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Transmission Potential of Rickettsia felis through Cutaneous Inoculation of Infectious Flea Feces

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TRANSMISSION POTENTIAL OF *RICKETTSIA FELIS* THROUGH CUTANEOUS INOCULATION OF INFECTIOUS FLEA FECES

A Thesis

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Master of Science

in

The Department of Pathobiological Science

by

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ABSTRACT

*Rickettsia felis*, the causative agent of flea-borne spotted fever, is an emerging pathogen of the transitional group Rickettsiae and an important cause of febrile illness in Africa. Since the organism’s original discovery in the early 1990s, much research has been directed towards elucidating transmission mechanisms within the believed primary host and reservoir, the cat flea (*Ctenocephalides felis*). However, while a growing number of human cases are being reported throughout the world, a definitive transmission mechanism from arthropod host to vertebrate host resulting in clinical disease has not been found. Several possible mechanisms, including bite of infected arthropods and association with infectious arthropod feces, are currently being investigated. This current study was undertaken to examine the role of infectious cat flea feces in dissemination of the organism to vertebrates. It was hypothesized that if cat fleas excrete viable *R. felis* during feeding, then the feces are capable of producing infection in vertebrates through cutaneous inoculation. Feces of cat fleas infected with *R. felis* were analyzed for the presence of the organism, and these potentially infectious feces were then used to intradermally inoculate naïve BALB/c mice. The results of this research show that *R. felis* is present in high numbers in infected cat flea feces post-exposure to an infectious blood meal, and these bacteria are presumed viable due to the detection of rickettsial transcripts within the feces. Detectable amounts of *R. felis* were found in the skin of mice inoculated both with *R. felis* from culture and *R. felis*-infected flea feces, and rare animals injected with *R. felis* from culture showed possible systemic dissemination. The response of mice inoculated with *R. felis*-infected cat flea feces is primarily a neutrophilic dermatitis with positive anti-*Rickettsia* IgG titers at 14 days post-exposure. No mice developed any overt clinical or physical signs. This study demonstrates that cutaneous inoculation with infectious arthropod feces is an effective transmission medium for the organism
to mice. Further work is needed to define the role of this route of exposure in the epidemiology of the human disease.
CHAPTER 1: *Rickettsia felis*: A REVIEW OF TRANSMISSION MECHANISMS OF AN EMERGING PATHOGEN

1.1. Introduction

*Rickettsia felis*, an obligate intracellular bacterium of the transitional group *Rickettsia*, is the causative agent of emerging flea-borne spotted fever [1]. This organism was first associated with human disease in a patient from Texas in 1994 [2], and human cases have since been reported on every continent except for Antarctica [3]. The widespread nature of the disease is likely secondary to the believed primary vector and reservoir host, the cat flea (*Ctenocephalides felis*), which shares a similar pervasive range (Figure 1). While much work has been done to investigate the spread of *R. felis* between cat fleas and to vertebrate hosts, a definitive transmission mechanism that produces a rickettsemic host with clinical signs that mimic the human disease has yet to be found.

Recent studies have associated *R. felis* to infection and febrile illness in Africa, with up to 15% of patients with fever of unknown origin having detectable levels of *R. felis* in their blood via PCR analysis [4]. There have also been recent outbreaks of flea-borne rickettsiosis in the United States, including Texas, California, and Hawaii, where data has shown *R. felis* to be more prevalent in arthropods and mammals via PCR analysis in the area than *R. typhi* (the etiologic agent of murine typhus) [5-7]. Murine typhus is another flea-borne rickettsioses, of the typhus group *Rickettsia*, causing a disease clinically indistinguishable from *R. felis*. This agent also has endemic foci in southern California and south Texas [4]. Given the similarities in clinical presentations and location of outbreaks, it is evident how many of these cases could be confused

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with each other, as well as other similar rickettsial diseases. The advent of more sophisticated diagnostic techniques has aided in the distinction of some of these cases [8], allowing for a clearer clinical picture of flea-borne spotted fever.

Figure 1. Reported distribution of *R. felis* in the world. *R. felis*-positive arthropods have been reported in (blue): Afghanistan, Albania, Algeria, Argentina, Australia, Canada, Chile, Colombia, Croatia, Cyprus, Czech Republic, Democratic Republic of Congo, Ethiopia, Gabon, India, Indonesia, Israel, Italy, Ivory Coast, Japan, Lebanon, Malaysia, Morocco, Panama, Peru, Portugal, Romania, United Kingdom, and Uruguay. Along with infected arthropods, human cases of *R. felis* have been reported in (red): Australia, Brazil, China, France, Germany, Kenya, Laos, Mexico, New Zealand, South Korea, Spain, Taiwan, Thailand, Tunisia, and the United States. Human cases without detection of infected arthropods have been reported in (green): Egypt, Nepal, and Sweden.
1.2. Background

Rickettsiosis is caused by bacteria of the genus *Rickettsia*, which includes the spotted fever group (SFG), typhus group (TG) and, a more recent classification, transitional group (TRG) [9]. *Rickettsia* spp. are most commonly divided into the SFG or TG based on their vector of transmission, antigenic characteristics, optimal growth temperatures, percent G+C DNA contents, and clinical features [10]. Bacteria associated with the SFG are usually transmitted to vertebrates via the bites of hard ticks, while members of the TG are predominantly transmitted by contamination of mucous membranes, conjunctivae, and/or open wounds with the infectious feces of lice and fleas [11]. *Rickettsia felis* was originally characterized as a typhus-like *Rickettsia* due to the fact that the first human case was originally misdiagnosed as murine typhus and the organism was initially isolated from a laboratory flea colony. Additionally, early analysis of the 17-kDA and citrate synthase genes of *R. felis* supported a TG classification [12]. However, later analysis revealed the presence of the *ompA* gene and a 17-kDA gene having more similarity to the SFG rather than TG [1]. There has been some debate in the literature as whether to classify *R. felis* as TRG *Rickettsia* or a SFG variant. While some agree with the creation of a third group of *Rickettsia* spp. (TRG), others still classify *R. felis* as a SFG *Rickettsia* sp. The latter classification for *R. felis* as a SFG-variant coincides with other organisms that also are transmitted by arthropods other than ticks (e.g. *R. felis*-like organisms and *R. hoostraalii*) [11]. The difficulty in even classifying this emerging pathogen helps display part of the obstacles that many have had in distinguishing *R. felis* from other related bacteria in both clinical and laboratory settings.

1.3. Clinical Disease

The clinical manifestation of several rickettsioses, specifically *R. felis* and *R. typhi*, have many similarities, including headache, chills, fever, myalgia, and malaise, with a large number of
patients presenting with a maculopapular rash [4]. Few cases have presented with an “eschar,”
which is a single crusted, cutaneous lesion surrounded by a halo, thought to represent the site of
inoculation via an arthropod [13]. It has been reported that the percentage of patients that present
with rashes and eschars (75% and 13%, respectively) is higher in cases of R. felis compared to R.
typhi [4]. Rarely, R. felis has also been associated with neurologic signs (including a
polyneuropathy-like syndrome and subacute meningitis), pneumonia, and gastrointestinal
symptoms [14]. To date, there have been no reports of R. felis causing more serious
complications or death [1]. However, the similarity of flea-borne spotted fever symptoms to R.
typhi and other vector-borne diseases, as well as the lack of specific diagnostocs, has potentially
led to an underdiagnosis of R. felis in many human cases.

As stated previously, R. felis has been reported as an emerging cause of fever of unknown
origin in Africa. However, given the fact that R. felis has also been detected in skin swabs from
afebrile patients in Africa [15], it has been suggested that the organism is ubiquitous in the area
and its true pathogenecity has been questioned [16]. To explain the variable presentations, it has
been proposed that patients in Africa exhibit a more chronic form of the disease, with disease-
free intervals interspersed with periods of relapse (similar to malaria- which shares a common
epidemiology to R. felis in certain areas of Africa) [14]. Adding to the perplexing nature of R.
felis in Africa, several studies performed in areas of outbreaks have surprisingly not been able to
detect R. felis in local cat fleas [14]. The organism was, however, able to be detected in multiple
species of mosquitoes, including several Anopheles spp. Survey studies in the area have shown a
correlation between the locations R. felis-infected mosquitoes and human infections [17], as well
as a correlation between infected mosquitoes and prevelance of R. felis in ape feces [18],
suggesting a potential role for alternate hosts in the disease ecology in Africa.
One of the strongest correlations between *R. felis* and human disease in Africa came from a recent case study that described a vesicular fever in an 8-month old girl in Senegal [15]. The patient erupted in vesicles and ulcers over her entire body and presented with a fever. Swabs were performed of the cutaneous lesions, which were found to be qPCR-positive for *R. felis*, although qPCR results were negative in the blood samples. Additionally, sera samples collected from time points prior to infection, as well 40-days post-presentation, were negative for *R. felis* antibodies via IFA and Western blot analysis. Given the lack of seroconversion of the patient, this was described as a primary infection of *R. felis* causing the clinical cutaneous presentation. Researchers proposed the term “yaaf” to identify the clinical entity, the Senegalese word for vesicle [15]. Another case of a primary infection was described previously in the Yucatan, with similar lesions, suggesting the specific cutaneous lesions may be pathognomonic for *R. felis* [19].

The inability to isolate *R. felis* from blood, even in acutely ill patients, has been thought to preclude a definitive link between the organism and disease. However, these recent case studies have shown that there is a possible alternative route to disease that does not include circulating blood-borne rickettsial organisms.

1.4. Transmission to Arthropods

To date, thirty-nine species of arthropods have been associated with *R. felis*, including several different species of fleas, ticks, lice, and mosquitoes [20]. However, the cat flea (*Ctenocephalides felis*) has been shown to serve as not only the primary vector, but seemingly the reservoir of *R. felis* in the environment as well [3, 21]. The maintenance of *R. felis* within laboratory colonies of cat fleas has been extensively studied and was originally attributed mainly to vertical transmission, or the transmission of pathogen from parent to offspring [12, 22]. Strong evidence for this mechanism was given when *R. felis* was found to be present in both male and
female cat flea reproductive tissue, including the ovaries and epithelial sheath of the testes [23]. *Rickettsia felis* was first shown to undergo transovarial transmission, with detection of *R. felis* in freshly-deposited cat flea eggs [12], followed by the exhibition of *R. felis* in newly emerged unfed adult cat fleas, demonstrating transstradial transmission [24]. However, vertical transmission of *R. felis* to the progeny of cat fleas has reported to be highly variable, with several studies demonstrating the inability of cat fleas to maintain vertical transmission of *R. felis* when exposed as adults [22, 25, 26]. While observed variability in vertical maintenance is likely a laboratory artifact, this lack of transmission to progeny during infection bioassays suggests alternate mechanisms to introduce and maintain *R. felis* in vector populations likely exist.

The ability to undergo frequent horizontal (infectious) transmission has been shown to be more prevalent in virulent rickettsiae species [27]. Multiple mechanisms for horizontal transmission have been elucidated for *R. felis* within cat fleas, as well as other invertebrate hosts. A prerequisite to successful horizontal transmission is oral acquisition of *R. felis*. This was demonstrated by an experiment exposing uninfected cat fleas to an *R. felis*-infected bloodmeal in an artificial host system, where cat fleas were shown not only to be able to acquire the infection, but also remain persistently infected for up to 28 days post-exposure [22]. Demonstration of transmission through a shared blood meal was confirmed via an experiment where cat fleas, both infected and uninfected, fed on an artificial host. Uninfected cat fleas became infected with *R. felis* at varying rates (3.3-40.0%), as early as 24-hours post-exposure to infected fleas [26]. In this experiment, it was also shown that cat fleas could become infected through mating with infected cat fleas without exposure to any infectious bloodmeal. Cofeeding, which is the successful horizontal transmission of pathogens between actively blood-feeding arthropods in the absence of a disseminated vertebrate infection, has also been demonstrated in
cat fleas infected with *R. felis*. Infected (donor) cat fleas were placed in either the same capsules (co-fed) or different capsules (cross-fed) as uninfected (recipient) cat fleas on an uninfected murine host. Recipient cat fleas were shown to acquire the infection in both co-fed and cross-fed models in absence of the murine hosts becoming rickettsemic. Interestingly, the experiment also revealed that infected cat fleas were able to transmit *R. felis* to naïve rat fleas (*Xenopsylla cheopis*) [3].

There has been further investigation into the specific mechanism of horizontal transmission of *R. felis* between cat fleas. Support for salivary transmission was found when *R. felis* was detected via qPCR in the salivary gland of cat fleas that had been feeding on cats for 2-4 days [28]. Definitive evidence for salivary gland localization within cat fleas was given when rickettsial organisms were visualized via IFA in salivary glands in previously uninfected cat fleas 7-14 days post-exposure to an infectious blood meal [29]. Given these findings, as well as the previously discussed studies on co-feeding, there is strong evidence for *R. felis* transmission through infectious saliva in cat fleas (e.g. biological transmission). Recent evidence for mechanical transmission has also been demonstrated in cat fleas. Previously uninfected cat fleas were shown to be infectious to naïve cat fleas as early as 24 hours post-exposure to an infectious blood meal, indicating early-phase transmission. In addition, *R. felis* was not able to be detected in the salivary glands of these infectious cat fleas and the organism was shown to be released from contaminated mouthparts during probing [30].

While *R. felis* is primarily transmitted by *C. felis*, multiple field studies have demonstrated molecular detection of the infectious agent in not only other species of fleas, but also ticks, mites, and mosquitoes [13, 30]. However, it is unclear whether these other arthropods contribute to the ecology of *R. felis*, or if their *R. felis*-infection is transient and insignificant in
transmission [21]. Recently, ticks exposed to *R. felis* maintained rickettsiae for one generation, but transmission was not stable [31]. Likewise, *Anopheles gambiae* mosquitoes demonstrated the ability to sustain an infection for up to 15 days, but stable transmission was not observed [32]. Various genotypes of *R. felis* have also been isolated from several of these other non-flea arthropods [21], including a novel strain of *R. felis* that was identified in the non-blood-feeding booklouse, *Liposcelis bostrychophila* [33, 34]. This strain of *R. felis* (str. LSU-Lb) has been shown to have the ability to not only infect cat fleas, but also undergo vertical transmission within these arthropods as well [25]. Genetic variation was not only found between strains isolated from different hosts (e.g. cat flea vs. booklouse), but also from strains isolated from the same host at different geographic locations [27]. Interestingly, *R. felis* seems to have a different effect on the host depending on the vector. As stated previously, vertical transmission of *R. felis* in *C. felis* has been highly variable, suggesting that this organism has a negative fitness effect on the arthropod population, requiring additional horizontal transmission for pathogen maintenance. However, in the booklouse, *R. felis* has been shown to be maintained 100% transovarially, and clearance of the organism from adults actually resulted in decreased longevity, fecundity, and non-viable egg production [33, 35]. Given the variation reported within arthropods, further work investigating the effect of strain variation within various arthropod species must still be done.

1.5. Transmission to Vertebrates

While several transmission mechanisms within arthropods have been described in relation to infection with *R. felis*, the means by which vertebrates acquire an infection from these arthropods remains unclear. Transmission of flea-borne pathogens is often multifactorial, with each species having several transmission routes to ensure maintenance [20]. The most common route of flea-borne pathogen transmission to vertebrates is through the bite of an infected
arthropod. Evidence that this could be a possible infection route for *R. felis* in *C. felis* was given by demonstrating the organisms within the salivary glands of fleas [28, 29]. Further, naïve cats exposed to cat fleas infected with *R. felis* seroconverted after four months, and *R. felis* DNA was detected via qPCR in the blood of 5/16 of these cats [36]. However, definitive culture of the organism from the blood of exposed cats could not be obtained. A survey study that sampled over 100 cats from several states in the United States found none to have detectable levels of *R. felis* in the blood, however, one cat did have detectable levels on the skin and another on the gingiva [37]. The cutaneous presence of *R. felis* in one feline patient, combined with the lack of circulating organisms, is reminiscent of the cutaneous presentation of human patients in Africa and the Yucatan, although no cutaneous lesions were reported in the cat.

Another possible mechanism for vertebrate infection is via infectious vector feces. Excretion of viable rickettsiae in feces of infected arthropods has been found to be crucial in the transmission for other species, including *Rickettsia prowazekii* and *Rickettsia typhi*. The most common form of exposure to infectious arthropod feces is through cutaneous inoculation, either through the deposition of arthropod feces at the bite site or contamination of broken skin or wounds with feces. Transmission of *R. prowazekii* has been demonstrated to occur via scarification of a louse bite site with rickettsiae-laden feces [38], and cutaneous inoculation of feces from fleas infected with *R. typhi* has been shown to create infections in rat and man (with as little as 0.2 mg of flea feces producing infection) [39, 40]. A closely related bacteria, *Bartonella hensalae*, has also been shown to use this transmission mechanism for dissemination to vertebrates. Feces from cat fleas infected with the bacterium caused cats to become bacteremic 1-2 weeks post intradermal injection, as well as caused seroconversion by 20 weeks post-injection [41].
There is evidence to suggest that *R. felis* is also transmitted via infectious flea feces. Egg-free feces from *R. felis*-infected *C. felis* fleas was assessed at days 2-28 post-exposure to an infectious blood meal. *R. felis* gDNA was detected at most time points throughout the study via qPCR amplification of the 17-kDa gene. Additionally, there is evidence that these are viable, transcriptionally active rickettsial organisms because a *R. felis* transcript was detected in the feces at 21 days post-exposure to an infectious bloodmeal [22]. Further work with vertebrates must be performed to determine if this is a possible *R. felis* transmission mechanism *in vivo*.

It is difficult to study transmission of *R. felis* to vertebrate hosts because a definitive host with appropriate clinical signs and bacteremia has not been found. Several animals, including cats, dogs, opossums, raccoons, rodents, and humans, have been found to be either seropositive or PCR-positive for *R. felis* DNA. Additionally, the cat flea lacks true host specificity, and *R. felis*-infected arthropods have been recovered from cats, dogs, rodents, opossum, hedgehogs, horses, sheep, goats, gerbils, and monkeys [30]. Given the lack of a definitive mammalian host, many research experiments have looked at transmission of *R. felis* from cat fleas to vertebrates using rodents, including mice and rats. Information about choice of mouse strain could be taken by previous experiments performed on related species, such as *Rickettsia parkeri*, a member of the SFG rickettsiae. Several strains of inbred mice, including A/J, BALB/c, C3H/HeJ, and C3H/HeN, were studied to determine their response to intravenous and intradermal inoculation of *R. parkeri* [42]. The only strain to show pathology consistent with sustained infection was C3H/HeN, which exhibited marked facial edema and splenomegaly, as well as characteristic eschar-like lesions. Given this information, the C3H/HeN strain was more extensively studied with regards to *R. felis* infection. Post-intravenous inoculation with a high-dose of *R. felis* (1x10^6 organisms), C3H/HeN mice had detectable levels of rickettsial DNA in the spleen and liver as
early as 1 day post-inoculation. Levels decreased to 50% or less by 6- and 14-days post injection. *Rickettsia felis* DNA was never detected in the blood of the mice, and no mice exhibited any overt clinical signs of illness or pathology [Macaluso, Unpublished]. This mouse strain was also used in the previously described cofeeding experiment, where mice received an interdermal inoculation with $5 \times 10^9$ rickettsiae from culture. Mice in this study also did not show any evidence of clinical signs or *R. felis* DNA in their blood, although other organs including liver and spleen were not tested for presence of *R. felis* gDNA [3].

A recent study looked at another mouse strain, BALB/c, in regards to the ability to acquire an *R. felis* infection. This study actually utilized mosquitoes, *Anopheles gambiae*, to examine transmission to vertebrates. This approach was undertaken to further investigate the paradox of the low number of infected cat fleas found in areas of high *R. felis* prevalence in Africa. Natural bites from *R. felis*-infected *A. gambiae* mosquitoes were shown to produce a transient rickettsemia in BALB/c mice, confirmed via qPCR analysis of the blood [32]. The bacteremia was present in a majority of the mice both 1 and 2 days after being exposed to infected mosquitoes, but disappeared by day 3. However, even though this mouse model was able to acquire an infection, no clinical signs or physical changes were reported in these mice. While several of these mouse models have shown some promise, it is apparent that a definitive laboratory model that mimics the clinical disease in humans has yet to be found.

1.6. Discussion

It is clear that there is still much to be understood about *Rickettsia felis*. While the cat flea is still believed to be the primary reservoir and vector of the organism worldwide, the discovery of multiple arthropods that harbor the pathogen reveals the need to do more extensive field research, including analysis of all possible arthropods in the area of reported human disease. A
more complete picture of the possible vectors of human disease could propel research in the right direction. In addition, given that multiple transmission mechanisms within C. felis have been elucidated, it is possible that the transmission of R. felis within and amongst other arthropods is equally as complicated and multifactorial. Experiments that include transmission of the organism to multiple vectors might more closely mimic what is happening in nature. The most perplexing question that remains to be answered is how humans are acquiring the infection. While bites from infected cat fleas were previously thought to be the most likely mechanism, multiple laboratory experiments have not been able to produce a rickettsemic vertebrate with clinical signs that mimic a human infection through this route. It is important to note, given the findings in Africa of non-rickettsemic patients that exhibit clinical signs, an appropriate laboratory model may also not show evidence of R. felis infection in the blood. Also, mammals may simply be asymptomatic reservoirs. Further research with vertebrates, such as association with other R. felis-infected arthropods or contact with infectious arthropod feces, might aid in answering these questions and discovering a definitive, disease-causing transmission mechanism from arthropod to human.
CHAPTER 2: TRANSMISSION POTENTIAL OF Rickettsia felis TO VERTEBRATES VIA CUTANEOUS INOCULATION OF INFECTIOUS FLEA FECES

2.1. Introduction

*Rickettsia felis* is a gram-negative, intracellular bacterium of the transitional group Rickettsiae and the causative agent of flea-borne spotted fever [1]. Clinical presentations of this disease in humans vary widely, ranging from asymptomatic carriers in Africa [15, 16], to mild fevers and dermal lesions [4], to finally the most severe manifestations including pneumonia and neurologic signs [14]. Diagnosis of the disease has previously been through somewhat indirect methods, including PCR of both skin lesions [15] and blood from diseased individuals [4], as well as serology. However, the inability to isolate *R. felis* from the blood of acutely ill patients and lack of a definitive link between the organism and disease has led some to question the pathogenicity of the organism [16]. An alternative route to disease that does not include blood-borne rickettsial organisms has been proposed, termed “yaaf,” after the Senegalese word for vesicle [15]. This suggestion is based on a case report from Africa where an infant girl erupted in vesicles and ulcers over her body and presented with a fever. While the cutaneous lesions from the girl were found to be PCR-positive for *R. felis*, blood samples were negative for the organism via PCR and no rickettsial antibodies were detected via serology (including IFA and western blot analysis). A similar case report from the Yucatán described an adult woman who presented with cutaneous lesions, along with fever, myalgia, and hearing loss. The skin lesions were PCR-positive for *R. felis*, while blood samples showed no amplification of rickettsial genes. The patient also contained no detectable antibodies to *Rickettsia* in acute serum samples, but convalescent sera contained antibodies to *R. akari, R. rickettsia,* and *R. typhi* [19]. These reports suggest the possibility of a cutaneous manifestation of the disease that does not depend on a rickettsemic vertebrate host.
Adding to the perplexing nature of *R. felis* is the lack of a definitive transmission method between arthropod and vertebrates. The organism has been long believed to be transmitted to humans by the cat flea (*Ctenocephalides felis*) since its first isolation from a laboratory flea colony in 1992 [12]. Strong evidence for *C. felis* as the primary vector and reservoir of *R. felis* has been presented, including the finding of numerous *R. felis*-positive cat fleas in areas of human outbreaks of flea-borne rickettsiosis [5-7]. While *R. felis* has been demonstrated in cat flea salivary glands [28, 29], alternative routes of transmission other than arthropod bites, including transmission through infectious flea feces, remain to be investigated. Excretion of viable rickettsiae in feces of infected arthropods has been found to be crucial in the transmission for other related species, including *Rickettsia prowazekii* [38] and *Rickettsia typhi* [39, 40]. Additionally, previous work has demonstrated that potentially viable *R. felis* organisms are excreted in the feces by *C. felis* [22]. Thus, it was hypothesized that if cat fleas excrete viable *R. felis* after exposure to the organism, then the feces would be capable of producing infection in vertebrates through cutaneous inoculation. This study was undertaken to further investigate the presence of *R. felis* in infected cat flea feces and the role of these infectious flea feces in the transmission of *R. felis* to vertebrates. In this experiment, the feces of cat fleas previously exposed to *R. felis*-infected bloodmeals were examined for the presence of *R. felis* DNA and RNA. The potentially infectious flea feces were then intradermally inoculated into naïve BALB/c mice, and the effects of these feces were compared to *R. felis*-free feces, as well as to *R. felis* from culture, over a period of 14 days.
2.2. Materials & Methods

2.2.1. Source and strains of bacteria, fleas, and mice

The *R. felis* strain used was originally obtained from the Louisiana State University cat flea colony (LSU; passage 3) and maintained in *Ixodes scapularis* embryonic cell line (ISE6) in modified L15B growth medium as previously described [43]. Rickettsial infections within culture were monitored weekly using the Diff-Quik staining procedure [43]. Newly-emerged, *Rickettsia*-uninfected cat fleas were obtained from Elward II (El-Labs, Soquel, Ca). Five to ten-week-old, mixed-sex, BALB/c mice were obtained from Louisiana State University (Division of Laboratory Animal Medicine) and used as a murine model organism.

2.2.2. Ethics Statement

This study was carried out in accordance with the following: Animal Welfare Act (9 CFR Ch. 1 Subpart C 2.31 (c) 1-8), Guide for the care and use of Agriculture Animals in Agricultural Research and Training (Chap. 1), and the Public Health Service Policy on Human Care and Use of Laboratory Animals (Section IV.B. (1-8)). All animal research was performed under the approval of the LSU Institutional Animal Care and Use Committee (IACUC; Protocol Number: 15-115).

2.2.3. Cat flea bloodmeal treatments in the artificial dog unit

Cat fleas were pre-fed 3-4 days on uninfected, defibrinated bovine blood (HemoStat Laboratories, Dixon, CA) within an artificial dog unit as previously described [44]. Cat fleas were divided into cages of ~200 fleas each (mixed-sex). All flea cages were changed prior to exposure to either infectious or control bloodmeal. Prior to infection, a portion of cat fleas from each cage were tested via quantitative real-time polymerase chain reaction (qPCR) of the *R. felis* ompB gene [8] and determined to be *R. felis*-negative. Following 24 hours of pre-feeding with
heat-inactivated bovine blood, cat fleas were starved for 5-6 hours, and then given a *Rickettsia felis*-infected bloodmeal. To prepare the infectious bloodmeal, following enumeration by the *Bac*Light viability staining kit (Molecular Probes, Carlsbad, CA), intact *R. felis*-infected ISE6 cells (containing $5 \times 10^9$ rickettsiae) were pelleted by centrifugation at 13,000 x $g$ for ten minutes, and then resuspended in 600 $\mu$L of HI bovine blood. Cat fleas were allowed to feed on the *R. felis*-infected bloodmeal for 48 hours, and then switched to an uninfected bloodmeal for the remainder of the study. To generate uninfected cat fleas and feces, 2 mL of unaltered (i.e. without rickettsiae) bovine blood was used as a control treatment.

2.2.4. Collection of flea feces

Following either exposure to infectious bloodmeals or control bloodmeals, cat flea cages were changed once every seven days for one month, at days 7, 14, 21, and 28-post exposure. Control cages were always changed before infected cages to avoid cross contamination. At the time of cage change, the remaining live cat fleas were collected and transferred to clean cages. A portion of the live cat fleas were collected for future qPCR analysis to determine presence of rickettsial infection. The dirty cages were inspected both grossly and under a dissection microscope to remove all dead cat fleas, larvae, and eggs, so only flea feces remained (“prepared” feces). Flea feces was then scraped from the cages, weighed, and kept in three separate microcentrifuge tubes for DNA extraction, RNA extraction, and extra for future mouse inoculations. Flea feces saved for DNA extraction was stored in 500 $\mu$L of sterile PBS and stored at $-20^\circ$C. Flea feces saved for RNA extraction was stored in 1 mL of TRIzol and stored at $-80^\circ$C. If flea feces was to be used for a vertebrate experiment, collection and qPCR analysis of feces was performed within 24 hours of experiment. While qPCR analysis was being performed, the extra flea feces was placed into a clean flea cage and put back into the artificial dog unit.
overnight until the next day when the vertebrate experiment was performed (to ensure bacterial viability). All flea feces used for vertebrate experiments was taken from 14-days post-exposure to infectious bloodmeals for standardization of the inoculations mice received.

2.2.5. Transmission experiments in BALB/c mice

Eighteen (18) mixed-sex, 5 to 10-week-old BALB/c mice were used for each replicate of the vertebrate experiments (Figure 2). Fifteen (15) experimental mice were injected intradermally with either *R. felis* from culture (cell-free lysate) or *R. felis*-infected cat flea feces, mixed with warmed L15B total media, to a total volume of 100 µL. Three (3) control mice were injected with either L15B total media alone (as a control for *R. felis* from culture) or *R. felis*-free cat flea feces (as a control for *R. felis*-infected flea feces). Six (6) mice, including 5 experimental and 1 control, were sacrificed at each of the following time points: 24 hours post-injection, 48 hours post-injection, and 14 days post injection. Mice that were kept for 14 days were monitored daily for any physical or clinical signs. Both experimental models, including injection of mice with *R. felis* from culture and *R. felis*-infected flea feces, included three replicates. For intradermal inoculations, both from culture and from feces, an infectious dose of 1x10^6 *R. felis* was calculated per mouse. The infectious dose was based on preliminary data using high passage *R. felis* (p. 8) [Legendre, Unpublished] where the average number of *R. felis* organisms per milligram of flea feces was found to be approximately 4x10^4. Given that cat fleas have been reported to produce ~0.77 mg of feces per day [45], an infective dose that aimed to mimic a natural infection was calculated based on the amount of feces that could be produced by five fleas living on a mouse for a week. Inoculations from *R. felis* from culture were enumerated via the backlight BacLight viability staining kit, and inoculations from infectious flea feces were based on qPCR analysis results. For control mice, either L15B total media alone or *R. felis*-free
Figure 2. Experimental methods for vertebrate inoculations
flea feces (confirmed via qPCR) mixed with L15B total media was used. For inoculations, mice were sedated with Isoflurane and a patch of hair was shaved in the intrascapular region. Mice were inoculated using a U-100 insulin syringe (Beckton, Dickinson and Company, Franklin Lakes, NJ). A sharpie marker was drawn around the resulting intradermal bleb to mark the site of inoculation (Figure 3).

![Figure 3. Intradermal inoculation of BALB/c mice. Site of intradermal inoculation on mice (red arrow), for both *R. felis* from culture and *R. felis*-infected cat flea feces. A sharpie mark was utilized to delineate the inoculation site for future sample collection.](image)

Mice were kept in separate cages based on sex, and control mice were kept separated from experimental mice. As previously stated, mice were humanely euthanized with carbon dioxide at 24 hours, 48 hours, and 14 days post-injection. Blood was obtained via cardiac puncture and full necropsies were performed. Samples from skin both at the inoculation site (i.e. within the sharpie mark) and away from the inoculation site were aseptically collected and saved for DNA extraction (microcentrifuge tubes at -20°C), RNA extraction (1 mL of TRIzol at -80°C), and histopathologic evaluation (biopsy cassettes in 10% formalin). Sections of heart,
spleen, and liver were also collected and saved for DNA extraction and histopathologic evaluation.

2.2.6. Detection of *Rickettsia felis* in fleas, flea feces, and mice

Previously collected fleas were surface sterilized prior to DNA extraction, via washing with 10% bleach for 5 minutes, 70% ethanol for 5 minutes, and finally sterile distilled water for 5 minutes (three times). Fleas were then placed in separate 1.7 mL microcentrifuge tubes and crushed with sterile plastic pestles in a liquid nitrogen bath. Genomic DNA (gDNA) was extracted using Qiagen DNeasy Tissue Kit (Qiagen, Germantown, MD) according to the manufacturer’s instructions for tissue samples and eluted in 30 µL PCR-grade H₂O. For extractions from cat flea feces, a portion (10 mg) of “prepared” flea feces was mixed with 500 µL of sterile PBS, followed by the standard protocol for extractions of non-nucleated blood according to the manufacturer’s instructions. For extraction from mouse tissue, tissue disruption and homogenization were performed by combining tissue samples with two sterile stainless-steel beads in a 1.7 mL Safe-Lock microcentrifuge tube containing proteinase K and Buffer ATL for 2 cycles of 3 minutes at 30 Hz in a TissueLyser (Qiagen, Valencia, CA) [42], followed by the standard tissue extraction protocol according to the manufacturer’s instructions. A negative environmental control (DNA extraction reagents without a biologic sample) was utilized for each DNA extraction process. All gDNA preparations were stored at -20°C until further analysis was performed. Quantitative PCR for detection of the rickettsial *ompB* gene [8], the *C. felis* 18S rRNA gene [22], and mouse *cfd* gene [42] was performed. The qPCR was performed with a LightCycler 480 Real-Time PCR system (Roche), and results were presented as quantified rickettsial copy numbers per sample. For cat flea feces, the rickettsial copy number per sample was used to calculate the number of organisms per milligram of feces. For all results, if qPCR
results showed amplification very late in the cycle (> cycle 35), the amplified product was visualized on 1.5% agarose gel to obtain a band for confirmation. The bands were cut from the gel and purified using Wizard SV Gel and PCR Clean-Up System (Promega). Purified samples were submitted for sequencing by the dye terminator method on a 3130 Genetic Analyzer (Applied Biosystems) at LSU (School of Veterinary Medicine). Nucleotide similarities were compared using the GenBank database. Environmental controls and control mice were also subjected to the same process to ensure there was no contamination of samples.

In order to examine the potential viability of *R. felis* found in both flea feces and mouse skin, rickettsial RNA was isolated from both “prepared” flea feces and mouse skin (both at and away from the inoculation sites), as previously described, with minor modifications [3]. Approximately 20 mg of “prepared” feces was combined with 1 mL of TRIZol, and mouse skin was combined with 1 mL of TRIZol and two stainless steel beads for tissue disruption and homogenization (as previously described with a TissueLyzer), prior to extraction. Extraction was performed using chloroform for phase separation, followed by removal of the aqueous phase (containing RNA), and finally RNA precipitation and wash with isopropanol and 75% ethanol, respectively. RNA samples were treated twice with DNase I (Promega) according to the manufacturer’s instruction, followed by RNA clean-up using RNA Clean & Concentrator (Zymo Research). The treated RNA samples were then used to synthesize cDNA, using iScript cDNA Synthesis Kit (Bio-Rad). To confirm the absence of DNA contamination, no-RT controls were included for all samples. Viability of the rickettsial organisms was determined via qPCR amplification of *R. felis* ompB from prepared cDNA samples.
2.2.7. Histopathology and Immunohistochemistry

After formalin fixation, tissue samples were paraffin-embedded and sections were cut for both hematoxylin and eosin (H&E) staining and immunohistochemistry (IHC) with a polyclonal anti-\textit{Rickettsia} antibody (diluted 1/1000) as previously described [42]. Tissue samples analyzed included any specimens that showed amplification of \textit{R. felis} ompB via qPCR, as well as sections from control mice (inoculated with either culture media alone or \textit{R. felis}-free cat flea feces). Samples were examined by a board-certified veterinary anatomical pathologist. The degree of dermatitis and panniculitis (inflammation of subcutaneous fat) were classified as either absent (0; no lesions noted), mild (+; rare to infrequent at high-power), moderate (++; change is found in multiple high-power fields or large foci are present in selected areas), marked (+++; changes are frequently observed in multiple high-power fields or change is severe in focal areas), or severe (++++; changes are similar to those seen in the previous category, with the addition of extensive necrosis). Any extensions into the superficial dermis, as well as the type of inflammatory cells present, were also noted. Immunohistochemistry samples were graded similarly: 0 (no rickettsial organisms seen), + (rare organisms seen), ++ (moderate numbers of organisms seen), and +++ (many organisms seen).

2.2.8. Enzyme-linked immunosorbent assay (ELISA) on mouse sera

Indirect ELISAs to detect anti-rickettsial IgG were performed on serum samples taken from mice sacrificed at 14 days post-injection as previously described, with minor modifications [31, 46]. Serum samples, mixed with blocking buffer (1% bovine serum albumin in 0.1% Tween-20) to a 1:32 dilution, were added to half of the wells of a 96-well \textit{R. parkeri} antigen-coated plate (Fuller Laboratories, Fullerton, CA). These dilutions were then added to the other half of the wells, containing 50 µL of blocking buffer, to obtain a 1:64 dilution. Serum from a
mouse previously inoculated with *R. parkeri* and wells without serum were used as positive and
negative controls, respectively. After a 1-hour incubation, the plate was washed three times with
washing buffer (0.1% Tween-20 in PBS). A secondary antibody (goat anti-mouse IgG
conjugated to horseradish peroxidase diluted 1:1000 in blocking buffer) was added and allowed
to incubate for 1 hour in the dark. Plates were then washed three times, followed by addition of
the TMB Membrane Peroxidase Substrate (KPL, Gaithersburg, MD). After 30-40 minutes of
incubation in the dark (time length depended on the color reached by the positive controls), the
reaction was stopped with 2N sulfuric acid. Optical densities (ODs) were immediately read with
a Spectramax M2 spectrophotometer (Molecular Devices, Sunnyvale, CA) at 450 nm minus the
absorbance at 650 nm. Samples were run in duplicate and the mean ODs were calculated.
Samples were considered positive if the mean of the net ODs was greater than the mean OD of
the negative controls plus three standard deviations.

2.2.9. Statistical Analysis

All statistical analysis was performed using GraphPad Software (Prism 5 for Mac OS X,
La Jolla, California). For all comparisons, a *P*-value of <0.05 was considered significantly
different.

2.3. Results

2.3.1. Detection and Quantification of *Rickettsia felis* in cat flea feces

Based on two replicates, all experimental cat fleas taken at all time points [7, 14, 21, and
28 days post-exposure (DPE)] of the study were positive for *R. felis* genomic DNA (via qPCR
amplification of *R. felis* ompB), depicting a 100% infection rate of cat fleas following exposure
to an infectious bloodmeal. This infection was detected as early as 7 days post-exposure and
remained for the entirety of the study (28 days post-exposure). No cat fleas from control cages
showed amplification of rickettsial DNA. Similarly, “prepared” flea feces taken from all time points from experimental cages was positive for *R. felis* genomic DNA, at varying rates (Table 1). The overall mean from both replicates for all time points was $1.34 \times 10^6$ organisms per milligram of flea feces, with a range of $9.93 \times 10^4$ to $3.5 \times 10^6$ organisms per milligram of flea feces. The results from both trials were statistically analyzed to evaluate if there was a significant difference in the number of rickettsial organisms present in the flea feces at different time points (Figure 4). No significant differences were found via one-way ANOVA ($P < 0.05$) between any of the time points.

Table 1. *R. felis* in infectious cat flea feces.

<table>
<thead>
<tr>
<th>FECAL SAMPLE</th>
<th>R. FELIS DNA</th>
<th>R. FELIS RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 DPE</td>
<td>$4.02E5 (3.02E5 – 5.02E5)$</td>
<td>+</td>
</tr>
<tr>
<td>14 DPE</td>
<td>$1.5E6 (9.93E4 – 2.99E6)$</td>
<td>+</td>
</tr>
<tr>
<td>21 DPE</td>
<td>$1.26E6 (4.34E5 – 2.1E6)$</td>
<td>+</td>
</tr>
<tr>
<td>28 DPE</td>
<td>$2.19E6 (8.87E5 – 3.5E6)$</td>
<td>+</td>
</tr>
</tbody>
</table>

DNA results shown in *R. felis* number of organisms per mg of feces (based on qPCR amplification of *R. felis* ompB). A “+” under RNA results indicates there was amplification of rickettsial cDNA prepared from isolated RNA samples. Data based on two replicates.

To assess the viability of rickettsiae, and thus potential infectivity, the cat flea feces were analyzed for the presence of rickettsial transcripts. Flea feces collected at all time points in both replicates showed amplification of *R. felis* ompB from prepared cDNA, indicating that rickettsial transcripts, and potentially viable rickettsiae, were present at all time points (Table 1). The amount of rickettsial RNA was not assessed in this study. All no-RT samples were negative for presence of *R. felis* gene products.
2.3.2. Detection of *Rickettsia felis* in mouse tissue

For all three replicates, gDNA extractions were performed on all tissues (including skin at and away from the inoculation site, heart, liver, spleen, and blood) from 24- and 48-hours post-intradermal inoculation and were analyzed via qPCR for *R. felis* ompB amplification (Table 2). For the mice injected with *R. felis* from culture, at 24- and 48-hours post inoculation, 68% (17/25) of skin samples taken from the inoculation site were positive for *R. felis* gDNA, while 16% (4/25) of skin samples taken away from the inoculation site were positive. Two samples from the heart (8%) and one blood sample (4%) out of 25 also were qPCR-positive for *R. felis* ompB gene amplification. No liver or splenic samples taken from any time points were positive (0%; 0/25). One of the gDNA extraction environmental controls for 1 time point (correlating to 5 experimental mice at 24-hours post-inoculation) showed minor amplification of *R. felis* ompB,
and therefore the corresponding experimental mice were not included in any of the presented data. All other control mice and environmental controls were negative. For the mice injected with _R. felis_-infected flea feces, at 24- and 48-hours post injection, 33% (10/30) of skin samples taken from the inoculation site were positive, and 13% (4/30) of skin samples taken away from the inoculation site were positive. No liver, splenic, heart, or blood samples from any time points from mice injected with _R. felis_-infected flea feces were qPCR-positive for _R. felis_ ompB gene amplification. No experimental controls or control mice were positive. All samples that showed late amplification (> cycle 35) were run on a gel to obtain a band for confirmation. Sequencing revealed 100% identity to the _R. felis_ ompB gene (Ascension KX090279.1) for all samples that were considered positive. No bands were visualized for environmental controls or control mice.

Table 2. Mouse samples positive for _R. felis_ gDNA via qPCR.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Samples positive for <em>R. felis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>R. felis</em> from culture</td>
</tr>
<tr>
<td>Skin at inoculation site</td>
<td>17/25 (68%)</td>
</tr>
<tr>
<td>Skin away from inoculation site</td>
<td>4/25 (16%)</td>
</tr>
<tr>
<td>Heart</td>
<td>2/25 (8%)</td>
</tr>
<tr>
<td>Spleen</td>
<td>0/25 (0%)</td>
</tr>
<tr>
<td>Liver</td>
<td>0/25 (0%)</td>
</tr>
<tr>
<td>Blood</td>
<td>1/25 (4%)</td>
</tr>
</tbody>
</table>

Samples are from mice sacrificed at 24- and 48-hours post-intradermal inoculation. Data based on three replicates. All control mice (injected with either L15B total media or _R. felis_-free flea feces) and environmental controls were negative for the presented data.
Detection of *R. felis* gDNA at the site of inoculation was significantly higher in mice injected with *R. felis* from culture compared to mice injected with *R. felis*-infected flea feces (unpaired t-test, *P* < 0.05). However, no statistically significant difference was found in *R. felis* qPCR detection in skin away from the inoculation site between experimental groups.

The data presented represents three replicates of mice injected with *R. felis*-infected flea feces that was collected and analyzed within 24 hours of inoculation, while being maintained in the artificial dog unit until time of injection. The first trial (not included in the data) used previously frozen *R. felis*-infected flea feces. No tissue samples from any of the time points from this trial showed amplification of *R. felis* ompB via qPCR. This was presumed due to lack of bacterial viability (after freezing and thawing), so future experiments all used unfrozen, fresh “prepared” feces.

To assess potential viability of the rickettsial organisms present, RNA isolation and cDNA synthesis, followed by amplification of *R. felis* ompB from cDNA, was performed on the skin samples that tested positive for *R. felis* gDNA via qPCR. No rickettsial transcripts were detected in any of the skin samples tested from 24- and 48-hours post-inoculation.

2.3.3. Gross Pathology

One mouse injected with *R. felis* from culture (at 48-hours post-injection) had a pinpoint, erythematous lesion at the injection site (Figure 5). This lesion did correlate with a skin sample that tested positive for *R. felis* gDNA via qPCR and IHC. All other tissues and all other necropsies on mice (including mice injected with *R. felis* from culture and *R. felis*-infected flea feces) were unremarkable (Figure 6). No mice developed any overt physical or clinical signs throughout the course of the experiments.
Figure 5. Pinpoint lesion on mouse infected with *R. felis* from culture. One mouse injected with *R. felis* from culture had a pinpoint, erythematous, scab-like lesion (red arrow) at the site of inoculation. This correlated with skin the tested positive for *R. felis* gDNA via qPCR and IHC.

Figure 6. Representative photo of mouse skin post-intradermal inoculation. With one exception (shown in Figure 5), all mice showed no visible gross lesions at either 24 hours, 48 hours, or 14 days post-intradermal inoculation with either *R. felis* from culture or *R. felis*-infected flea feces.
2.3.4. Histopathology and Immunohistochemistry (IHC)

All sections of tissue that showed amplification of rickettsial DNA via qPCR, including 35 sections of skin and two sections of heart from mice sacrificed 24- and 48-hours post-inoculation, were analyzed via histopathology and IHC. Control sections from corresponding tissues were also analyzed. For mice injected with *R. felis* from culture, histopathological evaluation revealed mild to occasionally moderate, deep dermatitis and panniculitis predominated by macrophages and lymphocytes, with lower numbers of neutrophils and rare plasma cells and mast cells (Figure 7). Some edema was also noted, and occasional inflammatory cells infiltrated the panniculus carnosus muscle (myositis). The control mice from this portion of study (that were injected with L15B total media alone) showed no histopathological changes. Using the scoring system as previously described, the mean histopathological score for mice injected with *R. felis* from culture (1.69+) was significantly greater than that of the control mice (0) at the inoculation site via an unpaired t-test (*P* < 0.05).

Mice injected with *R. felis*-infected flea feces showed moderate to occasionally severe, suppurative deep dermatitis and panniculitis, with lower numbers of mononuclear cells. Extension into the muscle (myositis) was also occasionally seen. Few samples had focal to extensive liquefactive necrosis (Figure 8), characterized by pyknotic and karyorrhectic debris mixed with viable and degenerate neutrophils and extensive fibrin deposition. Control mice from this portion of the study (that were injected with *R. felis*-free cat flea feces) showed similar histopathologic changes (Figure 9), with the exception of liquefactive necrosis, which was absent in the control sections. A significant difference was not found between the mean histopathological scores for experimental mice (2.37+) and control mice (3+). Compared to skin sections from mice injected with *R. felis* from culture (1.69+), mice injected with *R. felis*-infected
Figure 7. Histopathology of mouse skin post-inoculation with *R. felis* from culture. Photomicrographs of an H&E stained skin section taken from the site of inoculation (100x; Inset: 400x). There is mild to moderate deep dermatitis and panniculitis, characterized by predominantly mononuclear cells (including macrophages and lymphocytes- depicted in the inset), with fewer neutrophils and scattered mast cells and plasma cells. There is also cell infiltration into the underlying panniculus carnosus muscle (myositis- red circle).
Figure 8. Histopathology of mouse skin post-inoculation with *R. felis*-infected flea feces. Photomicrographs of an H&E stained skin section of mouse tissue taken from the site of inoculation (100x; Inset 400x). There is severe suppurative dermatitis and panniculitis with liquefactive necrosis, characterized by a dense band subjacent to the panniculus carnosus of viable and degenerate neutrophils mixed with karyorrhectic and pyknotic cellular debris.
Figure 9. Histopathology of mouse skin post-inoculation with *R. felis*-free flea feces. Photomicrographs of an H&E stained skin section taken from the site of inoculation. A. (100x) Mice injected with *R. felis*-free flea feces showed moderate to marked, deep dermatitis and panniculitis, consisting predominantly of neutrophils (B- 400x), and fewer mononuclear cells.

flea feces (2.37+) showed a greater histopathological score (unpaired t-test, *P* < 0.05). No histopathological changes were noted in any of the heart sections examined.

For immunohistochemistry, only few samples showed rare positively staining coccobacilli (Figure 10). Three samples (3/25; 12%) of skin taken from the inoculation site from mice injected with *R. felis* from culture revealed rare organisms (+), and one sample (1/30; 3%) from the inoculation site from a mouse infected with *R. felis*-infected feces had rare organisms (+). The organisms were visualized both within macrophages and neutrophils, and occasionally appeared to be extracellular. Positive and negative control slides showed appropriate labeling.
Figure 10. Anti-*Rickettsia* immunohistochemistry of mouse skin post-inoculation with *R. felis* from culture.

Photomicrographs of a skin section stained with a polyclonal anti-*Rickettsia* antibody. A: The skin sections revealed mild to moderate histiocytic to lymphocytic dermatitis (100x). B and C: Rare positive, brown-staining rickettsial organisms (red arrows) were seen within macrophages, neutrophils, and possibly the extracellular space (1000x; oil immersion).

2.3.5. ELISA Results

Serum from mice sacrificed at 14-days post-inoculation were analyzed via indirect ELISAs to detect the presence of rickettsial antibodies. At a 1:32 dilution, 8/10 (80%) mice injected with *R. felis*-infected flea feces had measurable levels of anti-*Rickettsia* IgG, whereas
only 1/9 (11%) of animals injected with *R. felis* from culture had detectable levels. At a 1:64 dilution, only 2/10 (20%) of mice injected with *R. felis*-infected feces had detectable levels, and no mice with injected with *R. felis* from culture had measurable anti-*Rickettsia* IgG titers. Anti-*Rickettsia* IgG was not detected in any control mice at significant levels (greater than 3 standard deviations above the mean ODs of the negative control). The number of mice with a measurable IgG response at a 1:32 dilution was significantly higher in the *R. felis*-infected feces group compared to the *R. felis* from culture group (unpaired t-test, *P* < 0.05).
CHAPTER 3: DISCUSSION & FUTURE DIRECTIONS

In this study, cutaneous inoculation with feces from cat fleas infected with *R. felis* was found to be an effective transmission medium for the organism to mice, supported both by the detection of high numbers of viable rickettsiae in infected flea feces, as well as the presence of *R. felis* gDNA within the skin of mice inoculated with infectious feces. The response of these animals to intradermally injected *R. felis*-infected flea feces is characterized by primarily a neutrophilic dermatitis, as well as a positive anti-*Rickettsia* IgG response at 14 days post-exposure. However, there were no physical or clinical changes associated with the transmission of *R. felis* through infectious cat flea feces, and no systemic dissemination of the organism was documented through this route.

The first step in determining transmission of *R. felis* through infectious arthropod feces included confirming the presence of viable *Rickettsia* in cat flea feces. This study found that *R. felis* gDNA was present in high numbers in cat flea feces as early 7 days-post exposure (DPE) to an infectious bloodmeal and remained present for the rest of the experiment (up to 28 DPE). These findings confirm what was shown in a previous study where rickettsial gDNA was found in cat flea feces at all time points post-exposure to an infectious bloodmeal [22]. The average number of rickettsiae (based on qPCR amplification of the *R. felis* ompB gene) in this study was found to be $1.34 \times 10^6$ organisms per milligram of flea feces. This number is higher than what was found in a preliminary study ($4 \times 10^4$ organisms/milligram) using high passage *R. felis* (LSU; passage 8) for cat flea infections [Legendre, Unpublished]. Because the data from the preliminary study was used in the calculation of the infectious dose for mice attempting to mimick a “natural” infection, it is possible that the dose used for inoculation of mice was
actually lower than what could be expected in nature. Future studies examining varying inoculation dosages might result in different findings in vertebrate inoculations.

The presence of rickettsial transcripts within the cat flea feces at all time points, indicating potentially viable organisms, is a novel finding, as a previous experiment showed rickettsial transcripts were present only at 21 DPE [22]. This discrepancy is likely due to the advent of more sensitive RNA techniques that have developed since previous experiments were performed. Additionally, even though all time points had evidence of potentially viable *R. felis* organisms, there is the potential that feces taken from different DPE may have varying effects upon inoculation into vertebrates. This assumption is based on previous work done with *R. typhi*, where rats demonstrated seroconversion after intraperitoneal injection with flea feces only when the feces was obtained over 10 days post-flea infection [39]. For this reason, all vertebrate experiments were performed using feces taken from 14 DPE. This time point was chosen as opposed to 7 DPE because it has been suggested that early detection of *R. felis* gDNA in flea feces might be secondary to lysis of heavily infected midgut epithelial cells in fleas in early infection, as opposed to live, actively replicating, organisms [22, 23]. Later time points (i.e. 21 DPE or 28 DPE) were not chosen for ease of experimental conditions. It is possible that the use of flea feces taken from varying time points post-exposure to an infectious bloodmeal may have produced different results in vertebrate inoculations.

Overall, *R. felis* from culture seemed to persist and possibly disseminate more efficiently in mice than *R. felis* within infectious flea feces. This is supported by the increased number of mice that had detectable levels of *R. felis* at the site of inoculation 24- and 48-hours post inoculation (68% compared to 33%), as well as the rare animals that had detectable levels of *R. felis* within the heart and blood when injected with *R. felis* from culture. While dissemination in
the animals injected with *R. felis* from culture could not be confirmed via staining of the organisms with IHC, it is suspected that the low number of bacteria present (detected via qPCR amplification) precluded immunohistochemical labeling, as IHC is not as sensitive as PCR for the detection of most organisms. The increased persistence and possible dissemination in the animals injected with *R. felis* from culture is likely due to a decreased immune response to the bacteria alone compared to the bacteria within flea feces. Mice injected with *R. felis*-infected flea feces exhibited a more severe cutaneous reaction (characterized by a moderate to severe suppurative dermatitis) compared to the mice injected with *R. felis* from culture. Additionally, a positive anti-*Rickettsia* IgG response (at a 1:32 dilution) was found more commonly in mice injected with *R. felis*-infected feces than *R. felis* from culture (80% vs. 20%, respectively). These findings suggest that the flea feces acted as a potential adjuvant and increased the animals’ immune responses to the injected bacteria, leading to an increased clearance and lack of dissemination.

The low numbers of organisms detected via qPCR amplification is also thought to be the reason that no rickettsial transcripts were found in the skin of mice injected with *R. felis* from culture or *R. felis*-infected flea feces. As RNA is much more difficult to extract and detect than DNA, our conclusion remains that viable rickettsiae were intradermally inoculated into the mice. This idea is supported by the fact that detection of *R. felis* gDNA in the skin of mice injected with *R. felis*-infected flea feces was precluded when the flea feces were previously frozen, corroborating the thought that only viable rickettsiae could persist in the skin within detectable limits of qPCR amplification for 24 to 48 hours. Future studies with a higher inoculation dose that would increase the levels of bacteria within the skin, and thus likely increase the chances of RNA detection, would help confirm this hypothesis.
BALB/c mice were chosen as a murine model organism for vertebrate inoculations based on the previous findings that this strain produced a detectable rickettsemia post-intraperitoneal injection with *R. felis* from culture [32]. BALB/c mice are particularly well known for their demonstration of Th2-biased immune responses [47]. Rickettsial bacteria are intracellular organisms which target endothelial cells and macrophages [48], and therefore primarily induce a Th1 immune response, requiring the generation of cytotoxic T lymphocytes for protective immunity. In this sense, BALB/c mice could possibly be immunodeficient in regards to rickettsial killing if they favor a Th2 response. This work is clearly a preliminary study, and future use of different vertebrates (including alternate strains of mice, other rodents, and even larger vertebrates) will likely produce varying results.

The lack of physical changes, especially at the site of cutaneous inoculation with *R. felis*–infected flea feces, was a surprising finding. However, the histologic changes noted in the skin of the injected mice were all deep within the dermis and underlying subcutaneous tissue, with no evidence of epidermal changes, which supports the lack of gross findings. An ideal animal model for studying a disease would mimic the physical and clinical signs observed in the human disease. However, given the wide range of described clinical presentations for human flea-borne spotted fever, it is difficult to accurately assess if an animal model is appropriate. It could be suggested that the response of the mice in this study injected intradermally with *R. felis*-infected flea feces most closely resembles the “yaaf” disease entity, supported by the amplification of *R. felis* gDNA from skin at the inoculation site and anti-*Rickettsia* IgG titers 14 days post-injection. However, these mice clearly lacked cutaneous lesions or any other systemic signs. While the most common reported accompanying symptom in humans was a fever, and mouse body temperatures were never assessed, mice never showed any clinical signs that warranted further
physical examination (e.g. ruffled fur, lethargy, squinted eyes). Lack of epidermal changes, and thus gross lesions, could be secondary to the route of inoculation (intradermal injections) of the infectious flea feces. Another cutaneous route of inoculation, such as introduction of infectious flea feces into open dermal wounds or puncture of the epidermis with an infectious flea feces-laden object, could produce similar lesions to what is observed in the human disease.

Overall, this study shows that high numbers of viable rickettsiae are excreted in cat flea feces infected with *R. felis*. These viable bacteria, along with the flea feces, can be introduced into vertebrates via intradermal injection and produce a measurable response, consisting of a moderate to severe suppurative dermatitis and a positive anti-*Rickettsia* IgG titer. Future work with varying inoculation doses, additional vertebrate models, and alternative cutaneous infection routes may prove helpful in determining the role of these infectious arthropod feces in the epidemiology of human flea-borne spotted fever.
REFERENCES


VITA

Kelsey Porter Legendre is the daughter of Ms. Cynthia Ann Williams and Dr. James Robert Young. She was born in Metairie, Louisiana in 1989 and is the younger sister of Ryan Lewis Young. Kelsey attended Louisiana State University in Baton Rouge, Louisiana and majored in Animal Science while pursuing her dream of veterinary medicine. She graduated in 2007, and was accepted to Louisiana State University School of Veterinary Medicine (LSU SVM). During that time, her passion for clinical pathology led her to pursue advanced training in the form of a combined clinical pathology residency and Master of Science degree at LSU SVM. She met her husband, Michael Paul Legendre, during her undergraduate time at LSU and they are expecting their first child in April of 2018. Kelsey will complete her residency in the spring of 2018 and hopes to pursue a career in a clinical pathology diagnostic laboratory.