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Retrospective Surveillance for the Co-existence and Risk Factors of Tick-Borne Infections in South Korea

국내 주요 진드기 관련 질환의 병합감염 유병률 및 위험인자에 대한 후향적 조사

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The Department of Internal Medicine

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Retrospective Surveillance for the Co-existence and Risk Factors of Tick-Borne Infections in South Korea

By

Jeong-Han Kim

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Professor _____ Chairman

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ABSTRACT

Retrospective Surveillance for the Co-existence and Risk Factors of Tick-Borne Infections in South Korea

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Introduction: Scrub typhus, also called tsutsugamushi disease, is an endemic acute febrile infectious disease in South Korea. Scrub typhus, severe fever with thrombocytopenia syndrome (SFTS) and human granulocytic anaplasmosis (HGA) share a common point that they are transmitted by arthropod bites mostly during outdoor activities and there are considerable overlaps of epidemiologic and clinical features. I aimed to investigate the co-infection of scrub typhus with the recently emerging tick-borne infection, HGA and SFTS in South Korea in a retrospective cohort.

Methods: This study was conducted in subjects who were ≥18 years old and clinical or laboratory confirmed to have scrub typhus. The subjects were enrolled in a retrospective cohort in 2006. SFTS virus (SFTSV) infection was confirmed by a reverse transcriptase polymerase chain reaction (RT-PCR) to amplify partial L segment of SFTSV for molecular diagnosis. HGA was confirmed by a nested PCR to amplify 16S rRNA gene of *Anaplasma phagocytophilum*. Direct sequencing of the positive PCR products was performed to confirm these infections. Clinical features of co-infected subjects were described.

Results: Two-hundred seven patients with scrub typhus were finally included for the analysis. Scrub typhus was confirmed in 146 patients by PCR and in 20 patients

by serologic assays. The other 40 patients were clinical scrub typhus which was not definitely confirmed by laboratory assays. Among the subjects, 7 patients were positive for *A. phagocytophilum*. BLAST matching showed 98.2 - 99.8% of similarity with *A. phagocytophilum* 16S rRNA gene sequences deposited in GenBank. Five patients were confirmed to be co-infected with scrub typhus by PCR and 2 patients were confirmed by serologic assay. The suspected regions of infection were western areas in 5 patients and eastern areas in 2 patients. The co-infected patients had not different clinical manifestations with scrub typhus infection only. There was no mortality. All the study subjects were negative for SFTSV.

Conclusions: I confirmed the co-infections of HGA with scrub typhus in South Korea although the frequency was low. This finding suggests that HGA may be more prevalent than expected and more active diagnostic approach is needed in febrile patients in South Korea.

Keywords: scrub typhus, severe fever with thrombocytopenia syndrome, SFTSV, human granulocytic anaplasmosis, *Anaplasma phagocytophilum*, co-infection

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INTRODUCTION

Scrub typhus, also called tsutsugamushi disease, is an acute febrile infectious disease by *Orientia tsutsugamushi* which is transmitted through a bite of infected chigger mites. It is endemic in Asia-Pacific region and highly burdened in South Korea affecting more than 10,000 people every year. Characteristic eschar and maculopapular rash after outdoor activities in September to November provide critical clues of diagnosis to clinicians. The case-fatality rate of scrub typhus was median 6.0% in untreated and 1.4% in treated cases [1]. Diagnosis and treatment of highly burdened scrub typhus may be complicated by other tick and mite borne infections which share some epidemiologic risks and clinical features. Severe fever with thrombocytopenia syndrome (SFTS) and human granulocytic anaplasmosis (HGA) are recently emerging infections in South Korea [2-4].

SFTS is a highly fatal infection with case-fatality rate ranging from 7.3% to 32.6% [2, 5, 6], which was first reported in China in 2011 [7], and subsequently reported in South Korea [3] and Japan [8]. A recent report suggested that SFTS might have existed before 2008 in South Korea [9, 10]. The causative SFTS virus (SFTSV) is transmitted though tick-bites and *Haemaphysalis longicornis* is known as a main vector. SFTS occurred from April to December, and the peak incidence was shown from July to September in South Korea [2]. Specific treatment and effective vaccine are not available yet. Although SFTSV and *O. tsutsugamushi* are

not transmitted by same vectors, these two diseases share a common point that they are transmitted by arthropod bites mostly during outdoor activities. And there are considerable overlaps of epidemiologic and clinical features between two diseases. Considering the increasing incidence of SFTS, the differentiation of SFTS from scrub typhus is important during the overlap season and there was a recent study regarding this question [11]. However, in addition to the differential diagnosis between two diseases, co-infection of scrub typhus and SFTS may also be an important clinical consideration. A recent study reported that 23% of patients of clinically suspected scrub typhus were SFTS-positive and, among the 17 patients with SFTS-positive confirmed by PCR, 7 patients were seropositive to scrub typhus [12]. There was a case report of co-infection [13]. In other report, one of 21 patients with SFTS was serologically suggestive of scrub typhus [11]. But, it is uncertain whether co-infection of scrub typhus and SFTS were present in the past.

HGA, caused by obligate intracellular bacterium *Anaplasma phagocytophilum*, is an acute febrile, tick-borne infection transmitted by *Ixodes* species ticks including *I. scapularis*, *I. ricinus* and *I.persulcatus*, and usually occurs from late spring to autumn [14, 15]. HGA was first reported in the United States in 1990 [14] and subsequently reported in countries in Europe [16]. HGA is recognized as an important pathogen in US and many parts of Europe because HGA cases markedly increased since 1990 [17]. In Asian region, HGA was reported in China [18] and Japan [19]. First case of HGA in South Korea was

reported in 2013 [4]. However, serological or molecular evidence suggested that the infection might have existed before 2013 [20-22]. A recent study of retrospective sample test for detection of HGA suggested that the infection existed in South Korea at least since 2006 [23]. *A. phagocytophilum* has been detected in *Ixodes nipponensis*, *I. persulcatus* and *H. longicornis* in South Korea [24]. The epidemiologic features of HGA are similar to SFTS. When SFTS was first reported in China, HGA was suspected first because the clinical features of SFTS and HGA were similar [7]. As in the case of SFTS, HGA and scrub typhus share similar epidemiologic features. There is no data about the co-infection with HGA and scrub typhus. Before performing prospective evaluation for co-infection, retrospective evaluation for co-infection may be needed and useful.

In this study, I aimed to investigate the co-infections of scrub-typhus with the recently emerging tick-borne infections, HGA and SFTS, in South Korea in a retrospective cohort.

MATERIALS AND METHODS

Study subjects

This retrospective cohort study was conducted in subjects who were ≥18 years old and were clinical or laboratory confirmed to have scrub typhus. Clinical scrub typhus was defined as a case with typical eschar or maculopapular rash, fever and therapeutic response to treatment such as doxycycline or azithromycin without laboratory confirmation. Definitive laboratory diagnosis of scrub typhus was confirmed either by a serologic assay or by polymerase chain reaction (PCR). The serological diagnosis was established either through a four-fold or greater rise of immunoglobulin G titer in paired samples by indirect immunofluorescence antibody assay. PCR targeting variable domains I and II of 56-kDa antigen gene of *O. tsutsugamushi* was performed using a set of primers (forward: TTTCGA ACG TGT CTT TAA GC; reverse: ACA GAT GCA CTA TTA GGC AA; 1,151 bp). And confirmed diagnosis was done if the sequenced PCR products were to match reference genotypes as described in a previous study [25]. Serum samples were prospectively collected from eight hospitals distributed nationwide (Sanggye Paik Hospital, Ilsan Paik Hospital, Pusan Paik Hospital, Dongguk University Ilsan Hospital, Dankook University Hospital, Namwon Medical Center, Chonbuk National University Hospital, and Sunlin Hospital) in 2006. These cases were part of the previous published study [25].

Laboratory diagnosis of STFS

I performed a reverse transcriptase polymerase chain reaction (RT-PCR) to amplify the partial L segment of SFTSV for the molecular diagnosis as previously described [26]. Template RNA was extracted from the 140 μL serum samples of scrub typhus patients using QlAamp Viral RNA Mini kit (Qiagen Inc., Mainz, Germany) according to manufacturer's instructions. The extracted RNA was stored in the elution buffer at −70℃ until performing RT-PCR assay. The quality and quantity of RNA were evaluated using NanoDrop 2000 (Thermo Scientific™) with 2 μL aliquots of extracted RNA. I used GoTaq® 1-Step RT-qPCR System (Promega™) and partial L segments primer pairs for real-time RT-PCR were as follow: forward primer forward primer (5′-AGTCTAGGTCATCTGATCCGTTYAG-3′), reverse primer (5′-TGTAACTTCGCCCTTTGTCCAT-3′), probe (5′-CAATGACAGACGCCTTCCATGGTAATAGGG-3′). The real-time RT-PCR mixture contained 10 μL of 2x RT-PCR Buffer, 400 nM of 25X RT-PCR Enzyme Mix, 800 nM of primer mix, 100 nM of probe mix, 3.7 μL of nuclease-free water and 5 μL RNA template in a total volume of 20 μL. RT-PCR was performed on 7500 Real Time PCR System (Applied Biosystems) with an reverse transcription at 45℃ for 15 minutes, 1 cycle at 95℃ for 10 minutes for Taq polymerase activation, and 40 cycles consisted of denature step at 95℃ for 15 seconds and annealing-

extension step 60℃ for 60 seconds. The cut-off threshold cycle (Ct) value for a positive sample set at 35 cycles as previously suggested [26]. Genomic RNA sample from confirmed SFTS patients was used as positive control. If RT-PCR results were positive, direct sequencing were performed to confirm SFTSV using a BigDye Terminator Cycle Sequencing Kit (PerkinElmer Applied Biosystems, Warrington, United Kingdom).

Laboratory diagnosis of HGA

I performed the nested PCR to detect 926-bp of *A. phagocytophilum* 16S rRNA gene in serum as previously described [27, 28]. Template DNA was extracted from 200 μL serum samples of scrub typhus patients using the DNeasy Blood and Tissue kit (Qiagen, Melbourne, Australia) according to manufacturer's instruction. The extracted DNA was preserved at −70℃ before the nested PCR assay. The quality and quantity of DNA were evaluated using NanoDrop 2000 (Thermo Scientific^{TM}) with 2 μL aliquots of extracted DNA. PCR amplification was performed with the AccuPower® PCR Premix Kit (Bioneer, Daejeon, Korea).

First PCR was done using a set of outer primer pairs: EE1-F (5'-TCCTGGCTCAGAACGAACGCTGGCGGC-3'), EE2-R (5'-AGTCACTGACCCAACCTTAAATG-3'). The first PCR mixture consisted of template DNA 5 μL, forward primer 2 μL (10 pmole/μL), reverse primer 2 μL (10 pmole/μL) and dextrose water 11 μL in total volume of 20 μL. The PCR

amplification was performed on C1000 Touch™ Thermal Cycler (Bio-Rad) with an initial denaturation at 95℃ for 5 min, followed by 35 cycles consisting of denaturation 95℃ for 30 second, annealing at 60℃ for 1 min, and extension at 72℃ for 1 min. Nested PCR was performed using 2 μL of corresponding primary products as templates. Inner primer pairs for nested PCR were as follow: EE3-F (5'-GTCGAACGGATTATTCTTTATAGCTTGC-3'), EE4-R (5'-CCCTTCCGTTAAGAAGGATCTAATCTCC-3'). The PCR mixture consisted of primary product 2 μL, forward primer 1 μL (10 pmole/μL), reverse primer 1 μL (10 pmole/μL) and dextrose water 16 uL in total volume of 20 μL. The PCR amplification was performed with an initial denaturation at 95℃ for 5 min, followed by 35 cycles consisting of denaturation 95℃ for 30 second, annealing at 55℃ for 1 min, and extension at 72℃ for 1 min. The nested PCR products were electrophoresed on a 2.0% agarose gel, stained with ethidium bromide and visualized under UV light. The genomic DNA sample from confirmed HGA patient [4] was used as a positive control.

The Positive PCR products were sequenced by the ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). To confirm the HGA, I used a nucleotide Basic Local Alignment Search Tool (BLAST) search (http://blast.ncbi.nlm.nih/gov/Blast.cgi) to match reference genotypes of *A. phagocytophilum*. The sequences were aligned and analyzed using the CLUSTAL Omega (ver. 1.2.1) multiple sequence alignment programs and was corrected using BioEdit (ver. 7.2.5). Phylogenetic tree was constructed by neighbor-joining

methods in MEGA 6.0 and performed with 1,000 bootstrap replicates.

Clinical data collection

I collected the clinical information of the subjects via electronic medical record. Clinical information included demographic variables, history of arthropod bite, suspected region of exposure, season, duration of fever before the visit to hospital, presence of symptoms and signs, laboratory parameters at the initial presentation, clinical response to antibiotics and in-hospital mortality. I divided the possible geographical locations of infection into western and eastern areas of South Korea. Western area where mostly consist of plain rice field included Seoul metropolitan area, Gyeonggi, Chungbuk, Chungnam, Jeonbuk and Jeonnam provinces. Eastern area where mostly consist of hilly and mountainous area included Kangwon, Gyeongbuk and Gyeonnam provinces. I defined leukocytosis as white blood cell count > $10 \times 10^3/mm^3$, leukopenia as white blood cell count < $4 \times 10^3/mm^3$, anemia as hemoglobin < 11 g/dL, thrombocytopenia as platelet count < $150 \times 10^3/mm^3$ and severe thrombocytopenia as platelet count < $50 \times 10^3/mm^3$. The Acute Physiology and Chronic Health Evaluation (APACHE) II score were calculated within 24 hours of initial admission. Glasgow Coma Scale (GCS) were also calculated and I defined altered mentality as the GCS < 15.

Statistical analysis

The lower and upper limits of the 95% confidence interval for the prevalence of co-infection were calculated by Wilson procedure with a correction for continuity [29].

Ethics statement

This study was approved by the Institutional Review Board at Boramae Medical Center (30-2018-57). All personal identifiers were anonymized for confidentiality before data processing was performed. All clinical research was in compliance with the principles expressed in the Helsinki Declaration.

RESULTS

Patients

During the study period, a total of 207 patients were enrolled for analysis. Among these patients, laboratory diagnosis of scrub typhus was confirmed in 146 patients by PCR and in 21 patients by serologic assays. Remained 40 patients were classified as clinical scrub typhus which was not definitely confirmed by laboratory assays. The clinical and demographical characteristics of patients were shown in Table 1. The median time from the first onset of fever to sample collection was 6 days (range, 0-37). All patients were diagnosed in autumn season and most of them (85.0%) were from western area of South Korea. There was no mortality case and all patients had improved after appropriate treatment.

Table 1. Demographic and Clinical characteristics of study subjects (n=207)

Variable	Values
Age, years, median (range)	63 (18-91)
Sex, male, *n* (%)	89 (43.0)
Comorbidity	
Hypertension, *n* (%)	44 (21.3)
Diabetes mellitus, *n* (%)	25 (12.1)
CVA, *n* (%)	9 (4.3)
Asthma/COPD, *n* (%)	7 (3.4)
Congestive heart failure, *n* (%)	7 (3.4)
Chronic liver disease, *n* (%)	6 (2.9)
Solid tumor, *n* (%)	6 (2.9)
None, *n* (%)	128 (61.8)
Seasonal occurrence	
Spring - summer (Mar - August), *n* (%)	0 (0)
Autumn (September - December), *n* (%)	207 (100)
Geographical location	
Western area, *n* (%)	176 (85.0)
Eastern area, *n* (%)	31 (15.0)
Onset of illness to admission, median (range)	6 (0-36)
Mortality, in-hospital, *n* (%)	0 (0)
Symptoms	
Fever, *n* (%)	230 (100)

Chill, *n* (%)	197 (95.2)
Headache, *n* (%)	175 (84.5)
General weakness, *n* (%)	173 (83.6)
Myalgia, *n* (%)	172 (83.1)
Nausea/vomiting, *n* (%)	72 (34.8)
Arthralgia, *n* (%)	67 (32.4)
Lymphadenopathy, *n* (%)	60 (29.0)
Abdominal pain, *n* (%)	53 (25.6)
Altered mentality, *n* (%)	13 (6.3)
Skin presentation	
Eschar, *n* (%)	200 (96.6)
Skin rash, *n* (%)	185 (89.3)
White blood cell, /mm^3, mean ±SD (range)	6,813 ±3,928 (950-23,900)
Leukocytosis (> 10,000 /mm^3), *n* (%)	32 (15.5)
Leukopenia (< 4000 /mm^3), *n* (%)	47 (22.7)
Hemoglobin, g/dL, mean ±SD (range)	12.9 ±1.6 (8.0-18.8)
Anemia (< 11 g/dL), *n* (%)	17 (8.2)
Platelet, (10^3/mm^3), mean ±SD	132 ±55 (25-471)
Thrombocytopenia (< 150,000 /mm^3), *n* (%)	132 (63.8)
Severe thrombocytopenia (< 50,000 /mm^3), *n* (%)	7 (3.4)
C-reactive protein, mg/dL, mean ±SD (range)	7.2 ±5.5 (0.2-28.8)
AST, IU/L, mean ±SD (range)	108 ±107 (17-1,039)
AST > 40 IU/L, *n* (%)	172 (83.1)
ALT, IU/L, mean ±SD (range)	95 ±111 (12-868)

ALT > 40 IU/L, *n* (%)	150 (72.5)
Total bilirubin, mg/dL, mean ±SD (range)	0.86 ±0.99 (0.17-12.4)
LDH, mean ±SD (range)	665 ±238 (126-1861)
APACHE II score, median (range)	6 (0-22)
Mortality, in-hospital, *n* (%)	0 (0)

All values are Number. (%), the mean ± standard deviation or median (range).

ALT, alanine transaminase; AST, aspartate transaminase; APACHE II, Acute Physiology and Chronic Health Evaluation II; COPD, chronic obstructive pulmonary disease; CVA, cerebrovascular accident; LDH, lactate dehydrogenase; MAP, mean arterial pressure; SD standard deviation

Co-infection of scrub typhus and HGA

Among the 207 patients, 7 patients showed positive results of nested PCR for 16S rRNA gene of *A. phagocytophilum*. BLAST analysis revealed that 16S rRNA gene sequence from these patients showed high identity with that of *A. phagocytophilum* in GenBank. The sequences similarity ranged from 98.2% to 99.8%. The obtained sequences were deposited in GenBank under accession numbers MK271302 – MK271308.

The clinical characteristics of co-infected patients were shown in Table 2. Four patients were male, and the median age was 70 years (range: 38-76). The suspected regions of mite exposure were western areas in 5 patients (2 Gyeonggi, 2 Chungnam and 1 Jeonbuk) and eastern areas in 2 patients (2 Gyeongbuk). The longest time from first fever onset to sample collection was 12 days. All patients showed fever with other non-specific clinical manifestation such as chill, headache, myalgia and general weakness. All patients were identified to have maculopapular rash and 6 of 7 patients showed typical eschar. In laboratory tests, leukopenia and thrombocytopenia were seen in only one and two patient, respectively. Five patients were confirmed to be infected with scrub typhus by PCR and 2 patients were confirmed by the serologic assay. There was no mortality case, although 3 patients were received azithromycin. The calculated prevalence of co-infection was from 1.5 to 7.1% (95% confidence interval).

Phylogenetic tree analysis using the 16s RNA gene sequence is shown in

Figure 1. The seven strains in this study were closely clustered with each other and constituted a clustered group with other human isolates in South Korea. In comparison with the strains from other countries, the study sequences were close to a human isolate from the United States and clustered with previously published sequences except some strains of China and Japan.

Table 2. Clinical findings of 7 Patients with HGA co-infection

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7
Age, year	38	76	42	75	66	70	76
Sex	Female	Female	Male	Male	Female	Male	Male
Region	Western	Western	Western	Western	Eastern	Eastern	Western
Time from first fever onset to sample collection, days	5	3	10	12	3	11	9
Comorbidities	None	None	None	None	None	COPD	Solid tumor
Clinical symptoms and signs							
Fever	+	+	+	+	+	+	+
Chill	+	+	+	+	+	+	+
Headache	+	+	+	+	+	+	+
Myalgia	+	+	+	+	+	+	+
General weakness	+	+	+	+	+	+	+
Joint pain	-	-	-	-	+	+	+
Nausea/Vomiting	-	-	-	-	-	-	-
Abdominal pain	+	-	-	-	+	+	+

Rash	+	+	+	+	+	+	+
Eschar	+	+	+	-	+	+	+
Local lymphadenopathy	+	+	-	-	+	+	-
Altered mentality	-	-	-	+	-	-	-
Laboratory findings							
WBC (/mm^3)	7,640	3,070	6,860	12,580	5,000	2,3600	7,000
Platelet (1,000/mm^3)	198	170	160	111	85	299	171
Hemoglobin (g/dL)	9.8	14.0	13.9	12.4	13.1	14.2	15.1
AST (IU/L)	39	81	84	89	165	51	111
ALT (IU/L)	40	57	53	62	112	52	83
CRP (mg/dL)	9.91	5.39	2.36	18.9	7.11	23.23	NA
LDH (IU/L)	407	575	731	634	1059	775	572
O.tsutsugamushi IgG titer							
Initial	0	0	320	160	0	0	40
Follow-up	0[a]	5120	5120	1280	0[b]	0[c]	320
PCR for *O. tsutsugamushi*	+	+	-	-	+	+	+
PCR for *A. phagocytophilum*	+	+	+	+	+	+	+

Treatment	AZT	DC	DC	AZT	DC	AZT	DC
Outcome	Survived	Survived	Survived	Survived	Survived	Survived	Survived

ALT, alanine transaminase; AST, aspartate transaminase; AZT, azithromycin; COPD, chronic obstructive lung disease; CRP, C-reactive protein; DC, doxycycline; LDH, lactate dehydrogenase; NA, not available; PCR, polymerase chain reaction; WBC, white blood cell

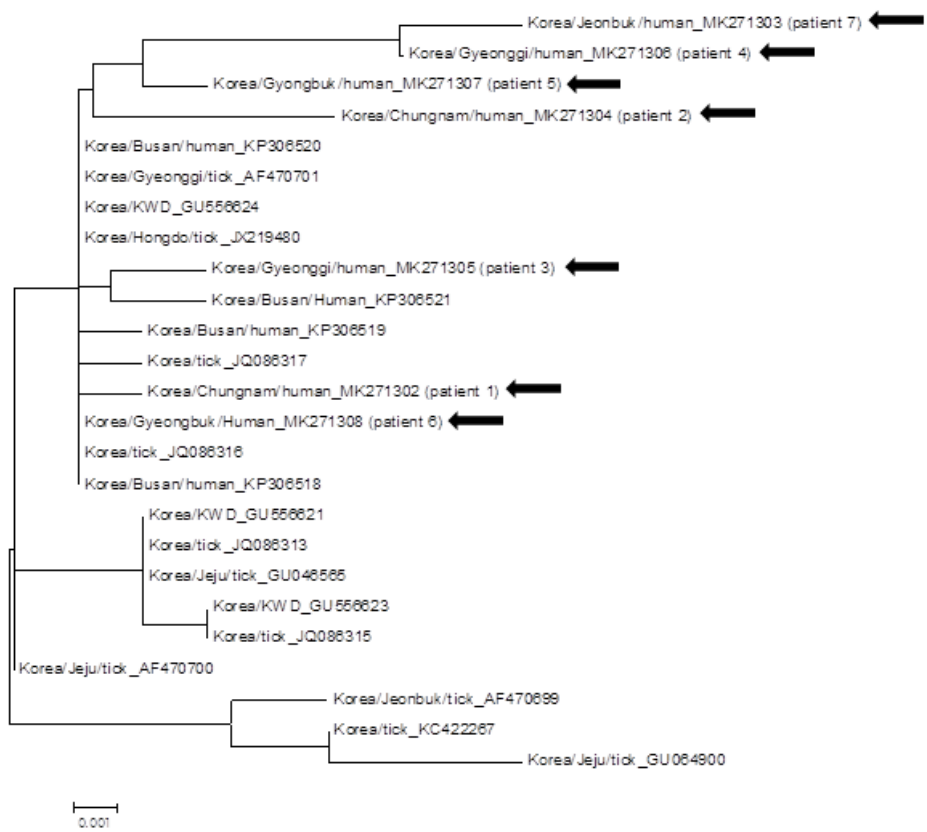
[a]Serologic assay of paired sample was done 22 days after from initial assay.

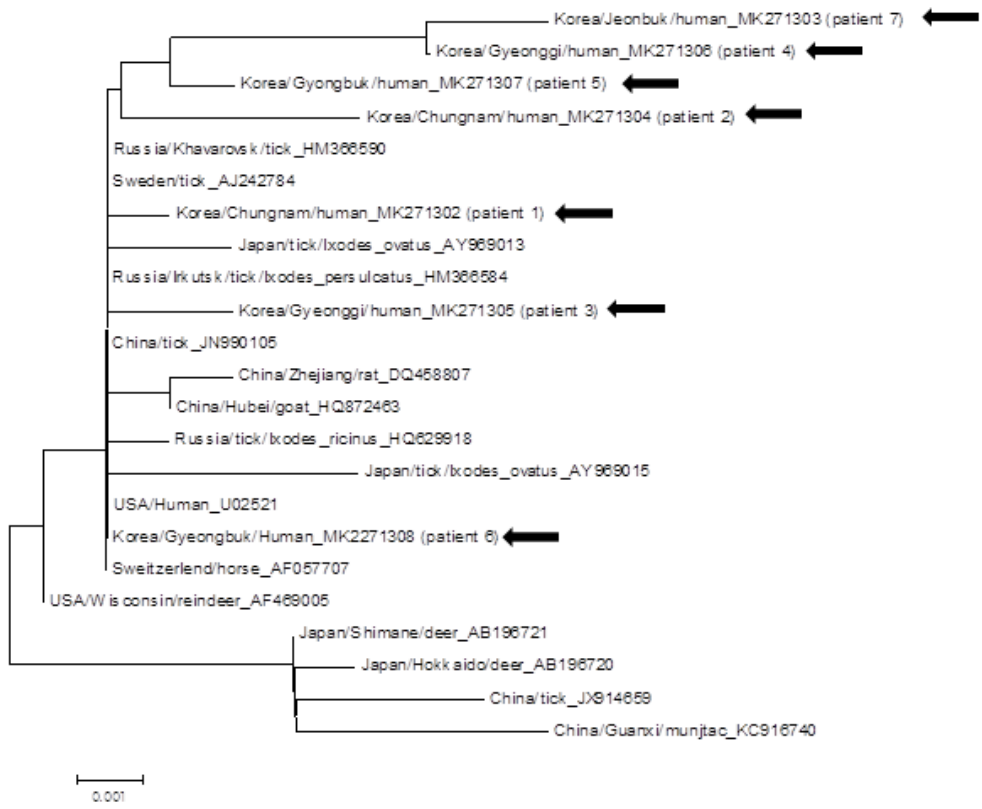
[b, c]Serologic assay of paired sample was done 15 days after from initial assay.

Figure 1 Phylogenetic tree of *Anaplasma phagocytophilum* 16s RNA gene

The tree was constructed using neighbor-joining methods based on the 16S rRNA gene sequences of *Anaplasma phagocytophilum* from this study and other strains from South Korea (upper) and from other countries (below). The sequences obtained in this study are marked with arrows. The scale bar represents the evolutionary distance.

KWD, Korean water deer

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Korea/Gyeonggi/human_MK271306 (patient 4)
Korea/Gyongbuk/human_MK271307 (patient 5)
Korea/Chungnam/human_MK271304 (patient 2)
Russia/Khavarovsk/tick_HM366590
Sweden/tick_AJ242784
Korea/Chungnam/human_MK271302 (patient 1)
Japan/tick/Ixodes_ovatus_AY969013
Russia/Irkutsk/tick/Ixodes_persulcatus_HM366584
Korea/Gyeonggi/human_MK271305 (patient 3)
China/tick_JN990105
China/Zhejiang/rat_DQ458807
China/Hubei/goat_HQ872463
Russia/tick/Ixodes_ricinus_HQ629918
Japan/tick/Ixodes_ovatus_AY969015
USA/Human_U02521
Korea/Gyeongbuk/Human_MK2271308 (patient 6)
Sweitzerlend/horse_AF057707
USA/Wisconsin/reindeer_AF469005
Japan/Shimane/deer_AB196721
Japan/Hokkaido/deer_AB196720
China/tick_JX914659
China/Guarxi/munjtac_KC916740
0.001

Co-infection of scrub typhus and SFTS

All patients in this study showed negative results of RT-PCR for SFTS. The calculated prevalence of co-infection was from 0 to 2.3% (95% confidence interval).

DISCUSSION

This is a first report regarding the co-infection of scrub typhus with HGA. A previous study published in 2002 suggested that co-infection might exist because six seropositive samples for *A. phagocytophilum* were also seropositive for *O. tsutsugamushi* [20]. However, because of the possibility of cross-reactivity of serological test, this study could not confirm the co-infection. Different from this study, I could confirm co-infection of scrub typhus with HGA using PCR method.

Theoretically, co-infection may occur through bites of multiple mites and ticks carrying each different pathogen or the bite of one vector carrying multiple pathogens. Co-infection of HGA with other tick-borne diseases mediated by same vectors has been well documented [30]. For example, *A. phagocytophilum* shares the same vector with *Borrelia burgdorferi* and co-infections of both pathogens have been identified where HGA and Lyme disease is endemic [31-33]. But the chigger mites were not known to transmit HGA. Multiple exposures to different vectors in a short time interval may be a more reasonable explanation. The HGA is mainly transmitted by *Ixodes* species which is distributed in nationwide of South Korea except Gyeongnam province [34]. The causative agent of scrub typhus is transmitted by chigger mites which is distributed in nationwide and more densely distributed in western and southwestern area of South Korea [35]. Although they do not share same vectors, co-infection may be possible in areas where mite

chiggers and ticks live at the same place and when the patient was exposed simultaneously to mite and tick through long outdoor activities. There are many evidences that wild rodents such as *Apodemus agrarius* are the reservoir of *A. phagocytophilum* in United States and South Korea [28, 36]. The enzootic cycle including *Ixodes* ticks and rodents do critical role to maintain *A. phagocytophilum* prevalence [37]. The mites, as main vector for *O. tsutsugamushi*, reside in relatively humid and grassland environment, consistent with where rodent, the main host of chigger mite, live [38]. This enzootic commonality supports the possibility of a co-infection.

Although it was a low frequency, this study finding showed that HGA was not uncommon and suggests HGA is rather prevalent without easy point-of-diagnosis. Compared to scrub typhus, HGA might have been missed or neglected during clinical practices in South Korea. HGA is often not considered as a differential diagnosis and the diagnostic assay for HGA has not been routinely available in most Korean hospitals. A recent surveillance study by Korea Centers for Disease Control and Prevention reported that the sera of 598 clinically suspected HGA patients collected in 2017 were positive in (58/598, 9.3%) for IgM or IgG IFA and in (24/298, 8.1%) for nested PCR [39]. Both this finding and current study suggest that HGA may be more prevalent than previously known and more active diagnostic approach is needed in febrile patients in South Korea.

It has been known that many cases of HGA are asymptomatic to mild illness [40]. Doxycycline is the primary treatment for scrub typhus and HGA and response to treatment is favorable. Azithromycin has not been proven to have a therapeutic effect on HGA [41], in contrast to scrub typhus [42]. Considering the clinical outcome of all patients was good even though 3 patients were received azithromycin treatment, it seems that co-infection dose not increase the clinical severity. Still, caution may be needed when azithromycin is prescribed as an initial treatment for scrub typhus in endemic area of HGA in the immunocompromised patients whose HGA infection may be more severe and even fatal.

Most patients in this study showed normal leukocyte and platelet counts, although most HGA patients were known to show transient leukopenia or thrombocytopenia. Generally, transient laboratory abnormality of HGA patients may return to normal after 7 days of disease onset [40]. Significant leukopenia and thrombocytopenia may not have been seen because the sample was taken after considerable period time of the onset of fever. Another possible explanation is that HGA were subclinical and asymptomatic and clinical features of scrub typhus dominated over those of HGA. All patients showed skin rash, which is different from the general clinical manifestation of HGA [43]. Conversely, considering skin rash is a rare clinical manifestations in patients with HGA, the presence of a rash in HGA may indicate that the patients has a co-infection with other ticks and mites borne diseases.

Regarding the co-infection of scrub typhus with SFTS, I did not observe any co-infection case including clinical scrub typhus in contrast to previous study finding [12] . Considering the ecologic perspective of vectors for both diseases, the co-infection is not much likely than in case of HGA. In the contrast to chigger mites, *H. longicornis,* predominant vector for SFTSV, reside mainly in shrubs and bigger mammals such as sheep, rather than rodents, are main host [44]. There had been controversy whether *Ixodes* species transmit SFTSV [45]. This difference of ecological features between these vectors lowers the likelihood of co-infection. However, caution must be taken in interpretation of current study results. *H. longicornis* is more prevalent in southeastern area than western area of South Korea. In agreement with this, the incidence of SFTS was high in eastern and southeastern mountainous area of South Korea, while the incidence of SFTS was low in western area in correlation with the low prevalence rate of tick [46]. A previous study suggesting co-infection was conducted in the southeastern region of South Korea [12]. But this study subjects were mainly in the western and southwestern region of South Korea. Due to the regional disparity of study patients, the co-infection case may not be observed in this study. Co-infection may be possible if the patient is exposed at the site where chigger mite and *H. longicornis* live simultaneously. Also, this study samples were collected mainly in 2006. Although there was evidence that HGA had been present at least since 2006 in South Korea [23], it is uncertain whether there was an epidemic of SFTS in 2006.

This study has a few limitations. First, this study is only for the past situation, therefore, I could not be sure that the situation of co-infection is still observable at current point. For evaluation of the current situation and risk factor of co-infection, prospective surveillance study is needed. Second, I could not check the change of antibody titer for HGA because I could not obtained paired samples from study patients. There may be serologically diagnosed HGA patients who could not be confirmed by PCR. However, cross-reactivity is a major concern for the specificity of serologic test, this study results would be valuable because PCR detects only the presence of a specific pathogen.

Conclusion

I confirmed the co-infections of HGA with scrub typhus in South Korea although the frequency was low. This finding suggests that HGA may be more prevalent than expected and more active diagnostic approach is needed in febrile patients in South Korea.

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국문 초록

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서론: 쯔쯔가무시증은 초원열이라고도 부르며, 국내에서 가을철에 주로 유행하는 급성 발열성 감염병이다. 쯔쯔가무시증과 중증열성혈소판감소증후군과 인간 과립구 아나플라마 증후군은 야외활동과 관련이 있고 매개곤충에 의해서 전파가 된다는 공통점을 가지고 있고, 역학적인 특징과 임상적 특징에서 상당히 유사한 점이 많다. 이 질환 군들 사이의 병합감염이 과거에 존재 하였는지에 대해, 본 연구를 통해 살펴보았다.

방법: 본 연구는 2006년에 실험실적 방법으로 임상적으로 혹은 실험실적으로 쯔쯔가무시증이 진단된 18세 이상의 성인을 대상으로 진행하였다. 중즐열성혈소판감소증후군 진단을 위해 SFTS 바이러스의 L 분절의 특정부위를 증폭하는 역전사 중합효소연쇄반응을 이용하였다. 인간 과립구 아나플라마 증후군 진단을 위해 아나플라즈마의 특정 유전자를 검출하는 이중 중합효소연쇄반응을 이용하였다. 중합효소연쇄반응에서 양성이 확인될 경우 확진을 위해서 직접순서결정법 및 BLAST 분석을 시행하였다.

결과: 207명의 쯔쯔가무시증 환자가 본 연구에 포함 되었다. 이 중 146명은 중합효소연쇄반응검사를 통해 확진되었고, 21명은 혈청학적인 방법으로 확진되었다. 남은 40명은 실험실적 검사 방법으로 확진되지 않은 쯔쯔가무시 의증으로 분류되었다. 7명의 환자가 아나플라즈마의 16S rRNA 유전자를 검출하기 위한 이중 중합효소 연쇄반응에 양성을 보였

다. BLAST 분석 결과 환자에서 얻어진 결과물의 16S rRNA 유전자 염기서열은 GenBanck의 아나플라즈마 16S rRNA 유전자 염기서열과 높은 동일성 (98.2-99.8%)을 보였다. 병합감염이 확인된 환자와 쯔쯔가무시증 단독 감염 환자간의 임상적 차이는 없었다. 병합감염 환자 중에서 사망한 경우는 없었다. 본 연구에 포함된 모든 환자에서 중증열성혈소판감소증후군 동반은 확인되지 않았다.

결론: 낮은 빈도이지만 국내에서 쯔쯔가무시증과 인간 과립구 아나플라마 증후군의 병합감염이 확인되었다. 이러한 결과는 국내에 인간 과립구 아나플라마 증후군은 드물지 않다는 것을 보여주며, 초기 진단되지 못한 채 유행하고 있음을 시사하고 더 적극적인 진단적 접근이 필요하다.

주요어: 쯔쯔가무시증, 중증혈소판감소증후군, 인간 과립구 아나플라즈마 증후군, 아나플라즈마, 병합감염

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