Human Infection with *Candidatus* Neoehrlichia mikurensis, China

Hao Li,[1] Jia-Fu Jiang,[1] Wei Liu,[1] Yuan-Chun Zheng, Qiu-Bo Huo, Kun Tang, Shuang-Yan Zuo, Kun Liu, Bao-Gui Jiang, Hong Yang, and Wu-Chun Cao

To identify *Candidatus* Neoehrlichia mikurensis infection in northeastern China, we tested blood samples from 622 febrile patients. We identified in 7 infected patients and natural foci for this bacterium. Field surveys showed that 1.6% of ticks and 3.8% of rodents collected from residences of patients were also infected.

Candidatus Neoehrlichia mikurensis was detected in 1999 in *Ixodes ricinus* ticks in the Netherlands and referred to as an *Ehrlichia* spp.–like agent (*1*). It was then classified as a new member of family *Anaplasmataceae* on the basis of ultrastructure and phylogenetic analysis (*2*). The agent was detected in ticks and small wild mammals in Europe and Asia (*1–6*) and has recently been reported to infect humans, especially immunocompromised patients in Europe (*7–10*). However, no cases of infection have been identified outside Europe. Moreover, the agent has not yet been isolated in pure culture, and its antigens are not available.

To investigate human infections with tick-borne agents in China, we initiated a surveillance study at Mudanjiang Forestry Central Hospital (Mudanjiang, China). This hospital is one of the largest hospitals treating patients with tick-borne infectious diseases in northeastern China, where various tick-borne agents have been detected in ticks and animal hosts (*11–15*).

The Study

During May 2–July 30, 2010, a total of 622 febrile patients, who had histories of recent tick bites and sought treatment at Mudanjiang Forestry Central Hospital (Figure 1) were screened for the infections of tick-borne agents. When patients were admitted, peripheral blood samples were collected and treated with EDTA. DNA as extracted by using the QIAmp DNA Blood Mini Kit (QIAGEN, Germantown, MD, USA).

Author affiliations: Beijing Institute of Microbiology and Epidemiology, Beijing, People's Republic of China (H. Li, J.-F. Jiang, W. Liu, K. Tang, S.-Y. Zuo, K. Liu, B.-G. Jiang, H. Yang, W.-C. Cao); and Mudanjiang Forestry Central Hospital, Mudanjiang, People's Republic of China (Y.-C. Zheng, Q.-B. Huo)

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Figure 1. Location of Mudanjiang, Heilongjiang Pro-vince, China, where *Candidatus* Neoehrlichia mikurensis was detected.

For a broad-range assay, a nested PCR specific for the 16S rRNA gene (*rrs*) was used to detect organisms in the family *Anaplasmataceae*. For positive samples, 2 heminested PCRs were used to amplify the entire *rrs* gene. For further confirmation, a nested PCR specific for the 60-kDa heat shock protein gene (*groEL*) was performed. Detailed cycling conditions for all amplifications are described in the Technical Appendix (wwwnc.cdc.gov/EID/pdfs/12-0594-Techapp.pdf).

Seven patients were found to be infected with *Candidatus* N. mikurensis by amplifications of the *rrs* and *groEL* genes. Amplified *rrs* gene (1,501 bp) and partial *groEL* gene (1,230 bp) sequences from these patients were identical. These sequences were also identical to genes of *Candidatus* N. mikurensis detected in ticks and rodents in the Asian region of Russia (*5*).

Serum samples were collected from patients during the acute (2–12 days after onset of illness) or convalescent (34–42 days after onset of illness) phases of illness. All samples were negative for IgG against *Anaplasma phagocytophilum*, *Ehrlichia chaffeensis*, *Borrelia burgdorferi*, *Rickettsia heilongjiangensis*, and tick-borne encephalitis virus when tested by indirect immunofluorescence assay.

[1]These authors contributed equally to this article.

All 7 patients were farmers residing in the villages in Mudanjiang. Their median age was 41 years (range 29–67 years) and 5 were men. None had been vaccinated against tick-borne encephalitis. The patients had onset of illness during May 20–July 13, 2010. The median time from the tick bite to the onset of illness and from the onset of illness to the physician visit was 8 days (range 2–35 days) and 7 days (range 1–12 days), respectively.

All patients were otherwise healthy, and none had a history of underlying immunocompromised conditions. Fever, headache, and malaise were reported for all 7 patients. Other major manifestations included nausea (5/7), vomiting (5/7), myalgia (4/7), and stiff neck (4/7). Less common symptoms were arthralgias (2/7), cough (2/7), diarrhea (1/7), confusion (1/7), and erythema (1/7). Skin erythema (multiple and oval) was seen on the neck of 1 patient.

Laboratory test results showed leukopenia in 1 patient, leukocytosis in 1 patient, thrombocytopenia in 2 patients, and anemia in 2 patients. Serum levels of alanine aminotransferase and aspartate aminotransferase were within reference ranges for all patients. Wright–Giemsa stained peripheral blood smears did not show morulae or other blood parasites.

To identify local natural foci, we performed a field investigation on infections of *Candidatus* N. mikurensis in ticks and rodents from areas of residences of the patients. During May–July 2010, a total of 516 host-seeking ticks, including 316 *I. persulcatus*, 187 *Haemaphysalis concinna*, and 13 *Dermacentor silvarum*, were collected on vegetation and individually examined. *Candidatus* N. mikurensis DNA was detected in 6 (1.9%) *I. persulcatus* and 2 (0.8%) *H. concinna* ticks, but no DNA was detected in *D. silvarum* ticks (Table).

A total of 211 rodents of various species were captured by using snap traps. After rodent species was identified, spleen specimens were collected for DNA extraction and PCR. Eight rodents of 3 species, 5 (4.6%) *Clethrionomys rufocanus*, 2 (5.7%) *Rattus norvegicus*, and 1 (33.3%) *Tamias sibiricus*, were positive for *Candidatus* N. mikurensis (Table).

Nucleotide sequences of *rrs* and *groEL* genes of 8 ticks and 8 rodents were identical to each other and to sequences obtained from the 7 patients. Phylogenetic analysis of *rrs* genes showed that nucleotide sequences identified were identical to those of *Candidatus* N. mikurensis from Japan and the Asian region of Russia but different from sequences from Europe (99.6%–99.8% similarity) (Figure 2, panel A). Similar phylogenetic relationships were observed in a neighbor-joining tree based on *groEL* gene nucleotide sequences. In comparison with sequences from humans and ticks in Europe, the *groEL* gene sequences identified in the study showed 97.6%–98.4% similarity (Figure 2, panel B).

Table. Prevalence of *Candidatus* Neoehrlichia mikurensis in ticks and rodents, Mudanjiang, China

Species	No. positive/no. tested (%)
Tick	
Ixodes persulcatus	6/316 (1.9)
Haemaphysalis concinna	2/187 (0.8)
Dermacentor silvarum	0/13 (0)
Total	8/516 (1.6)
Rodent	
Clethrionomys rufocanus	5/109 (4.6)
Rattus norvegicus	2/35 (5.7)
Apodemus peninsulae	0/30 (0)
Apodemus agrarius	0/25 (0)
Mus musculus	0/9 (0)
Tamias sibiricus	1/3 (33.3)
Total	8/211 (3.8)

Conclusions

We have detected *Candidatus* N. mikurensis DNA in blood samples from 7 patients collected during the period of acute illness, which suggests that this bacterium was the etiologic agent of the infections. Our findings demonstrated human infections with *Candidatus* N. mikurensis in China. The *rrs* and *groEL* gene nucleotide sequences of this *Candidatus* N. mikurensis variant were identical to those obtained from ticks and rodents in the Asian region of Russia, which have not been reported to cause human infection.

Unlike reported cases in elderly or immunocompromised patients in whom disease developed (*7–10*), all 7 patients in our study had relatively mild disease. Major clinical manifestations and laboratory findings of the cases in our report, such as leukocytosis, were not similar to those of previously reported cases. It is noteworthy that the patients reported in this study were previously healthy. Thus, their clinical manifestations might be typical of *Candidatus* N. mikurensis infection in an otherwise healthy population. However, the number of cases in our study was limited, and clinical data were not inclusive. Clinical characteristics of *Candidatus* N. mikurensis infection should include detailed descriptions of additional cases.

Our finding of a *Candidatus* N. mikurensis variant in 1.6% of ticks and 3.8% of rodents tested suggested natural foci of the bacterium in Mudanjiang. Therefore, clinical diagnosis of *Candidatus* N. mikurensis infection should be considered in patients who have been exposed to areas with high rates of tick activity. It is noteworthy that *Candidatus* N. mikurensis was originally detected in *R. norvegicus* from Guangzhou Province in southeastern China (*4*), thereby indicating the potential threat to humans in areas other than northeastern China.

In summary, we identified *Candidatus* N. mikurensis as an emerging human pathogen in China. Further studies should be conducted to isolate this bacterium and investigate its epidemiologic, genetic, and pathogenic features. To

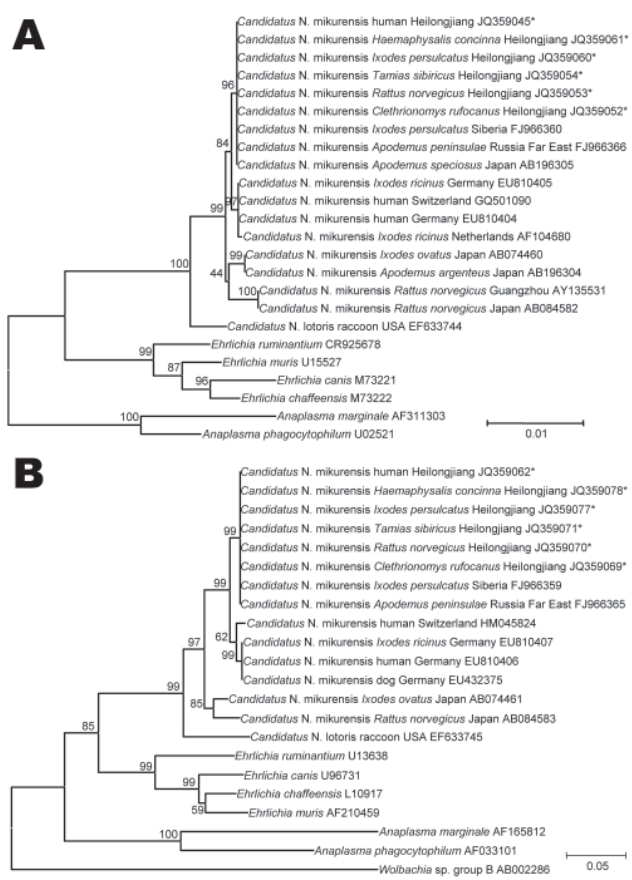

Figure 2. A) Neighbor-joining trees based on the 16S rRNA gene (*rrs*) and B) the 60-kDa heat shock protein gene (*groEL*) of *Candidatus* Neoehrlichia mikurensis, China, generated by using Molecular Evolutionary Genetics Analysis software version 4.0, (www.megasoftware.net/) the maximum composite-likelihood method, and bootstrap analysis of 1,000 replicates. Asterisks indicate nucleotide sequences of *Candidatus* N. mikurensis determined in this study. Numbers on branches indicate percentage of replicates that reproduced the topology for each clade. Scale bars indicate estimated evolutionary distance. A total of 1,303 positions for *rrs* and 953 positions for *groEL* were analyzed. Sources of *Candidatus* N. mikurensis sequences are shown between species names and GenBank accession numbers.

guide diagnostic testing and treatment, physicians should be aware that human *Candidatus* N. mikurensis infections are in Heilongjiang Province and that PCR can be used as a diagnostic technique for identifying suspected infections.

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Mr Li is a medical student in the State Key Laboratory of Pathogen and Biosecurity, Beijing Institute of Microbiology and Epidemiology. His research interests include the microbiology, epidemiology, and ecology of vector-borne diseases.

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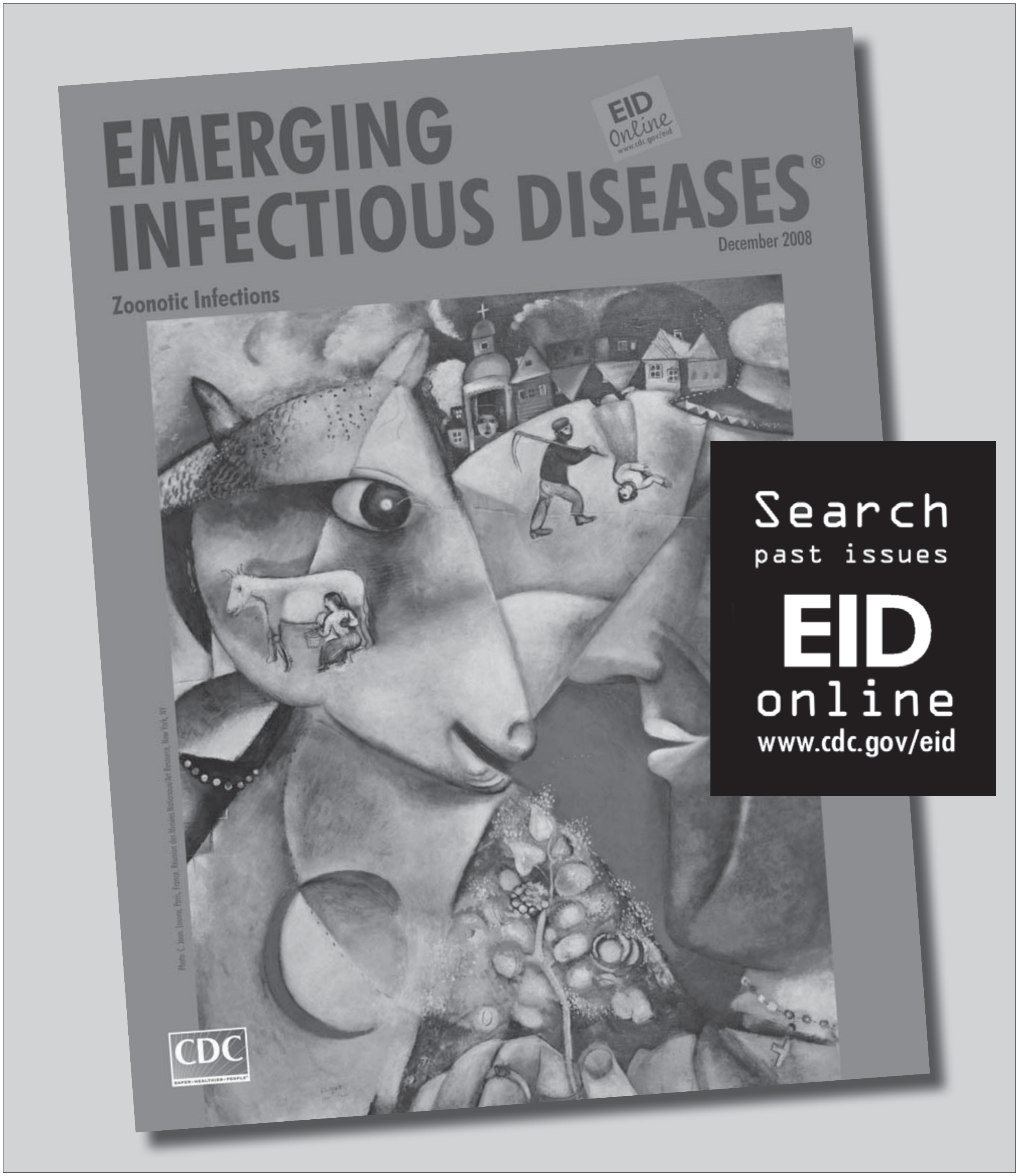
Address for correspondence: Wu-Chun Cao, State Key Laboratory of Pathogen and Biosecurity, Beijing Institute of Microbiology and Epidemiology, 20 Dong-Da St, Fengtai District, Beijing 100071, People's Republic of China: email: caowc@nic.bmi.ac.cn



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Technical Appendix

PCR, morphologic, and serologic procedures used for detection of *Candidatus* Neoehrlichia mikurensis, Mudanjiang, China

PCR

For broad-range assay, a nested PCR specific for the 16S rRNA (*rrs*) gene was used to detect all known species of the family *Anaplasmataceae* (Technical Appendix Table). PCR amplifications were performed in a 30-µL reaction volume in GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA).

For initial amplification, the reaction mixture contained 0.8 µmol/L each of primers Eh-out1 (*1*) and 3–17U, 200 mmol/L of each dNTP, 1 unit of Taq polymerase, 3 µL of 1× PCR buffer, and 3 µL of purified DNA. Cycling conditions were an initial 5-min denaturation at 94°C; 40 cycles at 94°C for 40 s, 55°C for 40 s, and 72°C for 1 min 45 s; and a final extension at 72°C for 7 min.

For nested amplification, the components were similar to those used in the initial amplification, except that 0.5 µmol/L of EHR16SD and 0.5 µmol/L of EHR16SR (*2*) were used as primers and 1 µL of the primary PCR product was used as template. Cycling conditions were 94°C for 5 min; 35 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s; and a final at 72°C extension for 7 min. Nested amplicons were directly sequenced by using primers EHR16SD and EHR16SR.

For positive samples, 2 heminested PCRs were performed to amplify the entire *rrs* gene. Components and conditions in the 2 PCRs were similar to those in the nested PCR, except that primers Eh-out1 and Eh-out2U were used to amplify 5′-end fragments, and primers Eh-out2fU and 3–17U were used to amplify 3′-end fragments. Amplified 5′-end fragments were sequenced by using primer Eh-out2U, and amplified 3′-end fragments were sequenced by using primers Eh-out2fU and CNM1050f.

For confirmation of identification of *Candidatus* Neoehrlichia mikurensis, a nested PCR specific for the 60-kDa heat shock protein (*groEL*) gene was performed. Components in the nested PCR were the same as those used in amplification of the *rrs* gene. Primers HS3-f and HSVR (*3*) was used for the initial amplification. Primers groEL-2f and groEL-2r were used for nested amplification. Cycling conditions were 94°C for 5 min; 35 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min 30s; and a final extension at 72°C for 7 min. Nested amplicons were sequenced by using primers groEL-Sf and groEL-Sr.

All positive amplicons were purified by using E.Z.N.A Gel Extraction Kit (Omega Bio-Tek, Norcross, GA, USA). These amplicons were then sequenced by using an automated DNA sequencer (3730 DNA Sequencer; Applied Biosystems).

To minimize risk for contamination, template isolation and PCR were performed by using specified pipettor sets in separate rooms. Certified DNA/RNase–free filter barrier tips were used to prevent aerosol contamination. All PCRs were performed with appropriate controls.

Morphologic Examination of Peripheral Blood Smears

Fresh peripheral blood smears from patients with PCR-confirmed *Candidatus* Neoehrlichia mikurensis infection were stained with Wright–Giemsa (BaSO Diagnostics, Inc., Zhuhai, China) and examined with a light microscope (BX43; Olympus, Center Valley, PA, USA) for intracellular morulae.

Serologic Testing

Serum samples from patients with PCR-confirmed *Candidatus* Neoehrlichia mikurensis infection were tested by using an indirect immunofluorescence assay for IgG against *Anaplasma* *phagocytophilum* (*4*), *Ehrlichia chaffeensis* (*Ehrlichia chaffeensis* IFA IgG Substrate Slide; Focus Diagnostics, Inc., Cypress, CA, USA), *Borrelia burgdorferi* (established in our laboratory), tick-borne encephalitis virus (*5*), and *Rickettsia heilongjiangensis* (*6*).

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Technical Appendix Table. Nucleotide sequences of primers used for detection of *Candidatus* Neoehrlichia mikurensis by PCR, China*

Gene	Primer	Sequence (5′→3′)	Reference

Technical Appendix Table. Nucleotide sequences of primers used for detection of *Candidatus* Neoehrlichia mikurensis by PCR, China*			
rrs	Eh-out1 (ap)	TTGAGAGTTTGATCCTGGCTCAGAACG	(*1*)
	Eh-out2U (ap, s)	CACCTCTACACTAGGAATTCCACTATC	(*1*) modified
	3–17U (ap)	WAAGGWGGTAATCCAGC	(*1*) modified
	EHR16SD (ap, s)	GGTACCYACAGAAGAAGTCC	(*2*)
	EHR16SR (ap, s)	TAGCACTCATCGTTTACAGC	(*2*)
	Eh-out2fU (ap, s)	GATAGTGGAATTCCTAGTGTAGAGGTG	(*1*) modified
	CNM1050f (s)	TAACCCTTGTCCTTAGTTGCC	This study
groEL	HS3-f (ap)	ATAGTYATGAAGGAGAGTGAT	(*3*)
	HSVR (ap)	TCAACAGCAGCTCTAGTWG	(*3*)
	groEL-2f (ap)	AAAGTTTAAGAGTTCGCCTC	This study
	groEL-2r (ap)	TCTACTTCGCTTGAACCACC	This study
	groEL-Sf (s)	TACAGTTGAAGAAAGTAAGGG	This study
	groEL-Sr (s)	CAAAATAAGGCGATAGATAACC	This study

**rrs,* 16S rRNA; ap, amplification primer; s, sequencing primer; *groEL*, 60-kDa heat shock protein.