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Author(s)	Fukui, Yuichi; Ohkawa, Seigo; Inokuma, Hisashi
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## Detection of morulae in peripheral blood neutrophils from two dogs with *Anaplasma phagocytophilum* infection in Japan

Yuichi Fukui<sup>1,2)</sup>, Seigo Ohkawa<sup>3)</sup> and Hisashi Inokuma<sup>2,4,\*</sup>

<sup>1)</sup> Komachi Animal Hospital, Tsukuba, Ibaraki 305-0033, Japan

<sup>2)</sup> United Graduate School of Veterinary Science, Gifu University, Gifu, 501-1193, Japan

<sup>3)</sup> Ohkawa Animal Hospital, Moriya, Ibaraki, 302-0118, Japan

<sup>4)</sup> Department of Veterinary Clinical Science, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080-8555, Japan

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### Abstract

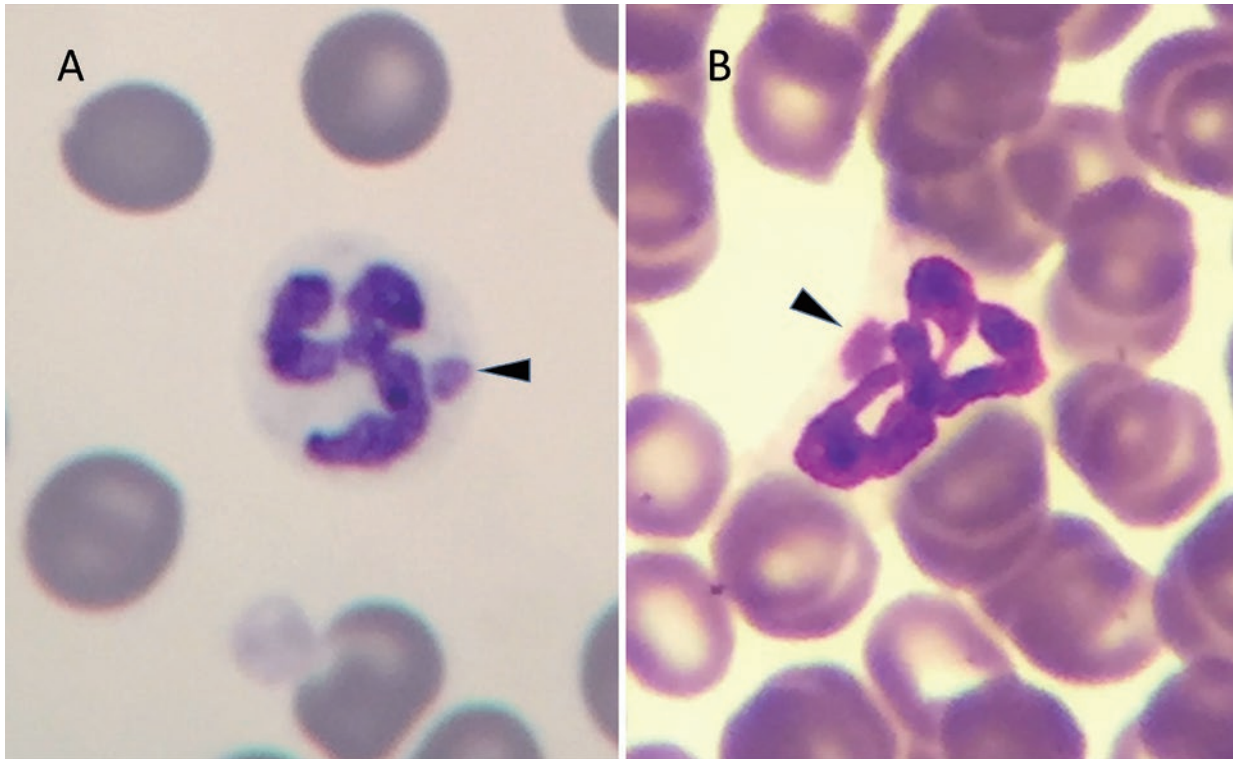
Morulae are cytoplasmic inclusions found in granulocytes of mammals infected by *Anaplasma phagocytophilum*. Two dogs presented with acute anorexia and lethargy in 2018, and both exhibited fever and thrombocytopenia as clinicopathological findings. Hematological examination of blood smears revealed cytoplasmic inclusions in neutrophils, which were confirmed as *A. phagocytophilum* infection with serological and molecular methods. Sequencing of partial 16S rRNA genes revealed highest homologies to the *A. phagocytophilum* sequence from a case of canine granulocytic anaplasmosis in Japan. This is the first report of morulae appearance with canine granulocytic anaplasmosis in Japan.

Key Words: *Anaplasma phagocytophilum*, dogs, morulae

*Anaplasma phagocytophilum* is a tick-borne zoonotic pathogen and an obligate bacterium that infects granulocytes, particularly neutrophils, in mammals<sup>3,12)</sup>. *A. phagocytophilum* proliferates in membrane-bound intracytoplasmic inclusions called morulae<sup>11)</sup>, which can be observed in neutrophils by hematological examination of Romanowsky-stained peripheral blood smears<sup>1,2)</sup>. Canine *A. phagocytophilum* infection is referred to as canine granulocytic anaplasmosis (CGA) and was first reported in the United States in 1982. Since then, it has been detected in Europe, Africa, and Asia<sup>2)</sup>. Common clinical signs of CGA are anorexia, lethargy, and fever, and the

most common clinicopathological abnormality is thrombocytopenia. Morulae have been detected in neutrophils from 36-100% of CGA cases reported to date. Recent studies have identified *A. phagocytophilum* in ticks, deer, cattle, and human patients in mainland and Hokkaido, Japan, using molecular methods<sup>7,10,14,15)</sup>. We previously reported the first case of CGA and a phylogenetic analysis of *A. phagocytophilum* in Ibaraki, Japan<sup>4)</sup>. However, blood smears were not analyzed at the microscopic level, and thus morulae could not be confirmed in the previous case. In the present report, we detected for the first time in Japan two CGA cases with morulae in neutrophils.

\*Hisashi Inokuma, DVM, PhD, Department of Clinical Veterinary Medicine, Obihiro University of Agriculture and Veterinary Medicine, Inada, Obihiro, Hokkaido 080-8555, Japan  
Phone: +81-155-49-5370. E-mail: inokuma@obihiro.ac.jp  
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**Fig. 1.** Detection of morulae of *Anaplasma phagocytophilum* in the cytoplasm of neutrophils in peripheral blood smear specimens ( $\times 1000$ ) from Case 1 (A) and Case 2 (B). Arrowheads indicate morulae-containing neutrophils. Diff-Quik staining (Sysmex Inc., Kobe, Japan).

Case 1, an 11-year-old male mixed-breed dog, presented to a local clinic in Tsukuba, Ibaraki, with anorexia and lethargy which continued for two days in May 2018. The routine vaccinations and heartworm and flea/tick chemoprophylaxis were up-to-date. The dog was slightly febrile (rectal temperature:  $39.3^{\circ}\text{C}$ ; normal range:  $37.7\text{--}39.2^{\circ}\text{C}$ ) on clinical assessment. Blood analysis indicated thrombocytopenia at  $102 \times 10^9 /\text{L}$  (normal range:  $200\text{--}500 \times 10^9 /\text{L}$ ), and examination of blood smears revealed pale basophilic intracytoplasmic inclusions in neutrophils resembling morulae of *A. phagocytophilum* at a rate of one to 20-50 (Fig. 1A). Antibacterial therapy with orbifloxacin (5 mg/kg, subcutaneously on Day 1 and orally from Day 2, SID) was initiated for two weeks. General health condition improved from Day 2 and no abnormalities were observed after the end of treatment (thrombocyte count:  $252 \times 10^9 /\text{L}$  on Day 29).

Case 2, a six-year-old male mixed-breed dog, presented to a local clinic in Moriya, Ibaraki, with anorexia and lethargy which continued for two days in May 2018. The routine vaccinations and heartworm chemoprophylaxis were up-to-date, however flea/tick chemoprophylaxis was not performed. The dog was febrile (rectal temperature:  $39.9^{\circ}\text{C}$ ) and had thrombocytopenia ( $100 \times 10^9 /\text{L}$ ). A peripheral blood sample was submitted to IDEXX Laboratories Inc. (Tokyo, Japan) for vector-borne pathogen-screening PCR to detect *Anaplasma*, *Babesia*, *Bartonella*, *Ehrlichia*, *Hepatozoon*, *Leishmania*, *Neorickettsia*, *Rickettsia*, and hemotropic mycoplasmas on Day 1, but the results were all negative. The dog was treated with subcutaneous injections of 1.5 mg/kg prednisolone and 5 mg/kg enrofloxacin on Day 1; oral prednisolone (1 mg/kg SID) and orbifloxacin (4 mg/kg SID) were administered from Day 2 to Day 8. The dog recovered temporarily, with an increase in thrombocyte count to  $235 \times 10^9 /\text{L}$ .

L on Day 5, but relapsed on Day 10, with a return of lethargy and fever (rectal temperature: 40.5°C). Blood examination revealed severe thrombocytopenia at  $29 \times 10^9/L$  and similar inclusions in neutrophils on Day 10 as was observed in Case 1 at a rate of one to 50-100 (Fig. 1B). Oral doxycycline (10 mg/kg SID) was initiated for four weeks from Day 10. The dog rapidly recovered again and thrombocyte count increased to  $240 \times 10^9/L$  on Day 18. No relapse was observed after the end of treatment.

Both dogs had been reared outdoors and had not traveled abroad. Moreover, there was no evidence of tick bites on Day 1. Blood smears of both dogs were stained with Diff-Quik staining (Sysmex Inc., Kobe, Japan) and Giemsa stain was not performed.

Peripheral blood samples from Case 1 on Day 1 and Day 29, and Case 2 on Day 1, Day 10, and Day 18, were preserved at -20°C until further analysis.

Serological examination for antibody detection was performed using an indirect fluorescent antibody (IFA) method with the *A. phagocytophilum* IFA Substrate Slide (Veterinary Medical Research & Development, Pullman, WA, USA). A 1:200 dilution of fluorescein isothiocyanate-labeled anti-canine IgG antibody (Rockland, Gilbertsville, PA, USA) was used as the second antibody. Sera were used at a 1:20 dilution and then titrated in serial 2-fold dilutions to determine end titers. IFA testing revealed that Case 1 had antibodies against *A. phagocytophilum*, with titers of 1:160 on Day 1 and 1:320 on Day 29. Case 2 also had antibodies against *A. phagocytophilum*, with titers of <1:20 on Day 1, 1:2560 on Day 10, and  $\geq 1:10240$  on Day 18. Serological examination for *Ehrlichia canis* and severe fever with thrombocytopenia syndrome virus (SFTSV), which symptoms resembled to CGA, were also investigated in both sera. IFA for *E. canis* was performed with *E. canis* IFA Substrate Slide (Veterinary Medical Research & Development, Pullman, WA, USA) and the same procedure as *A. phagocytophilum*. IFA testing

revealed that both cases did not have antibodies against *E. canis* with titers of <1:20. IgM and IgG ELISA were based on the SFTSV antigen obtained from culture cells infected with SFTSV as described by Gokuden *et al.*<sup>5)</sup>. The result of IgM and IgG ELISA against SFTSV revealed that both cases did not exceed the cut-off optical density value (0.5) enough to demonstrate infection of SFTSV.

Genomic DNA was extracted from whole blood using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA). A partial sequence of the 16S rRNA gene was amplified with fd1/EHR16SR primer pairs<sup>6)</sup>. PCR amplification, electrophoresis, purification, and direct sequencing were performed as previous described<sup>4,6)</sup>. Homology searches of PCR products were performed using BLAST (National Center for Biotechnology Information). A phylogenetic tree was constructed based on alignments of 16S rRNA sequences using the sequence analysis software MEGA7<sup>8)</sup>. The neighbor-joining method was used to construct a phylogenetic tree. The stability of the tree was estimated by bootstrap analysis of 1000 replications using the same program.

Positive PCR products were obtained from both cases on Day 1 and Case 2 on Day 10. Partial 16S rRNA gene sequences from both cases (744 bp) were 99.3% identical to that of *A. phagocytophilum* detected from a dog with CGA in Japan (LC334014) (Fig. 2). These sequences have been deposited in GenBank with accession numbers LC435049 and LC435050.

The dogs presented with fever, thrombocytopenia, and morulae in neutrophils, all of which are typical symptoms of *A. phagocytophilum*<sup>2)</sup>. This allowed us to consider the possibility of CGA before receiving results for definitive diagnoses. For small animal practitioners, this information would be beneficial for quick treatment of the disease. Morulae are found in neutrophils for only a short period of time in the acute phase of the disease and can easily go unrecognized<sup>9)</sup>. The detection of morulae alone is not sufficient for the definitive

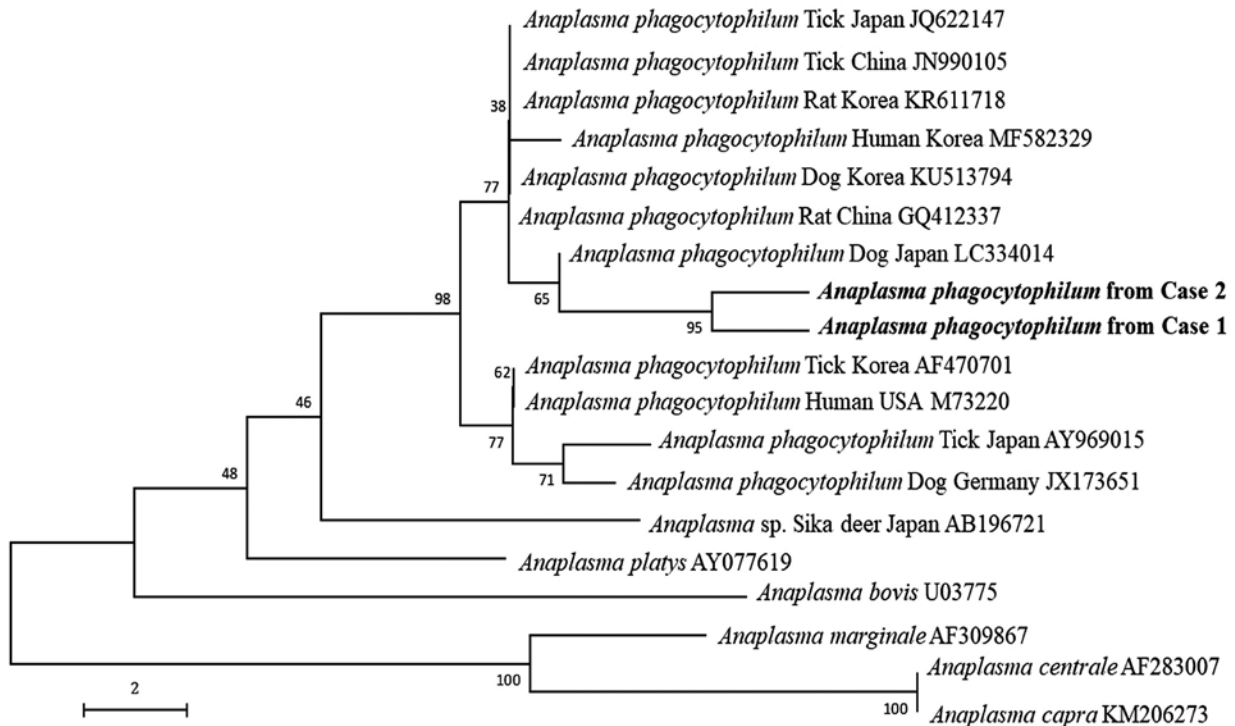


Fig. 2. Phylogenetic relationships of *Anaplasma phagocytophilum* from this study within the genus *Anaplasma* based on the 16S rRNA gene. The tree was analyzed using nucleotide sequences by the neighbor-joining method and was supported by 1000 bootstrap replications.

diagnosis of *A. phagocytophilum* infection because these morulae cannot be distinguished from those of *Ehrlichia ewingii*<sup>2)</sup>. Moreover, we could not demonstrate that these morulae were *A. phagocytophilum* by IFA. Since relying on the detection of morulae alone is insufficient, elevations in antibody titer to *A. phagocytophilum* and/or positive *A. phagocytophilum* specific-PCR results are additionally required to diagnose human *A. phagocytophilum* infection, which is referred to as human granulocytic anaplasmosis (HGA)<sup>1)</sup>. Acute and convalescent serologic testing with the IFA method for *A. phagocytophilum* IgG, and demonstrating a four-fold change or seroconversion, are generally required to confirm HGA<sup>1)</sup> and CGA<sup>2)</sup>. In the present study, both cases fitted criteria for conventional diagnosis of CGA and confirmed no evidences of Ehrlichiosis or SFTSV infection. Thus, these morulae were speculated to originate from *A. phagocytophilum*. Moreover, we confirmed seroconversion, elevation

of antibody titers, and negative PCR results during convalescent periods for both cases. In Case 1, the antibody titer on Day 1 was already elevated and this might indicate that the dog had experienced subclinical infection by *A. phagocytophilum* before Day 1 since most infected animals are subclinical and self-limited<sup>2)</sup>. In contrast, remarkable elevation of antibody titers from Day 1 to Day 10 was observed in Case 2, suggesting that the dog was infected by the pathogen for the first time and relapsed with severe fever and thrombocytopenia because of the defective treatment on Day 10.

Doxycycline is the drug of choice for treating CGA. While fluoroquinolones have been reported to be effective in vitro, one study reported that it led to relapse of the infection in HGA patients<sup>13)</sup>. Both dogs were first administered fluoroquinolone antibiotics (orbifloxacin or enrofloxacin) to treat *A. phagocytophilum* infection, but the outcomes differed. Case 1 was treated with single therapy

of fluoroquinolone and had the antibody for *A. phagocytophilum*, and this might result in rapid recovery. In contrast, Case 2 was treated with a combination of fluoroquinolone antibiotics and prednisolone, which is immunosuppressive, and this may have resulted in relapse of the disease, consistent with a report involving an immune-deficient mouse infection model<sup>13)</sup>. We considered that fluoroquinolones were not recommended for treating CGA as well as HGA.

*A. phagocytophilum* from the present two cases showed highest identity to the genotype detected from a CGA case in Ibaraki, Japan, where the present cases also lived<sup>4)</sup>. This previously reported genotype was closer in identity to genotypes isolated from rats, ticks, humans, and dogs in Korea and China than those isolated from cattle and ticks in Japan<sup>4)</sup> (Fig. 2). *A. phagocytophilum* is mainly transmitted by hard ticks<sup>2,3,11,12)</sup>, although there was no evidence of tick bites in both cases. Epidemiological studies of vectors in Ibaraki will be needed to address this potential threat.

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### Conflict of interest

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