Anaplasma phagocytophilum–infected Ticks, Japan

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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. persulcatus, 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.

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 QIAaamp DNA mini kit (Qiagen Inc., Valencia, CA, USA). For detection of A. phagocytophilum DNA, a nested PCR using primers designed based on the highly conserved region of p44/msp2 paralogs (p3726 [5′-GCTAAG-GAGTTAGCTTATGA-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. UG, Utsukushigahara; TK, Takabachi; MZ, Mizugazuka; TN, Tennyosan; UM, Utsukushinomori.
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 States 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. phagocytophilum* are unique and distinct from those of

### 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                           | Male  |
| *I. persulcatus*        |              |                |                                   |       |
| Yamanashi, 2004        |              |                |                                   |       |
| TN                     | 0/2          | 0/4            | 0/6                               | 0/6   |
| UM                     | 2/16         | 2/17           | 4/33                              | 4/33  |
| Nagano, 2004           |              |                |                                   |       |
| UG                     | 0/3          | 0/0            | 0/3                               | 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)                          | 0/24  |
| MZ                     | 0/22 (2)     | 0/9 (1)        | 0/31 (3)                          | 0/31  |
| Total                  | 2/21         | 2/21           | 7/17                              | 11/14 |
| *I. ovatus*            |              |                |                                   |       |
| Yamanashi, 2004        |              |                |                                   |       |
| TN                     | 0/8          | 0/8            | 0/16                              | 0/16  |
| UM                     | 0/9          | 0/3            | 0/12                              | 0/12  |
| Nagano, 2004           |              |                |                                   |       |
| UG                     | 0/1          | 0/2            | 0/3                               | 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)                          | 1/48  |
| MZ                     | 0/26 (2)     | 0/21 (2)       | 0/47 (4)                          | 0/47  |
| Total                  | 0/18         | 0/13           | 9/33                              | 10/159|

*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.
A. phagocytophilum 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. phagocytophilum 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. phagocytophilum, 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.

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