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Short Communication

Detection and Characterization of *p44/msp2* Transcript Variants of *Anaplasma phagocytophilum* from Naturally Infected Ticks and Wild Deer in Japan

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SUMMARY: *Anaplasma phagocytophilum* is an obligate intracellular bacterium and causes a febrile illness in humans and livestock. In nature, this bacterium is sustained in a tick-mammal cycle. Several *p44/msp2*-related genes are expressed from a single expression locus by gene conversion. In this study, we obtained 119 cDNA sequences of *p44/msp2* transcripts from *A. phagocytophilum* in 6 *Haemaphysalis* ticks and 3 wild sika deer (*Cervus nippon*) in Japan. These 119 sequences were classified into 36 different variant sequences based on their similarities. The 36 cDNA sequences were phylogenetically grouped into 2 major clusters—tick- and deer-associated. The tick-associated sequences were further classified into 4 distinct subclusters, suggesting that *A. phagocytophilum* in ticks seems to selectively express specific *p44/msp2* transcripts, such as the transcripts in the 4 subclusters that were closely related to previously identified *p44/msp2* genes. The deer-associated sequences were also grouped into 4 subclusters, but these transcripts were probably more diverse than the transcripts derived from ticks. This might be due to the relatively nonselective expression of *p44/msp2* in deer or the strain differences in *A. phagocytophilum* from ticks and deer in separate geographic regions or both. Thus, this study may contribute to the understanding of *A. phagocytophilum p44/msp2* expression in nature in Japan.

Anaplasma phagocytophilum is an obligate intracellular bacterium and a causative agent of human granulocytic anaplasmosis (HGA) and also infects granulocytes in sheep, horses, dogs, cattle, and deer (1,2). In nature, the organism is sustained in a life cycle alternating between tick vectors and mammalian hosts. This bacterium possesses a *p44/msp2* multigene family encoding multiple 44-kDa major outer membrane proteins (3,4). The 113 *p44/msp2* paralogous genes, including pseudogenes, are distributed throughout the genome and contain a hypervariable region flanked by 5′- and 3′- end conserved regions. *A. phagocytophilum* generates antigenic variation by producing of a variety of P44 surface proteins due to a unidirectional gene-conversion mechanism in which the *p44/msp2* copies are recombined into a single expression site in the genome to avoid the immune defense system (5–7). Previous studies have analyzed the structure of the *p44/msp2* expression site in *A. phagocytophilum* strains obtained from infections in different animal species (8). Recently, we characterized the structure of *A. phagocytophilum p44/msp2* expression sites from naturally infected ticks in Japan (9), and identified the *p44/msp2* cassettes within the expression sites, which are probably transcribed. In this study, we successfully detected and characterized *p44/msp2* transcript variants of *A. phagocytophilum* from *Haemaphysalis* ticks and wild sika deer (*Cervus nippon*).

A total of 171 ticks (*Haemaphysalis megaspinosa*, 51; *H. flava*, 10; *H. formosensis*, 9; and *H. kitaokai*, 2 in Wakayama Prefecture; *H. formosensis*, 68; *H. longicornis*, 23; *Amblyomma testudinarium*, 5; and *H. yeni*, 3 in the Goto islands of Nagasaki Prefecture) were collected by flagging in 2009. Total RNA was extracted from the salivary gland of each tick using RNAspin Mini Kit (GE Healthcare Life Sciences, Uppsala,

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Sweden). Wild deer were obtained by hunting in Shizuoka Prefecture in 2010–2011, as described elsewhere. Total RNA was extracted from the spleens of the deer using TRIzol Reagent (Invitrogen, Carlsbad, Calif., USA). After RNA preparation, all the RNA samples were treated with 5 units of recombinant DNase I (Takara Bio, Inc., Otsu, Japan) for 20 min at 37°C and reverse transcribed to synthesize cDNA by using the ReverTra Ace-α-RT-PCR kit (Toyobo Biochemicals, Osaka, Japan). The reverse transcription (RT) reaction was performed for 20 min at 42°C according to the manufacturer's protocol. To characterize *p44/msp2* transcripts, cDNA prepared from the salivary glands of ticks and the spleens of deer were amplified by nested polymerase chain reaction (PCR) and first-step PCR alone, respectively. The primer pairs used for the *p44/msp2* amplification were p3726 and p4257 for the first-step PCR (approximately 450–500 bp) and p3761 and p4183 for the second-step PCR (approximately 400 bp), as described previously (10). To confirm the quality of RNA preparation and to eliminate DNA contamination in the RNA preparations, RT-PCR was performed with or without reverse transcriptase, which targets the tick 18S rRNA or sika deer mitochondria (mt)-16S rRNA, as well as the *p44/msp2* mRNA. The primers Tick18S-63f (forward: 5′-CGA AAC CGC GAA TGG CTCA) and Tick18S-567r (reverse: 5′-GGC TGC TGG CAC CAG ACT), which amplify a 500-bp fragment of tick 18S rRNA, and primers Sika-16Sf (forward: 5′-GGA TAC AAC CTT AAC TAG AG) and Sika-16Sr (reverse: 5′-GAG AAC AAG TGA TTA TGC TAC), which amplify a 490-bp fragment of sika deer mt-16S rRNA, were newly designed in this study. By using nested RT-PCR targeting the *A. phagocytophilum p44/msp2* mRNA, we successfully detected 400-bp amplicons from 6 *Haemaphysalis* ticks (*H. formosensis*, 3; *H. megaspinosa*, 2; and *H. longicornis*, 1) out of 171 ticks. Additionally, 450-bp amplicons of *p44/msp2* cDNA were detected from total RNA prepared from 3 fresh deer spleens by single RT-PCR (shown in Fig. 1 as representatives). All *p44/msp2* cDNA amplicons obtained were gel-purified and sequenced into the pCR2.1 vector using the TA Cloning Kit (Invitrogen), and the recombinant plasmids were introduced into *Escherichia coli* DH5α (Toyobo Biochemicals). The recombinant *p44/msp2* cDNA clones were randomly selected, and the inserted cDNAs of the respective clones were all sequenced. A total of 119 *p44/msp2* cDNA clone sequences (74 from ticks and 45 from deer) were obtained and aligned by Clustal X software. The recombinant cDNA clones with identical sequences were determined and grouped. Thus, we eventually found 36 different *p44/msp2* cDNA clone sequences with distinctive base sequences.

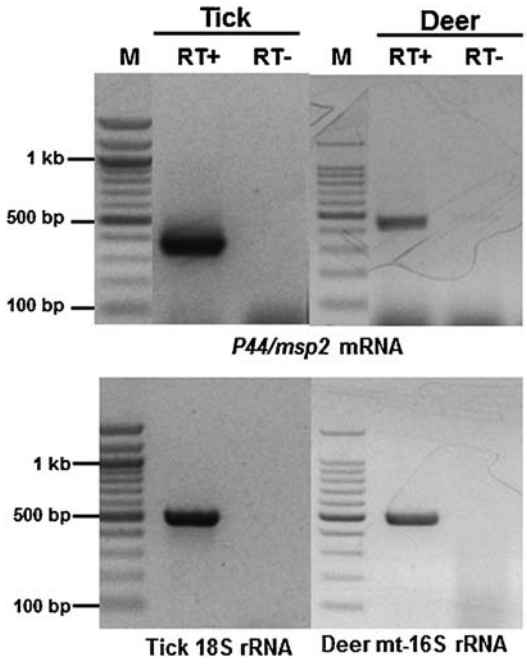

Fig. 1. RT-PCR detection of *p44/msp2* transcripts from *A. phagocytophilum* in tick salivary gland and deer spleen. RT-PCR targeting *p44/msp2*, tick 18S rRNA, and sika deer mitochondria (mt)-16S rRNA were performed with (RT+) or without (RT−) reverse transcriptase to detect *p44/msp2* transcripts, to confirm the qualities of RNA preparation, and to eliminate DNA contamination in the RNA preparation. Amplified product sizes are approximately 400 bp and 450 bp for *p44/msp2* mRNA from ticks and sika deer, respectively, and 500 bp and 490 bp for tick 18S rRNA and sika deer mt-16S rRNA, respectively. The figure shows one example of positive samples from ticks and deer. M, 100-bp size marker.

A phylogenetic tree was constructed by the neighbor-joining method with 1,000 bootstrap resamplings based on 335-bp to 398-bp *p44/msp2* cDNA clone sequences of *A. phagocytophilum* from ticks and sika deer in this study (Fig. 2). The *p44/msp2* cDNA sequences in the tree were clearly separated into 2 large clusters (A from ticks and B from sika deer in Fig. 2), and the similarities between these 2 clusters were relatively low (3.8–36.0%). Most of the cDNA sequences in cluster A (ticks) were further grouped into 4 distinctive subclusters (a1 to a4 in Fig. 2), and the similarities among those subclusters were 14.2–66.8%. The tick-associated cDNA sequences within each subcluster, except for a sequence of Tick35-12-mRNA-Goto-Hform in subcluster a1, were very similar or identical to each other (similarities: 99.5–100% in a1, 99.2–100% in a2, 99.4–100% in a3, and 99.5–100% in a4). Additionally, the tick-associated cDNA sequences were not obviously segregated into tick species as well as the individual ticks. In cluster B (sika deer), *p44/msp2* cDNA sequences could also be grouped into 4 subclusters (b1 to b4 in Fig. 2), and the similarities among the subclusters were 22.7–99.7%. Some of the deer-associated cDNA sequences were very similar or identical to each other, i.e., (i) 16 cDNA sequences within subcluster b2, except for a sequence of Sika29-4-mRNA-Fuji (similarities, 98.2–100%); (ii) 13 cDNA sequences, including Sika7-1-mRNA-Izu and Sika7-22-mRNA-Izu, within subcluster b3 (similarities, 99.7–100%); and (iii) 4 cDNA sequences, including Sika7-2-mRNA-Izu and Sika7-18-mRNA-Izu, within subcluster b3 (similarities, 99.4–100%). However, the deer-associated *p44/msp2* cDNA sequences were likely to be more diverse than the tick-associated cDNA sequences, as described above. In addition, those deer-associated cDNA sequences were not segregated into the 3 deer individuals.

By BLASTN search, the closest relatives for each of the 36 different *p44/msp2* cDNA sequences in this study were identified (Table 1). The tick-associated *p44/msp2*

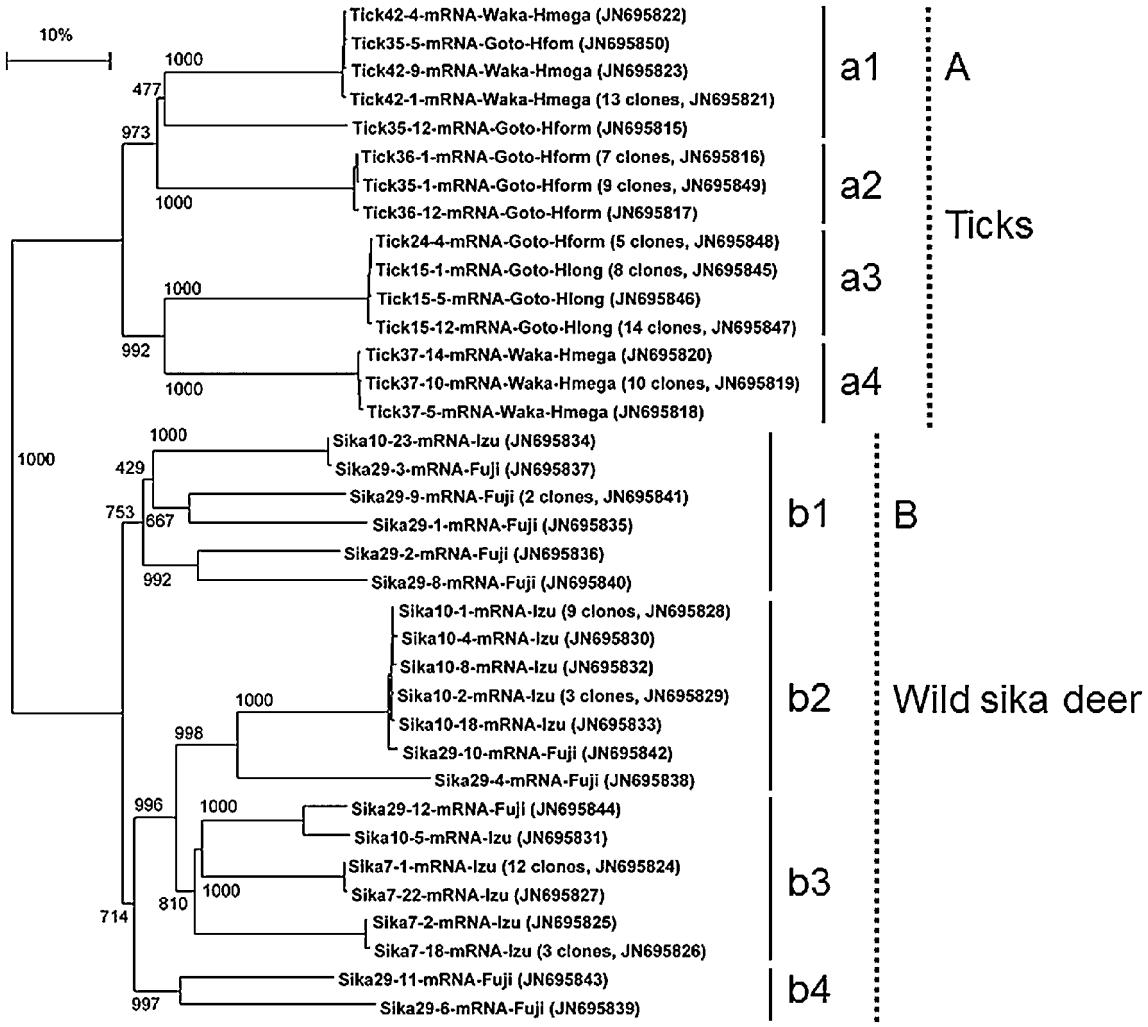

Fig. 2. Phylogenetic classification of *p44/msp2* cDNA clone sequences from *A. phagocytophilum* in tick salivary glands and wild sika deer. The tree was constructed based on 335-bp to 398-bp *p44/msp2* cDNA clone sequences by the neighbor-joining method with 1,000 bootstraps resamplings. The *p44/msp2* cDNA sequences of *A. phagocytophilum* were clearly separated into two clusters; A for ticks and B for wild sika deer. The cDNA sequence members in each cluster could be further grouped into 4 subclusters; a1 to a4 for cluster A (ticks) and b1 to b4 for cluster B (wild sika deer). The number of *p44/msp2* cDNA clones with identical sequences and/or accession numbers are shown in parentheses. A horizontal bar indicates 10% sequence divergence.

cDNA sequences in subclusters a2 and a3 were very similar or identical to Tick-162-10L-Iwate-ff-Ip (accession no. FJ600590) and Tick-162-12L-Iwate-ff-Ip (FJ600596), respectively (similarities, 98.3–100%) that were found in *p44/msp2* expression locus of *A. phagocytophilum* in *Ixodes persulcatus* ticks in our previous study (9). The cDNA sequences in subclusters a1 and a4 were also very similar or identical to *p44–35E/p44–62* and *p44–20* genes, respectively (similarities, 99.5–100%), which were located on the *A. phagocytophilum* genome from US human isolates HZ (CP000235). In deer-associated *p44/msp2* cDNA sequences, BLASTN search showed that, unlike tick-associated cDNA sequences, several subclusters from deer included multiple closest relatives of previously identified *p44/msp2* multigenes from Japanese sika deer, as described by Kawahara et al. (11), with much lower similarities (Table 1). Indeed, the *p44/msp2* cDNA sequences in subcluster b1 had the 3 closest relatives of SS14–3, SS14–7, and SS14–9, and the similarities were 68.4–73.0%. The cDNA sequences in subcluster b2 included the 2 closest relatives of SS14–1 and SS14–9, and the similarities were 74.2–91.5%. The closest relative for cDNA sequences in subcluster b3 was SS14–3 (similarities, 70.0–71.7%). The cDNA sequences in subcluster b4 had the 2 closest relatives of SS14–3 and SS14–5, and the similarities were 72.3–73.0%. Thus, the results suggest that *p44/msp2* transcript variants of *A. phagocytophilum* detected in this study seem to be newly identified *p44/msp2* repertories.

In Japan, *A. phagocytophilum* has been identified by PCR amplification in naturally infected ticks of *I. persulcatus, I. ovatus*, and *H. megaspinosa* (10,12–14), and in naturally infected mammals such as wild deer, wild boars, and cattle (11,13,15–17). In this study, we demonstrated for the first time that *H. longicornis* and *H. formosensis* ticks are naturally infected with *A. phagocytophilum*, suggesting additional potential vectors. Furthermore, we successfully characterized, for the first time, *p44/msp2* transcripts of *A. phagocytophilum* from salivary glands of naturally infected ticks and from spleens of wild sika deer in Japan. The

Table 1. Characterization of *p44/msp2* transcript variants from *A. phagocytophilum* in *Haemaphysalis* ticks and wild sika deer

p44/msp2 cDNA clone	Tick/deer species	Subcluster in Fig. 2	Closest relative	Similarity (%)/ accession no.
Tick15-1-mRNA-Goto-Hlong (8 clones)[1)]	*H. longicornis*	a3	Tick-162-12L-Iwate-ff-Ip	391/392 (99.7) FJ600596
Tick15-5-mRNA-Goto-Hlong	ibid	a3	Tick-162-12L-Iwate-ff-Ip	390/392 (99.5) FJ600596
Tick15-12-mRNA-Goto-Hlong (14 clones)	ibid	a3	Tick-162-12L-Iwate-ff-Ip	390/392 (99.5) FJ600596
Tick24-4-mRNA-Goto-Hform (5 clones)	*H. formosensis*	a3	Tick-162-12L-Iwate-ff-Ip	417/417 (100) FJ600596
Tick35-1-mRNA-Goto-Hform (9 clones)	ibid	a2	Tick-162-10L-Iwate-ff-Ip	394/395 (99.7) FJ600590
Tick35-5-mRNA-Goto-Hform	ibid	a1	*p44–35E*	379/380 (99.7) CP000235
Tick35-12-mRNA-Goto-Hform	ibid	a1	*p44–62*	392/392 (100) CP000235
Tick36-1-mRNA-Goto-Hform (7 clones)	ibid	a2	Tick-162-10L-Iwate-ff-Ip	395/395 (100) FJ600590
Tick36-12-mRNA-Goto-Hform	ibid	a2	Tick-162-10L-Iwate-ff-Ip	460/468 (98.3) FJ600590
Tick37-5-mRNA-Waka-Hmega	*H. megaspinosa*	a4	*p44–20*	373/374 (99.7) CP000235
Tick37-10-mRNA-Waka-Hmega (10 clones)	ibid	a4	*p44–20*	374/374 (100) CP000235
Tick37-14-mRNA-Waka-Hmega	ibid	a4	*p44–20*	373/374 (99.7) CP000235
Tick42-1-mRNA-Waka-Hmega (13 clones)	ibid	a1	*p44–35E*	378/380 (99.5) CP000235
Tick42-4-mRNA-Waka-Hmega	ibid	a1	*p44–35E*	379/380 (99.7) CP000235
Tick42-9-mRNA-Waka-Hmega	ibid	a1	*p44–35E*	379/380 (99.7) CP000235
Sika7-1-mRNA-Izu (12 clones)	*Cervus nippon*	b3	SS14–3	315/444 (70.9) DQ020151
Sika7-2-mRNA-Izu	ibid	b3	SS14–3	312/443 (70.5) DQ020150
Sika7-18-mRNA-Izu (3 clones)	ibid	b3	SS14–3	311/444 (70.0) DQ020151
Sika7-22-mRNA-Izu	ibid	b3	SS14–3	314/444 (70.7) DQ020151
Sika10-1-mRNA-Izu (9 clones)	ibid	b2	SS14–1	391/426 (91.8) DQ020151
Sika10-2-mRNA-Izu (3 clones)	ibid	b2	SS14–1	390/426 (91.5) DQ020144
Sika10-4-mRNA-Izu	ibid	b2	SS14–1	390/426 (91.5) DQ020151
Sika10-5-mRNA-Izu	ibid	b3	SS14–3	304/435 (69.9) DQ020146
Sika10-8-mRNA-Izu	ibid	b2	SS14–1	390/426 (91.5) DQ020144
Sika10-18-mRNA-Izu	ibid	b2	SS14–1	388/425 (91.3) DQ020144
Sika10-23-mRNA-Izu	ibid	b1	SS14–3	319/453 (70.5) DQ020144
Sika29-1-mRNA-Fuji	ibid	b1	SS14–7	290/424 (68.4) DQ020150
Sika29-2-mRNA-Fuji	ibid	b1	SS14–9	314/438 (71.6) DQ020151
Sika29-3-mRNA-Fuji	ibid	b1	SS14–3	339/465 (73.0) DQ020146
Sika29-4–mRNA-Fuji	ibid	b2	SS14–9	316/426 (74.2) DQ020152
Sika29-6-mRNA-Fuji	ibid	b4	SS14–3	357/489 (73.0) DQ020146
Sika29-8-mRNA-Fuji	ibid	b1	SS14–9	325/459 (70.9) DQ020146
Sika29-9-mRNA-Fuji (2 clones)	ibid	b1	SS14–3	342/483 (70.9) DQ020146
Sika29-10-mRNA-Fuji	ibid	b2	SS14–1	386/426 (90.5) DQ020144
Sika29-11-mRNA-Fuji	ibid	b4	SS14–5	297/411 (72.3) DQ020146
Sika29-12-mRNA-Fuji	ibid	b3	SS14–3	312/435 (71.7) DQ020146

[1)]: The number of identical cDNA clones obtained in this study are shown in parenthesis.

p44/msp2 transcripts obtained in this study were phylogenetically quite distinguishable between tick- and deer-associated ones. Only specific *p44/msp2* mRNA species such as the distinctive subclusters a1 to a4 shown in Fig. 2 seem to be transcribed selectively from the multiple *p44/msp2* repertories of *A. phagocytophilum* existing in the salivary glands of *Haemaphysalis* ticks, but such selective expression of the *p44/msp2* multigene family in wild sika deer does not appear to be strictly regulated. Furthermore, because mammalian host specificity of *A. phagocytophilum* and the *p44/msp2* sequence diversity has been reported (2), the differences between the sequences from ticks and deer might also be due to the strain variation of *A. phagocytophilum* in separate geographic regions in Japan. Thus, our study provides significant information for better understanding of *p44/msp2* expression of *A. phagocytophilum* in different life environments between naturally infected ticks and wild deer.

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Conflict of interest None to declare.

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