Characterization of 'Candidatus Neoehrlichia lotoris' (family Anaplasmataceae) from raccoons (*Procyon lotor*)

Michael J. Yabsley,^{1,2} Staci M. Murphy,² M. Page Luttrell,²
Benjamin R. Wilcox,^{1,2} Elizabeth W. Howerth³ and Ulrike G. Munderloh⁴

Correspondence

Michael J. Yabsley
myabsley@uga.edu

¹Daniel B. Warnell School of Forestry and Natural Resources, University of Georgia, Athens, GA 30602, USA

²Southeastern Cooperative Wildlife Disease Study, Department of Population Health, University of Georgia, Athens, GA 30602, USA

³Department of Pathology, College of Veterinary Medicine, University of Georgia, Athens, GA 30602, USA

⁴University of Minnesota, Department of Entomology, St Paul, MN 55108, USA

Recently, a novel ehrlichial organism was isolated from a raccoon (*Procyon lotor*) and the isolate (RAC413) was infectious to two naïve raccoons but not laboratory mice, rats or rabbits. In this study, amplification and sequencing of four gene targets (16S rRNA gene, *groESL*, *gltA* and *rpoB*) confirmed that the novel ehrlichial organism was a member of the family *Anaplasmataceae* and was most closely related to, but distinct from, 'Candidatus Neoehrlichia mikurensis' TK4456^R and IS58. RAC413 shared the highest sequence similarity with members of the genus *Ehrlichia* (94.2–95.1, 80.9–83.1, 67.9–71.9 and 39.9–40.7 % similarity for the 16S rRNA gene, *groESL*, *gltA* and *rpoB*, respectively). No sequence variation in three sequences (16S rRNA gene, *groESL* and *gltA*) was observed between the RAC413 isolate and five additional sequences amplified from blood of naturally infected raccoons from several geographically isolated populations in the south-eastern USA. Serum samples from four experimentally infected raccoons did not react to *Ehrlichia canis*, *Ehrlichia chaffeensis*, *Anaplasma marginale* or *Anaplasma phagocytophilum* antigens in an immunofluorescence assay or an *Ehrlichia ewingii* peptide in an ELISA format. On the basis of the distinctive molecular and serological characteristics and apparent host specificity of this ehrlichial organism, it is proposed that this organism be designated 'Candidatus Neoehrlichia lotoris' (reference strain RAC413^R).

INTRODUCTION

Members of the family *Anaplasmataceae* (genera *Aegyptianella*, *Anaplasma*, *Ehrlichia*, *Neorickettsia* and *Wolbachia*) are obligately intracellular bacteria that reside within membrane-enclosed vacuoles (Dumler *et al.*, 2001). This family also includes two *Candidatus* status genera, 'Candidatus Xenohalictis', detected in abalone (*Halictis* spp.) (Friedman *et al.*, 2000), and 'Candidatus Neoehrlichia', detected in ticks and rodents from Europe and Asia (Schouls *et al.*, 1999; Pan *et al.*, 2003; Brouqui

et al., 2003; Sanogo *et al.*, 2003; Kawahara *et al.*, 2004; Beninati *et al.*, 2006; Tabara *et al.*, 2007). 'Candidatus Neoehrlichia mikurensis' is distinguished from other genera and species based on sequence analysis of the partial 16S rRNA, citrate synthase (*gltA*) and *groESL* genes of several strains (Kawahara *et al.*, 2004; Brouqui *et al.*, 2003). Ultrastructural analysis of a single strain (TK4456^R) of this group revealed bacteria in endothelial cells of experimentally infected rats that were consistent with members of the family *Anaplasmataceae* (Kawahara *et al.*, 2004).

Sequences that were similar to or identical to 'Candidatus Neoehrlichia mikurensis' strains TK4456^R and IS58 have been detected in *Ixodes ricinus* ticks in the Netherlands (*Ehrlichia*-like sp. Shotti variant), *I. ricinus* in Russia and Italy (called 'Candidatus Ehrlichia walkerii') and *Ixodes persulcatus* and *Ixodes ovatus* from Japan (Schouls *et al.*,

Abbreviation: IFA, immunofluorescence assay.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *groESL*, *gltA* and *rpoB* gene sequences of 'Candidatus Neoehrlichia lotoris' RAC413^R are EF633744–EF633747, respectively.

Details of reference sequences and similarity matrices for the 16S rRNA gene, *groESL*, *gltA* and *rpoB* sequences are available as supplementary material with the online version of this paper.

1999; Alekseev *et al.*, 2001; Brouqui *et al.*, 2003; Sanogo *et al.*, 2003; Pan *et al.*, 2003; Kawahara *et al.*, 2004; Rar *et al.*, 2008). To date, natural infections have been reported from brown or Norway rats (*Rattus norvegicus*) from China and Japan, small Japanese field mice (*Apodemus argenteus*), large Japanese field mice (*Apodemus speciosus*), grey red-backed voles (*Myodes rufocanus bedfordiae*) and Smith's red-backed voles (*Eothenomys smithii*) in Japan, an East European field vole (*Microtus rossiaemeridionalis*) from Russia and a bank vole (*Myodes glareolus*) from Italy (Pan *et al.*, 2003; Kawahara *et al.*, 2004; Beninati *et al.*, 2006; Naitou *et al.*, 2006; Tabara *et al.*, 2007; Rar *et al.*, 2008).

In the USA, an ehrlichial organism closely related to 'Candidatus Neohrlichia mikurensis', based on a partial 16S rRNA gene sequence, was previously detected in 53 % of tested raccoons (*Procyon lotor*) from the Piedmont region of Georgia (Dugan *et al.*, 2005). Recently, this organism was isolated in ISE6 tick cell culture (Munderloh *et al.*, 2007) and the culture was infectious to raccoons but failed to produce a detectable infection in two strains of laboratory mice, laboratory rats or laboratory rabbits (Yabsley *et al.*, 2008a). The organism was commonly detected in tick-infested raccoon populations (prevalence of 46–95 %) but was not found in 137 rodents of 10 species (Yabsley *et al.*, 2008a). Herein, we characterize this organism more fully and propose that it should be designated 'Candidatus Neohrlichia lotoris'.

METHODS

Animals and procedures. Six, 10-week-old raccoons (RAC413, RAC414, RAC974, RAC975 and two negative controls) were acquired from a commercial source (Ruby Fur Farms, New Sharon, IA, USA). Raccoons were infected either by inoculation of infected blood (RAC413 and RAC414) or by inoculation of infected ISE6 tick cells (RAC974 and RAC975) as described previously (Munderloh *et al.*, 2007; Yabsley *et al.*, 2008a). Data on inoculation methods and infection dynamics in these raccoons have been provided previously (Munderloh *et al.*, 2007; Yabsley *et al.*, 2008a).

Serological testing. Plasma samples were tested for antibodies reactive with *Ehrlichia chaffeensis*, *Ehrlichia canis*, *Anaplasma marginale* and *Anaplasma phagocytophilum* by immunofluorescence assay (IFA) as described previously (Comer *et al.*, 2000; Yabsley *et al.*, 2003; Little *et al.*, 1998) and with *Ehrlichia ewingii* using an experimental peptide ELISA (IDEXX Laboratories). For IFA, plasma samples were tested at a 1:64 dilution in 0.01 M PBS. Slides were either purchased commercially from Focus Technologies, (*E. chaffeensis* and *A. phagocytophilum*) or made in our laboratory (*A. marginale*, *E. canis* and RAC413 organism). To make IFA slides, infected cultures were harvested, washed twice in PBS, applied to slides, allowed to dry and fixed with acetone. Fluorescein isothiocyanate (FITC)-labelled rabbit anti-raccoon immunoglobulin G (Kirkegaard & Perry Laboratories) diluted 1:50 in PBS was used as a conjugate. During the final wash, slides were counterstained with 1.65 % eriochrome black T. Positive controls for the IFA included serum samples from raccoons experimentally inoculated with either *E. chaffeensis* (titres of 1:128 and 1:1024), *E. canis* (1:512) or *A. phagocytophilum* (1:256 and 1:1024) (Yabsley *et al.*, 2008b) and serum from an *A. marginale*-infected deer (1:256) (combined with an FITC-labelled anti-deer antibody; Kirkegaard &

Perry Laboratories). Serum from a dog experimentally infected with *E. ewingii* was used as a positive control for the *E. ewingii* ELISA. Negative control serum samples were collected from young laboratory-raised raccoons, laboratory-raised orphaned fawns and purpose-bred young beagles. Two additional negative serum controls were collected from two raccoons that had been inoculated with 1×10^6 uninfected ISE6 tick cells.

Molecular characterization and phylogenetic analyses. Partial 16S rRNA, *groESL*, *gltA* and RNA polymerase β -subunit (*rpoB*) genes were amplified and sequenced from the culture isolate RAC413 (Inokuma *et al.*, 2001; Taillardat-Bisch *et al.*, 2003; Kawahara *et al.*, 2004). In addition, we investigated potential intraspecific variation by analysis of partial 16S rRNA gene, *groESL* and *gltA* sequences from five wild raccoons from geographically diverse locations in Florida ($n=2$), Georgia ($n=2$) and South Carolina ($n=1$). Resulting sequences were aligned with those from related organisms in GenBank using CLUSTAL_X. Phylogenetic analyses of the RAC413 sequences and related organisms were conducted using the MEGA program using the neighbour-joining algorithm with Kimura's two-parameter model (GenBank accession numbers are provided in Supplementary Table S1, available in IJSEM Online).

RESULTS AND DISCUSSION

All four raccoons, regardless of exposure route or inoculum, became PCR-positive for 'Candidatus N. lotoris', and the organism was successfully isolated from each raccoon into ISE6 tick cells (Munderloh *et al.*, 2007; Yabsley *et al.*, 2008a). Plasma samples from each sampling date from the four experimentally infected raccoons did not react with *E. chaffeensis*, *E. canis*, *E. ewingii*, *A. marginale* or *A. phagocytophilum* by IFA or ELISA. Beginning on day 16 p.i. (RAC413), day 21 p.i. (RAC414) or day 28 p.i. (RAC974 and RAC975), an antibody reaction to the culture isolate of strain RAC413^R was observed at a dilution of 1:64. Subsequent sampling periods also maintained an antibody response at only a 1:64 dilution. In positive samples, fluorescence was localized to clusters of intracellular organisms; no fluorescence was observed for samples from raccoons inoculated with uninfected ISE6 tick cells.

The partial 16S rRNA gene sequences (1379 bp) from all six samples of the raccoon ehrlichial agent (isolate RAC413^R and samples from five wild raccoons) were identical. The sequences were most similar (98.4–98.8 %) to those of 'Candidatus N. mikurensis' strains TK4456^R and IS58 (Kawahara *et al.*, 2004) (complete similarity matrix shown in Supplementary Table S2). The level of similarity between the two 'Candidatus N. mikurensis' strains was 99.3 %. The highest similarities to members of a currently described genus were with *Ehrlichia* (94.2–95.1 %), followed by *Anaplasma* (92.7–93.1 %). The similarities between members of the genera *Ehrlichia* and *Anaplasma* ranged from 92.6 to 93 % and the similarity between *Aegyptianella pullorum* (for the 574 available nucleotides deposited as GenBank accession no. AY125087) and members of the genus *Anaplasma* ranged from 95.2 to 96.4 % (Rikihisa *et al.*, 2003).

Partial *groESL* (1226 bp) and *gltA* (520 bp) sequences from isolate RAC413^R and organisms from five wild raccoons were identical. The sequence analysis supported the findings of the 16S rRNA gene sequence analysis; the *groESL* sequences were most similar (90.3 and 90.6%, respectively) to '*Candidatus N. mikurensis*' strains TK4456 and IS58 (see Supplementary Table S3 for complete similarity matrix). The *gltA* sequence of RAC413^R was most similar (84.4%) to that of *Ehrlichia* sp. Belluno ('*Candidatus Ehrlichia walkerii*'; GenBank accession no. AY098729), which is most closely related to '*Candidatus N. mikurensis*' by phylogenetic analyses (Yabsley *et al.*, 2008a) (see Supplementary Table S4 for similarity matrix). The genetic similarities between isolate RAC413^R and members of the genus *Ehrlichia* was only slightly higher than the similarities between the genera *Ehrlichia* and *Anaplasma* for the *groESL* (80.9–83.1 and 71.1–76.5%, respectively) and *gltA* (67.9–71.9 and 57.1–67.3%, respectively) genes. The partial *rpoB* (781 bp) sequence from RAC413^R was 39.9–40.7% identical to those of *Ehrlichia* species, 36.0–37.7% identical to those of *Anaplasma* species and 35.6–36.4% identical to those of *Neorickettsia* species; no *rpoB* sequences were available from '*Candidatus N. mikurensis*' for comparison.

Phylogenetic trees inferred from comparison of the 16S rRNA gene (Fig. 1) and *groESL* (Fig. 2) sequences provided similar topology and indicated a close relationship of '*Candidatus N. lotoris*' RAC413^R with '*Candidatus N. mikurensis*'. Similar to previous studies (Kawahara *et al.*, 2004; Brouqui *et al.*, 2003), '*Candidatus N. mikurensis*' was distinct from other genera in the family *Anaplasmataceae* and formed a well-supported sister clade to the genus *Ehrlichia*.

In the current study, we have shown by analysis of several gene targets that an ehrlichial organism recently isolated from a raccoon from the US state of Georgia (Munderloh *et al.*, 2007) is closely related to, but distinct from, '*Candidatus N. mikurensis*' TK4456^R and IS58. 16S rRNA gene and *groESL* sequences similar to those of '*Candidatus*

N. mikurensis' have been detected from a diverse group of rodents and *Ixodes* species from Italy, Japan, the Netherlands and Russia (Schouls *et al.*, 1999; Alekseev *et al.*, 2001; Brouqui *et al.*, 2003; Pan *et al.*, 2003; Kawahara *et al.*, 2004; Beninati *et al.*, 2006; Naitou *et al.*, 2006; Rar *et al.*, 2008). All of these current sequences from this group have been lumped together in a single candidate species ('*Candidatus N. mikurensis*'). Additional samples from other hosts over a wide geographical range and sequence data from other gene targets are necessary to determine whether '*Candidatus N. mikurensis*' represents a group of several distinct species or a single, genetically diverse species. In contrast, no differences in sequences of three genes (16S rRNA gene, *groESL* and *gltA*) were observed between '*Candidatus N. lotoris*' RAC413^R and five samples from naturally infected raccoons from three US states.

One of the characteristics of '*Candidatus N. mikurensis*' TK4456^R is a lack of serological cross-reactivity with *A. phagocytophilum* or *Ehrlichia muris* antigens by IFA (Kawahara *et al.*, 2004). Similarly, no serological cross-reactivity with *E. chaffeensis*, *E. canis*, *E. ewingii*, *A. marginale* or *A. phagocytophilum* antigens was noted in four raccoons experimentally infected with '*Candidatus N. lotoris*' RAC413^R. Serological cross-reactions between species of *Ehrlichia*, *Anaplasma* and *Neorickettsia* have been reported (Comer *et al.*, 2000; Rikihisa, 1991), but the level of cross-reactivity is highly variable. The lack of cross-reactivity between '*Candidatus N. lotoris*' and other ehrlichial organisms may be due to the fact that raccoons develop only a weak immune response to '*Candidatus N. lotoris*' antigens. Western blot analysis may yield more information about the immunological response of raccoons to '*Candidatus N. lotoris*'.

Members of the family *Anaplasmataceae* (*Aegyptianella*, *Anaplasma*, *Ehrlichia*, *Neorickettsia*, *Wolbachia*, '*Candidatus Neoehrlichia*' and '*Candidatus Xenohalictis*') are obligately intracellular organisms and, although they have similar morphological characters, some genera exhibit unique biological characteristics. Members of the genera

Fig. 1. Phylogenetic tree based on the 16S rRNA gene sequences of '*Candidatus Neoehrlichia lotoris*' RAC413^R and related organisms. Numbers at nodes indicate percentages of bootstrap support based on 1000 replicates. Bar, 0.02 substitutions per nucleotide position.

Fig. 2. Phylogenetic tree based on the *groESL* sequences of 'Candidatus Neoehrlichia lotoris' RAC413^R and related organisms. Numbers at nodes indicate percentages of bootstrap support based on 1000 replicates. Bar, 0.05 substitutions per nucleotide position.

Aegyptianella, *Anaplasma* and *Ehrlichia* infect a wide range of vertebrate hosts, but all with known life cycles utilize ticks as vectors (Dumler *et al.*, 2001), while members of the genus *Neorickettsia* infect fish and mammalian hosts and utilize a variety of flukes as vectors (Dumler *et al.*, 2001). In contrast, *Wolbachia* and 'Candidatus Xenohalictis' do not utilize vectors for transmission; *Wolbachia* species infect a variety of arthropods and nematodes and are believed to be transmitted primarily transovarially (Dumler *et al.*, 2001), while 'Candidatus Xenohalictis californiensis' is transmitted directly from one host to another (Friedman *et al.*, 2000). Currently, little is known about the natural history of 'Candidatus N. mikurensis' and 'Candidatus N. lotoris'. Many sequences related to, or identical to, 'Candidatus N. mikurensis' have been detected in *Ixodes* ticks, and infections of raccoons with 'Candidatus N. lotoris' were associated with tick-infested populations, so these organisms are probably transmitted by ixodid ticks, similar to *Ehrlichia* and *Anaplasma*. Future studies should attempt transmission studies and cultivation of 'Candidatus N. mikurensis' strains to characterize the proposed genus 'Candidatus Neoehrlichia' further.

Description of 'Candidatus Neoehrlichia lotoris'

'Candidatus Neoehrlichia lotoris' [lo.tor]'is. L. gen. n. *lotoris* of a clothes-washer, of *Procyon lotor* (a raccoon)]. The reference strain is RAC413^R. Isolated from a captive raccoon experimentally infected with 'Candidatus N. lotoris' by blood inoculation from a naturally infected raccoon from Clarke Co., Georgia, USA (Munderloh *et al.*, 2007). Not infectious to two strains of laboratory mice (*Mus musculus*, strains BALB/c and C3H), laboratory rats (*Rattus norvegicus*, Wistar strain) or laboratory rabbits (*Oryctolagus cuniculus*, New Zealand White) in limited inoculation trials (Yabsley *et al.*, 2008a). Host cells within raccoons are not known. Morphology of organisms in ISE6 tick cells is similar to that of other members of the family *Anaplasmataceae*. Member of the family *Anaplasmataceae* based on 16S rRNA gene, *groESL* and *gltA* sequences and growth within membrane-bound cytoplasmic vacuoles. Organisms are Gram-negative, highly pleomorphic (compact coccus-like and elongated, wavy forms; 0.5–4 µm), stain light to dark purple with Giemsa and tend to cluster

on the side of the endosome nearest the host cell nucleus. Ultrastructurally, the organism has an outer unit membrane separated from the inner cell wall by a thin periplasmic space, characteristic of the family *Anaplasmataceae*. In some cases, individual bacteria are wrapped in two sets of membranes. Analysis of the 16S rRNA gene sequence revealed a novel 2 bp insertion compared with members of the genus *Ehrlichia* (AT at base 784 of *E. chaffeensis* Arkansas^T; GenBank accession no. AF416764). Analysis of *gltA* sequence revealed a novel deletion compared with members of the genera *Ehrlichia* and *Anaplasma* (6 bp deletion at base 1011 of *E. chaffeensis* Arkansas^T; GenBank accession no. AF304142) and a 2 bp insertion compared with other members of the family *Anaplasmataceae* (TTG at base 744 of *E. chaffeensis* Arkansas^T; AF304142). The G+C content of four sequenced genes of 'Candidatus N. lotoris' RAC413^R is 38.1 mol%. Can be differentiated from 'Candidatus N. mikurensis' on the basis of unique 16S rRNA gene, *groESL* and *gltA* sequences.

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