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Detection of Antibodies to *Anaplasma phagocytophilum* and *Ehrlichia chaffeensis* Antigens in Sera of Korean Patients by Western Immunoblotting and Indirect Immunofluorescence Assays

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Two hundred seventy one serum samples from South Korean patients were tested to detect antibodies against *Anaplasma phagocytophilum* (the human granulocytic ehrlichiosis agent) and *Ehrlichia chaffeensis* (the human monocytic ehrlichiosis agent) by indirect fluorescent-antibody assay (IFA) and the Western blot assay. These sera were collected from patients with symptoms of high fever. The rate of seropositivity for *Orientia tsutsugamushi* was 50.9% by IFA at the Public Health & Environmental Research Institute and National Institute of Health in South Korea. By IFA, 30 (11.1%) and 39 (14.4%) of the serum samples reacted with *A. phagocytophilum* and *E. chaffeensis* antigens, respectively. By the Western blot assays, 24 (8.9%) and 29 (10.7%) of the serum samples reacted with purified *A. phagocytophilum* and *E. chaffeensis* protein antigens, respectively. This report strengthens other evidence regarding the presence of *A. phagocytophilum* and *E. chaffeensis* infections in humans in South Korea.

Because of South Korean agricultural practices and Korean geography, various types of ticks and arthropod vectors are commonly present and every year transmit the agents of several vector-borne diseases, such as scrub typhus (tsutsugamushi disease), Lyme disease, and murine typhus (*Rickettsia typhi*) (8, 10, 14, 18–22). It is estimated that not less than 10,000 patients are treated for tick- or arthropod-borne diseases such as tsutsugamushi disease or spotted fever every year in South Korea.

Human anaplasmosis (formerly human granulocytic ehrlichiosis [HGE]) and human monocytic ehrlichiosis (HME) are emerging infectious diseases transmitted by ticks. The etiological agents of HGE and HME are *Anaplasma phagocytophilum* and *Ehrlichia chaffeensis*, respectively, and these have been classified as members of the family *Anaplasmataceae* (11, 13).

HME and HGE were first reported and described in the United States (2, 9). However, serologic evidence of *A. phagocytophilum* infection has been found broadly (3). Seroepidemiologic and molecular studies have shown that these causative agents are also present in Asia (6, 17). Nevertheless, the tick-borne diseases HGE and HME have not yet been reported in South Korea.

The diagnosis of these diseases depends on evaluation of clinical, laboratory, and epidemiological data. *A. phagocytophilum* and *E. chaffeensis* infections are characterized by the presence of intracytoplasmic inclusions called morulae within leukocytes of human or animal peripheral blood smears. However, since it is difficult to detect *A. phagocytophilum* and *E. chaffeensis*, examination of blood smears for morulae is not a sensitive approach for laboratory diagnosis. Thus, testing of serum for antibodies against *A. phagocytophilum* and *E. chaffeensis* is important.

In this study, we tested human patients in South Korea for antibodies against *A. phagocytophilum* and *E. chaffeensis* using the indirect fluorescent-antibody assay (IFA) and the Western blot assay.

MATERIALS AND METHODS

Human sera. The Public Health & Environmental Research Institute (PHERI) and the National Institute of Health (NIH) in South Korea kindly provided unpaired serum specimens from 271 patients with symptoms of high fever. All serum specimens were initially tested for *Orientia tsutsugamushi* antibodies by IFA at PHERI or NIH (Fig. 1), and 138 (50.9%) were IFA positive for antibodies to the scrub typhus agent. Strains Karp, Kato, Giliam, and Boryong were used, with an IFA titer cutoff of 128 used for immunoglobulin G (IgG) and a cutoff of 10 used for IgM.

Preparation of antigen by in vitro culture of *E. chaffeensis* and *A. phagocytophilum*. The Webster strain of *A. phagocytophilum* (the HGE agent) was propagated in HL-60 cells (a human promyelocytic leukemia cell line) in RPMI 1640 medium (GIBCO-BRL) supplemented with 1% fetal bovine serum (GIBCO-BRL) and 2 mM L-glutamine (GIBCO-BRL) in an incubator at 37°C with 5% CO_2 (15).

The *E. chaffeensis* Arkansas strain was propagated in DH82 cells (a dog macrophage cell line) in Dulbecco's minimal essential medium (GIBCO-BRL) supplemented with 10% fetal bovine serum (GIBCO-BRL) and 2 mM L-glutamine (GIBCO-BRL) in an incubator at 37°C with 5% CO_2 (14).

Cell number and viability were checked manually by trypan blue staining. The infection rate was monitored by examination of cytocentrifuged (Cytospin 3 cytocentrifuge; Shandon, Pittsburgh, Pa.) preparations by Leuko-Stat staining (HEMA 3; Biochemical Science Inc., Swedesboro, N.J.).

IFA. IFA was performed by a previously described procedure (26). Briefly, *A. phagocytophilum*-infected HL-60 cells and *E. chaffeensis*-infected DH82 cells were preloaded onto slides, fixed with acetone, and used as antigens. For antibody titrations, twofold serial dilutions of human serum were prepared, starting at a dilution of 1:80, in phosphate-buffered saline (PBS; pH 7.4) with 0.5% nonfat dry milk (PBSM). A fluorescein isothiocyanate-labeled goat anti-human IgG-IgA-IgM (IgGAM; Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) conjugate at a 1:50 dilution in PBSM was used as the secondary antibody. Before fluorescence microscopic examination, the antigen slides were counterstained with 0.005% Evans blue and mounted for examination.

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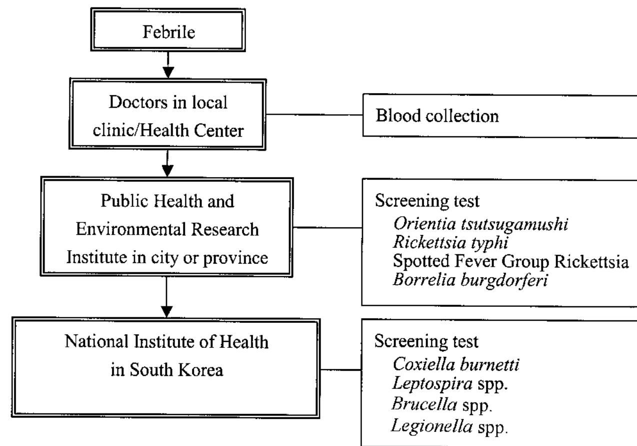

FIG. 1. Flow diagram for diagnosis of ehrlichiosis or anaplasmosis from febrile patients by PHERI and NIH in South Korea.

Western blot assay. The Western blot assay was performed by previously described procedures (1, 4, 12, 28). Antigens were prepared from *A. phagocytophilum* and *E. chaffeensis* purified by a gradient centrifugation method. Normal HL-60 cell and DH82 cells were used as negative antigen controls. Human serum was diluted 1:100 in PBSTM (1% normal goat serum diluted with 0.1 M PBS with 0.05% Tween 20 and 0.5% nonfat dry milk). Alkaline phosphatase-labeled goat anti-human IgGAM (Kirkegaard & Perry Laboratories, Inc.) was used as a secondary antibody at a 1:5,000 dilution in PBSTM. 5-Bromo-4-chloro-3-indolyl phosphate–nitro blue tetrazolium chloride was used as the substrate for alkaline phosphatase and color development.

Statistical analysis for independence of tests. The independence of the test results for *O. tsutsugamushi* with regard to positive or negative IFA or Western blotting results for *A. phagocytophilum* and *E. chaffeensis* was assessed by the χ^2 test.

RESULTS

IFA. Of the 271 serum samples submitted, 30 (11.1%) and 39 (14.4%) reacted with *A. phagocytophilum* and *E. chaffeensis* in the IFA, respectively. The IFA titers of positive sera are shown in Tables 1 and 2. Among the serum samples that showed positive IFA reactions with *A. phagocytophilum*, 15 (5.5%) reacted with both *O. tsutsugamushi* and *E. chaffeensis*, 7 (2.6%) reacted only with *O. tsutsugamushi*, 3 (1.1%) reacted only with *E. chaffeensis*, and 5 (1.8%) reacted only with *A. phagocytophilum*. Among the 39 serum samples that were positive for *E. chaffeensis* by IFA, 14 (5.2%) also reacted with *O. tsutsugamushi* and 7 (2.6%) reacted only with *E. chaffeensis* (Fig. 2). Overall, the IFA results were independent from those obtained for *O. tsutsugamushi* when the results were analyzed by χ^2 tests for *A. phagocytophilum* ($P < 0.01$) or *E. chaffeensis* ($P < 0.002$), or both ($P < 0.001$).

Western blot assay. The Western blot assay was performed with the sera with positive reactions by IFA. The results of the Western blot assay for representative serum samples that reacted with purified *A. phagocytophilum* or *E. chaffeensis* antigen are shown in Fig. 3A and B. The IFA titers of sera with positive reactions by the Western blot assays were higher than those of sera with negative reactions by the Western blot assays. Among the 30 and 39 serum specimens reactive with *A. phagocytophilum* and *E. chaffeensis* in the IFA, respectively, 24 (80.0%) and 29 (74.4%) reacted only with purified *A. phagocytophilum* and *E. chaffeensis* antigens, respectively, and not with negative control antigens prepared from uninfected HL-60 cells or DH-82 cells (Tables 1 and 2). When purified *A. phagocytophilum* antigen was used, all 24 serum specimens reacted with an approximately 44-kDa antigen, presumably major surface protein 2 (*Msp*2). The reaction patterns of these sera could be divided into three groups; 11 serum specimens reacted only with the 44-kDa antigen, 8 reacted with the 44-kDa antigen plus one antigen between 60 and 85 kDa, and 5 reacted with the 44-kDa antigen plus two antigens between 60 and 160 kDa (Fig. 3A). When purified *E. chaffeensis* antigen was used, the reaction patterns of 32 serum specimens could also be divided into three groups, with each group minimally defined by reaction with an antigen of approximately 28 kDa; 15 serum specimens reacted only with an antigen of approximately 28 kDa, 10 reacted with two antigens between 28 and 35 kDa, and 4 reacted with three or four antigens between 26 and 100 kDa (Fig. 3B). A weak 120-kDa antigen was detected in only three serum specimens. Overall, the Western blotting results were independent from those obtained for *O. tsutsugamushi* when the results were analyzed by χ^2 tests for *A. phagocytophilum* ($P < 0.05$) or *E. chaffeensis* ($P < 0.002$), or both ($P < 0.001$).

TABLE 1. Patient sexes and sex ages IFA titers, and Western blot assay results for sera reacted against *A. phagocytophilum* and *O. tsutsugamushi*[a]

Sample no.	Sex	Age (yr)	*A. phagocytophilum* IFA titer	*A. phagocytophilum* Western blot result (kDa)[b]	IFA titer for *O. tsutsugamushi*
2	F	36	160	Neg	128
6	M	77	320	44	Neg
10	F	78	640	44	128
19	F	49	320	44	128
29	F	50	320	44	512
37	F	58	160	Neg	Neg
55	U	U	640	44, 68	Neg
77	U	U	320	44, 68, 70	Neg
7	F	65	160	Neg	512
9	U	U	320	44, 60, 120	4,096
11	U	U	160	44	4,096
14	U	U	160	44	8,192
16	U	U	640	44	Neg
21	U	U	320	44, 68	4,096
43	F	71	320	44, 85	8,192
46	M	59	160	Neg	4,096
53	F	74	160	44, 60	Neg
58	U	U	320	44	8,192
67	U	U	320	44, 60, 85	4,096
71	M	84	320	44	4,096
80	F	44	160	44	Neg
87	F	88	160	44, 85	1,280
93	F	65	320	44, 70	Neg
106	U	U	80	Neg	4,096
111	F	67	320	44	16,384
126	U	U	640	44, 60, 85	4,096
136	M	69	320	44, 60, 85	4,096
156	F	70	320	44, 68	4,096
160	M	84	320	NP	4,096
167	M	59	320	44, 85	4,096

[a] Abbreviations: F, female; M, male; U, unknown; NP, not performed; Neg, negative.
[b] Approximate molecular size of reactive antigen.

DISCUSSION

Although IFA has both advantages and disadvantages, it is the most widely used method for the identification of *E.*

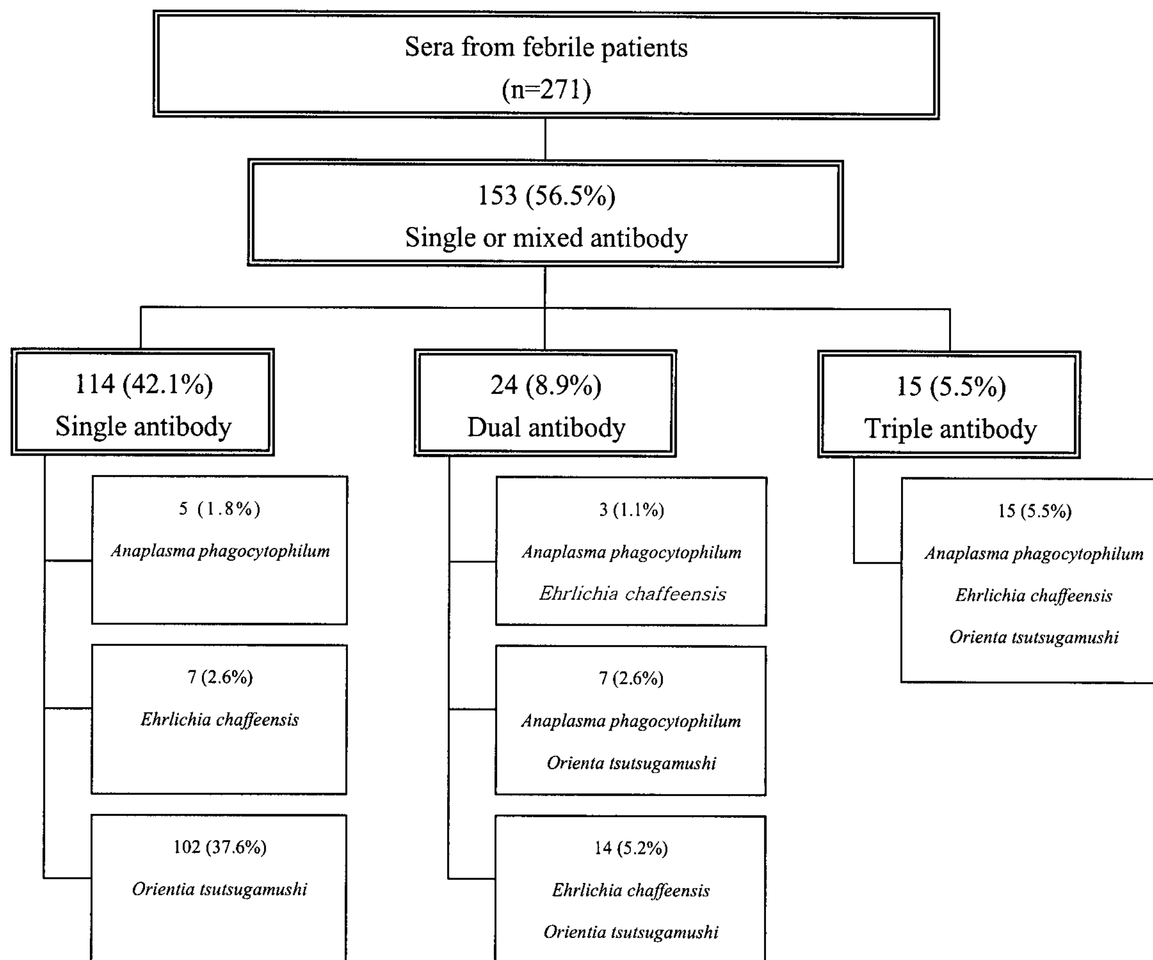

FIG. 2. *E. chaffeensis*, *A. phagocytophilum*, and *O. tsutsugamushi* antibodies in human sera collected from patients in Jeonnam and Jeonbuk, South Korea, in 2001 and 2002. n, number of patients examined.

chaffeensis and *A. phagocytophilum* antibodies in both acute- and convalescent-phase human sera after infection. In this study, we used IFA as a screening test and the Western blot assay for confirmation.

Among all of the serum specimens submitted for this study, 138 showed positive reactions for *O. tsutsugamushi* by IFA. The relatively higher rate of IFA positivity for *O. tsutsugamushi* seems to reflect the fact that all serum specimens were collected from patients with febrile responses typical for scrub typhus. However, this may also indicate cross-reactivity between *A. phagocytophilum*, *E. chaffeensis*, and *O. tsutsugamushi*. It is well recognized that serum samples from patients with HGE cross-react with *E. chaffeensis* (24). Bunnell et al. (5) suggested that suspected cross-reactions between *A. phagocytophilum* and *Borrelia burgdorferi* antigens in a murine model were not significant enough to confuse the results of specific serological tests. In the present study, 102 serum specimens were IFA positive only for *O. tsutsugamushi*, suggesting that cross-reactions between *E. chaffeensis* or *A. phagocytophilum* and *O. tsutsugamushi* are also not significant and that HGE or HME may exist as mixed infections or subsequent to infection with *O. tsutsugamushi* in patients with acute fever. Moreover, the independence of the results obtained by both IFA and the Western blot tests indicates that the concurrent positive reactions are unlikely to be due to cross-reactions. It would be useful to assess seroconversion or fourfold increases in titers between paired serum specimens, which was not possible for the single serum samples tested in the present study.

Although IFA is the most sensitive method, it can give inconsistent results because of antigenic diversity and various technical factors among different laboratories. For example, use of the MRK strain of *A. phagocytophilum* (*Ehrlichia equi*) as the antigen for IFA revealed a high degree of variability even if tests were performed in the same laboratory (1). Western blot assays are generally thought to provide more detailed information than IFA about the specific reactive antigens. Therefore, we confirmed the IFA results by Western blot assays using purified *A. phagocytophilum* and *E. chaffeensis* antigens. All sera that reacted against *A. phagocytophilum* in Western blot assays recognized an antigen of approximately 44 kDa that is presumed to represent *Msp*II. The reaction patterns of those sera could be further divided into three groups, depending on the overall number of antigens detected, further enforcing the likelihood of the addition of specificity with Western blot assays. Likewise, when purified *E. chaffeensis* antigens were used in a Western blot assay, all sera reacted against an

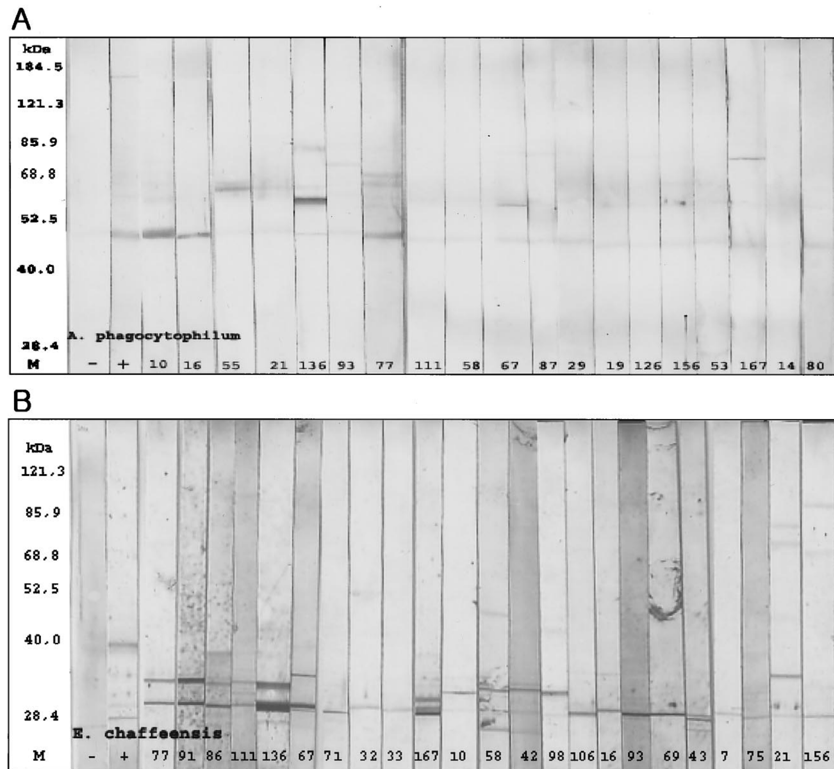

FIG. 3. Western blot assay of electrophoretically separated gradient-purified *A. phagocytophilum* (A) and *E. chaffeensis* (B) antigens reacted with IFA-positive sera. Lanes: M, protein size marker; −, negative control; +, positive control; the lane numbers represent sample numbers. (A) Twenty-five serum specimens (83.3%) reacted with an *A. phagocytophilum* antigen of approximately 44 kDa presumed to be *Msp*2. The reaction patterns were divided into three groups: 11 serum specimens reacted only with the 44-kDa antigen, 8 reacted with the 44-kDa antigen and another antigen between 60 and 85 kDa, and 5 reacted with the 44-kDa antigen and two other antigens between 60 and 160 kDa. (B) Twenty-nine (74.4%) serum specimens reacted with purified *E. chaffeensis* antigens. The reaction patterns of 32 serum specimens were also divided into three groups: 15 reacted only with antigens of 28 kDa, 10 reacted with two antigens between 28 and 35 kDa, and 4 reacted with three or four antigens between 26 and 100 kDa.

antigen of approximately 28 kDa that is presumed to represent the p28 major membrane protein family. These reaction patterns could also be divided into three groups: the first group reacted only with an antigen of approximately 28 kDa; the second group reacted with two antigens that showed bands between 28 and 35 kDa, which may reflect detection of the *E. chaffeensis* p28 family of membrane proteins; and the third group reacted with three or four antigens which showed bands between 26 and 120 kDa, also reflecting the detection of higher-molecular-size antigens, such as the immunodominant protein gp120 (29) and conserved heat shock proteins of questionable specificity for *E. chaffeensis*.

Such variable reactions in Western blot assays have been demonstrated before with both *A. phagocytophilum* and *E. chaffeensis*. Unver et al. (25) reported that IFA-positive sera of HME patients reacted not only with the p28 family of major surface proteins but also with several other native antigens of *E. chaffeensis*, while Chen et al. (9a) showed variable degrees of genus, species, and strain specificity. However, further studies are needed to verify these Western blot assay results, perhaps by using variant *A. phagocytophilum* and *E. chaffeensis* isolates that may further validate and standardize this serological approach.

In order for these serological results to make sense, several ecological factors need to be considered. *O. tsutsugamushi* is transmitted through the bites of trombiculid mites, but not through the bites of ticks. However, the concurrence of antibodies to several vector-borne bacteria could be predicted, since both the trombiculid mites and ticks that transmit *Ehrlichia* and *Anaplasma* spp. share many of the same habitats. Likewise, an association of specific ticks and *Ehrlichia* or *Anaplasma* spp. is expected. *A. phagocytophilum* has been detected in *Ixodes persulcatus* ticks and in the blood of humans after tick bites in China (6, 30), consistent with the results of the present report. Although the *I. persculcatus* group that transmits *A. phagocytophilum* is present in South Korea, the classical tick vector for *E. chaffeensis*, *Amblyomma americanum*, is not. However, *E. chaffeensis* or a similar species has been detected in *Amblyomma testudinarium* and *Haemaphysalis yeni* ticks in South China (7), *Boophilus microplus* ticks in Tibet (27), and *Ixodes ovatus* ticks in Japan (23); and we have found evidence of both *E. chaffeensis* and *A. phagocytophilum* in both *Haemaphysalis longicornis* and *I. persulcatus* ticks from South Korea (17a). Although a definitive role of these causative agents and potential tick vectors in human infections cannot be assumed from these data, the presence of the causative agents and potential tick vectors with the capacity to bite humans suggests that the serological data reflect a previously

TABLE 2. Patient sexes and sex ages, IFA titers, and Western blot assay results for sera reacted against *E. chaffeensis* and *O. tsutsugamushi*[a]

Sample no.	Sex	Age (yr)	*Ehrlichia chaffeensis* IFA titer	*Ehrlichia chaffeensis* Western blot result (kDa)[b]	IFA titer for *O. tsutsugamushi*
10	F	78	320	28, 32	128
20	M	76	320	28	128
42	F	62	320	26, 28, 32	Neg
75	U	U	160	28	Neg
77	U	U	320	28, 32	Neg
91	F	71	320	28, 32	128
98	F	74	320	28, 32	128
7	F	65	160	28	512
14	U	U	160	Neg	8,192
16	U	U	160	28	Neg
21	U	U	320	28, 35, 70, 80	4,096
32	F	67	320	28	1,024
33	U	U	160	28	1,024
38	U	U	160	Neg	Neg
43	F	71	160	28	8,192
46	M	59	320	26	4,096
47	F	62	320	26	2,048
54	F	49	320	28, 35	32,768
57	F	42	160	28	Neg
58	U	U	320	26, 28, 32	8,192
67	U	U	320	28, 35	4,096
69	U	U	160	28	4,096
71	M	84	160	28	4,096
86	U	U	320	28, 32	4,096
87	F	88	160	28	1,280
93	F	65	160	28	Neg
99	U	U	160	Neg	512
106	U	U	160	28	4,096
108	U	U	160	Neg	Neg
109	F	84	80	Neg	Neg
111	F	67	320	28, 32	16,384
115	U	U	320	Neg	4,096
136	M	69	320	28, 32	4,096
147	F	66	160	Neg	2,048
148	M	57	160	Neg	Neg
154	U	U	160	NP	2,048
156	F	70	320	28, 68, 100	4,096
166	F	75	160	NP	512
167	M	59	320	28, 30	4,096

[a] Abbreviations: F, female; M, male; U, unknown; NP, not performed; Neg, negative.
[b] Approximate molecular size of reacting antigen.

unrecognized but emerging problem in South Korea and perhaps more broadly across Asia.

Many rickettsial diseases have been studied in South Korea, and the geographical distribution of *O. tsutsugamushi* was recently investigated. However, only one report has described the incidence of *Ehrlichia* or *Anaplasma* sp. infection in humans (16). To confidently confirm human exposure and the presence of HME and HGE in South Korea, much more study is needed, including the isolation and identification of *Ehrlichia* or *Anaplasma* strains from infected humans.

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