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Isolation and characterization of a novel *Rickettsia* species (*Rickettsia asembonensis* sp. nov.) obtained from cat fleas (*Ctenocephalides felis*)

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A novel rickettsial agent, '*Candidatus* Rickettsia asembonensis' strain NMRCii^T, was isolated from cat fleas, *Ctenocephalides felis*, from Kenya. Genotypic characterization of the new isolate based on sequence analysis of five rickettsial genes, *rrs*, *gltA*, *ompA*, *ompB* and *sca4*, indicated that this isolate clustered with *Rickettsia felis* URRWXCal2. The degree of nucleotide similarity demonstrated that isolate NMRCii^T belongs within the genus *Rickettsia* and fulfils the criteria for classification as a representative of a novel species. The name *Rickettsia asembonensis* sp. nov. is proposed, with NMRCii^T (=DSM 100172^T=CDC CRIRC RAS001^T=ATCC VR-1827^T) as the type strain.

Members of the genus *Rickettsia* are Gram-negative, obligate intracellular bacteria belonging to the order *Rickettsiales*. To date, the genus *Rickettsia* comprises 26 rickettsial species with validly published names and several other uncharacterized rickettsial strains detected mainly in ticks, fleas, lice, mites and leeches (Fournier & Raoult, 2009; Kikuchi *et al*., 2002; Parola *et al*., 2013). Rickettsial agents of unknown pathogenicity were identified by molecular typing in *Ctenocephalides felis*, *Ctenocephalides canis* and *Xenopsylla cheopis* fleas from western Kenya and were designated '*Candidatus* Rickettsia asemboensis'strains F30 and F82 (Jiang *et al*., 2013). Analysis of partial sequences of the *rrs*, *gltA*, *ompA*, *ompB*, *sca4* and 17 kDa antigen genes of the rickettsial strains F30 and F82 detected in fleas demonstrated that substantial sequence differences existed between rickettsial strains F30/F82 and *Rickettsia felis*, which was the closest relative among rickettsial species with validly published names (Jiang *et al*., 2013). Similar or very closely related partially characterized rickettsial strains have been reported worldwide, as summarized in Table 1. With the description of this agent and other *Rickettsia felis*-like organisms worldwide, a need existed to isolate '*Candidatus* R. asembonensis' in cell culture and conduct microscopic, biological and whole genome sequencing to describe it as a member of a novel species.

Accordingly, we isolated a novel *Rickettsia* species designated as *Rickettsia asembonensis* sp. nov. strain NMRCii^T (formerly known as '*Candidatus* Rickettsia asemboensis') from cat fleas collected from dogs in western Kenya (Luce-Fedrow *et al*., 2015). We subsequently sequenced the whole genome of this agent and deposited it in GenBank as '*Candidatus* Rickettsia asemboensis' strain NMRCii^T (Jima *et al*., 2015). The fleas were collected from dogs in Asembo division of Siaya County, western Kenya, and were identified morphologically using standard entomological keys. The fleas used for isolation of the new rickettsia were determined to be *C. felis* and speciation was confirmed by amplification and sequencing of two genes targeting the 18S rRNA and cytochrome oxidase II (COII) (Roucher *et al*., 2012; Whiting, 2002). For

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Abbreviations: SFG, spotted fever group; TG, typhus group; WGS, whole genome sequence; MLST, Multi-locus sequence typing.

The GenBank/EMBL/DDBJ accession numbers for the *rrs, gltA*, 17 kDa antigen, *sca4, ompA* and *ompB* genes of strain F30 from *Ctenocephalides canis* are JN315967–JN315972. Those for the *rrs*, *gltA*, 17 kDa antigen, *sca4* and *ompA* genes of strain F82 from *C. felis* are JN315973–JN315977. The accession numbers for the whole genome sequence of *Rickettsia asembonensis* strain NMRCii^T and the plasmid pRAS01 are JWSW00000000 and CP011517, respectively.

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Table 1. Similar or closely related partially characterized rickettsia strains from GenBank showing per cent sequence similarities of six genes to those of strain NMRCii^{T}

Uncultured *Rickettsia* species	Vector	Country	16S rRNA	*gltA*	*ompA*	*ompB*	Sca4	17 kDa	Reference
'*Candidatus* R. asemboensis' F30	*Ctenocephalides canis*	Kenya	100	100	99.8	99.9	100	100	Jiang *et al.* (2013)
'*Candidatus* R. asemboensis' F82	*Ctenocephalides felis*	Kenya	100	–	99.9	–	100	100	Jiang *et al.* (2013)
Rickettsia sp. RF2125	*Ctenocephalides canis*	Thai-Myanmar	–	99.3	–	99.7	–	–	Parola *et al.* (2003)
Rickettsia sp. 'Synosternus'	*Synosternus pallidus*	Senegal	–	100	–	100	–	–	Roucher *et al.* (2012)
Uncultured R. sp. Clone Hf56-2	*Archaeopsylla erinacei*	Germany	–	–	–	100	–	–	Gilles *et al.* (2009)
Rickettsia sp. SE313	*Echidnophaga gallinacea*	Egypt	–	99.7	–	–	–	100	Loftis *et al.* (2006)
R. endosymbiont of *C. felis* isolate F143	*C. felis*	Thailand	–	99.7	–	–	–	100	Foongladda *et al.* (2011)
R. endosymbiont of *C. felis* isolate F144	*C. felis*	Thailand	–	99.4	–	–	–	100	Foongladda *et al.* (2011)
Rickettsia sp. cf1 and 5	*C. felis*	US	–	99.5	–	–	–	100	Reeves *et al.* (2005)
Uncultured *R.* sp. clone ARV5606	*C. felis*	Peru	–	99.7	–	–	–	99.7	Forshey *et al.* (2010)
Rickettsia sp. cf9	*C. felis*	US	–	–	–	99.9	99.8	–	Accession numbers: DQ379483, DQ287314
R. sp. R14	*Ceratophylus fasciatus*	India	–	99.7	–	99.9	–	–	(Accession numbers: HM370112, HM370112 .)
Uncultured *R.* sp. clone HL2a	*C. felis*	Malaysia	–	99.7	–	–	–	–	Tay *et al.* (2014)
Rickettsia sp. FS27	*Orchopeas horwadi*	USA	–	-	–	–	–	99.7	. Accession number: DQ395097
Rickettsia sp. J28p	*C. felis*	Peru	–	99.7	–	–	–	–	Accession number: LN831076
Rickettsial strain from *C. felis*	*C. felis*	Equador	99.9	99.7	–	100	100	100	Oteo *et al.* (2014)
Rickettsia sp.	*C. felis*	Colombia	100	100	–	100	–	–	Faccini-Martínez *et al.* (2016)
Rickettsia sp.	*Xenopsylla ramesis*	Israel	100	99.7	100	100	–	100	Rzotkiewicz *et al.* (2015)

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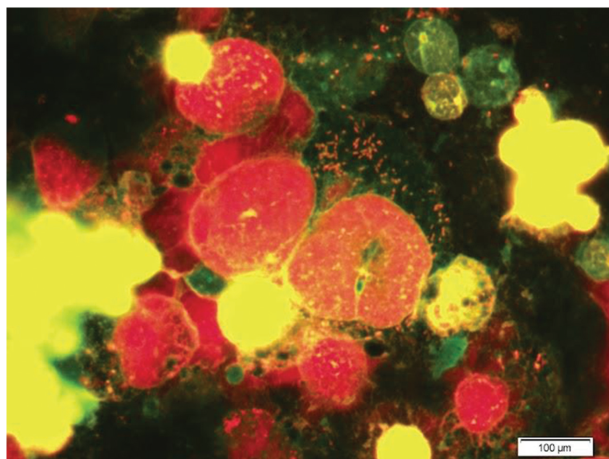

Fig. 1. Acridine orange staining of strain NMRCii^T in *Aedes albopictus* C6/36 cells at 14 days post-infection. Rickettsiae were not observed in negative control cells. The image was captured using an Olympus BX43 microscope and DP72 camera (×1000). Bar, 100 μm.

the 18S rRNA, a 1347 bp sequence generated was 100 % similar to that of *C. felis* (KC177274) and *C. canis* (AF423914) and a 636 bp sequence generated for the COII was 100 % identical to *C. felis* (KF684919–KF684921 and KF684929–KF684932).

The agent was successfully isolated from a pool of *C. felis* fleas in pure culture using the *Aedes albopictus* C6/36 and *Drosophila melanogaster* S2 cell lines grown aerobically at 25 °C in Schneider's *Drosophila* medium supplemented with 5 % heat-inactivated fetal bovine serum (Luce-Fedrow *et al.*, 2015). Based on phylogenetic analysis of the fragments of three housekeeping genes, 17 kDa antigen gene (386 bp), *gltA* (1186 bp) and *ompB* (536 bp), the sequences of strain NMRCii^T were determined to be identical to the rickettsial strains F30 and F82, initially detected in fleas by molecular typing (Luce-Fedrow *et al.*, 2015).

Microscopic characterization of strain NMRCii^T was performed by transmission electron microscopy using a JEM 100 CX II transmission electron microscope (JEOL). The morphology of the agent following culture in C6/36 cells was typical of other *Rickettsia* species (Fig. 1). There were mainly round forms with a few bacilli and no rickettsiae could be seen in the nuclei of infected cells (Luce-Fedrow *et al.*, 2015). There were no marked cytopathic/cytotoxic changes to cells infected with strain NMRCii^T (Luce-Fedrow *et al.*, 2015).

Attempts to subculture NMRCii^T from the infected C6/36 cells into Vero and L929 cell lines incubated at 37 °C (5 % CO_2) in Dulbecco's modified Eagle's medium (supplemented with 5 % fetal bovine serum) were unsuccessful. During each attempt, the Vero and L929 cells were monitored for growth of strain NMRCii^T for a minimum of 40 days using acridine orange staining and the *R. asembonensis*-specific qPCR assay, Rasem (Jiang *et al.*, 2013). The agent was not detectable by either acridine orange staining or by the Rasem qPCR assay in the Vero or L929 cells. In concurrent experiments, growth of the agent in C6/36 cells was observed by both acridine orange staining and the Rasem qPCR assay. Attempts to establish strain NMRCii^T from C6/36 into embryonated chicken eggs incubated at 35 °C were unsuccessful after three serial yolk sac passages.

Immunocytochemistry was performed on strain NMRCii^T-infected and uninfected S2 and/or C6/36 cells with known human sera positive for anti-spotted fever group (SFG) (*Rickettsia parkeri*) antibodies and known human sera positive for anti-typhus group (TG) (*Rickettsia typhi*) antibodies. S2 and C6/36 cells were confirmed to be positive for *R. asembonensis* sp. nov. by acridine orange staining and our species-specific qPCR assay (Rasem). S2 cells were prepared for immunocytochemistry, as previously described (Luce-Fedrow *et al.*, 2012), and incubated with the primary antibody (human anti-SFG rickettsiae-specific IgG developed following infection with *R. parkeri*). In strain NMRCii^T-infected S2 cells incubated with the anti-SFG rickettsia sera, many rickettsiae were visualized by immunofluorescence both extracellularly (Fig. 2a) and intracellularly (Fig. 2b);

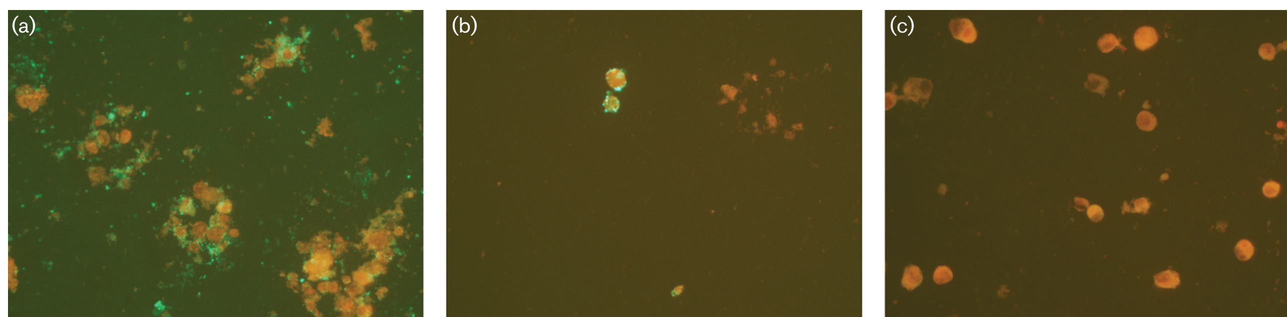

Fig. 2. Immunofluorescence staining of strain NMRCii^T antigen with anti-SGF-specific (*R. parkeri*) antibody-containing serum showing rickettsiae both extracellularly (a) and intracellularly (b) (×400). (c) Uninfected S2 cells incubated with anti-SGF-specific (*R. parkeri*) antibody-containing serum (×400).

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Table 2. *Rickettsia* species from GenBank showing levels of sequence similarity (%) of five genes to those of strain NMRCii^T

NE, Non-existent; –, nucleotide sequences were not available.

Rickettsia species	*rrs*	*gltA*	*ompA*	*ompB*	*sca4*
R. felis URRWXCal2	99.5	98.0	92.5	95.9	91.4
R. australis Philips 32^T	99.2	95.4	91.2	89.1	89.3
R. akari MK (Kaplan)^T	98.4	94.7	–	89.1	88.6
R. africae ESF-5	98.9	94.5	87.88*	91.1	88.9
R. aeschlimannii MC16^T	98.9	94.6	87.8*	91.8	88.5
R. bellii OSU 85-389	99.1	86.8	NE	71.2	64.6
R. conorii Malish 7^T	99.1	94.5	88.1*	91.1	88.4
R. heilongjiangensis 054^T	98.9	94.5	87.1*	90.7	88.4
R. honei RB	99.0	94.5	87.3*	90.8	89.1
R. japonica YH^T	99.1	94.3	87.3*	90.2	88.5
R. massiliae Mtu1^T	99.2	94.6	87.8*	91.7	88.8
R. montanensis OSU.85-930	98.7	94.9	87.8*	91.2	89.1
R. parkeri Maculatum 20^T	98.9	94.5	87.3*	91.0	88.9
R. peacockii Rustic	99.2	94.5	87.1*	90.8	88.5
R. prowazekii Brein1^T	98.2	90.7	NE	81.8	80.4
R. raoultii Khabarovsk^T	99.0	94.7	88.1*	91.7	89.1
R. rhipicephali 3-7-6^T	99.1	94.7	87.8*	91.8	89.2
R. rickettsii R	99.2	94.1	87.8*	90.5	87.6
R. sibirica RH05	98.9	94.5	–	90.9	88.4
R. slovaca 13-B	99.1	94.5	87.6*	91.2	89.3
R. tamurae AT-1^T	99.0	94.3	–	90.0	95.6
R. typhi Wilmington^T	98.1	90.3	NE	81.9	80.6

*Less than 50 % coverage.

no rickettsiae were observed in uninfected S2 cells (Fig. 2c). A few rickettsiae were observed with anti-TG-specific (human IgG against *R. typhi*) sera. Both infected and uninfected cells were also incubated with normal human sera as a negative control; no rickettsiae were observed in the infected cells (data not shown). It was observed that strain NMRCii^T reacts to both SFG and TG but much more so to the SFG rickettsia. From these results, its antigenic/serological classification could not be confirmed.

For additional genotypic characterization, DNA from *Rickettsia* sp. strain NMRCii^T was extracted from the C6/36 cells using the DNeasy blood and tissue kit (Qiagen) according to the manufacturer's instructions. PCR amplification and sequencing of five signature genes, *rrs*, *ompA*, *ompB*, *gltA* and *sca4*, were attempted as previously described (Fournier *et al.*, 2003; Jiang *et al.*, 2013). To determine the taxonomic position of the new isolate, sequences of the following genes were analysed: *rrs* (1512 bp), *gltA* (1303 bp), *ompA* (1505 bp), *ompB* (4261 bp) and *sca4* (1539 bp). The sequences of strain NMRCii^T showed 100 % similarity to those of strains F30 and F82 for the *rrs*, *gltA* and *sca4* genes. Sequence comparisons of the *ompA* and *ompB* genes showed that the novel isolate shared 99.86 and 99.98 % similarity, respectively, with the partially characterized strains F30 and F82 initially detected in fleas. The differences observed were a result of non-synonymous nucleotide substitutions in two positions for the *ompA* gene and in one position for the *ompB* gene. We compared the sequences of strain NMRCii^T to other validly published *Rickettsia* species and the results are summarized in Table 2. Searches via the BLAST program revealed that *Rickettsia* sp. NMRCii^T shared the highest nucleotide sequence similarity with *R. felis* URRWXCal2. The sequence of the *rrs* gene showed 99.5 % similarity to *R. felis* URRWXCal2, indicating that this new isolate belongs within the genus *Rickettsia*. Sequence similarity between the new isolate and *R. felis* URRWXCal2 was 98, 92.5, 95.9 and 91.4 % for the *gltA*, *ompA*, *ompB* and *sca4* genes, respectively. Although *R. asembonensis* strain NMRCii^T is phylogenetically closely related to *R. felis*, the levels of similarity for the five genes were lower than the cut-offs proposed for *Rickettsia* species definition (Fournier *et al.*, 2003), and thus we conclude that strain NMRCii^T represents a novel species.

Finally, purified bacterial genomic DNA of strain NMRCii^T was subjected to next-generation sequencing using a MiSeq sequencer and reagents (Illumina) and *de novo* assembly of the whole genome sequence (WGS) with DeconSeq and a Roche GS *Deat Novo* assembler as described previously (Jima *et al.*, 2015). Whole genome sequencing showed the DNA G+C content of *R. asembonensis* strain NMRCii^T was 32.28 mol%. This is similar to the G+C content of other rickettsiae (Gillespie *et al.*, 2012). Analysis of the WGS of strain NMRCii^T revealed that its genome comprised a 1 381 774 bp circular chromosome (Jima *et al.*, 2015) and a 21 692 bp circular plasmid, designated as pRAS01 (CP011517). Thus, a unique plasmid may be associated with this novel rickettsia. Complete sequences of the five housekeeping genes, *rrs*, *gltA*, *ompA*, *ompB* and *sca4*, were extracted from the WGS of strain NMRCii^T for comparison with other validly published rickettsiae. Multi-locus sequence typing (MLST) analysis using concatenated fragments of the *rrs* (1512 bp), *gltA* (1303 bp), *ompB* (4261 bp) and *sca4* (1539 bp) genes revealed clustering of strain NMRCii^T with *R. felis* URRWXCal2 and the former (NMRCii^T) was placed in the same clade as *Rickettsia australis*, *Rickettsia akari* and *R. felis* (Fig. 3), all of which are members of the transitional rickettsia group (Gillespie *et al.*, 2007).

In conclusion, a rickettsial agent of unknown pathogenicity has been successfully isolated and maintained in culture at the Naval Medical Research Center (NMRC), Silver Spring, MD, USA. The sequencing results (MLST and WGS) have demonstrated that strain NMRCii^T is different from all other rickettsial species with validly published names. The name *Rickettsia asembonensis* sp. nov. is proposed to accommodate strain NMRCii^T.

Description of *Rickettsia asembonensis* sp. nov.

Rickettsia asembonensis [a.sem.bo.nen′sis. N.L. fem. adj. *asembonensis* from Asembo, a part of Siaya County,

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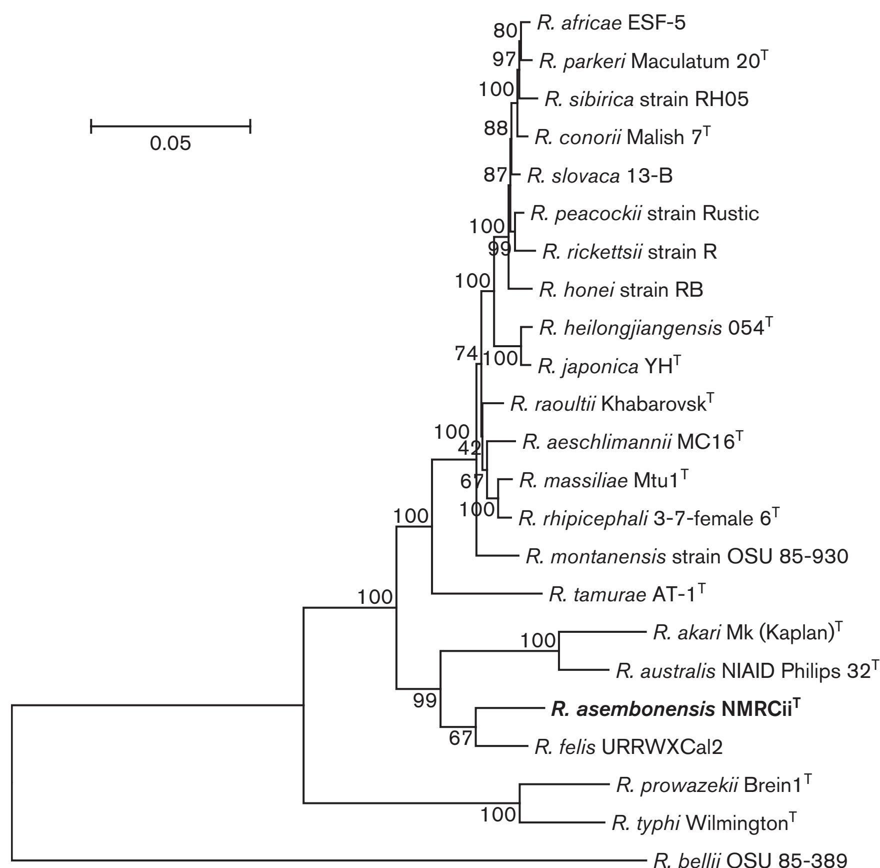

Fig. 3. MLST dendrogram for a 7956 bp fragment consisting of concatenated fragments of the *rrs* (1512 bp), *ompB* (3602 bp), *gltA* (1303 bp) and *sca4* (1539 bp) genes showing the phylogenetic position of strain NMRCii^T among *Rickettsia* species with validly published names. The evolutionary history was inferred by using the maximum-likelihood method (Tamura *et al.*, 2013).

western Kenya, where the *Ctenocephalides felis* fleas that yielded the first isolate were collected (Luce-Fedrow *et al.*, 2015)].

Obligate intracellular bacterium that grows aerobically in the *A. albopictus* C6/36 and *D. melanogaster* S2 cell lines at 25 °C in Schneider's *Drosophila* medium supplemented with 5 % heat-inactivated fetal bovine serum. The whole genome size is 1.46 Mb and has a G+C content of 32.28 mol%. Sequences of the *rrs*, *gltA*, *ompA*, *ompB* and *sca4* genes demonstrate that this rickettsia is different from all other rickettsial species with validly published names, with the most closely related being *R. felis*. It does not grow at 35–37 °C in cultures of Vero or L929 cells, or in embryonated chicken eggs. The cells possess surface antigens that react predominantly with anti-SFG serum raised against *R. parkeri* and to a lesser extent with anti-TG raised against *R. typhi*, as determined by indirect immunofluorescence.

The type strain, NMRCii^T (=DSM 100172^T=CDC CRIRC RAS001^T=ATCC VR-1827^T), was isolated from a pool of *C. felis* fleas collected from Asembo division, Siaya County, western Kenya, in 2013.

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System, a Division of the Armed Forces Health Surveillance Center; work unit # A1402.

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