Distribution of acute febrile disease in SFTS-suspected patient in Gwang-ju metropolitan area from 2015 to 2018

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초 록

자연환경계 발열성 질환에 대하여 원인체 규명률이 낮아 다양한 원인체들의 존재가 있을 것으로 보인다. 따라서 우리 원에 2015-2018 4년동안 SFTS로 의심되어 의뢰된 환자 혈액에서 SFTS, 쯔쯔가무시증, 신증후군출혈열, 발진열, 아나플라즈마증, 큐열에 대하여 조사를 수행하였다. 쯔쯔가무시증, 신증후군출혈열, 발진열에 대해서는 간접면역형광항체법으로 항체검사를 수행하였고, SFTS, 아나플라즈마증, 큐열에 대하여서는 항원을 검출하기 위하여 중합효소연쇄반응법을 이용하였다. 조사결과 총 529명의 SFTS의심환자 중에서 SFTS 30건 5.6%, 쯔쯔가무시증 45건, 8.5%, 신증후군출혈열, 22건 4.1%, 발진열 32건, 6.0%, 아나플라즈마증 2건 0.4%, 큐열, 3건 0.6%을 검출하였다. SFTS의 월별요인에 따른 단면연구결과는 5월과 6월이 다른 보다 높은 위험성을 보였다 ($P < 0.05$)

키워드 : 불명의 발열성 질환, 아나플라즈마증, 큐열, SFTS의심환자

INTRODUCTION

In Gwang-ju metropolitan area, the primary acute febrile illness for human that can be infected from acari(tick, mite), rodent include SFTS(Severe Fever with Thrombocytopenia Syndrome), Tsutsugamushi disease, HFRS(Hemorrahgic Fever with Renal Syndrome). According to data of our institute, identification rate for acute febrile illness was under 50%. Therefore, to clarify the causative agent of unknown acute febrile illness is important to establish the preven-tion strategy.

SFTS, HFRS are caused by *SFTSvirus* and *Hantavirus* in genus *Bunyaviridae,* res-pectively (1). SFTS is tick-borne pathogen, and HFRS is caused by virus-aerosol which are released simultaneously with rodent's excrement (1). Every year, SFTS occurs around 200 people and HFRS 500 people in Korea (2). In Gwangju and South Jella province, about 20 and 100 people are occurring, respectively. The laboratory diag-noses of the viruses include PCR targeting constructural genes (L, M, S segments) and virus isolation through Vero E6, DH82 cell culture and IFA for antibody testing (2).

Tsutsugamushi disease is the most common acute febrile illness that chigger mit

e mediates in Korea (3). It occurs 10,000 people every year in Korea (2). In Gwangju and South Jella province, about 1,000 people are occurring (2). The laboratory diagnoses of the viruses include PCR targeting 56KD type specific antigen gene and isolation through L929 cell culture and IFA for antibody testing (2).

Anaplasma spp. is tick-borne pathogen and intracellular bacteria that infects leukocytes and included in the family *Anaplasmataceae* (4,5). *Anaplasma* spp. is not communicable disease, yet (2). *Anaplasma* contains 6 species (6). *16SrRNA* gene sequencing analysis is one of the key methods to categorize *Anaplasma* species (7).

Coxiella burnetii is a human pathogen that causes the zoonotic disease Q-fever (8). The main route of transmission to humans if inhalation of contaminated areosols, less common, outbreaks have been reported ingestion of milk (9). It has reported all *Coxiella* isolates to the single *C. burnetii* species, and phylogenetic analysis, mainly based on *16SrRNA,* has placed *Coxiella* in the Class *Gammaproteobacteria* along with *Legionella, Francisella,* and *Rickettsiella* (9). It occurs 200 people every year in Korea (2). In Gwangju and South Jella province, about 20 people are occurring (2).

The laboratory diagnoses of *C. burnetii* include PCR targeting 16SrRNA, IS1111 and isolation through L929 cell culture and IFA for antibody testing (2). In PCR, IS1111 gene is more sensitive than any other gene (10).

With increasing climate changes, the burden of various vector-borne diseases is also increasing (11). Hence, it is necessary to clarify the causative agents of acute febrile illness in patients for taking preventive measures. Therefore, Retrospective study was conducted in blood sample of SFTS-suspected patient, from 2015 to 2018. For detection of antibody for Tsutsugamushi disease, HFRS, Murine typhus, IFA was used and, for detection of antigen for SFTS, Anaplasmosis, Q-fever, PCR was used.

MATERIALS AND METHODS

Sample collection and preparation

For 4 years (2015－2018), blood sample of SFTS-suspected patient were collected from five public health center. The blood samples were centrifuged to isolate blood cell and serum using 3000 rpm for 10 min in microcentrifuge (5427R, Eppendorf, USA). The isolated blood samples was maintained at 4 °C .

IFA for antibody detection for Tsutsugamushi disease, HFRS, Murine typhus

A total of 10 ㎕ of serum from each sample was used for serial dilutions of 1:16, 1:32, 1:64, 1:128, 1:256, 1:512, 1:1024, 1:2048 in PBS(pH 7.2). Diluted serum were deposited on an antigen (Tsutsugamushi disease, HFRS, Murine typhus) spot slide, incubated at 37 °C for 30 min in a humidified chamber, and then washed. the slide was washed for 3 min with PBS, and washed for 3 min with distilled water to remove PBS. And a total of 25 ㎕ fluorescein isothiocyanate (FITC)-conjugated goat antimouse IgG (Sigma St. Louis, USA) was added to a spot slide, incubated at 37 °C for 30 min in a humidified chamber, and then washed. the slide was washed for 3 min with PBS, and washed for 3 min with distilled water to r

emove PBS. After mounting medium (Sig ma, St Louis, USA), was added to a spot slide, and covered with coverslip, the slide s were examined for specific spots using a fluorescence microscope (Carl Zeiss, Oberk ochen, Germany). In Tsutsugamushi diseas e, Murine typhus, a cutoff titer of 1:256 w as used to identify seropositivity. In HFRS a cutoff titer of 1:512 was used to identify seropositivity.

Detection of SFTS specific RNA

RNA extraction from the serum were per formed by using viral RNA Mini Kit (Qiag en, Hilden, Germany) according to the man ufacturer's instructions. The RNA was stor ed 4 °C until the PCR was performed. For *SFTSvirus* detection, nested PCR was perfor med using M segment coding sequence (Ki m, 2018). The primer sequences of the first PCR were: forward, 5′-TCATCCTGACTATTYTAG CAATWG-3′;and reverse, 5′-TAAGTYACACT CACACCCTTGAA-3′. PCR was performed as follows:

reverse transcription at 50 °C for 30 min,

denaturation at 95 °C for 15 min, 40 cycl es of denaturation at 95 °C for 20 sec, ann ealing at 55 °C for 40 sec, and elongation at 72 °C for 30 sec. The second PCR was performed with primer:forward 5′-GATGA GATGGTCCATGCTGATTCTAA-3′;revers e, 5′-CTCATGGGGTGGAATGTCCTCAC-3′. P CR was performed as follows: denaturation at 95 °C for 15 min, 35 cycles of denaturat ion at 95 °C for 20 sec, annealing at 58 °C for 40 sec, and elongation at 72 °C for 7 min. The amplification products (560 bp) were analyzed after electrophoresis using 2% agarose gel stained with ethidium bro mide.

Detection of *Anaplasma phagocytophylum* and *Coxiella burnetii* specific DNA

DNA extraction from the blood cell were performed by using Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The genomic DNA was stored 4 °C until the PCR was performed. For *Anaplasma phagocytophylu m* detection, nested PCR was performed usin g *16S rRNA* gene coding sequence (Oh et a l, 2009). The primer sequences of the first P CR were: forward, 5′-AAGCTTAACACATGCAA GTCGAA-3′;and reverse, 5′-AGTCACTGACCCA ATAAATG-3′. PCR was performed as follow s: denaturation at 94 °C for 5 min, 40 cycles of denaturation at 94 °C for 1 min, annealing at 59 °C for 1 min, and elongation at 72 °C. The second PCR was performed with primer:f orward 5′-AGTCACTGACCCAACCTTAAAT G-3′;reverse, 5′-GTCGAACGGATTATTCTTTA A TAGCTTGC-3′. PCR was performed as follo ws: denaturation at 94°C for 5 min, 40 cycles of denaturation at 94 °C for 1 min, annealing at 56 °C for 1min, and elongation at 72 °C for 2 min. The amplification products (926 bp) we re analyzed after electrophoresis using 2% ag arose gel stained with ethidium bromide. For *Coxiella burnetii* detection, nested PCR was performed using IS1111 gene coding sequenc e (Murray et al, 2016). The primer sequences of the first PCR were: forward, 5′-TACTGGGTG TTGATATTGC′;and reverse, 5′-CCGTTTCAT CCGCGGTG-3′. PCR was performed as follo ws: denaturation at 95 °C for 10 min, 40 cycl es of denaturation at 95 °C for 30 sec, anneali ng at 52 °C for 45 sec, and elongation at 72 ° C for 45 sec. The second PCR was performed with primer:forward 5′-GTAAAGTGATCTAC TACACGA-3′;reverse, 5′-TTAACAGXGXTTGAA CGT-3′. PCR was performed as follows: denat uration at 95°C for 10 min, 35 cycles of denat

uration at 95 °C for 30 sec, annealing at 48 °C for 30 sec, and elongation at 72 °C for 30 sec. The amplification products (260 bp) were analyzed after electrophoresis using 2% agarose gel stained with ethidium bromide.

Sequence and phylogenetic analysis

The amplified PCR products were transported to Cosmogenetech (Daejeon, Korea) for sequencing via an ABI 3730XL DNA Analyzer (Applied Biosystems, Foster City, USA). The nucleotide sequences were aligned via ClustalW to a reference sequence obtained from the NCBI (National Center for Biotechnology Information) database and phylogenetic analyses were conducted using the MEGA6 program (http://megasoftware.net). A neighbor-joining tree with 1000 bootstrap replicates was constructed using the Kimura two-parameter model.

Distribution of acute febrile disease in SFTS-suspected patient, 2015-2018

From 2015 to 2018, the number of detections of SFTS, Tsutsugamushi disease, HFRS, Murine typhus, Anaplasmosis, Q-fever in the total number of requests was expressed as a percentage.

Cross-sectional study

To clarify the month variation with SFTS, a cross-sectional study was conducted. The odds ratio was calculated and the ticks having a significant prevalence rate ($P < 0.05$) were incorporated into a logistic regression model using SPSS software ver.25 (IBM, USA).

RESULTS

Detection of *Anaplasma phagocytophylum* and *Coxiella burnetii* specific DNA

The two sequence (15-4 patient, 16-2 patient) were 95% homologue to KR611719 (*Anaplsama phagocytophylum* AAIK4 strain) (Fig.1.).

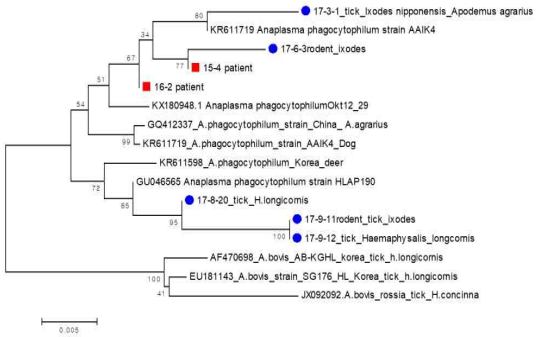

Fig. 1. Phylogenetic tree based on *16S rRNA* gene sequence of *Anaplasma phagocytophylum* of detected in SFTS-suspected patient, 2015-2018, in Gwang-ju metropolitan area. The phylogenetic tree was constructed through the neighbor-joining method with the Kimura two-parameter model (bootstrap 1,000) using MEGA 6.0. The GenBank accession numbers of each sequence of *Anaplasma phagocytopylum* species are indicated. (closed rectangle ■: 15-4 patient, 16-2 patient sequence)

The three sequence (17-7 patient, 18-18 patient, 18-148 patient) were 98% homologue to MG764422 (*Coxiella burnetii*) (Fig.2.).

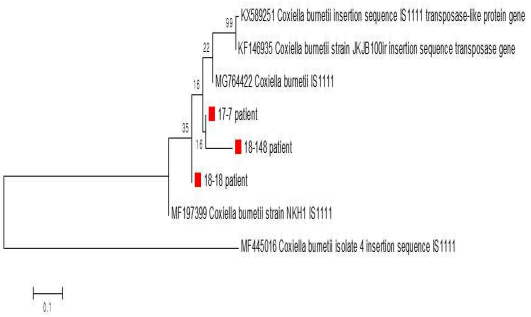

Fig. 2. Phylogenetic tree based on IS1111 gene sequenc

e of *Coxiella burnetii* of detected in SFTS-suspected patie nt, 2015-2018, in Gwang-ju metropolitan area. The phyloge netic tree was constructed through the neighbor-joining m ethod with the Kimura two-parameter model (bootstrap 1,0 00) using MEGA 6.0. The GenBank accession numbers of eac h sequence of *Coxiella burnetii* are indicated. (closed rect angle ■: 17-7 patient, 18-18 patient, 18-148 patient

Distribution of acute febrile disease in SFTS-suspected patient, 2015-2018

In 529 cases of suspected SFTS patient over t he four year period, SFTS had 30 cases (5.6%),d Tsutsugamushi disease 45 cases (8.5%), HFRS 2 2 (4.1%), Murine typhus 32 cases (6.0%), Anapla -smosis 2 cases (0.4%) and Q-fever 3 cases (0. 6%) (Table. 1). In 2015, SFTS had 6 cases,

Tsutsugamushi disease 6 cases, HFRS 4 case s, Murine typhus 15 cases, Anaplasmosis 0 case and Q-fever 0 case. In 2016, SFTS had 4 cases, Tsutsugamushi disease 9 cases, HFRS 4 cases, Murine typhus 4 cases, Anaplasmosis 2 cases an d Q-fever 0 case. In 2017, SFTS had 13 cases, Tsutsugamushi disease 12 cases, HFRS 10 cases, Murine typhus 0 case, Anaplasmosis 0 case and Q-fever 2 cases. In 2018, SFTS had 13 cases, T sutsugamushi disease 12 cases, HFRS 10 cases, Murine typhus 0 case, Anaplasmosis 0 case and Q-fever 2 cases.

Cross-sectional study for SFTS with m onthly factor

To assess the risk factors (Month) for tr ansmission of SFTS, the cross-sectional st udy were conducted. The distribution of th e SFTS by month variation showed a sign ificant difference in May (odds ratio 6.844, 95% CI: 1.310－35.756, P = 0.023), June (od ds ratio 4.734, 95% CI: 1.257－17.832, P = 0.022) (Table 2)

Table 2. Cross-sectional study between month nal factors and SFTS using logistic regression in SFTS-suspected patient, 2015-2018, in Gwan g-ju metropolitan area.

year	SFTS		
	OR (odd ratio)	95% CI	P-value
January	-	-	-
Februay	-	-	-
March	-	-	-
April	-	-	-
May	**6.844**	**1.310-35.756**	**0.023**
June	**4.734**	**1.257-17.832**	**0.022**
July	2.775	0.789-9.755	0.112
August	2.391	0.772-7.405	0.131
Septemer	1.000	-	-
October	1.688	1.437-6.521	0.448
November	3.750	0.420-33.490	0.448
December	-	-	-

Table 1. Distribution of acute febrile disease in SFTS-suspected patient, 2015-2018, in Gwang-j u metropolitan area.

year	total	Tsutsuga mushi disease	Murine typhus	SFTS	HFRS	Anaplas-mosis	Q-fever
		antibody	antibody	antigen	antibody	antigen	antigen
Case (%)	529	45 (8.5)	32 (6.0)	30 (5.6)	22 (4.1)	2 (0.4)	3 (0.6)
2015	65	6	15	6	4	0	0
2016	95	9	4	4	4	2	0
2017	180	12	13	13	10	0	2
2018	189	18	0	7	4	0	1

DISCUSSION

Global warming greatly affects vector(ticks, rodents) which are carriers of various acute febrile diseases (12, 13). Because of the low causality rate, it is presumed to exist unknown acute febrile illness. Therefore, it is essential to investigate various causative agents to establish preventive measures against acute febrile illness in humans. To clarify the investigation of acute febrile illness, The survey for SFTS, Tsutsugamushi disease, HFRS, Murine typhus, Anaplasmosis, Q-fever was conducted from blood of SFTS suspected-patient for 4 years (2015－2018) in Gwang-ju.

In 529 SFTS suspected-patient for 4 years, 2 cases (0.4%) of Anaplasmosis were confirmed (Fig.1., Table 1.) Therefore, Anaplamosis is believed to be one of the causes of an unknown febrile disease. According to Heo's study (14), 17 cases were confirmed using PCR, in Jeollabuk-do, South Korea, 2015-2018 (Heo, 2019). To detect *Anaplasma phagocytophylum* 16srRNA, The same primer as the one used in our study was used. The first patient with Human Anaplasmosis was reported at Korea in 2013, and since then, the number of suspected patients has incresed steadily (6).

3 cases (0.3%) of Q-fever were confirmed (Fig.2., Table 1.). In Park's a 2012 year survey, the Q-fever showed antibodies in 84 (9.1%)out of 923 slaughterhouse workers (Park, 2018). As the spread of the Q-fever is known to be diverse, such as air, food borne, Tick, the our results showed that the Q-fever could be one of th cause of the in monitoring acute febrile disease.

In SFTS suspected patient, because of the high proportion of Tsutsugamushi disease, Murine typhus (Table 1), it is judged that the SFTS's screening diagnosis should consider these deseases. In our region, a recent study by a local hospital also announced that SFTS and Tsutsugamushi disease are representative acute febrile disease and require identification because they have similar symptoms (9). In 4-years survey, HFRS, Murine typhus were steadily detected. In Gwang-ju, it is judged these diseases were endemic.

In cross sectional study for SFTS, May and June were more dangerous than other months ($P<0.05$). Our annual survey showed that May was at the peak of the ticks count due to a surge in the number of Haemaphysalis longicornis larva, indicating that this is consistent with patient data.

To prepare for unknown acute febrile disease epidemics, the various pathogens should be continuously monitored.

Conclusion

In SFTS-suspected patient, Anaplasmosis, Q-fever were confirmed using PCR in Gwang-ju city. Attention for unknown acute febrile illness should be paid. SFTS prevalence showed significance in May and June, when tick are collected a lot, requiring people's attention to outdoor activities during this period.

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Competing interests

The authors declare that they have no competing interests.

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