Ultrastructure and phylogenetic analysis of 'Candidatus Neoehrlichia mikurensis' in the family Anaplasmataceae, isolated from wild rats and found in *Ixodes ovatus* ticks

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A novel bacterium that infects laboratory rats was isolated from wild *Rattus norvegicus* rats in Japan. Transmission electron microscopy of the spleen tissue revealed small cocci surrounded by an inner membrane and a thin, rippled outer membrane in a membrane-bound inclusion within the cytoplasm of endothelial cells. Phylogenetic analysis of the 16S rRNA gene sequence of the bacterium found in *R. norvegicus* rats and *Ixodes ovatus* ticks in Japan revealed that the organism represents a novel clade in the family Anaplasmataceae, which includes the Schotti variant found in *Ixodes ricinus* ticks in the Netherlands and the *Ehrlichia*-like *Rattus* strain found in *R. norvegicus* rats from China. The novel clade was confirmed by phylogenetic analysis of *groESL* sequences found in *R. norvegicus* rats and *Ixodes ovatus* ticks in Japan. No serological cross-reactivity was detected between this bacterium and members of the genera *Anaplasma*, *Ehrlichia* or *Neorickettsia* in the family Anaplasmataceae. It is proposed that this new cluster of bacteria should be designated 'Candidatus Neoehrlichia mikurensis'.

The family Anaplasmataceae currently includes five genera: *Ehrlichia*, *Anaplasma*, *Neorickettsia*, *Aegyptianella* and *Wolbachia* (Dumler *et al.*, 2001; Rikihisa *et al.*, 2003). These are all obligately intracellular bacteria that are capable of infecting invertebrates and/or vertebrates. Species of the genera *Ehrlichia* and *Anaplasma* include the emerging

tick-borne human pathogens, such as *Ehrlichia chaffeensis* and *Anaplasma phagocytophilum*. As it is difficult to isolate and culture this group of bacteria, the ultrastructure and sequences of conserved genes, such as 16S rRNA, *groEL* and *gltA* (citrate synthase gene), are primarily used to identify and classify this group of bacteria. The present study describes a novel bacterium in the family Anaplasmataceae that was isolated from wild *Rattus norvegicus* rats by using laboratory rats. The bacteria were found in *R. norvegicus* rats and *Ixodes ovatus* ticks in Japan and represent a novel genetic cluster, based on phylogenetic analysis of 16S rRNA gene sequences that included the bacteria found in *R. norvegicus* rats in China (Pan *et al.*, 2003) and in *Ixodes ricinus* ticks in the Netherlands (Schouls *et al.*, 1999). Of note, infection of *I. ricinus* ticks with similar bacteria in Baltic regions of Russia was reported by Alekseev *et al.* (2001). A similar 16S

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *groESL* gene sequences of 'Candidatus Neoehrlichia mikurensis' determined in this study are respectively AB084582 and AB084583 for strain TK4456^R from a wild rat and AB074460 and AB074461 for strain IS58 from *I. ovatus* ticks.

Detailed primer sequences and phylogenetic tables are available as supplementary material in IJSEM Online.

rRNA gene sequence and a *gltA* (621 bp) sequence of the bacterium '*Candidatus Ehrlichia walkeri*' in *I. ricinus* ticks removed from asymptomatic humans in Italy (Brouqui *et al.*, 2003; Sanogo *et al.*, 2003) were reported recently, although whether the humans from whom ticks were removed were infected with this agent was not mentioned. There was no additional information, such as the mammalian host or light or electron microscopic description, available for '*Candidatus E. walkeri*'. The *groEL* sequence had not been determined for any of the above-mentioned bacteria.

Phylogenetic analysis based on *groEL* sequences of the bacteria in *R. norvegicus* rats and *I. ovatus* ticks in Japan confirmed this novel cluster in the family *Anaplasmataceae* by analysis of 16S rRNA gene sequences. So far, only *A. phagocytophilum sensu lato* DNA has been detected in wild rats (*Neotoma fuscipes*) (Castro *et al.*, 2001). This is the first member of the family *Anaplasmataceae* that is known to infect *Rattus* species. Establishing a new classification and determining its geographical distribution and tick and mammalian host species would facilitate future diagnosis and identification of this new group of bacteria. According to Murray & Stackebrandt (1995), '*Candidatus*' status is applied to an uncultured species for which relatedness has been determined and phenotypic properties, such as ultra-structure, are made available, but is not formally recognized by the International Code of Nomenclature of Bacteria. Thus, we propose to designate this novel taxon '*Candidatus Neoehrlichia mikurensis*'. The well-characterized reference strain is TK4456^R.

The characterization of a novel member of the family *Anaplasmataceae* in the present study contributes significantly to veterinary medicine and public health by providing a means for differential laboratory diagnostics.

Wild rat and tick specimens

In total, 15 rats (*R. norvegicus*) were captured on Mikura Island, southern Japan, in 1998, 1999 and 2003. Animals were anaesthetized with ether to collect blood specimens and after euthanasia, livers and/or spleens were collected aseptically from these rats and frozen at -20°C (specimens collected in 1998 and 1999) or -80°C (specimens collected in 2003) until DNA extraction was performed. Tissues from *R. norvegicus* rats were homogenized in sucrose/phosphate/glutamate (SPG) buffer (3.8 mM KH_2PO_4 , 7.2 mM K_2HPO_4 , 4.9 mM L-glutamate, 218 mM sucrose, pH 7.2). The 10% (w/v) spleen homogenate derived from rat MK01, which was captured in 2003 and was PCR-positive for '*Candidatus Neoehrlichia mikurensis*', was inoculated intraperitoneally into five 8-week-old Wistar male rats (Japan SLC) at 0.5 ml per rat. To determine the isolation of the bacterium, one rat was killed at each of the following time points: 10, 15, 20, 30 and 60 days after inoculation. Samples were subjected to PCR and electron microscopy. To determine the presence of cross-reacting antibodies, an indirect fluorescent antibody (IFA) test was performed on blood

specimens from these rats by using *Ehrlichia muris* and *A. phagocytophilum* antigen slides, as described previously (Kawahara *et al.*, 1999; Rikihisa *et al.*, 1997).

A total of 164 adult ticks (*I. ovatus*, 131 female and 33 male) were collected in Hokkaido, northern Japan, in 2000 and 2001 by flag sampling. Ticks were soaked for 10 min in a solution of 70% ethanol with 0.1% povidone/iodine and then rinsed with PBS supplemented with 0.5–1.0% calf serum. Fifty-four ticks collected in 2000 were grouped into 18 pools of three ticks each and ground in 0.6 ml SPG buffer with a glass pestle on a glass slide with a central depression. Each of the resulting tick homogenates was divided in half. One half was frozen at -20°C and used for DNA extraction and the other half was frozen at -80°C and used to isolate infectious agents. Of 110 ticks that were collected in the year 2001, 80 live ticks were divided vertically from the median line with a sterile razor blade under a dissection microscope. One half of each bisected tick was ground up individually in TRIzol buffer (Invitrogen Life Technologies). The other half of each tick was ground up individually in SPG buffer and frozen at -80°C for isolation of infectious agents. Thirty dead ticks were ground up individually in SPG buffer for PCR. The four tick homogenates that tested positive for '*Candidatus N. mikurensis*' by PCR were each inoculated intraperitoneally into two 3-week-old male BALB/c mice (Japan SLC) and two 5-week-old male CB-17/1cr-scid mice (CLEA Japan).

PCR detection of members of the family *Anaplasmataceae* in wild rats and ticks

DNA was extracted from the blood, liver and/or spleen of each rat, as well as from tick homogenates, by using a QIAamp DNA mini or tissue kit (Qiagen). RNA was extracted from ticks with TRIzol reagent (Invitrogen Life Technologies) and samples containing 1–5 µg RNA were subjected to reverse transcription in a 20 µl reaction mixture that contained 10 mM random hexamer, 0.5 mM each dNTP, 1 U RNase inhibitor and 200 U SuperScript II reverse transcriptase (Invitrogen Life Technologies) at 42°C for 50 min. The sequences and nucleotide positions of all the primers used for this study are available in Supplementary Table A in IJSEM Online. To amplify the 16S rRNA gene, an initial PCR was performed in a 25 µl reaction mixture that contained 2 µl extracted DNA or cDNA, forward primer EC12A and reverse primer EC9. All PCR products were sequenced. For nested amplification, a 1 µl aliquot of the primary PCR product was used as the template in a second 25 µl reaction mixture with the *E. muris*-specific primer pair EM87f and EM584r, the HF strain-specific primer pair HF51f and HF954r, the *A. phagocytophilum*-specific primer pair GE9f and GE10r or primer pair IS58-62f and IS58-594r, which were derived from the 16S rRNA gene sequence of '*Candidatus N. mikurensis*' strain IS58 that was obtained in this study. Both negative (distilled water) and positive [DNA extracted from *E. muris* strain HF (Shibata *et al.*, 2000) and *A. phagocytophilum* strain HZ (Rikihisa

et al., 1997)] controls were included in each set of amplification reactions.

Of spleen specimens that were isolated from 15 wild rats, seven tested positive for 'Candidatus N. mikurensis' by 16S rRNA gene-based PCR. Sequence analysis of DNA samples derived from wild rats showed that all of them (TK0003, TK0005, TK4456^R, TK4457, TK4458, MK01 and MK08) possessed identical sequences of 463 bp (base positions 141–642 of the *Escherichia coli* 16S rRNA gene). Of 128 pooled or unpooled tick (*I. ovatus*) specimens (164 ticks), three DNA and two cDNA specimens were positive for 'Candidatus N. mikurensis' and two other DNA and four other cDNA specimens were positive for HF strains, as determined by 16S rRNA-based PCR and DNA sequencing analysis. However, the *E. muris* and *A. phagocytophilum* 16S rRNA genes were not detected by PCR in any of the *I. ovatus* specimens analysed. Of five *I. ovatus* specimens that were found to be positive for 'Candidatus N. mikurensis', two sequences from samples collected in the year 2000 were identical to each other: IS58 and IS60 shared an identical sequence of 458 bp, spanning positions 168–654 of the *E. coli* 16S rRNA gene. Similarly, three sequences from samples collected in the year 2001 (IS091, IS108 and IS168) shared 99.6–99.8 % identity to IS58 within this region.

Isolation and ultrastructure of 'Candidatus N. mikurensis'

Spleen and liver specimens were cut into 1 mm cubes. These tissue and buffy coat specimens from wild rats and experimentally infected rats were fixed and processed as described elsewhere (Rikihisa *et al.*, 1985). Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a Philips model 300 electron microscope at 60 kV. Laboratory rats were inoculated with the spleen homogenate from wild rat MK01 that was PCR-positive for 'Candidatus N. mikurensis'. Blood, liver and spleen specimens from these laboratory rats were tested for the 'Candidatus N. mikurensis' 16S rRNA gene 10–60 days after inoculation by PCR. Specimens were nested PCR-negative up to day 15 after inoculation and only spleens of days 20 and 30 after inoculation had weak bands (i.e. were weakly positive) by nested PCR. However, blood, liver and spleen specimens from day 60 after inoculation were strongly positive by first-round PCR. Sequences of the resulting PCR products were identical to that of strain TK4456^R. Transmission electron microscopy of spleen specimens from the rat revealed small cocci in a membrane-bound inclusion within the cytoplasm of endothelial cells of the venous sinuses (Fig. 1). The bacteria contained fine DNA strands and ribosomes and were surrounded by a double bileaflet membrane: an inner membrane and a thin, rippled outer membrane with a narrow periplasmic space, which is characteristic of members of the family *Anaplasmataceae* (Rikihisa, 1991). The host cells were confirmed as endothelial cells lining the venous sinus, based on the presence of the unique cytoplasmic membrane canalicular system (Uehara & Miyoshi, 1999). To date, we have been unable to culture the

Fig. 1. Transmission electron micrograph of 'Candidatus Neoehrlichia mikurensis'. Organism is present as an inclusion in an endothelial cell lining the splenic sinus of a Wister rat 60 days after inoculation with the spleen homogenate from infected wild rat MK01. The organism contains fine DNA strands and ribosomes (A). Arrows indicate the characteristic thin, rippled, bileaflet outer membrane (B), irregular, narrow periplasmic space between inner and outer membranes (C) and canalicular system of the endothelial cell (D). Bar, 0.3 μ m.

bacterium. None of the serum samples from PCR-positive rats reacted with *A. phagocytophilum* or *E. muris* antigens by the IFA test. Four mice inoculated with tick homogenates that were PCR-positive for 'Candidatus N. mikurensis' did not develop any clinical signs and ehrlichial DNA was not detected in their spleens 10 days after inoculation, as determined by PCR analysis.

16S rRNA gene and *groESL* sequences of strains TK4456^R and IS58

We obtained almost-entire 16S rRNA gene sequences of strain TK4456^R from a rat captured in 1999 and strain IS58 from a tick pool collected in 2000, using the first PCR products (approx. 1459 bp) that were obtained with the EC9 and EC12A primer pair. Primer pairs used for the nested PCR were EC12A and IS58-52r; A17a and EM 584r; Ehr387 and EM924; EM749 and IS58-1345r; and IS58-1203f and EC9. The *groESL* sequences (approx. 1700 bp) from both strains TK4456^R and IS58 were obtained by performing nested PCR with primer pair HS1a and HS6a in the first round and primer pair HS43 and HSVR in the nested

that strains TK4456^R and IS58, together with the Schotti variant and the *Ehrlichia*-like *Rattus* strain from China (Pan *et al.*, 2003), create a new cluster between *Ehrlichia* and *Anaplasma* species (Fig. 2). The 16S rRNA gene sequence of 'Candidatus *E. walkerii*' (GenBank accession no. AY098730) is 1281 bp, which corresponds to positions 134–1416 of the 16S rRNA gene of *A. phagocytophilum* Webster^T (GenBank accession no. U02521). The 'Candidatus *E. walkerii*' sequence lacked most of the divergent

sequences that would be useful to classify members of the family *Anaplasmataceae* and therefore could not be included in the phylogenetic study. However, the 1281 bp sequence from 'Candidatus *E. walkerii*' was found to share 99.1, 99.1 and 99.0 % identity with the corresponding sequences of the TK4456^R, IS58 and Schotti variants, respectively.

No sequence other than the 16S rRNA gene sequence has been determined for the Schotti variant or the *Ehrlichia*-like

Fig. 3. Phylogram of 'Candidatus *Neoehrlichia mikurensis*' strains TK4456^R and IS58 and other members of the family *Anaplasmataceae*, based on *groEL* sequence comparison. GenBank accession numbers are shown in parentheses. Numbers above internal nodes indicate the percentage of 1000 bootstrap replicates that supported the branch.

Rattus strain. Sumner *et al.* (1997, 2000) showed that the phylogram generated from *groEL* sequences correlates well with the phylogenetic relationships that are inferred from analysis of 16S rRNA gene sequences and provides a useful tool for characterizing novel members of the family *Anaplasmataceae*. New *groESL* 1327 bp sequences, including *groES* (13 bp), the intergenic space (91 bp) and *groEL* (1223 bp), were obtained for strains TK4456^R and IS58. The 1220 bp *groEL* sequence of TK4456^R was 99.6, 82.5, 82.1, 75.8, 68.6 and 60.2% identical to those of IS58, *E. chaffeensis*, *E. ruminantium*, *A. phagocytophilum*, *Wolbachia* sp. and *N. sennetsu*, respectively. A phylogram based on *groEL* sequences showed that strains TK4456^R and IS58 create a novel cluster that is almost equidistant from *Ehrlichia* and *Anaplasma* species (Fig. 3).

The present study demonstrates, for the first time, the ultrastructure of a new bacterium isolated from a wild rat. Along with *E. ruminantium* (Rikihisa, 1991) and an HF strain that is related closely to *E. chaffeensis* (Okada *et al.*, 2001), '*Candidatus* N. mikurensis' is now the third member of the family *Anaplasmataceae* that is known to infect endothelial cells *in vivo*. Phylogenetic analysis based on *groEL* sequences of '*Candidatus* N. mikurensis' confirmed the new cluster that was inferred by analysis of 16S rRNA gene sequences. For the Schotti variant or the *Ehrlichia*-like *Rattus* strain, the results of phylogenetic analysis based on 16S rRNA gene sequences have not been confirmed by sequencing other genes. Brouqui *et al.* (2003) reported that the *gltA* sequence (621 bp) of '*Candidatus* E. walkeri' (GenBank accession no. AY098729) has 64% similarity to that of the most closely related organism, *E. ruminantium* (GenBank accession no. AF304146). This suggests that '*Candidatus* E. walkeri' is quite different from other *Ehrlichia* species.

The 16S rRNA gene sequence of the Schotti variant was detected in an *I. ricinus* tick in the Netherlands (Schouls *et al.*, 1999); a similar agent was also detected by PCR and subsequent reverse line-blot hybridization (no DNA sequence is available) in *I. ricinus* ticks in the Baltic regions of Russia (Alekseev *et al.*, 2001) and by PCR and sequencing in *I. ricinus* ticks collected from asymptomatic humans in northern Italy (Brouqui *et al.*, 2003; Sanogo *et al.*, 2003). No mammalian host of these bacteria has been identified in Europe or Russia. *I. ovatus* is found in various regions of Asia, including Russia and Japan (Yamaguchi *et al.*, 1971), and can be infected with the HF strain (Shibata *et al.*, 2000). *I. ricinus*, which is distributed widely in Europe, is known to be infected with *A. phagocytophilum*, which infects humans, horses, ruminants and rodents (Dumler *et al.*, 2001). Identification of this novel member of the family *Anaplasmataceae* would require the use of more specific PCR primers, such as those used in this study, to distinguish this group of bacteria from *A. phagocytophilum* or the HF strain.

Based on these findings from DNA sequence and electron

microscopy analyses, we propose to designate this group of bacteria as '*Candidatus* Neoehrlichia mikurensis'.

Description of '*Candidatus* Neoehrlichia mikurensis' Kawahara *et al.* 2004

Neoehrlichia (Ne.o.ehr.lic'hi.a. Gr. adj. *neos* new; N.L. fem. n. *Ehrlichia* name of a bacterial genus; N.L. fem. n. *Neoehrlichia* a new *Ehrlichia*; mi.ku.ren'sis. N.L. fem. adj. *mikurensis* referring to Mikura Island, southern Japan, where the organism was discovered).

Gram-negative pleomorphic cocci of 0.5–1.2 µm. Members of the α -*Proteobacteria*, placed phylogenetically within the family *Anaplasmataceae*. Not cultivated. Grows in membrane-bound inclusions within endothelial cells of the rat. Parasitic to rats and *Ixodes* ticks. Reference strain is TK4456^R.

'*Candidatus* Neoehrlichia mikurensis' [(α -*Proteobacteria*) NC; G–; C (approx. 0.5–1.2 µm in diameter); NAS (GenBank no. AB084582 and AB084583), ultrastructure, oligonucleotide sequence complementary to unique region of 16S rRNA gene 5'-AATTGTA(G/A)(T/C)TGTA GTTCTAT-3'; S (*Rattus*, cytoplasm of endothelial cells; *Ixodes* ticks); M]. Kawahara *et al.*, this study.

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