Identification of *Rickettsia felis* in the Salivary Glands of Cat Fleas

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ABSTRACT

Rickettsia felis, a flea-associated rickettsial pathogen, has been identified in many tissues, including the digestive and reproductive tissues, within the cat flea, *Ctenocephalides felis*. We utilized transmission electron microscopy and polymerase chain reaction to identify *R. felis* in the salivary glands of fed fleas and further define the distribution of *R. felis* within the arthropod host. We identified *Rickettsia*-like organisms in salivary glands using electron microscopy. Sequence analysis of portions of the *Rickettsia* genus-specific 17-kDa antigen gene and *R. felis* plasmid confirmed the morphological identification of *R. felis* in cat flea salivary glands. This is the first report of *R. felis* in tissues critical for horizontal transmission of rickettsiae. Key Words: Cat flea—*Ctenocephalides felis*—*Rickettsia felis*.

INTRODUCTION

Rickettsia felis IS AN INTRACELLULAR gram-negative bacterium associated predominantly with the cat flea, *Ctenocephalides felis*. Clinical reports, coupled with serological analysis and molecular detection of rickettsial DNA in human samples, implicate *R. felis* as a human pathogen (Parola et al. 2005). Horizontal transmission of *R. felis* between fleas and vertebrate hosts (including companion animals, rodents, and opossums) has been suggested as a potential source of human exposure (Azad et al. 1997, Case et al. 2006, Psaroulaki et al. 2006). Vertical transmission of *R. felis* is suspected to be the primary route of maintenance in colonized cat fleas (Azad et al. 1992, Wedincamp et al. 2002); however, the potential for horizontal transmis-

sion is evident as *R. felis* DNA was polymerase chain reaction (PCR) amplified in the blood of laboratory cats that were exposed to *R. felis*-infected cat fleas and subsequently seroconverted (Wedincamp et al. 2000). The acquisition mechanism of *R. felis* by vertebrates and uninfected fleas in nature is unknown.

The distribution of *R. felis* within fleas has been examined using both transmission electron microscopy (TEM) and PCR. Specific tissues in which *R. felis* has been identified in cat fleas include the midgut epithelial cells (adult and larval fleas), as well as adult flea muscle cells, fat body, tracheal matrix, ovaries, and the epithelial sheath of testes (Adams et al. 1990, Bouyer et al. 2001). However, the presence of *R. felis* in the salivary glands of the flea host has not been confirmed.

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Most of the studies characterizing the relationship between *R. felis* and fleas have utilized colonized fleas (Adams et al. 1990, Bouyer et al. 2001, Higgins et al. 1994, Moron et al. 2000, Pornwiroon et al. 2006, 2007, Wedincamp et al. 2000, 2002, 2003, Zavala-Castro et al. 2005). The objective of the current study was to identify *R. felis* in the salivary glands of cat fleas, which should allow expansion of studies relating to horizontal transmission of *R. felis*. In this report, we describe localization of *R. felis* in the salivary glands of fed fleas by TEM and PCR amplification of *R. felis* 17-kDa antigen gene, the conjugative plasmid pRF, but, consistent with the LSU strain of *R. felis*, not the smaller plasmid, pRF δ .

METHODS

Source of fleas

Rickettsia felis-infected *Ctenocephalides felis* were maintained at the Louisiana State University, School of Veterinary Medicine (LSU-SVM) on domestic shorthair cats as previously described (Henderson et al. 1993). The prevalence of rickettsial infection in the LSU colony of *C. felis* has been examined (Higgins et al. 1994), and we have recently identified *R. felis* prevalence in this colony to be approximately 94% (Pornwiroon et al. 2007). Unfed, *R. felis*-infected adult fleas were applied directly to the cat and allowed to feed for 2–4 days. A flea comb was used to brush/remove fleas off the cat host. Fleas were immobilized on ice and dissected under a standard dissecting microscope. Salivary glands were recovered in phosphate-buffered saline and stored on ice until placed into tissue fixative or frozen for subsequent DNA isolation.

Electron microscopy

Salivary glands from approximately 10 fed adult fleas were extirpated at LSU-SVM and placed in a fixative containing 1.25% formaldehyde, 2.5% glutaraldehyde, 0.03% CaCl₂, and 0.03% trinitrophenol in 0.05 M cacodylate buffer, pH 7.4 (Ito et al. 1981). The fixed samples were transported to University of Texas Medical Branch (UTMB), where they were post-fixed in 1% osmium tetroxide in 0.05 M cacodylate buffer (pH 7.2), stained *en bloc* for

30 min with 1% uranyl acetate in 0.05 M maleate buffer, pH 5.2 at 60°C. The fixed tissues were then dehydrated in a graded series of ethanol and embedded in Poly/Bed 812 (Polysciences). Ultrathin sections were cut with diamond knives on Reichert-Leica Ultracut S ultramicrotome, mounted on copper grids, and stained at room temperature for 5 min in 2% aqueous uranyl acetate followed by 3 min in lead citrate. TEM was carried out at UTMB with a Philips 201 transmission electron microscope (Philips Electron Optics) as previously described (Bouyer et al. 2001).

DNA isolation and PCR amplification

Salivary glands from approximately 20 fed adult fleas were collected separately into a 1.5-mL microcentrifuge tube, pelleted by centrifugation, and resuspended in 200 μ L of sterile phosphate-buffered saline. Genomic DNA was extracted from flea salivary glands using the DNeasy tissue kit (Qiagen) according to the manufacturer's protocol; an empty tube environmental blank treated the same as the sample was also used for genomic DNA extraction. For PCR amplification, 20 ng of genomic DNA from sample fleas, genomic DNA from *R. felis* (LSU; positive control for 17-kDa antigen gene and plasmid pRF) (Pornwiroon et al. 2006), environmental genomic DNA blank (genomic DNA isolation negative control), or water (PCR negative control) were used as templates for PCR in a final volume of 25 μ L. PCR products were amplified using PCR Master Mix (Promega) together with gene-specific oligonucleotide primers (400 nM for each primer) for the 17-kDa antigen gene (Williams et al. 1992), pRF (primers pRFc and pRFd), pRF δ (primers pRFa and pRFd), and with the addition of the primer combination (pRFa and pRFb) to amplify the approximately 160-basepair portion of pRF (Ogata et al. 2005). The specific annealing temperatures for the primer pairs were 55°C (Rr17.61p and Rr17.492n; pRFc and pRFd) as reported by Pornwiroon et al. (2006) and 50°C (pRFa and pRFd; pRFa and pRFb).

DNA sequencing and analysis

The PCR products were gel purified and cloned into pCR4-TOPO vector (Invitrogen)

according to the manufacturer's protocol. M13forward, M13reverse, and pRF36882p (Pornwiroon et al. 2006) was used in the sequencing reaction to obtain sequence data for the inserts. At least two clones of each PCR amplicon were sequenced by the dye terminator method on a 3130 Genetic Analyzer (Applied Biosystems) at LSU-SVM to generate a consensus sequence using VectorNTI software (Invitrogen). Nucleotide similarity comparisons were assessed using the GenBank database.

RESULTS AND DISCUSSION

In this report, we demonstrate *R. felis* in the salivary glands of fed cat fleas by microscopic and molecular analyses. The recovery of flea salivary glands is challenging, possibly limit-

ing examination of *R. felis* in these tissues. Also, the high prevalence of *R. felis* in the colony increased the likelihood of detecting rickettsiae in a limited number of flea salivary glands. In *C. felis* salivary glands, *R. felis* (LSU) had typical *Rickettsia* morphology (Fig. 1). Rickettsiae were located free in the cytosol of epithelial cells. Increased clearance of cytoplasmic contents around *R. felis*, usually described as "halo," was also observed. The findings are similar to our previous report for *R. felis* (LSU) in the tick-derived cell line ISE6 in which there were clear areas of cytosol associated with clusters of rickettsiae. Occasionally, we observed rickettsiae with altered morphology within membrane-bound cytoplasmic vacuoles. These rickettsiae usually had irregular shape, dense cytoplasm, enlarged periplasmic spaces and represented rickettsiae being destroyed within

FIG. 1. Ultrastructure of *Rickettsia felis* (Louisiana State University [LSU]) in cat flea salivary glands. Scale bar = 1 μ m. **(A)** Rickettsiae free in the cytosol (arrows) of several epithelial cells. Microvilli (MV) extending into the lumen (L) of the salivary gland. **(B)** Rickettsiae are clustered in a basal part of a cell close to the basal lamina (BL) in an epithelial cell from a different flea. Arrowheads indicate small vacuoles in the rickettsial cytoplasm.

the phagolysosomes (not shown). Also some rickettsiae contained electron-lucent vacuoles in their cytoplasm (Fig. 1B) as demonstrated for *R. felis* earlier (Bouyer et al. 2001, Pornwiroon et al. 2006).

The presence of *R. felis* in the *C. felis* salivary glands was confirmed by PCR amplification and sequencing of the *Rickettsia* genus-specific 17-kDa antigen gene and *R. felis* plasmid (Fig. 2). Nucleotide sequences for the amplified portions of the 17-kDa antigen (434 bp) gene and pRF plasmid (1,342 bp) were identical to the sequences reported previously for *R. felis* (LSU) (Pornwiroon et al. 2006) and to the sequences reported for *R. felis* in the GenBank database (accession numbers CP000053 and AF195118 for 17-kDa antigen gene). Similar to our previous study (Pornwiroon et al. 2006), the portion of pRF δ plasmid was not amplified in all samples tested, including both the environmental blank incorporated into the gDNA isolation procedure and the negative control for the PCR (water). Additionally, we were unable to am-

plify rickettsial DNA from whole *R. felis*-negative *C. felis* (Heska Corp., Loveland, CO; data not shown). However, the amplification of rickettsial DNA with the pRFa and pRFb primer set indicates that the pRFa primer is valid. While pRF has been recently confirmed by pulse-field gel electrophoresis and southern blot analysis in *R. felis* (LSU) (Baldrige et al. 2007a), additional experimental evidence supports the lack of pRF δ in *R. felis* (LSU) and other *R. felis* strains examined (Jiang et al. 2006), and recent interpretation of the genome sequence suggests that only one plasmid may be present in the genome (Gillespie et al. 2007).

Although there is potential for the fleas used in the current study to have acquired *R. felis* from the vertebrate host during the 3-day feeding period, we believe that this is not the case in this laboratory model for two reasons. First, the role of horizontal transmission for maintenance of *R. felis* is not clear, as horizontal transmission of viable *R. felis* via cat flea feeding has not been proven. Additionally, cat flea uptake

FIG. 2. Polymerase chain reaction (PCR) amplification of genes encoding *Rickettsia* genus specific 17-kDa antigen and plasmid from *Rickettsia felis* (Louisiana State University [LSU])–infected cat flea salivary glands. In cat flea salivary gland samples and cultured *R. felis* (LSU), portions of genes encoding the 17-kDa antigen and pRF (primers pRFc-d), but not pRF δ (primers pRFa-d), were amplified. The amplification of a portion of pRF with the primer pair pRF(a-b) confirms the inability to detect pRF δ by PCR amplification. An environmental blank processed at the same time as samples and water served as negative controls for the genomic DNA extraction and PCR, respectively. Marker (100 bp) sizes are listed to the left of each gel.

of *R. felis* during bloodmeal acquisition, either directly from the vertebrate host or through cofeeding with infected fleas, has not been demonstrated (Wedincamp et al. 2002). Cats seroconvert after controlled exposure to previously unfed, infected fleas, indicating horizontal transmission; however, overt disease was not observed, and viable rickettsiae have not been recovered from cats (Wedincamp et al. 2000). Second, a recent study assessing *R. felis* infection in the LSU colony of newly-emerged unfed adult cat fleas, using whole fleas, has demonstrated approximately 94% prevalence of *R. felis* within this population of fleas (Pornwiroon et al. 2007). The possibility that *R. felis* disseminates to the salivary glands from other tissues in accompaniment with bloodmeal acquisition, versus being constitutively present in cat flea salivary glands, is interesting but unlikely. For example, vertically maintained bacteria (e.g., *Wolbachia*) are often identified in the salivary glands of insect hosts (Dobson et al. 1999). The potential interaction of bacteria within the flea host is intriguing; *Wolbachia* spp. 16S rRNA sequences have recently been amplified from the LSU colony of fleas (Pornwiroon et al. 2007). *R. felis* infection does impact the species richness of the microbiota (Pornwiroon et al. 2007), and the interaction between rickettsiae and other members of the bacterial community in relation to vector competence is under investigation.

The association between rickettsial infection of specific arthropod tissues and potential for horizontal transmission is not clear. Some tick-borne spotted fever group *Rickettsia* not associated with human disease, for example, *Rickettsia peacockii*, have limited tissue distribution within the invertebrate hosts (*Dermacentor andersoni*) in nature and are maintained via vertical transmission within the tick rather than horizontal transmission to vertebrate hosts during feeding (Burgdorfer et al. 1981, Niebylski et al. 1997). Additionally, although *Rickettsia monacensis* has recently been associated with human infection (Jado et al. 2007), it can be identified in the salivary glands of *Ixodes scapularis* in the absence of horizontal transmission (Baldridge et al. 2007b).

In summary, we report here the first ultrastructural and molecular characterization of *R.*

felis in the salivary glands of cat fleas. The significance of *R. felis*, and other bacteria, in the salivary gland with respect to horizontal transmission is currently under investigation.

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