Human Granulocytic Anaplasmosis as a Cause of Febrile Illness in Korea Since At Least 2006

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Abstract. Human granulocytic anaplasmosis (HGA) is a tick-borne rickettsial zoonosis with fever, thrombocytopenia, and leukopenia. HGA has been reported in Korea in 2013 but it is uncertain how long it has existed. A retrospective study was conducted on patients who underwent bone marrow examination due to fever and cytopenia, with no clear hematologic or microbiologic causes, from 2003 through 2012. Laboratory diagnosis was made by detecting 16S rRNA genes of *Anaplasma phagocytophilum* from the stored blood samples. Among the 70 patients, five (7.1%) HGA cases were found, and the earliest case dated back to 2006. Two cases met the diagnostic criteria of hemophagocytic lymphohistiocytosis (HLH) and were fatal. Although HGA has been prevalent in Korea since at least 2006, it is not always diagnosed and has posed a possible lethal health risk to the people in Korea. HGA should be considered as a cause of fever with cytopenia or HLH.

INTRODUCTION

Rickettsial diseases are zoonoses caused by gram-negative, obligate intracellular bacteria, belonging to the order Rickettsiales.^{1,2} The three disease entities of rickettsial diseases are rickettsioses, anaplasmoses, and ehrlichioses, based on the causative microorganisms. The major clinical manifestations of rickettsial diseases are fever, headache, and rash. Historically, many of these diseases were major causes of febrile illness.³ In the past 25 years, due to advances in 16S rRNA analysis and cell culture assays, many new rickettsial diseases have been revealed as causes of febrile illness.¹ Of these diseases, human granulocytic anaplasmosis (HGA) and human monocytic ehrlichiosis (HME) are the most prevalent in the United States and European countries.

HGA and HME are tick-borne infectious diseases, caused by an intracellular organism, *Anaplasma phagocytophilum* and *Ehrlichia chaffeensis*, respectively.^{4–6} Major clinical symptoms of HGA and HME are nonspecific, including fever, myalgia, headache, and malaise, and the major laboratory findings are thrombocytopenia, leukopenia, and elevated levels of serum hepatic enzymes.^{4,6–8} HGA and HME are transmitted by ixodid ticks and its reservoir are wild and domestic mammals.^{5,6,8} HGA and HME were first identified in the United States^{7,9} and subsequently in European countries.^{10,11} Since 1990, the number of reported cases has increased in the United States.^{6,12} However, in Asia, HGA cases have been reported in China and Japan in 2008 and 2013, respectively.^{13,14}

In South Korea, *A. phagocytophilum* and *E. chaffeensis* have been frequently detected in ticks including *Haemaphysalis longicornis*, *Ixodes nipponensis*, and *Ixodes persulcatus* and in wild animals such as striped field mice, weasels, and Korean water deer (KWD).^{15–19} However, in South Korea, only one suspected HME case was serologically diagnosed in a U.S. Forces Korea soldier in 2000,²⁰ and only one microbiologically confirmed HGA case with clinical description has been reported in 2014.²¹ Because there have been no laboratories capable of providing HGA or HME diagnostics until 2014 and Korean clini-

cians have not been aware of HGA or HME, undiagnosed cases must have existed previously in South Korea.

Many of the causes of febrile illness involve the bone marrow (BM).²² Especially in the patients with cytopenia, an examination of the BM is commonly performed to find or diagnose the cause.²³ Because leukopenia and thrombocytopenia are common laboratory findings of HGA/HME, missed cases would undergo BM examination.

This study was conducted to investigate whether HGA or HME existed in Korea in the past and what proportion of patients with febrile illness of unknown etiology had these infections.

METHODS

Patients and blood samples. A retrospective study was conducted on patients who presented at a tertiary medical center in Korea between 2003 and 2012 and underwent a BM examination due to fever. Patients were enrolled in this study if there had been no clear hematologic or microbiologic causes of fever and if their leftover blood samples had been stored. The blood samples were stored at –70°C until polymerase chain reaction (PCR) was performed.

Laboratory diagnosis of HGA and HME. Laboratory diagnosis of HGA or HME was made according to the criteria of the U.S. Centers for Disease Control and Prevention (CDC), as follows.²⁴ A confirmed case was defined as the detection of *A. phagocytophilum* or *E. chaffeensis* DNA by PCR amplification in a blood sample obtained during the acute phase of illness. A probable case was defined as the identification of bacterial inclusions (morulae) in neutrophils or monocytes in peripheral blood by microscopic examination.

PCR amplification, nucleotide sequencing, and phylogenetic analysis. DNA was extracted from the blood samples using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). A nested PCR to detect the 16S rRNA gene from *A. phagocytophilum* or *E. chaffeensis* was performed as described previously.²⁵ First-round PCR was performed to detect both *Anaplasma* spp. and *Ehrlichia* spp. using a primer set of AE1-F (5'-AAGC TTAACACATGCAAGTCGAA-3') and AE1-R (5'-AGTCACTGA CCCAACCTTAAATG-3'). Second-round PCR was performed to amplify a 926-bp *A. phagocytophilum* and a 390-bp *E. chaffeensis* fragment of the 16S rRNA gene using species-specific primer sets as follows: *A. phagocytophilum*-specific

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primerset, AP-F (5'-GTCGAACGGATTATTCTTTATAGCTTGC-3') and AP-R (5'-CCCTTCCGTTAAGAAGGATCTAATCTCC-3'), and *E. chaffeensis*-specific primer set, EC-F (5'-CAA TTGCTTATAACCTTTTGGTTATAAAT-3') and EC-R (5'-TATA GGTACCGTCATTATCTTCCCTAT-3'). For the samples positive for *A. phagocytophilum* 16S rRNA gene, non-nested PCR targeting *msp2* gene was also performed according to a previous study.²⁶

If the nested or non-nested PCR was positive, direct sequencing of the PCR product was performed. Automated sequencing was performed using an ABI PRISM 3730XL DNA analyzer (Applied Biosystems, Foster City, CA). Sequencing reactions of the PCR products were performed using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit V.3.1 (Applied Biosystems). A nucleotide Basic Local Alignment Search Tool search (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) was performed to confirm *A. phagocytophilum* or *E. chaffeensis*.

The obtained sequences were aligned using CLC Sequence Viewer version 7.5 (CLC bio, Aarhus, Denmark). Evolutionary distances were estimated using the Jukes–Cantor method, and phylogenetic trees were constructed using the neighbor-joining method. The robustness of the trees was tested using 1,000 bootstrap replications. Sequences used for phylogenetic trees are described in the Supplemental Tables 1 and 2.

Examination of peripheral blood and BM aspirate smear. Stored peripheral blood and BM aspirate smear slides were reviewed under a microscope. At least 1,000 polymorphonuclear granulocytes per smear were evaluated for rickettsial morulae by inspecting Wright–Giemsa stained peripheral blood.

Diagnosis of hemophagocytic lymphohistiocytosis. A patient was diagnosed as hemophagocytic lymphohistiocytosis (HLH) if at least five of the eight following criteria were met: 1) fever; 2) splenomegaly; 3) cytopenias (affecting two or more of the three lineages in the peripheral blood): hemoglobin < 9.0 g/dL, platelets < 100 × 10⁹/L, neutrophils < 10⁹/L; 4) hypertriglyceridemia and/or hypofibrinogenemia; 5) hemophagocytosis in the BM, spleen, or lymph nodes; 6) low or absent NK-cell activity; 7) ferritin ≥ 500 µg/L; and 8) elevated soluble CD25 (soluble interleukin 2 (IL-2) receptor alpha).²⁷

Serologic testing for *A. phagocytophilum* by immunofluorescence assay. For the samples positive for *A. phagocytophilum* 16S rRNA gene, both IgG and IgM antibodies were examined using *Anaplasma phagocytophilum* (HGA) IFA IgG and IgM Antibody Kits, respectively, according to the manufac-

turer's instructions (Fuller Laboratories, Fullerton, CA). Each immunofluorescence assay (IFA) result was read under a fluorescent microscope and interpreted independently by three researchers.

Ethics consideration. The study protocol was approved by the institutional review board (no. E-2013002) and was performed according to the Declaration of Helsinki.

RESULTS

Patient characteristics. In total, 70 patients met the study inclusion criteria. Of these, five patients (7.1%) were tested positive using the nested PCR for *A. phagocytophilum*-specific primers (Table 1). The partial 16S rRNA gene sequences of the amplicons were deposited in the GenBank (accession nos. KP306518–306522); the lengths of the sequences were 850–923 bp. Morulae were found in the peripheral blood smear of patient 2 (Figure 2). None tested positive for *E. chaffeensis* by PCR. Patient 2 also tested positive for *A. phagocytophilum* by non-nested PCR targeting *msp2* gene; the sequence of the *msp2* amplicon was deposited in the GenBank (accession no. KY113316). The IFA detected IgG in patients 1, 2, 4, and 5 and IgM in patients 1, 4, and 5 (Table 2).

The median age of the five HGA patients was 32 years (range: 0.5–57), and all were male (Table 1). All were living in the southeastern part of Korea, as all the study patients were from a single center located in the most southeastern province of the Korean Peninsula. The earliest case dated back to 2006. The patients visited the hospital in various seasons: three in the spring, one in the fall, and one in the winter.

All the patients showed fever with or without nonspecific symptoms including myalgia, fatigue, and headache (Table 2). Two of five cases (40%) were fatal. The findings of BM aspirates and biopsies were diverse, from normal to hemophagocytosis (Table 1). Two patients (2 and 5) met the diagnostic criteria of HLH and subsequently died due to the infection (Tables 1 and 2).

Only patient 1 received doxycycline. The patient received it for 4 days, but fever and generalized vesicular rashes developed. Because Stevens–Johnson syndrome was suspected due to doxycycline, the therapy was stopped.

Clinical manifestations of fatal cases. Patient 2. In April 2008, fever and chills developed in a 32-year-old male resident of Busan, Korea. Ten days before the fever developed,

TABLE 1
Clinical characteristics and BM findings of patients with human granulocytic anaplasmosis, South Korea, 2003–2012

Patient no.	Age (year)	Year and month of presentation	Outdoor activity or tick bite	Doxycycline treatment	Outcome	Findings of BM examination	Duration of fever before BM examination (days)	Duration of fever till defervescence or death (days)
1	26	December 2006	Yes	Yes	Survival	Panhyperplastic marrow with erythroid hyperplasia	12	17
2	32	April 2008	Yes	No	Death	Hemophagocytosis*	9	12
3	57	May 2008	N/A	No	Survival	Reactive plasmacytosis	7	2
4	0.5	November 2008	N/A	No	Survival	Normal BM	N/A	N/A
5	53	April 2012	Yes	No	Death	First: hypercellular marrow with erythroid hyperplasia and reticulin fibrosis Second: hemophagocytosis*	1	9

BM = bone marrow; N/A = not available.

*Diagnostic criteria for hemophagocytic lymphohistiocytosis.

TABLE 2

Clinical and laboratory findings of five patients with human granulocytic anaplasmosis, South Korea, 2003–2012

Parameters	Patient no.				
	1	2	3	4	5
Clinical findings					
Fever*	+	+	+	+	+
Fatigue	+	–	–	–	+
Myalgia	–	–	–	–	–
Arthralgia	–	–	–	–	+
Headache	–	–	–	–	+
Abdominal pain	–	+	–	–	–
Nausea or vomiting	–	+	–	–	+
Cough	+	–	–	+	+
Dyspnea	+	–	–	+	+
Bleeding tendency	–	+	–	–	–
Splenomegaly*	+	+	–	–	+
Laboratory findings					
Leukopenia*	–	+	–	+	+
Anemia†	+	+	+	+	+
Thrombocytopenia*	–	+	–	–	+
Elevated level of aminotransferase	+	+	+	–	+
Elevated level of lactate dehydrogenase	+	+	+	+	+
Elevated level of ferritin*	+	+	+	N/A	+
Hypertriglyceridemia*	–	–	N/A	N/A	+
Elevated soluble IL-2 receptor alpha*	–	+	–	–	+
IgG ≥ 1:80 by IFA	+	+	–	+	+
IgM ≥ 1:16 by IFA	+	–	–	+	+

+ = yes; – = no; IFA = immunofluorescence assay for *Anaplasma phagocytophilum*; IL = interleukin; N/A = not available.

*Diagnostic criteria for hemophagocytic lymphohistiocytosis.

he picked tomatoes in a farm, located in a rural area ~40 km from Busan. On day 4 of the illness, he visited our emergency medical center due to a persistent high fever and an upper abdominal pain. The patient had a body temperature (BT) of 38.9°C, blood pressure (BP) of 92/54 mmHg, and a heart rate (HR) of 118 beats/minute. Petechiae were evident on his whole body, gingival bleeding was observed, and hepatosplenomegaly was also noted. Laboratory tests showed leukopenia (white blood cell [WBC] count: $1.02 \times 10^9/L$) and thrombocytopenia (platelet count: $3 \times 10^9/L$). Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), and C-reactive protein (CRP) were elevated. The serum ferritin level was $> 1,000,000 \mu g/L$, and serum soluble IL-2 receptor alpha was elevated to 8,890 U/mL. The BM aspirate revealed more active hemophagocytic histiocytes. These findings met the diagnostic criteria of HLH, and he was administered etoposide, prednisolone, and intravenous immunoglobulin. However, he deteriorated rapidly and died 2 days after receiving chemotherapeutic agents.

Patient 5. In April 2012, general weakness and poor oral intake developed in a 53-year-old male who lived in Busan, Korea. He was an electrician at a tunnel construction site, located at a mountain ~60 km from Busan. He visited a local clinic and then was referred to our hospital due to pancytopenia, cervical lymphadenopathy, but without fever. BM specimen was obtained: hypercellular marrow with erythroid hyperplasia and reticulin fibrosis was noted. Three weeks later, he developed a fever, chills, fatigue, cough, sputum, and dyspnea and visited our emergency medical center. The patient had a BT of 38.0°C, BP of 100/60 mmHg, and an HR of 86 beats/minute. Laboratory tests showed pancytopenia (WBC count: $0.4 \times 10^9/L$, hemoglobin: 5.2 g/dL, platelet

count: $5 \times 10^9/L$). The serum levels of AST, ALT, LDH, creatinine, and CRP were elevated. Serum triglyceride, ferritin, and soluble IL-2 receptor alpha were elevated to 921 mg/dL, 77,965 $\mu g/L$, and 39,600 U/mL, respectively. The second BM biopsy obtained showed a markedly increased number of hemophagocytic histiocytes. These findings met the diagnostic criteria of HLH, and he was administered etoposide, prednisolone, and intravenous immunoglobulin. However, he deteriorated rapidly and died the next day.

Phylogenetic analysis. Phylogenetic trees comparing the five human *A. phagocytophilum* strains in this study with various strains from Korea and other countries are shown in Figure 1. In comparison with the Korean strains (Figure 1A), the five strains in this study were not identical, but closely related to each other and to the first human isolate in Korea (Korea/Gangwon/2013, KF805344)²¹; a group of strains, which included two of the three KWD strains that were relatively distant from the five strains of this study, were also noted (Figure 1A). In comparison with the strains from other countries, the five strains of this study were close to a human isolate from the United States and were relatively distant from the five strains from China, Japan, and Italy, which included two of the three deer isolates and a muntjac isolate (Figure 1B). The *A. phagocytophilum* strains from KWD, deer, and muntjac, which are similar mammals and included in the family Cervidae, showed a tendency to be grouped separately from human strains.

DISCUSSION

HGA is prevalent in the United States and European countries, but not in Asian countries. HGA cases have been reported in China,¹³ Japan,¹⁴ and Korea,²¹ but few retrospective cases have been reported in Asia. Among 70 febrile patients, five HGA cases were confirmed over a 10-year period at a hospital in Busan. All these patients fulfilled the U.S. CDC criteria for laboratory-confirmed HGA. Therefore, this study showed that HGA could have been prevalent in Korea since at least 2006.

Among the five HGA cases in this study, it is notable that patient 4 is an infant (Table 1). Theoretically speaking, any infants may get tick-borne disease like HGA when they, for example, have outdoor activities with their parents and are bitten by infected ticks. And indeed, infants can actually get HGA although rarely.^{12,28,29}

Cases of anaplasmosis generally occur all year round and peak in summer.¹² The HGA cases of this study occurred in April, May, November, and December (Table 1). Because this study was conducted on patients who underwent BM examination and whose samples were stored, only a small number of severe cases were enrolled, and the month distribution might differ from those of previous studies.

BM findings of HGA and HGA-associated HLH have not been well described. In this study, the BM findings of five confirmed cases varied from normal to hemophagocytosis (Table 2). According to the HLH criteria, two patients were diagnosed as HLH. There have been previous reports discussing the increase in erythrophagocytic activity in BM in HGA.³⁰ In one report, macrophage activation syndrome associated with HGA was described, but no patients underwent BM biopsy; the serum levels of cytokine, ferritin,

FIGURE 1. Phylogenetic analysis of *Anaplasma phagocytophilum* strains. Phylogenetic trees for partial 16S rRNA gene sequences of *A. phagocytophilum* obtained from five patients with human granulocytic anaplasmosis in South Korea and various *A. phagocytophilum* strains from (A) Korea and (B) other countries. #1–#5 are five strains from this study. Trees were constructed using the neighbor-joining method. Locations (country/province or city), hosts, and GenBank accession numbers are indicated. Branch lengths of trees show evolutionary distances. Scale bars indicate 1.0% sequence distance. KWD = Korean water deer.

and triglycerides were evaluated only for diagnosing macrophage activation syndrome.³¹

HLH is a severe and fatal clinical syndrome characterized by uncontrolled inflammatory responses, of which the fatality rate is 52.1–79.6%.^{32,33} HLH can occur as primary and secondary forms, and infection is a major cause (~49%) of secondary HLH.³⁴ The most common pathogen is Epstein–Barr virus, but zoonotic diseases including many rickettsial diseases and severe fever with thrombocytopenia syndrome, a tick-borne viral disease emerging in East Asia, are important causes of HLH.^{35,36} The etiologic agent in a secondary HLH case may be difficult to identify in a timely manner, because many diagnostic tests are not available in every hospital and need to be referred to reference laboratories.

Owing to the severity of HLH, early treatment is recommended when there is a high clinical suspicion but some

diagnostic tests are still pending.^{32,33,37–39} The therapeutic agents for HLH are combinations of cytotoxic agents and immunosuppressive drugs, including etoposide, cyclosporine A, and dexamethasone (HLH-2004 protocol).³⁹ Treatment of secondary HLH depends on its cause, and targeted antimicrobial therapy is the cornerstone.³³ In this study, the two patients who died of HGA-associated HLH did not receive doxycycline treatment but only cytotoxic and immunosuppressive agents.

In addition, among various rickettsial diseases, only scrub typhus has been well known, and patients have been routinely evaluated only for scrub typhus in Korea. Scrub typhus is a notifiable disease in Korea, and the annual number of cases has increased continuously, from 238 in 1994 to 10,365 in 2013.⁴⁰ Because HGA is not a notifiable disease and has been virtually unknown compared with scrub

FIGURE 2. Wright-Giemsa stain of a peripheral blood film from patient 2 showing an intracytoplasmic rickettsial morula in a neutrophil (original magnification $\times 1,000$).

typhus in Korea and diagnostic testing for HGA has not been available, HGA would have been missed or neglected and some cases of clinically diagnosed scrub typhus might well have been actually HGA cases. This suggests that infectious causes of HLH, especially HGA, should be considered and treatable infectious diseases should be ruled out before administration of aggressive cytotoxic and immunosuppressive therapies.

In conclusion, HGA has been prevalent in Korea since at least 2006 and cases of fatal HGA-associated HLH were identified in this study. HGA should be considered as a cause of febrile illness with cytopenia or HLH in Korea. Further prospective studies are required to evaluate the incidence of HGA as an etiology of febrile illness or HLH.

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