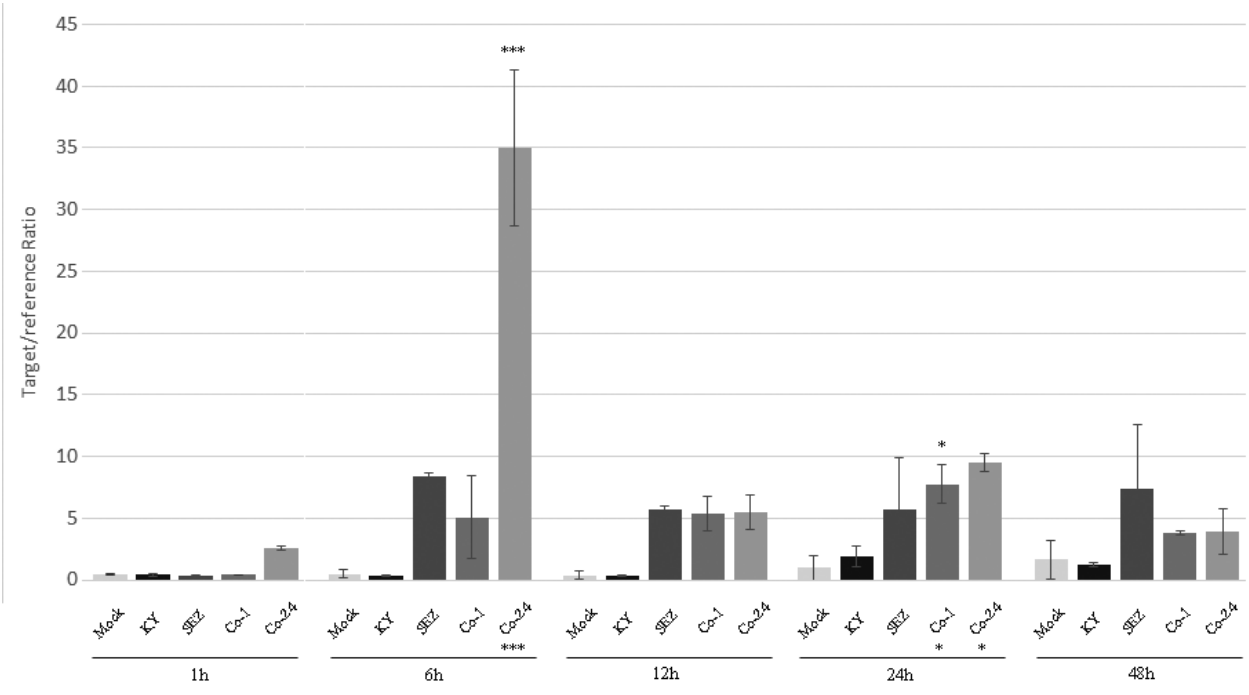
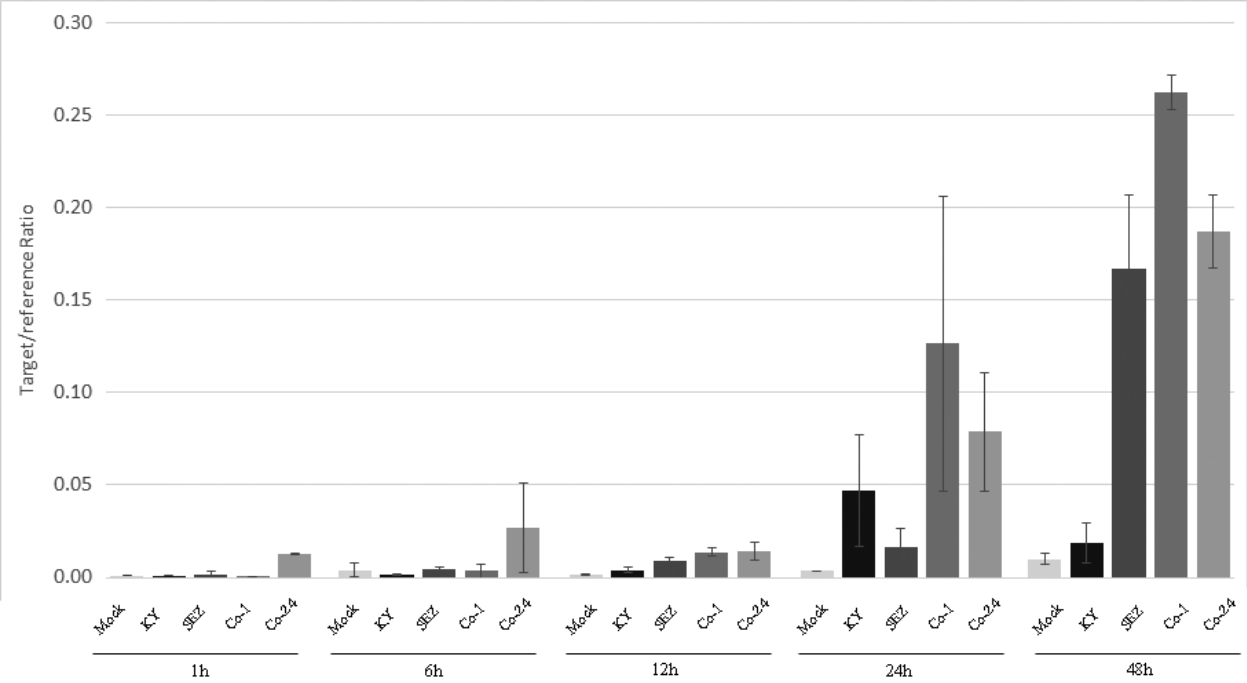


Figure A.19: Relative quantification of IFNβ mRNA expression with respect to GAPDH/HMBS reference genes in cells over time. The data is presented as mean and SD indicated by the error bars. KY indicates cells infected with Kentucky/5/02, SEZ indicates cells infected with SEZ, Co1 indicates cells co-infected with K/5/02 and SEZ 1h apart, and Co24 indicates cells co-infected with K/5/02 and SEZ 24h apart.



Discussion

In vitro model

The canine macrophage-like cell line DH82α was chosen for this study for several reasons. First, no permanent equine macrophage-like cell line exists. The only two available equine permanent cell lines are fetal horse kidney (FHK) and equine fibroblasts (E.Derm). Previous work demonstrated that the FHK cells were resistant to equine influenza virus infection (J. Daly, unpublished results). The E-Derm cell line, has life-span limitations and cells appear to become senescent sooner than other cell lines (maximum 40 passages) and fibroblasts are not a usual cell target for influenza virus. The DH82α canine cell line has been used as a model for replication of other equine viruses such as equine infectious anemia virus.[231] In addition, the dog is known to be a possible host for H3N8 equine influenza.[238] Previous work demonstrated that DH82α were susceptible to infection with equine influenza virus and they produced IL-6 in response to infection (J. Daly, unpublished data). If the response observed *in vitro* in DH82α cells resembles what has been observed *in vivo* in experimental infections in ponies, this cell line could be a good *in vitro* model without the need to infect live animals to perform multiple experiments. However, other *in vitro* models that need to be explored in the future include the use of the E-Derm cell line, or the use of primary equine tracheal epithelial cells or tracheal explants, which will represent more accurately what happens *in vivo* in horses. However, the maintenance of primary cultures is more challenging and higher variability is obtained. So different experiments could be performed first in DH82α cells, and then confirmed in primary equine cultures.

In addition, equine models can serve as a model of pathogen interactions in human disease. The majority of the investigations in people are carried out in mouse models, which are

not natural hosts for the influenza strains tested. Knowledge obtained from equine investigations, with the horse as a model with equine viruses and bacteria as adapted pathogens in their natural host, can serve to understand pathogen interactions that occur in cases of secondary bacterial infection in people or to understand cytokine expression.

Virus

The two strains used in this study were chosen because they have been reported to differ in virulence and in the host antiviral response that they induce. The Sussex/89 strain is a Eurasian strain that has a full length NS1 protein. Kentucky/5/02 is a Florida clade 2 strain with a truncated NS1 protein. A highly similar strain to K/5/02, Newmarket/5/03, induced a higher IFN response in infected ponies compared to Sussex/89.[239] The same results were observed *ex vivo* in equine tracheal explants, where N/5/03 and the closely related K/5-02 induced higher IFNβ response than Sussex/89.[239] Therefore, these two strains can differ in the interferon and cytokine response observed *in vitro*. The differences in the NS1 protein could be the cause of differences in ability to antagonize the host IFN response, however differences in other genes such as the PB1-F2 protein can have an effect on cytokine production. Therefore, further work to investigate the role of NS1 in cytokine induction needs to be performed using reverse genetics recombinant viruses differing only in the NS1 gene with the same backbone.

Bacteria

Streptococcus equi subsp. *zooepidemicus* was chosen because it is reported to be the most common opportunistic bacteria in adult horses with bronchopneumonia.[240] The D2a strain was chosen because it had been recently isolated from a tracheal wash in a horse with clinical

disease, which could be representative of the strains that are naturally circulating among horses. In addition, this strain had a high stimulation index (5.1), which is an index of bacterial classification based on their effect on stimulating cytokines in polymorphonuclear cells, with an index greater than 2 considered positive.[241]

Streptococcus spp. are extracellular pathogens therefore no intracellular growth could be measured. The total number of CFU recovered per well was used to measure a possible effect of co-infection in bacterial growth. We tried to minimize confounding factors due to overgrowth of the bacteria in the medium by washing the cells 1h after infection and replacing the supernatant every 12h, which could be a closer representation to the level of adhesion (total associated bacteria) because the non-attached bacteria would have been removed.[242] However, this procedure could have introduced variability due to manipulation.

We observed a difference in the bacterial growth in supernatants from the group co-infected with EIV and SEZ 24h apart compared to the other two groups (mono-infected with SEZ and co-infected with EIV and SEZ 1h apart). This difference could be related to multiple factors. First, the inoculation doses used were slightly different initially because the bacterial culture needed to be fresh each day and the only way we had to estimate the same amount of bacteria was the to measure the OD after 3h of growth. In both cases the OD was very similar (0.18 and 0.19), and CFU counts the next day revealed very similar counts ($7.2x\ 10^7$ CFU/ml and $8.2x\ 10^7$ CFU/ml), however this could have introduced a change and caused an initial higher bacterial load in the Co-24 group. Despite this, the 3 groups followed a similar bacterial growth curve until 12h post-infection. Then, the Co-24h group showed a decrease in CFU that was not observed in the other 2 groups. This could be explained by macrophages cleaning-up bacterial infection more efficiently when they were primed by viral infection 24h prior, or due to lower

attachment of bacteria to the cells and removal during the supernatant change at 12h. Despite the facts that the same methodology was used for the three groups and performed by the same operator, variability could have been introduced if the supernatant was not removed completely and then higher loads of bacteria replicating in the supernatant lead to higher bacterial measurements. Therefore, it is difficult to draw definitive conclusions from this experiment alone. The addition of another internal control group such a mono-infected with bacteria group inoculated at the same time as the Co-24 could have been helpful to make sure the variability was not produced by the different inoculation or the methodology. This should be considered if the experiment is repeated in the future.

In addition, in order to study the level of bacterial attachment to cells other techniques such as immunofluorescence analysis of cells should be used, which could help to determine if pre-infection with EIV leads to lower attachment of bacteria to the cell due to downregulation of receptors, which has been studied in swine tissues co-infected with influenza virus and *Streptococcus suis*.[243] Moreover, it would be interesting to perform investigations with other types of bacteria.

ELISA results

Unfortunately, the ELISA kits used for protein concentration determination had several reagents not appropriately conserved after their initial use. Subjectively, the colorimetric reaction observed for the standard recombinant protein was low in intensity and the standard curve generated after absorbance measurement revealed OD almost 10 fold lower than the manufacturer's guidance. Therefore, the results of the ELISA test could not be completely relied upon. However, the fact that no TNFα was detected and that IL-6 was only detected at 48h after virus infection was consistent with previous results performed at the University of Nottingham (J. Daly, unpublished results). Protein expression is delayed compared to mRNA expression due to the time needed for the cell to translate the mRNA into protein, which justifies why protein levels where too low until 48h after virus infection. Further evaluation of the kinetics of proteins in supernatants after virus infection is needed with new ELISA kits to draw any conclusion.

Cytokine expression

Surprisingly, SX/89 induced a higher TNFα and IL-6 response compared to K/5/02. If the differences seen are attributable to NS1 protein changes, we would expect that the virus with truncated NS1 protein (K/5/02) would induce a higher cytokine response, as has been observed in human models.[244] Further research with reverse genetic recombinant viruses varying only in the NS1 gene would definitively demonstrate if the difference observed is due to NS1 or other viral genes. Another explanation for the lower cytokine response in the NS1 truncated virus would be that truncation leads to attenuation, therefore the lower number of viral particles from lower replication rates would induce a lower inflammatory response. However, previous studies

with these strains have demonstrated that NS1 truncated viruses did not have lower replication rates.

Equine tracheal explants infected with different wild-type viruses showed that N/5/03 and the closely related K/5/02 induced the highest IFNβ response at 12 and 48h post-infection followed by Sussex/89, N1/93 and finally N/2/93,[239] which agreed with results from in vivo studies in ponies.[228] Our investigations in DH82α cells agreed with these studies, demonstrating that K/5/02 induced higher type I IFN response than Sussex/89 in our *in vitro* model. No previous studies have compared the induction of cytokines such as TNFα and IL6 by the two strains chosen for this study and further evaluation to correlate our *in vitro* findings with *in vivo* and equine tissue explants investigations is warranted.

In general, the effect of NS C-terminal truncation is attenuation of the virus due to inefficiency in blocking host gene expression, leading to higher levels of the antiviral responses such as type I IFN and other pro-inflammatory cytokines.[245–248] In order to be certain that the differences in cytokine responses are caused by differences in the NS1 gene, the use of truncated NS1 recombinant viruses with the same backbone is necessary to minimize variability due to the effect of differences between changes in other influenza genes among strains. In one study, NS1 truncation lead to increased host gene expression of inflammatory cytokines such as IL-6 and TNF-α, in human macrophages and in mice.[244] Similarly, another study demonstrated that NS1 truncation decreased the ability of swine influenza viruses to prevent type I IFN synthesis in pig cells.[247] Furthermore, these mutant viruses were attenuated *in vivo* in pigs.[247] Interestingly, the 2009 flu pandemic in people was caused by a novel swine-origin H1N1 influenza virus that possessed an 11aa C-terminal truncation in its NS1 protein, which was associated with a higher induction of pro-inflammatory cytokine response than human seasonal H1N1 viruses.[248] It was

demonstrated that C-terminal 11aa truncation in NS1 was responsible for inefficient blocking of host gene expression, nucleolar localization, and poly-A binding protein II (PABII) binding capacity; because the nuclear localization signal 2 and PABII binding domains are present at the C-terminal region of NS1.[248] The NS1 truncation did not significantly alter virus replication *in vitro* or *in vivo*, but surprisingly enhanced virus virulence in mice. Therefore it can be concluded that the C-terminal region of NS1 plays an important role on cellular gene expression modulation and viral pathogenicity.

Limited information is available regarding the effect of NS1 truncation in horses and the host innate immune response. Quinlivan et al. demonstrated that recombinant NS1 truncated viruses were attenuated in their ability to block the antiviral response, with higher amount of IFNβ being produced in equine primary cells compared to wild-type recombinant viruses.[230] Another study was conducted in equine tracheal explants infected with segment 8 (NS1 gene) reassortant viruses with a K/5/02 background.[239] The reassortant Sussex/89 and N/2/93 induced lower IFNβ expression than the parental K/5/02 and their respective wild-type strains. This agrees with human and swine studies where NS1 truncated viruses induced higher type I IFN host responses.[247] The difference between the reassortant and the wild type viruses could be explained due to differences in other genes between the wild type and the parental K/5/02 such as PB1-F2. Further studies are needed to confirm if the same response is observed for TNFα and IL6, which could provide some insight on why Sussex/89 produced a higher cytokine response than K/5/02 in our study.

A different cytokine expression response was observed in cells co-infected with K/5/02 and SEZ 1h apart or 24h apart. The peak of TNFα expression of the cells co-infected 24h apart was observed at 6h post-infection and was significantly higher than SEZ mono-infected cells and

cells co-infected 1h apart. On the other hand, the peak of TNFα in 1h apart co-infected cells was much later at 24h apart, but it was not significantly higher than SEZ mono-infected cells and it was lower than TNFα in the co-infected cells 24h apart at that time-point. A higher expression for IFNβ was observed in co-infected cells 1h apart at 48h post-bacterial infection compared to mono-SEZ infected and 24h apart co-infected cells, however this difference was not significant. Our results are in agreement with human and mice studies that report that inflammatory cytokines, such as TNFα and type I IFN, are excessively elevated after influenza virus and pneumococcus co-infection.[216,217] This cytokine unbalance has been linked to massive influx of neutrophils and macrophages that invade the lung, causing inflammatory damage, but not effectively clearing the bacteria in mice models. It is unknown if the higher levels of TNFα in the 24h apart co-infected group is associated with the lower bacterial counts obtained in this group. Further research is necessary to investigate if our results are repeatable in other *in vitro* models such as tracheal explants and to correlate it with *in vivo* studies, where other components of the immune system are present.

Detection of gene expression by messenger RNA (mRNA) quantification might not reflect the exact biologically active cytokine levels released by cells, however it allows rapid evaluation of the expression of several cytokines concurrently. Evaluation of protein production should be performed to evaluate if correlation with mRNA expression exists, and to determine the biological significance of the differences among groups observed.

Conclusion

The results of our studies constitute preliminary results confirming that DH82α cells can be used as an *in vitro* model to investigate the cytokine production after EIV infection. A higher expression of TNFα and IL6 was observed in Sussex/89 infected cells, whereas a higher IFNβ and IFNα expression was observed in K/5/02 infected cells. Co-infection with EIV and SEZ induced a higher cellular expression of TNFα, especially if co-infected 24h apart. In addition, lower bacterial growth in the supernatants of cells co-infected with K/5/02 and SEZ 24h apart was observed 48h post-infection compared to cells mono-infected with SEZ or cells co-infected with SEZ and K/5/02 1h apart. Further evaluation using equine *in vitro* models and *in vivo* experiments, using recombinant viruses and using other techniques to study bacterial attachment, are warranted to confirm the results of our studies.

Footnotes

a. Thermo Fisher Scientific, Paisley, UK

b. Sigma-Aldrich, Gillingham, UK

c. RNA isolation kit, Macherey-Nagel, Duren, Germany

d. PCR product purification kit, Quiagen, Manchester, UK

e. NanoDrop 2000, Thermo Scientific, Wilmington, USA

f. Source BioScience, Nottingham, UK

g. UGene, Unipro, Novosirirsk, Russia

h. StarLab, Blakelands, UK

i. New England BioLabs, Ipswich, MA, USA

j. Bioer XP thermos-cycler, Bioer Technology, Binjiang, China

k. LightCycler, Roche, West Sussex, UK

l. Excel 2010, Microsoft, Redmond, WA

m. http://www.genomics.agilent.com/biocalculators

n. R&D systems, Inc. Minneapolis, MN, USA

o. JMP Pro 11.0, SAS Institute Inc., Cary, NC

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