Anaplasma phagocytophilum–infected Ticks, Japan

Norio Ohashi,*† Megumi Inayoshi,*‡ Kayoko Kitamura,* Fumihiko Kawamori,*‡ Daizoh Kawaguchi,* Yuusaku Nishimura,* Hirotaka Naitou,* Midori Hiroi,*‡ and Toshiyuki Masuzawa*†

We report *Anaplasma phagocytophilum* infection of *Ixodes persulcatus* and *I. ovatus* ticks in Japan. Unique *p44/msp2* paralogs (and/or 16S rRNA genes) were detected in tick tissues, salivary glands, and in spleens of experimentally infected mice. These findings indicate the public health threat of anaplasmosis in Japan.

Anaplasma phagocytophilum (formerly known as the agent of human granulocytic ehrlichiosis), *Ehrlichia phagocytophila*, and *E. equi* (1) are tickborne human pathogens of veterinary importance. They cause an emerging infectious and febrile systemic illness now known as human granulocytic anaplasmosis. The first case of human infection by *A. phagocytophilum* was reported in 1994 (2). Since then, an increasing number of cases have been recognized in the United States. Severities of this disease range from asymptomatic seroconversion to death, and severe illness is frequently documented. In Europe, the first human cases of this disease were described in 1997 (3), and serologic and polymerase chain reaction (PCR) analyses suggest that *A. phagocytophilum* is distributed throughout Europe and in some parts of the Middle East and Asia (4–6).

In nature, *A. phagocytophilum* is believed to be maintained in a tick-rodent cycle. The known vectors for this agent are *Ixodes* ticks, i.e., *Ixodes scapularis* and *I. pacificus* in the United States, *I. ricinus* mostly in Europe, and *I. persulcatus* in Russia (7) and China (5). Exposure to *A. phagocytophilum*–infected tick bites is the most common route of human infection, except for perinatal transmission or contact with infected mammalian blood (8,9).

In Japan, several *Ixodes* species, such as *I. persulcautus*, *I. ovatus*, and *I. monospinosus*, are potential vectors for transmission of *Borrelia* spp., *Rickettsia* spp., or *Ehrlichia* spp. (10–12). However, little information is available regarding the ecologic and epidemiologic features of clinical cases of infection with *A. phagocytophilum* in Japan. We report infection with *A. phagocytophilum* in *Ixodes* ticks in central Japan determined by molecular epidemiologic approaches.

*University of Shizuoka, Shizuoka, Japan; †Center of Excellence Program in the 21st Century, Shizuoka, Japan; and ‡Shizuoka Institute of Environment and Hygiene, Shizuoka, Japan

The Study

In 2003 and 2004, a total of 273 unfed and adult *Ixodes* ticks (114 *I. persulcatus* and 159 *I. ovatus*) were collected in central Japan (Figure 1). Of these, 123 live ticks were dissected, and DNA was isolated from whole tissues of 73 ticks and salivary glands of 50 ticks by using the QIAamp DNA mini kit (Qiagen Inc., Valencia, CA, USA). For detection of *A. phagocytophlilum* DNA, a nested PCR using primers designed based on the highly conserved region of *p44/msp2* paralogs of (p3726 [5′-GCTAAGGAGTTAGCTTATGA-3′], p3761, p4183, and p4257) was conducted (12–14). Four (12.1%) of 33 *I. persulcatus* ticks collected at the Utsukushinomori (UM) site in Yamanashi Prefecture were positive by PCR (Table). Sixteen (7 *I. persulcatus* and 9 *I. ovatus*) (32%) of 50 salivary glands from ticks collected at the Takabachi and Mizugazuka sites in Shizuoka Prefecture were positive by PCR. Data indicated that *I. persulcatus* and *I. ovatus* in Japan are naturally infected with *A. phagocytophilum* and that ticks at certain sites are highly infected.

We further examined the infection of immunocompromised mice with *A. phagocytophilum* in ticks by using the procedure described previously (12). Briefly, whole tissues from 150 live ticks (55 *I. persulcatus* and 95 *I. ovatus*) were pooled and intraperitoneally injected into 15 ddY male mice (6–15 pooled ticks per mouse) treated with the

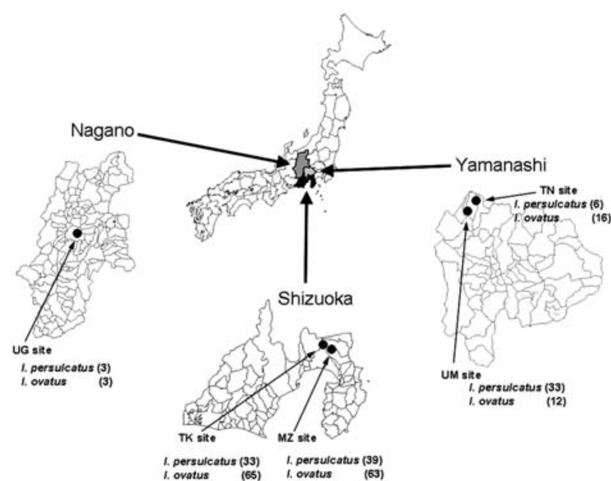

Figure 1. Areas in Shizuoka, Nagano, and Yamanashi Prefectures of Japan where *Ixodes persulcatus* and *I. ovatus* ticks were collected in 2003 and 2004. Closed circles indicate collection sites. Numbers of ticks collected at each site are shown in parentheses. UG, Utsukushigahara; TK, Takabachi; MZ, Mizugazuka; TN, Tennyosan; UM, Utsukushinomori.

Table. Polymerase chain reaction (PCR) detection of *A. phagocytophilum p44/msp2* paralogs from *Ixodes* ticks or spleens of mice experimentally infected with tick tissues

Collection site, year*	Whole tissue†		Salivary gland†	Experimental infection with ticks‡		Total
	Female	Male	Female	Female	Male	
I. persulcatus						
Yamanashi, 2004						
TN	0/2	0/4				0/6
UM	2/16	2/17				4/33
Nagano, 2004						
UG	0/3	0/0				0/3
Shizuoka, 2004						
TK			6/9			6/9
MZ			1/8			1/8
Shizuoka, 2003						
TK				0/14 (2)	0/10 (1)	0/24 (3)
MZ				0/22 (2)	0/9 (1)	0/31 (3)
Total	2/21	2/21	7/17	0/36 (4)	0/19 (2)	11/114 (6)
I. ovatus						
Yamanashi, 2004						
TN	0/8	0/8				0/16
UM	0/9	0/3				0/12
Nagano, 2004						
UG	0/1	0/2				0/3
Shizuoka, 2004						
TK			9/17			9/17
MZ			0/16			0/16
Shizuoka, 2003						
TK				0/32 (3)	1/16 (2)	1/48 (5)
MZ				0/26 (2)	0/21 (2)	0/47 (4)
Total	0/18	0/13	9/33	0/58 (5)	1/37 (4)	10/159 (9)

*TN, Tennyosan; UM, Utsukushinomori; UG, Utsukushigahara; TK, Takabachi; MZ, Mizugazuka.
†No. positive/no. examined. One hundred twenty-three ticks were dissected (whole tissues from 73 ticks and salivary glands from 50) were individually examined by PCR.
‡No. of positive mouse spleens/no. of ticks examined (no. of mice used). Six to 15 ticks were pooled and homogenized (55 *I. persulcatus* and 95 *I. ovatus*), and intraperitoneally injected into ddY male mice.

immunosuppressant cyclophosphamide. PCR was conducted with DNA isolated from blood and spleens of these mice. Only 1 of 9 spleens from *I. ovatus*-injected mice was positive by PCR (Table). We previously detected *Ehrlichia* spp. DNA in *I. ovatus*–injected mice, but did not detect *A. phagocytophilum* DNA in *I. ovatus*– or *I. persulcatus*–injected mice (12) because we used only a few immunocompromised mice, i.e., most had normal immune systems. Thus, we treated all 15 mice used in the present study with cyclophosphamide. Results indicate that *A. phagocytophilum* in *I. ovatus* can be infective for immunocompromised mice, although the efficiency of infection was low (1/95 [1.1%]).

The *p44/msp2* amplicons from 8 PCR-positive ticks and 1 PCR-positive mouse were cloned into a pCR2.1 vector with the TA Cloning Kit (Invitrogen, Carlsbad, CA, USA). Recombinant clones were randomly selected and 28 recombinant *p44/msp2* clones were sequenced with an ABI 3100-Avant Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). A phylogenetic tree was constructed based on the alignment of Japanese *p44/msp2* sequences and the most closely related paralogs (220–400 bp) by using ClustalX (http://www-igbmc.u-strasbg.fr/BioInfo/ClustalX/), followed by the neighbor-joining method with 1,000 bootstrap resamplings (Figure 2). In this tree, the *p44/msp2* sequences obtained from *I. ovatus* were located mostly in clusters different from those where sequences from *I. persulcatus* were located, except for Tick41-1. This finding suggests that *A. phagocytophilum* in *I. ovatus* may encode *p44/msp2* paralogs distinct from those of *A. phagocytophilum* in *I. persulcatus*. A previous study suggested that the *p44/msp2* sequences from the United States and the United Kingdom can be divided into 27 similarity groups based on >90% similarities of DNA sequences, and most sequences from the United Kingdom are distinguishable from those from the United State because of the similarities <79% (15). Of 28 Japanese *p44/msp2* sequences in this study, 11 sequences with similarities >85.6% to the previously identified paralogs were probably divided into 8 similarity groups (Figure 2). Of the remaining 17 sequences with similarities <73.1%, 11 members that were grouped into 2 distinctive clusters (Figure 2) and 6 members that were individually located (Figure 2, arrows) were distinguishable from the 8 similarity groups. Thus, some *p44/msp2* paralogs of Japanese *A. phagocytphilum* are unique and distinct from those of

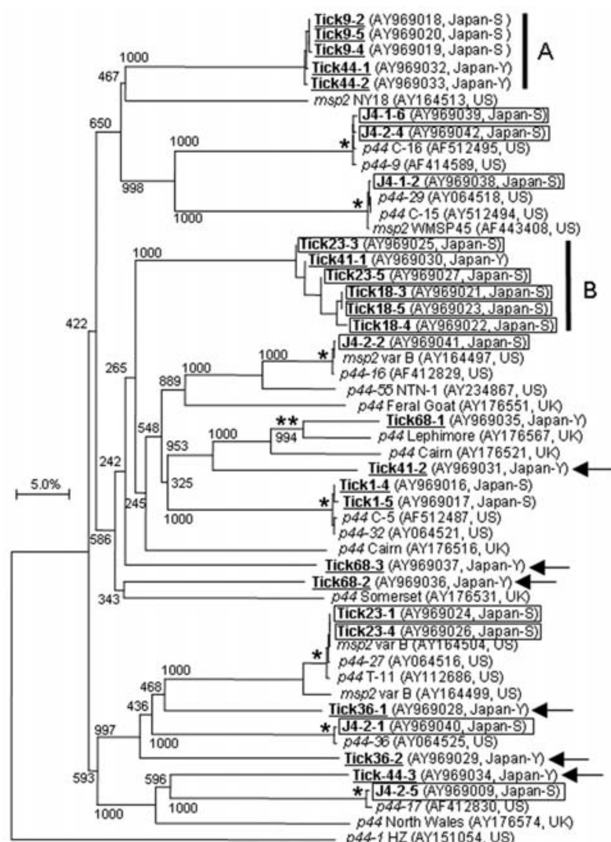

Figure 2. Phylogram of *Anaplasma phagocytophilum p44/msp2* including Japanese paralogs. A) Cluster from *Ixodes persulcatus*. B) Cluster from *I. ovatus*, except for Tick41-1. The tree was constructed using the neighbor-joining method. Numbers on the tree indicate bootstrap values for branch points. Japanese *p44/msp2* paralogs from *Ixodes persulcatus* and *I. ovatus* are underlined and boxed, respectively, in bold. A single star shows *p44/msp2* clusters with 99.2%–100% similarities and double stars show a cluster with 85.6% similarity. Two vertical bars and 6 arrows indicate Japanese *p44/msp2* clusters and paralogs, respectively, which are distinct from the previously identified *p44/msp2* (<73.1% similarity). A horizontal bar indicates percentage of sequence divergence. Accession numbers and location (Japan-Y [Yamanashi], Japan-S [Shizuoka], US [United States], and UK [United Kingdom]) are in parentheses.

A. phgocytophlium in other countries, although multiple copies of *p44* in the genome of an organism should be considered (13).

A partial sequence of the 16S rRNA gene of *A. phagocytophilum* (1.4 kb) from a *p44/msp2* PCR-positive mouse was amplified from spleen DNA with primers ER5-3, ER-R1, AP-F1, and AP-R1 (12), cloned, and sequenced. Similarities among 6 Japanese recombinant 16S rRNA sequences (GenBank accession nos. AY969010–AY969015) were 99.3%–99.6%. When compared with *A. phgocytophilum* human agent U02521, the similarities were 99.6%–99.8% between individual 16S rRNA cloned sequences and human agent U02521. Because we used pooled ticks to examine infection in mice, these sequence diversities may depend on genetic variants (or a heterogeneous population) of *A. phagocytophilum* from individual ticks. When the amplicon was directly sequenced, its sequence was identical with that of human agent U02521.

Conclusions

We demonstrated that *A. phagocytophilum* infects *Ixodes* ticks in Japan, that both *I. persulcatus* and *I. ovatus* ticks are naturally infected with *A. phgocytophilum*, that *A. phagocytophilum* may be transmitted by *Ixodes* ticks because of organisms in the salivary glands of unfed and female adult ticks, and that immunocompromised mice can be infected with *A. phagocytophilum*. This study provides new information on the ecologic, biologic, and public health significance of *A. phagocytophilum* and emphasizes the threat of anaplasmosis in Japan.

Acknowledgments

We thank Hiroki Kawabata for useful help in the field work.

This work was supported in part by grant H15-Shinkou-14 for research on emerging and reemerging infectious diseases from the Japanese Ministry of Health, Labor and Welfare to N.O. and T.M., grant H14-H15 from the Shizuoka Research Institute to N.O., and grant H14 from the president of the University of Shizuoka to N.O.

Dr Ohashi is associate professor at the Institute for Environmental Sciences, University of Shizuoka, Japan. His primary research interests are molecular biology, ecology, and epidemiology of zoonotic parasites, particularly tickborne pathogens.

References

1. Dumler JS, Barbet AF, Bekker CP, Dasch GA, Palmer GH, Ray SC, et al. Reorganization of genera in the families *Rickettsiaceae* and *Anaplasmataceae* in the order Rickettsiales: unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with *Ehrlichia* and *Ehrlichia* with *Neorickettsia*, descriptions of six new species combinations and designation of *Ehrlichia equi* and 'HGE agent' as subjective synonyms of *Ehrlichia phagocytophila*. Int J Syst Evol Microbiol. 2001;51:2145–65.
2. Chen S-M, Dumler JS, Bakken JS, Walker DH. Identification of a granulocytotropic *Ehrlichia* species as the etiologic agent of human diseases. J Clin Microbiol. 1994;32:589–95.
3. Petrovec M, Furlan SL, Zupanc TA, Strle F, Brouqui P, Roux V, et al. Human disease in Europe caused by a granulocytic *Ehrlichia* species. J Clin Microbiol. 1997;35:1556–9.
4. Keysary A, Amram L, Keren G, Sthoeger Z, Potasman I, Jacob A, et al. Serologic evidence of human monocytic and granulocytic ehrlichiosis in Israel. Emerg Infect Dis. 1999;5:775–8.
5. Cao WC, Zhao QM, Zhang PH, Dumler JS, Zhang XT, Fang LQ, et al. Granulocytic Ehrlichiae in *Ixodes persulcatus* ticks from an area in China where Lyme disease is endemic. J Clin Microbiol. 2000;38:4208–10.

6. Heo EJ, Park JH, Koo JR, Park MS, Park MY, Dumler JS, et al. Serologic and molecular detection of *Ehrlichia chaffeensis* and *Anaplasma phagocytophila* (human granulocytic ehrlichiosis agent) in Korean patients. J Clin Microbiol. 2002;40:3082–5.
7. Alekseev AN, Dubinina HV, van de Pol I, Schouls LM. Identification of *Ehrlichia* spp. and *Borrelia burgdorferi* in *Ixodes* ticks in the Baltic regions of Russia. J Clin Microbiol. 2001;39:2237–42.
8. Bakken JS, Krueth J, Lund T, Malkovitch D, Asanovich K, Dumler JS. Exposure to deer blood may be a cause of human granulocytic ehrlichiosis. Clin Infect Dis. 1996;23:198.
9. Horowitz HW, Kilchevsky E, Haber S, Aguero-Rosenfeld M, Kranwinkel R, James EL, et al. Perinatal transmission of the agent of human granulocytic ehrlichiosis. N Engl J Med. 1998;339:375–8.
10. Masuzawa T. Okada Y, Yanagihara Y, Sato N. Antigenic properties of *Borrelia burgdorferi* isolated from *Ixodes ovatus* and *Ixodes persulcatus* in Hokkaido, Japan. J Clin Microbiol. 1991;29:1568–73.
11. Fournier PE, Fujita H, Takada N, Raoult D. Genetic identification of rickettsiae isolated from ticks in Japan. J Clin Microbiol. 2002;40:2176–81.
12. Inayoshi M, Naitou H, Kawamori F, Masuzawa T, Ohashi N. Characterization of *Ehrlichia* species from *Ixodes ovatus* ticks at the foot of Mt. Fuji, Japan. Microbiol Immunol. 2004;48:737-45.
13. Zhi N, Ohashi N, Rikihisa Y. Multiple *p44* genes encoding major outer membrane proteins are expressed in the human granulocytic ehrlichiosis agent. J Biol Chem. 1999;274:17828–36.
14. Lin Q, Zhi N, Ohashi N, Horowitz HW, Aguero-Roenfeld ME, Raffalli J, et al. Analysis of sequences and loci of *p44* homologs expressed by *Anaplasma phagocytophilum* in acutely infected patients. J Clin Microbiol. 2002;40:2981–8.
15. Casey AN, Birtles RJ, Radford AD, Bown KJ, French NP, Woldehiwet Z, et al. Groupings of highly similar major surface protein (*p44*)-encoding paralogues: a potential index of genetic diversity amongst isolates of *Anaplasma phagocytophilum*. Microbiology. 2004;150:727–34.

Address for correspondence: Norio Ohashi, Laboratory of Environmental Microbiology, Institute for Environmental Sciences, University of Shizuoka, 52-1 Yada, Suruga, Shizuoka 422-8526, Japan; fax: 81-54-264-5793; email: ohashi@u-shizuoka-ken.ac.jp