Molecular Epidemiology on *Ehrlichia* Species from Ticks in Japan

（日本のマダニにおけるエーリキア属細菌の分子疫学的研究）

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ABSTRACT

Human ehrlichiosis is an emerging tick-borne bacterial disease caused by *Ehrlichia chaffeensis*, which is endemic to the USA. Although human ehrlichiosis has not been reported in Japan, the previous reports suggested that several Ixodid ticks carried Ehrlichia spp., which forms a monophyletic linage with *E. chaffeensis*. I focused this ehrlichial study on the clarification of the current status of ehrlichial presence in several tick species in Japan.

I describe the presence of ehrlichial agents in ticks in Japan by using isolation and molecular detection methods. A total of 1,237 ticks including *Ixodes* ticks and *Haemaphysalis* ticks were collected, and then tested for ehrlichial isolation and/or detection of ehrlichial DNA. Ehrlichial DNA such as *groEL* and 16SrRNA genes was detected in both *Ixodes* tick and *Haemaphysalis* tick. Sequencing analysis on *groEL* and 16SrRNA genes revealed that ehrlichial agents detected in *Ixodes turdus* were divided into two types. One type showed a high genetic similarity to *E. chaffeensis.* DNA of *Ehrlichia* sp. HF strain and '*Candidatus* Neoehrlichia mikurensis' was detected in *Ixodes ovatus*. Furthermore, *Ehrlichia* sp. HF strain was isolated from *I. ovatus* by intraperitoneal inoculation of ddY mice with the homogenate obtained from the PCR-positive ticks. It is considered that the ehrlichial agents are widely distributed in several tick in central and western Japan. Although *Amblyomma* tick is suggested to be the transmission vector for

E. chaffeensis in the USA, *Ixodes* ticks in Japan carry *Ehrlichia* sp., which is closely related to *E. chaffeensis*. The Ehrlichial agents detected in *Ixodes* ticks might be a novel *Ehrlichia* species. The life cycle of the *Ehrlichia* sp. in nature in Japan should be elucicaed.

In this study, *Candidatus* N. mikurensis was detected from *I. ovatus* ticks. *Candidatus* N. mikurensis is known as the pathogen for an emerging tick-borne infectious disease in Europe and China. Neoehrlichiosis, which is caused by *Candidatus* N. mikurensis, is primarily the disease in the immunosuppressed patients. The patients with neoehrlichiosis usually experience recurring fever accompanied by a variety of other symptoms including musculoskeletal pain, and suffer from deep-vein thrombosis. Since *I. ovatus* is widely distributed and the cases of tick-bite are ubiquitously reported in Japan, there is a potential that neoehrlichiosis might be endemic to Japan, necessitating the intensive surveillance on this infectious disease.

PREFACE

Infectious diseases caused by *Ehrlichiae* and *Neoehrlichiae* bacterium, Ehrlichiosis and Neoehrlichiosis, are emerging tick-borne infectious diseases. *Ehrlichia* and *Neoehrlichia* are gram-negative obligate intracellular bacteria that belong to the order *Rickettsiales*, family *Anaplasmaceae*. According to the current classification, the family Anaplasmaceae genera includes *Anaplasma*, *Ehrlichia, candidatus* Neoehrlichia, *Neorickettsia*, and *Wolbachia*. All these bacteria are transmitted to mammals including humans by ixodid tick bite (7) (Table 1).

Ehrlichia is known as zoonotic disease pathogen, with which human and domestic animals are infected, and an infection not only in the field of veterinary medicine but also that of clinical setting.

The genes of *E. chaffeensis*, *E. canis*, *E. ewingii*, and *E. muris* like agent (EMLA) have been detected in human ehrlichiosis patients. *Ehrlichia chaffeensis, E. canis,* and *E. ewingii* have also been detected in canine ehrlichiosis. *Ehrlichia ruminantium* causes diseases known as heartwater and cowdriosis in cattle, sheep, goats, and some wild

ruminants in sub-Saharan Africa and the Caribbean regions (Table 2).

These pathogens are the small gram-negative obligate intracellular bacteria and are localized in membrane-bound vacuoles (morulae) in the cytoplasm of the patient's blood cells (neutrophils, monocytes, macrophages, and erythrocytes) or endothelial cells of blood vessels. Microscopic images of the morulae look like intracytoplasmic inclusions resembling mulberries colored from dark blue to purple with Giemsa staining.

Ehrlichia chaffeensis was first identified as a human pathogen causing human monocytic ehrlichiosis (HME) in the USA (1). The first case of HME was reported in 1986 in the USA. The patient was diagnosed as having HME by the detection of intracytoplasmic inclusions in the monocytes of the patient (5). In 1991, *E. chaffeensis*, the causative agent of this disease, was demonstrated to be different from the other known species of *Ehrlichia* by the 16S rRNA gene analysis (1). HME cases were reported mainly in the southeastern, south central and mid-Atlantic USA (28). 4,364 HME cases were confirmed and reported to the United State Centers for Disease Control and Prevention

(US CDC) in Atlanta, GA from 2003 to 2010 (24, 25). In North America, the lone star tick (*A. americanum*) and white-tailed deer were recognized as the main vector and host of *E. chaffeensis* by detecting *E. chaffeensis* DNA in the ticks and the deer in nature as well as by the transmission experiments reported (2, 8, 23). Other tick species, in addition to *A. americanum*, can be the vectors for *E. chaffeensis*. *Ehrlichia chaffeensis* DNA has been detected in *Ixodes pacificus* and *Dermacentor variabilis* in the USA (20). It was also detected in *Haemaphysalis longicornis* and *Ixodes persulcatus*, *Rhipicephalus sanguineus*, *Amblyomma testudinarium* and *Haemaphysalis yeni*, and *Amblyomma parvum* in South Korea, Cameroon, China, and Argentina, respectively (3, 19, 21, 27, 41). However, the role of these tick species in the maintenance of *E. chaffeensis* in native habitats has not yet been fully clarified.

Ehrlichia species HF strain that is closely related to *E. chaffeensis* was isolated from ticks of *I. ovatus* in Japan and was confirmed to be unique to Japan (36). Several *Ehrlichia* variants closely related to HF strains were later identified in *I. ovatus* in various parts of Japan (10, 15, 36). The *Ehrlichia* variants were also detected in the wild mice such as

Apodemus argenteus, *Apodemus speciosus*, and *Eothenomys smithi* and in a sick dog with signs of anorexia, depression, moderate thrombocytopenia, and anemia (10, 14, 26, 38). The *Ehrlichia* HF strains cause fatal infection in immunocompetent laboratory mice. These bacteria were detected in mononuclear cells in small blood vessels of some organs, including the spleen, liver, and thymus, in which a finding of severe necrotic lesions was demonstrated (10, 36).

The bacteria of a new candidate species, "*Candidatus* Neoehrlichia mikurensis", was initially discovered in wild rodents and *I. ovatus* ticks in Japan (17). Later "*Candidatus* N. mikurensis" was found in the *I. ricinus* and canine blood in Europe and in *I. persulcatus* and rodents in Asian Russia (6, 33, 42). This clade also includes the bacteria formerly referred to as *Ehrlichia*-like "Schotti variant" detected in *I. ricinus*, *I. persulcatus* and *Ehrlichia* sp. and "Rattus strain" detected in the rats *Rattus norvegicus* in China (29, 35, 37).

Recently, it was found for the first time that *Candidatus* N. mikurensis causes diseases in humans. "*Candidatus* N. mikurensis" DNA was detected in the blood samples of febrile

patients in Sweden, Germany, Switzerland, and Czechia (9, 30, 42, 44). In most cases, the disease developed in immunocompromised individuals. Three of six patients were splenectomized and had lymphoma or lymphocytic leukemia in their history (30, 44). Two patients were diagnosed as having *Candidatus* N. mikurensis infections. One had undergone immunosuppressive therapy for suspected chronic inflammatory demyelinating polyneuropathy and the other patient had been previously healthy (42).

Thus, although Japanese *Ixodes* ticks possess several *Ehrlichia* spices, neither of patients with ehrlichiosis nor neoehrlichiosis have been reported. On the other hand, Japanese spotted fever (JSF) and Scrub typhus (ST) are known as endemic rickettsiosis in Japan. Although JSF and ST share similar clinical features in Japan, these diseases differ in seasonality, geographic distribution, physical signs, and severity. Patients with rickettsial diseases often do not show rash and eschar, and the sensitivity of the serologic tests for diagnosis of rickettsial diseases can be low in the acute phase of illness. A substantial number of rickettsial diseases may be underdiagnosed (34).

The typical signs and symptoms of rickettsiosis and ehrlichiosis are similar (e.g., fever,

rash, and eschar). Both ehrlichiosis and rickettsiosis are endemic to the USA. Although over 200 cases of rickettsiosis are reported annually in Japan, ehrlichiosis has not been reported so far. Significant number of patients with fever of unknown origin in Japan, where tick borne disease are endemic, are left undiagnosed. The rickettsiosis-endemic areas may have a potential for other tick-borne diseases like ehrlichiosis or neoehrlichiosis.

In this study, I focused my study on ehrlichiosis and neoehrlichiosis, which might be prevalent in Japan. I investigate the prevalence of *Ehrlichia* species in ticks by detection of ehrlichial DNA and/or the isolation of *Ehrlichia* species from ticks collected in multiple regions in Japan to evaluate the potential of *Ehrlichia* species to cause human ehrlichiosis in Japan.

Table 1. The microorganisms classified in the genus of the *Anaplasmaceae* family

Phylum	Class	Order	Family	Genus
Protebacterica	*α-Ploteobacteria*	*Rickettsiales*	*Anaplasmataceae*	*Anaplasma*
				Ehrlichia
				Candidatus Neoehrlichia
				Neorickettsia
				Wolbachia

Table 2. Characteristics of *Ehrlichia* and *Neoehrlichia*.

Species	Infected cells	Distribution area	Primary vectors	Main hosts	Diseases
Ehrlichia chaffeensis	Monocytes / macrophages	USA, Africa, South America, Asia	*Amblyomma americanum*	White-tailed deer	Human monocytic ehrlichiosis Canine ehrlichiosis
Ehrlichia canis	Monocytes / macrophages	Worldwide	*Rhipicephalus sanguineus, Dermacentor variabilis*	Dogs wild canids	Canine monocytic ehrlichiosis
Ehrlichia ewingii	Granulocytes	USA, Africa, Asia	*Amblyomma americanum*	White-tailed deer, dogs	Human ewingii ehrlichiosis Canine ehrlichiosis
Ehrlichia muris	Monocytes / macrophages	Eurasia	*Haemaphysalis* spp., *Ixodes* spp.	Small rodents	Murine splenomegaly
Ehrlichia ruminantium	Endothelial cells, neutrophils, macrophages	Africa, Caribbean	*Amblyomma* spp.	Cattle, sheep, goats, and some wild ruminants	Heartwater in ruminants
Candidatus Neoehrlichia mikurensis	Endothelial cells, granulocytes	Eurasia	*Ixodes* spp.	Small rodents	Human neoehrlichiosis

Summary

Ehrlichiosis is a tick-borne bacterial disease caused by pathogens of the *Ehrlichia* genus. Although human ehrlichiosis has not been reported in Japan, *Ehrlichia* spp., which are closely related to *Ehrlichia chaffeensis*, were detected in several species of ixodid ticks. The presence of *Ehrlichia* spp. in ticks in Japan was studied by using isolation and molecular detection methods. In total, 1,237 ticks were collected from vegetation in western, central, and eastern parts of Japan. The ticks were tested for detection of ehrlichial DNA with a nested polymerase chain reaction and/or isolation by inoculation of mice with the homogenate. Ehrlichial DNA was detected in 29 of these ticks. The ehrlichial DNAs, *groEL* and 16S rRNA genes, detected in *Ixodes turdus* showed a high similarity to those of *E. chaffeensis* with 94.7% and 99.2% identity, respectively. *Ehrlichia* sp. HF and *Candidatus* Neoehrlichia mikurensis were also detected in *I. ovatus*. Furthermore, *Ehrlichia* sp. HF was isolated from laboratory mice that were intraperitoneal inoculated with *I. ovatus* tick homogenate. Some ehrlichial agents detected in *Ixodes* ticks might be a previously unknown *Ehrlichia* species. In this study, *Candidatus* N. mikurensis was detected in *I. ovatus* ticks as well. Because *I. ovatus* is distributed widely and cases of its tick bite in humans are ubiquitously reported in Japan, there is a potential that ehrlichiosis might be endemic to Japan, necessitating intensive surveillance of this infectious disease.

Introduction

Human ehrlichiosis is a tick-borne bacterial disease caused by obligate intracellular bacteria, *Ehrlichia chaffeensis*, *E. ewingii*, and *E. muris*. Neoehrlichiosis is also a tick-borne bacterial disease caused by *Candidatus* Neoehrlichia mikurensis. These species of bacteria are commonly present in nature in a life cycle between wildlife mammals and some species of ticks (*Ixodes*, *Haemaphysalis*, and *Amblyomma* species), and they may cause asymptomatic infections or mild diseases in wildlife mammals (26, 28, 38, 47).

Ehrlichia chaffeensis, an obligatory intramonocytic bacterium, causes human monocytic ehrlichiosis (HME) (1, 5). *Ehrlichia chaffeensis* was discovered for the first time in 1986 in the monocytes of a patient with fever, headache, pharyngitis, nausea, vomiting, and dehydration with multiple tick bites (1, 5, 28). *Ehrlichia chaffeensis* has been detected in *Amblyomma americanum* ticks collected in the field, and the wild white-tailed deer is well known to be its reservoir in the USA (12, 23, 28).

Ehrlichia muris-like agent (EMLA), which was detected in febrile patients with tick bites in Minnesota and Wisconsin in the USA, has recently been recognized as a novel pathogenic *Ehrlichia* species (31). EMLA was related genetically to *E. muris*, a species of *Ehrlichia* isolated from *Haemaphysalis* ticks and wild rodents in Japan (16, 18). However, despite the use of the term EMLA in several previous reports, it has recently been formally named *E. muris eauclairensis* (31).

Candidatus N. mikurensis was first isolated from wild rats captured in Mikura Island, Izu Archipelago, Japan in 2004 (17). It is prevalent among ticks and wild rodents in Europe and Asia (17, 45). *Candidatus* N. mikurensis is also a novel tick-borne disease agent for neoehrlichiosis. The disease caused by *Candidatus* N. mikurensis was first

discovered in an immunocompromised patient with febrile episodes in Sweden in 2010 (44). *Candidatus* N. mikurensis infections have been reported not only from Europe but also from Asia (22). These patients were diagnosed as having neoehrlichiosis by detection of *Candidatus* N. mikurensis genes in their blood with the use of polymerase chain reaction (PCR), followed by subsequent sequencing of the DNA amplified (22, 42, 44).

Ticks collected in Japan have already been reported to possess *Ehrlichia* species that are closely related to *E. chaffeensis*, *E. muris*, and *Candidatus* N. mikurensis (10, 13, 16, 17, 26, 36, 39).

Both ehrlichiosis and rickettsiosis are endemic to the USA. Although over 200 cases of rickettsiosis are reported annually in Japan, ehrlichiosis has not yet been reported. The rickettsiosis-endemic area may have the potential for other tick-borne infectious diseases. However, neither patients with HME nor those with *Candidatus* N. mikurensis infection have been reported in Japan so far. Ehrlichiosis may have the potential to be endemic to Japan. Because previous investigations surveyed limited areas, further investigation in a wider area is required. The aim of this study was to investigate the prevalence of *Ehrlichia* species in ticks by detection of ehrlichial DNA and/or the isolation of *Ehrlichia* species from ticks collected in multiple regions of Japan to evaluate the potential for human ehrlichiosis in Japan.

Material and methods

Tick collection and preparation

Ticks were collected by use of a flagging method in Akita, Miyagi, Yamagata, Fukushima, Chiba, Nagano, Shizuoka, Tokushima and Yamaguchi prefectures, Japan, from 2013 to 2016 (Fig. 1). The tick species were identified morphologically (Yamaguti et al., 1971). The ticks collected were then soaked in 70% ethanol with 0.1% povidone-iodine for 10 min and rinsed with phosphate buffered saline solution (PBS) containing 0.5-1.0% calf serum. The ticks were homogenized in tubes using a pestle with sucrose-phosphate-glutamate (0.0038 M KH_2PO_4, 0.0072 M K_2HPO_4, 0.0049 M L-glutamate, 0.218 M sucrose, pH 7.2) at a volume of 0.3 mL per one tick. Then, 0.2 mL of the tick samples homogenized was applied for the extraction of DNA using a High Pure PCR Preparation kit (Roche Diagnostics, Mannheim, Germany). The remaining 0.1 mL of the tick sample was stored at -80ºC for further study. DNA extracted from the ticks was also stored at -80ºC until use.

Detection of ehrlichial DNA

DNA extracted from each tick sample was individually tested for amplification of the ehrlichial genome with PCR targeting a conserved region of the *groEL* and 16S rRNA genes (Table 1) (13, 38). When the specific DNA was amplified, the nucleotide sequence of the amplified DNA was determined with cycle sequencing using a BigDye Terminator v1.1 cycle sequencing kit and Genetic Analyzer 3130 (Applied Biosystems, Foster City, CA). Alignments were performed using MEGA 6.0 software with bootstrap support (1,000 replications), and their phylogenetic relationships were analyzed with the

neighbor-joining method (40).

Isolation of ehrlichial agent in mice

Three 5-week-old male ddY mice (Japan SLC, Shizuoka, Japan) were inoculated intraperitoneally with each tick homogenate, which was ehrlichial *groEL* gene-positive by PCR, per one sample. The mice were observed for 2 weeks or until clinical signs such as ruffled fur appeared or they died. When mice showed the clinical signs, they were sacrificed by excess isoflurane, and spleen and liver were aseptically collected. The organs removed were weighed, and their homogenates were prepared in sucrose-phosphate-glutamate buffer as a 10% w/v concentration. They were applied for the additional propagation of pathogens using ddY mice, in which the ehrlichial *groEL* gene was amplified with PCR for confirmation of isolation. The nucleotide sequence of the PCR product was also determined. *Ehrlichia* sp. HF565 strain was used for ehrlichial isolation as positive controls.

Ethical considerations

The animal experiments were performed in strict accordance with the Regulation for Animal Experimentation of the Chiba Prefectural Institute of Public Health. The protocol of the experiments, in which mice were used, was approved by the Institutional Animal Care and Use Committee of the Chiba Prefectural Institute of Public Health (numbers 28-3 and 29-2). All the mice infected with pathogens were handled in biosafety level 2 animal facilities in accordance with the biosafety guidelines of the Chiba Prefectural Institute of Public Health. The mice were inoculated with tick homogenates while taking all efforts to minimize any potential pain and distress.

Results

Ticks collection

In total, 1,237 ticks (84 *I. turdus*, 350 *I. ovatus*, 99 *Haemaphysalis megaspinosa*, 55 *H. flava*, 516 *H. longicornis*, 107 *H. kitaokai*, 15 *H. cornigera*, and 11 *A. testudinarium*) were captured from vegetation in western, central, and eastern parts of Japan (Fig. 1, Fig. 2, Table 2).

Phylogenetic analysis of ehrlichial *groEL* genes

Ehrlichial *groEL* fragments were detected in 29 tick samples. The ehrlichial *groEL* gene was amplified in the following tick species of *I. turdus*, *I. ovatus*, *H. megaspinosa*, *H. flava*, and *H. longicornis*, but not in *H. kitaokai, H. cornigera*, and *A. testudinarium* (Tables 2 and 3). The ehrlichial *groEL* gene-positive rate in *I. turdus* was the highest among those of the tick species tested. The ehrlichial *groEL* gene fragments were detected in the ticks collected in Chiba, Nagano, Shizuoka, and Fukushima prefectures, but not in those collected in Akita, Yamagata, Miyagi, Tokushima, and Yamaguchi prefectures (Fig. 1, Table 3). The novel sequences of *groEL* genes were deposited in the DNA Data Bank of Japan (DDBJ) (Table 4).

The nucleotide sequences of the *groEL* gene fragments amplified in the 29 ticks were determined, revealing that the ehrlichial *groEL* genes detected in *I. ovatus* were divided into two genotypes (Fig. 3, Table 4). One type including Io30, Io67, Io82, Io86, Io143, Io181, Io184, Io191, Io193, Io198, Io199, and Io202 was positioned in a cluster to which *Ehrlichia* sp. strain HF565 belonged, being that the sequence identities of the *groEL* genes amplified from these *I. ovatus* were 100% identical to that of HF565. The other type of

ehrlichial *groEL* genes detected in *I. ovatus* Io3, Io106, Io114, and Io145 was positioned in a cluster to which *Candidatus* N. mikurensis was included (Fig. 3), being that the sequence identities of the *groEL* genes amplified from these *I. ovatus* were 98.6% identical to that of *Candidatus* N. mikurensis (Acc. No. AB074461).

Ehrlichial *groEL* genes detected in *I. turdus* were also divided into two genotypes. The *groEL* genes amplified from *I. turdus* Itp3, It19, It20, and It40 were closely related to that of *E. chaffeensis* (Acc. No. CP000236), whereas *groEL* genes amplified from *I. turdus* Itp1, It1, It24, It25, and *H. megaspinosa* Hm28 were positioned in a cluster with *Candidatus* Ehrlichia shimanensis (Acc. No. AB074462). Novel ehrlichial *groEL* genes sequences, which were amplified from *H. megaspinosa* Hm37, *H. flava* Hf34, and *H. longicornis* Hl9, clustered with those of *Ehrlichia* sp. yonaguni206 (Acc. No. HQ697591) and *E. ewingii* (Acc. No. AF195273).

Phylogenetic analysis of ehrlichial 16S rRNA genes

The 16S rRNA gene fragments amplified from 12 ticks were sequenced. The novel sequences of 16S rRNA genes were also deposited in the DDBJ under the accession numbers assigned (Table 4). Ehrlichial 16S rRNA genes detected in *I. ovatus* were divided into two genotypes (Fig. 4). One type including Io30, Io67, Io82, Io86, Io143, Io191, and Io202 was positioned in a cluster to which *Ehrlichia* sp. strain HF565 (Acc. No. DQ647318) belonged, being that the sequence identities of the 16S rRNA genes amplified from these *Ehrlichia* of *I. ovatus* were 100% identical to that of *Ehrlichia* sp. strain HF565. Another type of ehrlichial 16S rRNA genes detected in *Ehrlichia* of *I. ovatus* Io106 and Io114 was positioned in a cluster to which *Candidatus* N. mikurensis (Acc. No. AB074460) was included (Fig. 4), being that the sequence identities of the 16S rRNA

genes amplified from these *Ehrlichia* of *I. ovatus* were also 100% identical to that of *Candidatus* N. mikurensis.

Ehrlichial 16S rRNA genes detected in *I. turdus* were also divided into two genotypes. The 16S rRNA genes amplified from It20 and It40 were closely related to that of *E. chaffeensis* (Acc. No. NR074500), whereas 16S rRNA genes amplified from It25 were positioned in a novel cluster.

Isolation of ehrlichial agent from ticks

Mice were inoculated with each of the homogenates of the 29 ehrlichial DNA-positive samples, composed of 16 *I. ovatus* and 9 *I. turdus* homogenates, for isolation. Ehrlichial organisms were isolated from 4 *I. ovatus* but not from *I. turdus* (Table 3). All the mice inoculated with each of the Io30, Io67, Io191, and Io202 samples developed clinical signs of ruffled fur and died within 9 to 11 days after inoculation (Fig. 5). The organisms isolated were confirmed to be *Ehrlichia* spp. by genetic analysis. *Candidatus* N. mikurensis-like *Ehrlichia* was not isolated from any samples.

Discussion

Ehrlichial DNA fragments, which were classified to *E. chaffeensis* or to *Candidatus* N. mikurensis, were detected in multiple tick species collected in central and eastern parts of Japan. The detected ehrlichial DNA fragments had diversities, some of which were closely related to *E. chaffeensis*, which is a causative agent of HME.

Ehrlichia sp. strain HF565-like *Ehrlichia* spp. was isolated from *I. ovatus* ticks collected in Nagano, Shizuoka, and Fukushima prefectures. The *Ehrlichia* sp. strain HF565 isolated was closely related to *E. chaffeensis* isolated in the USA. The present study suggests that *Ehrlichia* sp. strain HF565-like *Ehrlichia* is more widely distributed in Japan than was previously thought.

Candidatus N. mikurensis is a pathogen of neoehrlichiosis first isolated in Japan (18). However, no patient with neoehrlichiosis has been reported in Japan, whereas the DNA of *Candidatus* N. mikurensis was detected from immunodeficient patients in Europe and China (22, 42, 44, 45). Because *Candidatus* N. mikurensis was detected in *I. ovatus* collected in Chiba, Nagano, and Shizuoka prefectures, *Candidatus* N. mikurensis is also considered to be distributed more widely in Japan than was previously thought. The *Candidatus* N. mikurensis DNA sequence detected from *I. persulcatus* collected in areas where neoehrlichiosis patients were reported was identical to those amplified from neoehrlichiosis patients in China (43). Therefore, *I. persulcatus* is thought to be the vector of *Candidatus* N. mikurensis in China. *Ixodes ovatus* is widely distributed, and cases of its tick bite are ubiquitously reported in Japan (11). There is a potential that ehrlichiosis might be endemic to Japan, necessitating intensive surveillance of this infectious disease. To address this assumption, a prospective study is needed.

Ehrlichial DNA detected in *I. turdus* was more closely related to *E. chaffeensis* than to *Ehrlichia* sp. strain HF565. Ehrlichial *groEL* and 16s RNA DNAs detected in 4 samples of *I. turdus* showed a high similarity to those of *E. chaffeensis* strain Arkansas with 94.8% and 99.6% identity, respectively. Humans are reported to be bitten by *I. turdus* in Japan, suggesting that *I. turdus* may be a potential vector tick for HME in Japan (46).

Recently, EMLA and *E. muris eauclairensis* were detected from human ehrlichiosis patients in the USA (4,31, 32). *E. muris* was not detected in this study, although some species of ticks in Japan, and especially *H. flava*, possess *E. muris* (16). The total number of *H. flava* ticks collected accounted for only 4% of the total tick collection, and thus the number of samples was limited. Expanding the sampling area and increasing the number of ticks collected are needed to assess the prevalence of *E. muris* accurately.

There are many areas to be addressed in terms of HME and neoehrlichiosis in Japan. *Ehrlichia chaffeensis*, *E. muris eauclairensis*, and *Candidatus* N. mikurensis have been detected in patients outside Japan (1, 22, 31, 42). Since 2008, around 1,000 cases of *E. chaffeensis* infection have been reported annually in the USA (4). Since the discovery of *E. muris eauclairensis* in the USA, 115 cases of infection by this species have been reported in total (4). In total, 26 cases of *Candidatus* N. mikurensis infection have been reported from several European and Asian countries such as Sweden, Switzerland, Czech Republic, Germany, Poland, and China (45).

To evaluate the risk for ehrlichiosis more precisely, further studies are needed. In the present study, ticks were collected in a limited number of areas, and the total number of ticks collected was also limited. Thus, similar studies that undertake an expansion of the sampling areas and an increase in the number of ticks collected will be necessary and important in the future. Infectious *Candidatus* N. mikurensis has not been isolated and

cultivated, and its genome sequence has not yet been fully determined. In a previous study, in which the isolation of *Candidatus* N. mikurensis using rats was reported, neoehrlichial DNA was negative up to day 15 after inoculation. However, specimens from days 20-60 after inoculation became positive for *Candidatus* N. mikurensis DNA in PCR (17). Thus, in the present study, the time period for observation of the mice might have been too short. Furthermore, it seems to be highly likely that mice may be less sensitive than rats in the isolation of *Candidatus* N. mikurensis. The development of an isolation method for infectious *Candidatus* N. mikurensis is needed to enhance the research capacity for sequencing genomes, elucidating host-pathogens interactions, and developing diagnostics and therapeutics.

There is a risk of having HME or neoehrlichiosis patients in Japan, because it was confirmed that some tick species of Japanese *Ixodes* possess *ehrlichia* in Japan. It is speculated that the ehrlichial pathogens may be more widely prevalent in Japan than those shown in the previous studies (10, 13, 16-18, 26, 36, 38). The prospective study to identify the patients with ehrlichial diseases should be carried out with development of the diagnostic systems of and the surveillance system for ehrlichiosis.

Conclusions

The objective of this study was to investigate the prevalence of Ehrlichia species in ticks collected by detection of ehrlichial DNA and/or the isolation of Ehrlichia species from tick collected in multiple areas in Japan to evaluate the potential for human ehrlichiosis and neoehrlichiosis. The results of obtained were written described as follows.

1. Ehrlichial gene was detected from 29 of 1237 tick species in Japan. Frequency of ehrlichial DNA in *I. turdus* was the highest among the tick species tested.

2. Ehrlichial DNA detected in *I. turdus* was closely related to *E. chaffeensis*.

3. Two species of ehrlichia were detected in *I. ovatus*; *Ehrlichia* sp. HF and *Candidatus* Neoehrlichia mikurensis, and *Ehrlichia* sp. HF was isolated from *I. ovatus*.

4. These Ehrlichia are novel Ehrlichia species and had potential to be the ehrlichiosis pathogens in Japan. It is likely that human ehrlichiosis and neoehrlichiosis patients might be present in Japan.

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Figure legends

Fig. 1. Geographic areas where ticks were collected from June 2013 to June 2016. The areas, in which the ehrlichial DNA-positive ticks were found are shown in black, and those in which only ehrlichial DNA-negative ticks were found are shown in gray.

I. ovatus (Female)

I. turdus (Nymph)

H. flava (Female)

H. megaspinosa (Male)

Fig. 2-1. Morphological feature of ticks collected.

H. Kitaokai (Male)

H. longicornis (Female)

A. testudinarium (Female)

H. cornigera (Nymph)

Fig. 2-2. Morphological feature of ticks collected.

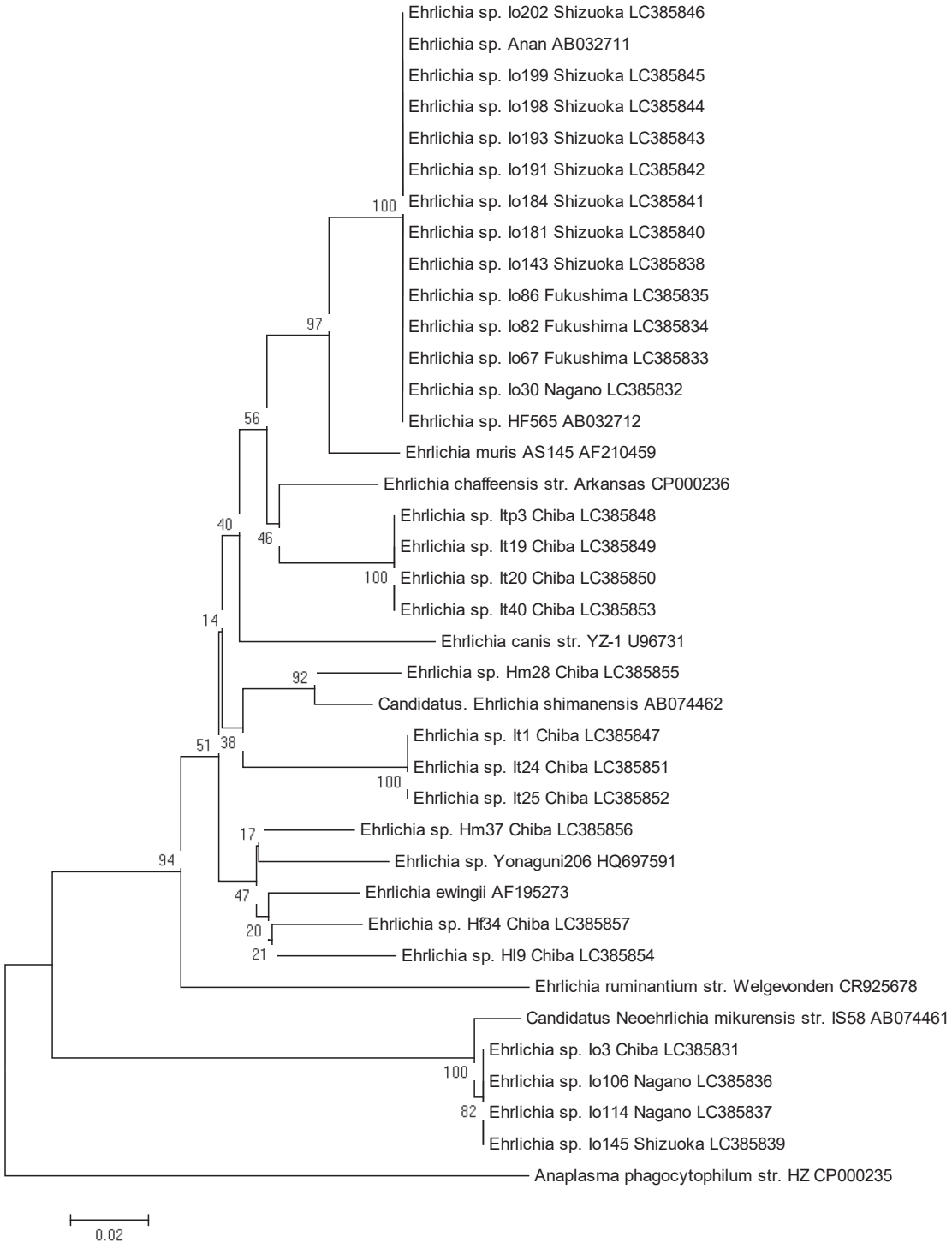

Fig. 3. Phylogenetic relationships between the *Ehrlichia* spp. genes based on the sequence of the 362-bp fragment of the *groEL* gene.

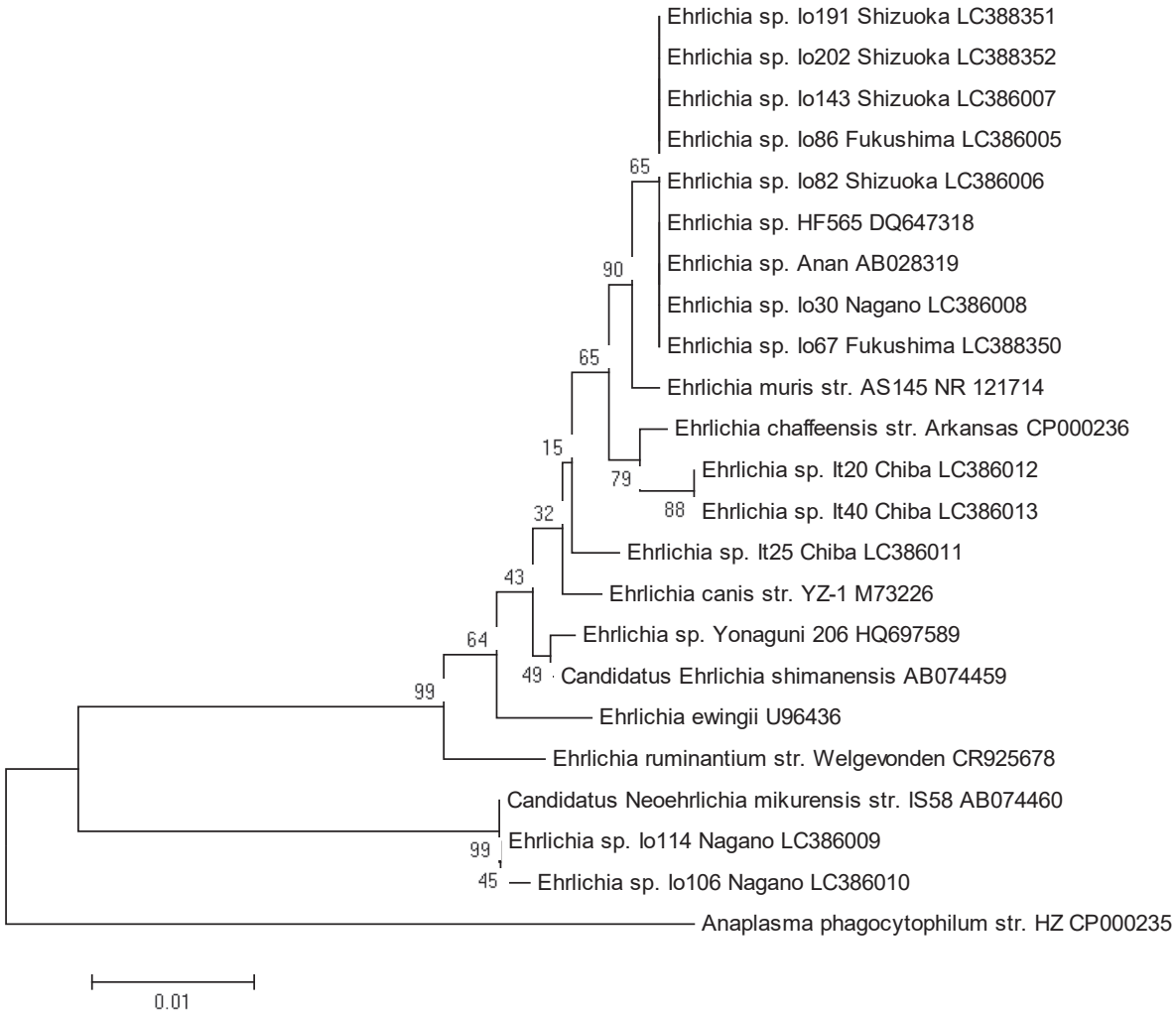

Fig. 4. Phylogenetic relationships between the *Ehrlichia* spp. genes based on the sequence of the 702-bp fragment of the 16s rRNA gene.

Fig. 5. The clinical signs of mice ruffled fur within 9 days after inoculation.

Table 1

Primer sets for the amplification of *groEL* and 16S rRNA of the ehrlichial species.

Target gene	PCR step	Length of PCR product	Primer name	Primer sequence 5′ -3′	Reference
groEL	1st	687 bp	gro607F	GAAGATGCWGTWGGWTGTACKGC	Tabara et al., 2007
			gro1294R	AGMGCTTCWCCTTCWACRTCYTC	
	Nest	444 bp	gro677F	ATTACTCAGAGTGCTTCTCARTG	
			gro1121R	TGCATACCRTCAGTYTTTTCAAC	
16S rRNA	2nd	1490 bp	ER5-3	TTGAGAGTTTGATCCTGG	Inayoshi et al., 2004
			ER-R1	GGAGGTAATCCAGCCGCA	
	Nest	910 bp	E16s-200F	GATCAGCCACACTGGAACTGAGA	Tabara et al., 2007
			E16S-1162R	CATTGTAGCACGTGTGTAGCCCA	

Table 2

Nested PCR-positive rate for the ehrlichial *groEL* gene in each tick species

Tick	Number of samples	Number of PCR-positive ticks	Positive rate (%)
I. turdus	84	9	10.7
I. ovatus	350	16	4.6
H. megaspinosa	99	2	2.0
H. flava	55	1	1.8
H. longicornis	516	1	0.2
H. kitaokai	107	0	0
H. cornigera	15	0	0
A. testudinarium	11	0	0
Total	1237	29	2.3

Table 3

Information on the ticks in which the ehrlichial DNA was detected.

Tick species	Sample ID	Sex (Stage)	Site (prefecture)	Nested PCR		Isolation
				groEL	16S rRNA	
I. turdus	Itp1	Unknown (Nymph)	Chiba	+	+	-
	Itp3	Unknown (Nymph)	Chiba	+[a]	+	-
	It1	Female	Chiba	+[a]	+	-
	It19	Unknown (Nymph)	Chiba	+[a]	+	-
	It20	Unknown (Nymph)	Chiba	+[a]	+[a]	-
	It24	Unknown (Nymph)	Chiba	+[a]	+	-
	It25	Unknown (Nymph)	Chiba	+[a]	+[a]	-
	It40	Unknown (Nymph)	Chiba	+[a]	+[a]	-
	It51	Unknown (Nymph)	Chiba	+	+	-
I. ovatus	Io3	Unknown (Nymph)	Chiba	+[a]	+	-
	Io30	Male	Nagano	+[a]	+[a]	+
	Io67	Female	Fukushima	+[a]	+[a]	+
	Io82	Male	Fukushima	+[a]	+[a]	-

	Io86	Male	Fukushima	+[a]	+[a]	-
	Io106	Female	Nagano	+[a]	+[a]	-
	Io114	Female	Nagano	+[a]	+[a]	-
	Io143	Female	Shizuoka	+[a]	+[a]	-
	Io145	Female	Shizuoka	+[a]	+	-
	Io181	Female	Shizuoka	+[a]	+	-
	Io184	Female	Shizuoka	+[a]	+	-
	Io191	Female	Shizuoka	+[a]	+[a]	+
	Io193	Male	Shizuoka	+[a]	+	-
	Io198	Female	Shizuoka	+[a]	+	-
	Io199	Female	Shizuoka	+[a]	+	-
	Io202	Female	Shizuoka	+[a]	+[a]	+
H. megaspinosa	Hm28	Male	Chiba	+[a]	-	-
	Hm37	Female	Chiba	+[a]	-	-
H. flava	Hf34	Female	Chiba	+[a]	-	-
H. longicornis	Hl9	Female	Chiba	+[a]	-	-

[a] The nucleotide sequence was determined.

Table 4

Information on the ehrlichial species used for the phylogenetic analysis, including the isolate determined in the present study.

Ehrlichia strain	Country	Year	Source	Accession number	
				groEL	16S rRNA
Ehrlichia chaffeensis str. Arkansas	U.S.A.	1991	Human	CP000236	CP000236
Ehrlichia sp. HF565	Japan: Fukushima	1994	Tick (*I. ovatus*)	AB032712	DQ647318
Ehrlichia sp. Anan	Japan: Tokushima	1994	Tick (*I. ovatus*)	AB032711	AB028319
Ehrlichia muris AS145	Japan: Aichi	1983	Wild mouse (*Eothenomys kageus*)	AF210459	NR121714
Ehrlichia canis str. YZ-1	Japan: China	2016	Dog	U96731	M73226
Ehrlichia sp. Yonaguni 206	Japan: Yonaguni Island	2012	Tick (*H. longicornis*)	HQ697591	HQ697589
Ehrlichia ewingii	U.S.A.	1999	Human/Dog	AF195273	U96436
Candidatus Ehrlichia shimanensis	Japan: Shimane	1999	Tick (*H. longicornis*)	AB074462	AB074459
Ehrlichia ruminantium str. Welgevonden	South Africa	1985	Tick (*A. hebraeum*)	CR925678	CR925678

Candidatus Neoehrlichia mikurensis str. IS58	Japan: Mikura Island	2000	Tick (*I. ovatus*)	AB074461	AB074460
Anaplasma phagocytophilum str. HZ	U.S.A.	2014	Human	CP000235	CP000235
Present study					
Ehrlichia sp. Itp3 Chiba	Japan: Chiba	2014	Tick (*I. turdus*)	LC385848	-[a]
Ehrlichia sp. It1 Chiba	Japan: Chiba	2016	Tick (*I. turdus*)	LC385847	-
Ehrlichia sp. It19 Chiba	Japan: Chiba	2016	Tick (*I. turdus*)	LC385849	-
Ehrlichia sp. It20 Chiba	Japan: Chiba	2016	Tick (*I. turdus*)	LC385850	LC386012
Ehrlichia sp. It24 Chiba	Japan: Chiba	2016	Tick (*I. turdus*)	LC385851	-
Ehrlichia sp. It25 Chiba	Japan: Chiba	2016	Tick (*I. turdus*)	LC385852	LC386011
Ehrlichia sp. It40 Chiba	Japan: Chiba	2016	Tick (*I. turdus*)	LC385853	LC386013
Ehrlichia sp. Io3 Chiba	Japan: Chiba	2013	Tick (*I. ovatus*)	LC385831	-
Ehrlichia sp. Io30 Nagano	Japan: Nagano	2015	Tick (*I. ovatus*)	LC385832	LC386008
Ehrlichia sp. Io67 Fukushima	Japan: Fukushima	2015	Tick (*I. ovatus*)	LC385833	LC388350
Ehrlichia sp. Io82 Fukushima	Japan: Fukushima	2015	Tick (*I. ovatus*)	LC385834	LC386006
Ehrlichia sp. Io86 Fukushima	Japan: Fukushima	2015	Tick (*I. ovatus*)	LC385835	LC386005
Ehrlichia sp. Io106 Nagano	Japan: Nagano	2015	Tick (*I. ovatus*)	LC385836	LC386010

Ehrlichia sp. Io114 Nagano	Japan: Nagano	2015	Tick (*I. ovatus*)	LC385837	LC386009
Ehrlichia sp. Io143 Shizuoka	Japan: Shizuoka	2015	Tick (*I. ovatus*)	LC385838	LC386007
Ehrlichia sp. Io145 Shizuoka	Japan: Shizuoka	2015	Tick (*I. ovatus*)	LC385839	-
Ehrlichia sp. Io181 Shizuoka	Japan: Shizuoka	2015	Tick (*I. ovatus*)	LC385840	-
Ehrlichia sp. Io184 Shizuoka	Japan: Shizuoka	2015	Tick (*I. ovatus*)	LC385841	-
Ehrlichia sp. Io191 Shizuoka	Japan: Shizuoka	2015	Tick (*I. ovatus*)	LC385842	LC388351
Ehrlichia sp. Io193 Shizuoka	Japan: Shizuoka	2015	Tick (*I. ovatus*)	LC385843	-
*Ehrlichi*a sp. Io198 Shizuoka	Japan: Shizuoka	2015	Tick (*I. ovatus*)	LC385844	-
Ehrlichia sp. Io199 Shizuoka	Japan: Shizuoka	2015	Tick (*I. ovatus*)	LC385845	-
Ehrlichia sp. Io202 Shizuoka	Japan: Shizuoka	2015	Tick (*I. ovatus*)	LC385846	LC388352
Ehrlichia sp. Hm28 Chiba	Japan: Chiba	2013	Tick (*H. megaspinosa*)	LC385855	-
Ehrlichia sp. Hm37 Chiba	Japan: Chiba	2013	Tick (*H. megaspinosa*)	LC385856	-
Ehrlichia sp. Hf34 Chiba	Japan: Chiba	2013	Tick (*H. flava*)	LC385857	-
Ehrlichia sp. Hl9 Chiba	Japan: Chiba	2013	Tick (*H. longicornis*)	LC385854	-

[a] The nucleotide sequence could not be determined.