Short Communication

Molecular Detection and Characterization of *p44/msp2* Multigene Family of *Anaplasma phagocytophilum* from *Haemaphysalis longicornis* in Mie Prefecture, Japan

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SUMMARY: *Anaplasma phagocytophilum* is an obligate intracellular bacterium that causes human granulocytic anaplasmosis (HGA), an emerging tick-borne infectious disease. This bacterium expresses various 44-kDa major outer membrane proteins encoded by the *p44/msp2* multigene family to avoid the host immune system. We previously detected *A. phagocytophilum p44/msp2* from the tick *Haemaphysalis longicornis* in Mie Prefecture, Japan in 2008. In this study, we further investigated a total of 483 *H. longicornis* ticks (220 adults and 263 nymphs) collected from the Mie Prefecture by PCR targeting *p44/msp2* to characterize the *p44/msp2* multigene family of *A. phagocytophilum*. Six of the 483 ticks tested were PCR-positive for *A. phagocytophilum p44/msp2*, and these positive individuals were at the nymph stage of the tick life cycle. Cloning, sequencing, and phylogenetic analyses of the amplicons revealed that the 11 *p44/msp2* clones obtained from the positive ticks shared a 54.9%–99.3% amino acid sequence similarity with the 27 previously identified clones from HGA patients in Japan. In particular, 6 *p44/msp2* clones displayed the highest similarities (97.2%–99.3%) with 3 previously identified clones (FJ417343, FJ417345, FJ417357). Thus, the data from this study provide important public health information regarding *A. phagocytophilum* infection transmitted by *H. longicornis* ticks, especially at the nymph stage.

Anaplasma phagocytophilum is an obligate intracellular bacterium that causes a tick-borne zoonosis termed human granulocytic anaplasmosis (HGA) (1). In nature, this bacterium is maintained through enzootic cycles between ticks and mammals. *A. phagocytophilum* expresses various 44-kDa major outer membrane proteins (P44) encoded by the *p44/msp2* multigene family consisting of 113-paralogous genes including pseudogenes (2). These paralogous genes have a central hypervariable region flanked by 5'- and 3'-conserved regions and are widely distributed in the genome. Some genes are recombined into a single expression site through gene conversion (3). Thus, *A. phagocytophilum* can express a variety of P44 proteins to avoid the host immune system (4). In Japan, HGA was first found and reported in 2013 (5). Additionally, *Haemaphysalis longicornis*, *H. megaspinosus*, *H. formosensis*, *Amblyomma*

testudinarium, *Ixodes persulcatus*, and *I. ovatus* ticks have been identified as potential vectors of HGA (6,7). However, the surveillance of *A. phagocytophilum*-infected ticks has been insufficient in Japan and the characterization of the *p44/msp2* multigene family of *A. phagocytophilum* remains unclear.

The aim of this study was to survey *A. phagocytophilum*-infected ticks, especially *H. longicornis* ticks, in Mie Prefecture, where one tick (nymph) infected with *A. phagocytophilum* was previously found (7). Furthermore, we attempted to characterize the *p44/msp2* multigene family of *A. phagocytophilum* in the infected ticks.

We collected unfed ticks by flagging 13 sites in forests or weedy areas in Shima Peninsula, Mie Prefecture, Japan, in June 2018 (Fig. 1). Tick species were morphologically identified, and 483 *H. longicornis* ticks (111 males, 109 females, and 263 nymphs) were selected for the detection of *A. phagocytophilum p44/msp2*. Total DNA was extracted from whole tissues of each tick using InstaGene Matrix kit (Bio-Rad Laboratories, Hercules, CA, USA). Nested PCR was performed with primer pairs (p3726 and p4257 for the first step, and p3761 and p4183 for the second step) based on the highly conserved regions of *p44/msp2* multigene family as described previously (6). The amplicons were gel-purified and cloned into the pCR2.1 vector using the TA Cloning kit (Thermo Fisher Scientific Inc., Waltham,

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Fig. 1. Location of tick collection sites in Shima Peninsula of Mie Prefecture, Japan. Circles and triangles show the sites where ticks were collected in 2018 and in 2008, respectively. Black-circles and -triangle indicate the collection sites of *H. longicornis* ticks that *A. phagocytophilum* *p44/msp2* were detected in 2018 and 2008, respectively.

MA, USA), and the recombinant plasmids were introduced into *Escherichia coli* DH5 α cells (Toyobo Biochemicals, Osaka, Japan). The recombinant *p44/msp2* clones were randomly selected and sequenced. The deduced amino acid sequences of *p44/msp2* clones were aligned and phylogenetically analyzed by MEGA 7 software.

Using nested PCR, we detected 400- to 450-bp amplicons of *p44/msp2* from 6 of the 483 *H. longicornis* ticks, representing a positive rate of 1.2%. These 6 positive ticks were all nymphs at the tick life stage. TA cloning of these amplicons yielded 11 *p44/msp2* clones from those positive ticks. Amino acid sequence analysis of the 11 clones revealed similarities of 36.6% to 100%. Nine of the 11 clones had different amino acid sequences, and the sequences of the remaining 2 clones were identical to those of the other 2 clones. The recombinant DNA experiment in this study was approved by the Committee of University of Shizuoka (no. 661-2303). A phylogenetic tree was constructed using the 9 different clones obtained in this study and 31 previously-identified *p44/msp2* clones (4 clones from one of 87 *H. longicornis* ticks in Mie Prefecture in 2008, and 27 clones from 2 HGA patients in Japan) (6,7) (Fig. 2). Except for clone Tick-Mie-HloN238-2 (accession no. MK120491), the presently-obtained clones were grouped into 4 clusters in the tree (Fig. 2). The 9 different clones displayed 42.3% to 100.0% similarities with the 31 previously-identified

clones. The 6 different clones in clusters 1, 2, and 3 were 97.2% to 99.3% similar to 3 clones—Kochi-h18-2 (FJ417343), Kochi-h18-4 (FJ417345), and Kochi-h18-27 (FJ417357)—obtained from a single HGA patient in Japan. The clone Tick-Mie-HloN86-2 (MK120483) obtained in this study was included in cluster 4 that consisted of 4 *p44/msp2* clones—Tick-165-1s-Mie-Hlo (JQ697944), Tick-165-4s-Mie-Hlo (JQ697945), Tick-165-7s-Mie-Hlo (JQ697946), and Tick-165-12s-Mie-Hlo (JQ697947)—from the *H. longicornis* tick from Mie Prefecture reported in 2008 (7). We further investigated the sequence similarities of a total of 207 clones including the currently- and previously-identified *p44/msp2* clones from ticks, with 27 clones obtained from 2 HGA patients in Japan (Table 1) (6–10). The *p44/msp2* clones from *H. longicornis*, *H. megaspinosus*, and *A. testudinarium* ticks displayed higher similarities with those from human origin (98.6%–100%), including the 4 clones with 99.3% similarity from *H. longicornis* ticks in this study.

P44 major outer membrane proteins encoded by the *p44/msp2* multigene family of *A. phagocytophilum* are immunodominant proteins recognized by the immune system of HGA patients (11). Barbet et al. suggested that the *p44/msp2* multigene family may be associated with *A. phagocytophilum* that causes infection in humans based on the comparison of P44 amino acid sequences of a human isolate (HZ strain) from the US with those detected from different origins worldwide,

Fig. 2. Phylogenetic tree of *A. phagocytophilum p44/msp2* clones from *H. longicornis* ticks and HGA patients in Japan. The tree was constructed based on the 112–130 amino acid sequences of 11 *p44/msp2* clones in this study (indicated by boldface) and 31 previously-identified clones including 4 clones from 1 *H. longicornis* ticks in Mie Prefecture in 2008 and 27 clones from 2 HGA patients in Japan, by maximum likelihood method with 1,000 bootstrap replications. Vertical bars with the numbers show clusters of *p44/msp2* clones that are closely-related to those from HGA patient (clusters 1–3) and a tick in 2008 (cluster 4). The scale bar indicates evolutionary distance.

Table 1. Comparison of *A. phagocytophilum p44/msp2* clones from ticks collected in different geographic locations with 27 clones from HGA patients in Japan

Tick species ¹⁾	Location	No. ticks tested	No. ticks positive for <i>p44/msp2</i> (%)	No. of <i>p44/msp2</i> clones compared	Maximum % similarity with HGA patients in Japan ²⁾	References
<i>H. formosensis</i>	Nagasaki (Goto islands)	37	16 (43.2)	21	78.5	8
<i>H. longicornis</i>	Okinawa (Yonaguni island)	72	2 (2.8)	13	100.0	7
	Nagasaki (Goto islands)	41	1 (2.4)	3	79.1	8
	Wakayama	43	1 (2.3)	5	67.3	7
	Mie (2008 survey)	87	1 (1.1)	4	55.6	7
	Mie (2018 survey)	483	6 (1.2)	11	99.3	This study
<i>H. megaspinosa</i>	Nagasaki (Goto islands)	2	1 (50.0)	3	100.0	7
	Wakayama	51	2 (3.9)	6	98.6	8
<i>A. testudinarium</i>	Kagoshima	12	3 (25.0)	12	100.0	7
<i>I. persulcatus</i>	Yamanashi	33	4 (12.1)	19	68.8	6, 9
	Shizuoka	17	7 (41.2)	23	69.7	6, 9
	Aomori	19	2 (10.5)	6	62.7	10
	Iwate	23	1 (4.3)	46	93.0	10
<i>I. ovatus</i>	Mie	5	1 (20.0)	5	50.7	7
	Shizuoka	33	9 (27.3)	18	70.6	6, 9
	Aomori	134	2 (1.5)	12	63.8	10

¹⁾ *H*, *Haemaphysalis*; *A*, *Amblyomma*; *I*, *Ixodes*.

²⁾ The similarity is based on amino acid sequences.

such as human patients, mammalian hosts, and ticks (12). The authors reported that the P44 sequences from the US-human HZ were similar to those from the 2 HGA patients in Japan, but the published sequences from ticks in Japan, based on the statistical analysis of the similarities, were significantly different from those of the US-human isolate (12). In China and Korea, *H. longicornis* ticks are suspected to be potential vectors for HGA (13, 14). In this study, the possibility of *H. longicornis* as a potential vector was analyzed by the comparison of P44 amino acid sequences from two HGA patients with all the 207 identified P44 sequences from ticks in Japan. The P44 sequences from *H. longicornis* ticks in Yonaguni island of Okinawa Prefecture and Mie Prefecture (7) had high similarities compared to those of human origin, suggesting that *H. longicornis* is also a potential vector of HGA in Japan. A large number of tick stages are probably larvae and nymphs, followed by adults in seasonal and local population. *A. phagocytophilum* is horizontally transmitted between ticks (vector) and wild mammals (reservoir), because vertical transmission is usually negative, indicating that most larvae are probably free of *A. phagocytophilum*. Therefore, the nymphs are the most potential vectors compared to the other tick life stages.

The current study provides important public health data regarding *A. phagocytophilum* infection transmitted by *H. longicornis* ticks, especially at the nymphal stage. Further studies on tick species, such as *H. megaspinosa* and *A. testudinarium* ticks, as potential vectors are warranted, as the P44 sequences detected from these 2 tick species are very similar to those from human origin.

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Conflict of interest None to declare.

REFERENCES

1. Rikihisa Y. Mechanisms of obligatory intracellular infection with *Anaplasma phagocytophilum*. Clin Microbiol Rev. 2011;24:469-89.
2. Dunning Hotopp JC, Lin M, Madupu R, et al. Comparative genomics of emerging ehrlichiosis agents. PLoS Genet. 2006;2:e213.
3. Lin Q, Zhang C, and Rikihisa Y. Analysis of involvement of the RecF pathway in *p44* recombination in *Anaplasma phagocytophilum* and in *Escherichia coli* by using a plasmid carrying the *p44* expression and *p44* donor loci. Infect Immun. 2006;74:2052-62.
4. Barbet AF, Meeus PF, Bélanger M, et al. Expression of multiple outer membrane protein sequence variants from a single genomic locus of *Anaplasma phagocytophilum*. Infect Immun. 2003;71:1706-18.
5. Ohashi N, Gaowa, Wuritu, et al. Human granulocytic anaplasmosis, Japan. Emerg Infect Dis. 2013;19:289-92.
6. Ohashi N, Inayoshi M, Kitamura K, et al. *Anaplasma phagocytophilum*-infected ticks, Japan. Emerg Infect Dis. 2005;11:1780-3.
7. Gaowa, Ohashi N, Aochi M, et al. Rickettsiae in ticks, Japan, 2007-2011. Emerg Infect Dis. 2013;19:338-40.
8. Gaowa, Wuritu, Wu DX, et al. Detection and characterization of *p44/msp2* transcript variants of *Anaplasma phagocytophilum* from naturally infected ticks and wild deer in Japan. Jpn J Infect Dis. 2012;65:79-83.
9. Wuritu, Gaowa, Kawamori F, et al. Characterization of *p44/msp2* multigene family of *Anaplasma phagocytophilum* from two different tick species, *Ixodes persulcatus* and *Ixodes ovatus*, in Japan. Jpn J Infect Dis. 2009;62:142-5.
10. Wuritu, Ozawa Y, Gaowa, et al. Structural analysis of a *p44/msp2* expression site of *Anaplasma phagocytophilum* in naturally infected ticks in Japan. J Med Microbiol. 2009;58:1638-44.
11. Zhi N, Ohashi N, Rikihisa Y, et al. Cloning and expression of the 44-kilodalton major outer membrane protein gene of the human granulocytic ehrlichiosis agent and application of the recombinant protein to serodiagnosis. J Clin Microbiol. 1998;36:1666-73.
12. Barbet AF, Al-Khedery B, Stuenkel S, et al. An emerging tick-borne disease of humans is caused by a subset of strains with conserved genome structure. Pathogens. 2013;2:544-55.
13. Jiang JF, Jiang BG, Yu JH, et al. *Anaplasma phagocytophilum* infection in ticks, China-Russia border. Emerg Infect Dis. 2011;17:932-4.
14. Lee SH, Park SY, Jang MJ, et al. Clinical isolation of *Anaplasma phagocytophilum* in South Korea. Am J Trop Med Hyg. 2017;97:1686-90.