Journal of Clinical Microbiology, Aug. 2002, p. 3082–3085 Vol. 40, No. 8
0095-1137/02/$04.00+0 DOI: 10.1128/JCM.40.8.3082–3085.2002
Copyright © 2002, American Society for Microbiology. All Rights Reserved.

Serologic and Molecular Detection of *Ehrlichia chaffeensis* and *Anaplasma phagocytophila* (Human Granulocytic Ehrlichiosis Agent) in Korean Patients

Eun-jeong Heo,[1] Jin-ho Park,[2] Ja-ryong Koo,[3] Man-suk Park,[4] Mi-yeoun Park,[4] J. Stephen Dumler,[2] and Joon-seok Chae[1]*

Department of Veterinary Internal Medicine and Clinical Pathology, College of Veterinary Medicine, Chonbuk National University, Jeonju, Jeonbuk 561-756,[1] and Mol-BioNet Research Institute, Scientec Lab Center Co., Ltd., Daeduk-Gu, Taejon 306-030,[3] and Division of Rickettsial & Zoonotic Diseases, Department of Microbiology, National Institute of Health, Seoul 122-701,[4] Korea, and Division of Medical Microbiology, Department of Pathology, The Johns Hopkins Medical Institutions, Baltimore, Maryland 21287[2]

Received 4 February 2002/Returned for modification 18 March 2002/Accepted 29 May 2002

Sera from 491 Korean patients with acute febrile diseases were tested for *Ehrlichia chaffeensis* and *Anaplasma phagocytophila* antibodies by indirect immunofluorescence assay (IFA), Western blotting, and TaqMan real-time PCR. Overall, 0.4% of sera reacted with *E. chaffeensis*, and 1.8% reacted with *A. phagocytophila* in IFAs. This is the first report of detection of antibodies to *A. phagocytophila* and *E. chaffeensis* in Korea and suggests the presence of *A. phagocytophila* and *E. chaffeensis* or antigenically similar species.

Human granulocytic ehrlichiosis (HGE) and human monocytic ehrlichiosis (HME) are emerging, tick-transmitted infectious diseases described in many areas of the United States and (for HGE) Europe. During a recent reclassification, the names "HGE agent" and "*Ehrlichia equi*" were merged with *Ehrlichia phagocytophila*, which was changed to *Anaplasma phagocytophila,* and the etiological agents of HGE and HME, *Anaplasma phagocytophila* and *Ehrlichia chaffeensis*, respectively, were moved into the family *Anaplasmataceae* (9).

HME was first reported in 1987, and *E. chaffeensis* was isolated 4 years later (8). HGE was identified in 1990 and reported in 1994 (6). HME and HGE were first described in parts of Europe in 1991 and 1995, respectively, and serologic evidence reveals that *A. phagocytophila* infections occur broadly across Europe (2, 4, 14, 15). Although definitive cases have not been identified outside of North America and Europe, additional seroepidemiologic and molecular studies suggest their presence elsewhere, including Africa and Asia (5, 20). There have been no reports of ehrlichiosis in both humans and animals in Korea. In this study, we describe the first serologic evidence for *E. chaffeensis* and *A. phagocytophila* infections in Korean subjects and confirm these findings with Western blots and TaqMan real-time PCR.

Sera of 491 febrile patients from Jeonbuk and Jeonnam Public Health and Environmental Research Institute (PHERI) and the National Institute of Health in Korea (KNIH) were collected from June through November 2000 and were submitted for routine tests for hemorrhagic fever with renal syndrome (HFRS), *Orienta tsutsugamushi*, leptospirosis, Lyme disease, and *Rickettsia typhi*. A total of 172 individuals were seropositive for *O. tsutsugamushi* (35.0%), and 21 (3.8%) were seropositive for *Leptospira* spp. The remainder were seronegative for these infectious agents. The intervals after fever when samples were obtained were not known.

Serologic tests for *A. phagocytophila* and *E. chaffeensis* were conducted at The Johns Hopkins University School of Medicine (21). *A. phagocytophila* was propagated in the HL-60 cell line in RPMI 1640 medium supplemented with 1% fetal bovine serum (FBS) and 2 mM L-glutamine, whereas *E. chaffeensis* was propagated in the DH82 cell line in EMEM (Gibco) medium supplemented with 5% FBS and 2 mM L-glutamine (11, 12, 21). The indirect immunofluorescence assay (IFA) was performed as described previously (21). Briefly, Teflon-coated slides with heavily infected HL-60 and DH82 cells were acetone fixed before use. Human sera were diluted 1:80 in phosphate-buffered saline (PBS) with 0.5% nonfat dry milk (PBSM), added to the antigen slides, and incubated at room temperature (RT) for 1 h. After washing, fluorescein isothiocyanate (FITC)-labeled goat anti-human immunoglobulin G (IgG), IgA, and IgM (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) were added at a dilution of 1:50 and incubated at RT for 1 h. After washing, the antigen slides were counterstained with 0.005% Evans blue and mounted for examination by fluorescence microscopy. All of the sera were tested for *O. tsutsugamushi* antibodies by IFA at PHERI or KNIH.

For the Western blot assay, *A. phagocytophila* and *E. chaffeensis* used as antigens were purified by Renografin density gradient centrifugation (1, 3, 22). Antigens were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and cut into antigen strips. Membranes were blocked with Tris-buffered saline (TBS; 20 mM Tris, 500 mM NaCl [pH 7.5]) containing 0.5% nonfat dry milk (NFDM) for 1 h. Human sera diluted 1:80 in 1% normal goat serum diluted with 0.1 M PBS, with 0.05% Tween 20 and 0.5%

* Corresponding author. Mailing address: Department of Veterinary Internal Medicine and Clinical Pathology, College of Veterinary Medicine, Chonbuk National University, Jeonju, Jeonbuk 561-756, Korea. Phone: 82-63-270-3881. Fax: 82-63-278-3884. E-mail: jschae@chonbuk.ac.kr.

TABLE 1. Sex and age characteristics and positive results of serological testing for *A. phagocytophila* and *E. chaffeensis* confirmed by Western blotting and real-time PCR

City and/or province	Sex[a]	Age (yr)[b]	IFA titer (Western blot result)[c]		Real-time PCR result[d]	IFA result for *O. tsutsugamushi*[e]
			A. phagocytophila	*E. chaffeensis*		
Seongbuk, Seoul	M	U	320 (−)	N	−	−
Paju, Gyeounggi	M	43	640 (+)	N	−	−
Youngcheon, Gyeongbuk	M	56	N	320 (+)	−	−
Muju, Jeonbuk	M	80	N	2,560 (+)	−	+
Jangsu, Jeonbuk	M	72	640 (−)	N	−	−
Gokseong, Jeonnam	M	47	2,560 (−)	N	−	+
Naju, Jeonnam	M	75	2,560 (−)	N	−	+
Muan, Jeonnam	F	53	1,280 (−)	N	−	+
Suncheon, Jeonnam	F	54	640 (−)	N	−	+
Yeongam, Jeonnam	F	49	1,280 (−)	N	−	+
Kangjin, Jeonnam	F	80	80 (−)	N	+	+

[a] M, male; F, female.
[b] U, unknown.
[c] N, negative in IFA test; −, negative in Western blot; +, positive in Western blot. There were no significant cross-reactions among *A. phagocytophila, E. chaffeensis*, and *O. tsutsugamushi*.
[d] DNA was purified and amplified with the *Ehrlichia* or *Anaplasma* sp. 16S rRNA gene fragment by real-time TaqMan PCR with genus-specific primers.
[e] All of the sera were tested by IFA for *O. tsutsugamushi* before being tested for *A. phagocytophila* and *E. chaffeensis*.

nonfat dry milk (PBSTM), were added to individual antigen strips and allowed to react at RT for 1 h. Following washes, alkaline phosphatase-labeled goat anti-human IgG, IgA, and IgM (KPL) were added, and this mixture was incubated for 1 h. After additional washes, BCIP-NBT (5-bromo, 4-chloro, 3-indolyl phosphate–nitroblue tetrazolium chloride) was used as a substrate for color development.

Real-time PCR was conducted by a procedure modified from that of Pusterla et al. (16). Genomic DNA was extracted from 200 μl of IFA-reactive sera with a QIAmp blood kit (Qiagen, Chatsworth, Calif.) according to the manufacturer's directions. The TaqMan probe and primers for the 16S rRNA gene of *Ehrlichia* and *Anaplasma* spp. were ESP-P (5′-6-FAM-ACGCGTTAAGCACTCCGCCTGG-TAMRA-3′), ESP-F (5′-ACGATGAGTGCTGAATGTGG-3′), and ECH-R (5′-TAACTGAGTTGTCGATCCAATGAAA-3′). PCR was performed in a 20-μl volume containing 450 nM each primer and 250 nM fluorescent-labeled probe; TaqMan Universal PCR master mix (Applied Biosystems) was added to each reaction and cycled in a Sequence Detection System 7700 (Applied Biosystems) thermal cycler under the following conditions: uracil *N*-deglycosylase digestion at 50°C for 2 min, AmpliTaq Gold preactivation at 95°C for 10 min, and 45 cycles at 95°C for 20 s and 55°C for 60 s. Positive and negative DNAs from organisms of related tick-borne diseases were tested to examine the specificity for TaqMan real-time PCR with the probe and primers designed for *Ehrlichia* and *Anaplasma* species (data not shown).

Of the 491 Korean sera, 9 reacted with *A. phagocytophila* and 2 reacted with *E. chaffeensis* by IFA; no cross-reactions between *A. phagocytophila* and *E. chaffeensis* were detected in these samples. Seven of the 11 samples were also positive for *O. tsutsugamushi* antibodies (Table 1). The reactive sera were from individuals between 43 and 80 years old (Table 1). Western blotting revealed that both *E. chaffeensis* IFA-positive sera reacted with the 28-kDa antigen of *E. chaffeensis*, and one of the nine *A. phagocytophila* IFA-positive sera reacted with the *A. phagocytophila* 44-kDa protein (Fig. 1).

An *Anaplasma* or *Ehrlichia* species 16S rRNA gene fragment was amplified by TaqMan real-time PCR with genus-specific primers from DNA of the nine positive sera. This serum was from an individual from Kangjin, Jeonnam, Korea. Sequence analysis was not performed.

In this report, we evaluated exposure to *A. phagocytophila* and *E. chaffeensis* or antigenically similar bacteria in human patients in Korea. The distribution of infections seems to be

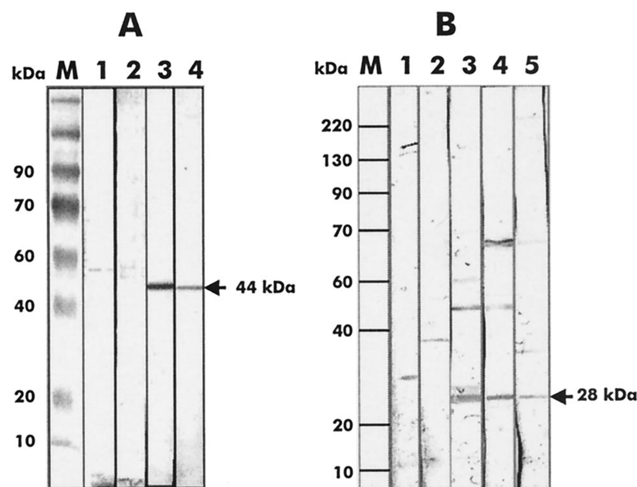

FIG. 1. Western blot with *A. phagocytophila* (A) and *E. chaffeensis* (B) as antigens reacted with IFA-positive serum of Korean patients with acute fever. (A) Lanes: M, size marker; 1, uninfected HL-60 cell antigen reacted with *A. phagocytophila* IFA-negative serum; 2, uninfected HL-60 cell antigen reacted with *A. phagocytophila* IFA-positive serum; 3, purified *A. phagocytophila* antigens reacted with monoclonal antibody 20B4, which detects *A. phagocytophila* Msp2 (44-kDa protein antigen; 4, purified *A. phagocytophila* antigens reacted with *A. phagocytophila* IFA-positive Korean human serum. (B) Lanes: M, size marker; 1, DH82 cell antigens reacted with *E. chaffeensis* IFA-negative serum; 2, DH82 cell antigens reacted with *E. chaffeensis* IFA-positive control serum from a U.S. patient; 3, purified *E. chaffeensis* antigen reacted with *E. chaffeensis* IFA-positive control serum from a U.S. patient; 4 to 5, purified *E. chaffeensis* antigen reacted with *E. chaffeensis* IFA-positive Korean human sera.

mainly in agricultural regions of Korea, where various ticks are commonly present. Since there is a high seropositive rate (35.0%) in Korea, especially in Jeonnam province, many patients are also *O. tsutsugamushi* seropositive. The next most common disease diagnosed was leptospirosis, with 3.8% of the sera positive. The serologic tests for *A. phagocytophila* and *E. chaffeensis* were positive mainly in patients who were middle age or older (43 to 80 years old).

Eleven (2.2%) of the 491 sera reacted with the antigens of *A. phagocytophila* and *E. chaffeensis* at an antibody titer of ≥1:2,560. The seropositive rate of *A. phagocytophila* (1.8%) was higher than that of *E. chaffeensis* (0.4%), but the difference was not significant.

We found that 6 of 11 *A. phagocytophila*-positive sera were also seropositive for *O. tsutsugamushi,* suggesting a cross-reaction. To evaluate this possibility, four samples seropositive only for *O. tsutsugamushi* were retested as seronegative in IFA with *A. phagocytophila* and *E. chaffeensis* antigens. This is further supported by the lack of *E. chaffeensis* or *A. phagocytophila* antibodies in the remaining 166 sera that were found reactive with *O. tsutsugamushi*. Thus, serologic cross-reactions between *E. chaffeensis* or *A. phagocytophila* and *O. tsutsugamushi* are probably not significant, and human ehrlichiosis sometimes exists as a mixed infection with scrub typhus. Serologic cross-reactions between *A. phagocytophila* and *E. chaffeensis* are frequent (10), but were not detected in any of these Korean samples.

Although two samples were positive with *E. chaffensis* antigen, we cannot exclude infection by serologically related species, such as *E. canis* or *E. muris*, which are known to be present in Asian animals. Further serological studies with a variety of antigens of *Ehrlichia* spp. may allow more precise identification of the infecting agent in these and similar cases.

The IFA is the most sensitive serodiagnostic method; however, inconsistent results may occur because of antigenic diversity and technical differences among laboratories. For example, *A. phagocytophila* (*E. equi*) MRK strain antigen revealed a high degree of variability, even when performed in the same laboratory, depending upon antigen preparation and method of analysis (21). Because of the potential for nonspecificity with serologic assays, we attempted confirmation by amplification of *Ehrlichia* and *Anaplasma* 16S rRNA gene fragments from the positive sera. The single Taqman PCR-positive sample had an *A. phagocytophila* IFA titer of 80, but was negative by *A. phagocytophila* Western blot. We suspect that the low titer represents early infection when circulating bacteria or DNA might be found. Nested PCR for the 16S rRNA gene of *A. phagocytophila* did not confirm this result, a phenomenon previously observed when serum is used as the template for amplification of *E. chaffeensis* or *A. phagocytophila* (7).

Immunoblot analyses showed specific antibodies for the agents, including one of nine sera reactive with the diagnostic 44-kDa *A. phagocytophila* antigen (1, 17) and both *E. chaffeensis* IFA-positive sera reactive with a diagnostic 28-kDa *E. chaffeensis* antigen band (3, 22). The reactions were generally weak and absent for most of the *A. phagocytophila* IFA-positive samples, perhaps due to the existence of antigenically divergent strains in Korean ticks. Although prior studies suggest that the *A. phagocytophila* 44-kDa antigen is a sensitive marker, previous studies have been conducted with sera obtained from a single specific location or that included only a small number of samples overall (13, 17, 22). Cultivation of the causative agents would provide definitive evidence and would help to devise the best serologic tests.

Although rickettsial diseases (in particular, those caused by *O. tsutsugamushi*) are well studied in Korea (18), there are no reports regarding the incidence of *Ehrlichia* spp. Although no definitive, culture-confirmed cases were identified, this report provides the first evidence that Koreans may be infected by *A. phagocytophila* and *E. chaffeensis* or antigenically similar organisms (19). Additional studies are needed to isolate and identify *Ehrlichia* and *Anaplasma* spp. from humans and animals in Korea to confirm their presence and capacity to cause disease.

We thank Chan-moon Jin and Mi-young Shin in Jeonbuk and Jeonnam Public Health and Environmental Research Institute and Hyeon Yim in Chonbuk National University Hospital for providing sera.

This study was supported by a grant of the Korea Health 21 R&D Project, Ministry of Health & Welfare, Republic of Korea (HMP-00-B-20200-0003 [J.S.C.]), and in part by grant RO1 AI44102-01A1 from the National Institutes of Health, Bethesda, Md. (to J.S.D.).

REFERENCES

1. **Asanovich, K. M., J. S. Bakken, J. E. Madigan, M. Aguero-Rosenfeld, G. P. Wormser, and J. S. Dumler.** 1997. Antigenic diversity of granulocytic *Ehrlichia* isolates from humans in Wisconsin and New York and a horse in California. J. Infect. Dis. **176:**1029–1034.
2. **Bakken, J. S., J. Krueth, R. L. Tilden, J. S. Dumler, and B. E. Kristiansen.** 1996. Serological evidence of human granulocytic ehrlichiosis in Norway. Eur. J. Clin. Microbiol. Infect. Dis. **15:**829–832.
3. **Brouqui, P., C. Lecam, J. Olson, and D. Raoult.** 1994. Serologic diagnosis of human monocytic ehrlichiosis by immunoblot analysis. Clin. Diagn. Lab. Immunol. **1:**645–649.
4. **Brouqui, P., J. S. Dumler, R. Lienhard, M. Brossard, and D. Raoult.** 1995. Human granulocytic ehrlichiosis in Europe. Lancet **346:**782–783.
5. **Cao, W.-C., Q.-M. Zhao, P.-H. Zhang, J. S. Dumler, X.-T. Zhang, L.-Q. Fang, and H. Yang.** 2000. Granulocytic ehrlichiae in *Ixodes persulcatus* ticks from an area in China where Lyme disease is endemic. J. Clin. Microbiol. **38:**4208–4210.
6. **Chen, S.-M., J. S. Dumler, J. S. Bakken, and D. H. Walker.** 1994. Identification of a granulocytotropic *Ehrlichia* species as the etiologic agent of human disease. J. Clin. Microbiol. **32:**589–595.
7. **Comer, J. A., W. L. Nicholson, J. W. Sumner, J. G. Olson, and J. E. Childs.** 1999. Diagnosis of human ehrlichiosis by PCR assay of acute-phase serum. J. Clin. Microbiol. **37:**31–34.
8. **Dawson, J. E., B. E. Anderson, D. B. Fishbein, J. L. Sanchez, C. S. Goldsmith, K. H. Wilson, and C. W. Duntley.** 1991. Isolation and characterization of an *Ehrlichia* sp. from a patient diagnosed with human ehrlichiosis. J. Clin. Microbiol. **29:**2741–2745.
9. **Dumler, J. S., A. F. Barbet, C. P. Bekker, G. A. Dasch, H. H. Palmer, S. C. Ray, Y. Rikihisa, and F. R. Rurangirwa.** 2001. Reorganization of genera in the families Rickettsiaceae and Anaplasmataceae in the order Rickettsiales: unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with *Ehrlichia* and *Ehrlichia* with *Neorickettsia*, descriptions of six new species combinations and designation of *Ehrlichia equi* and "HGE agent' as subjective synonyms of *Ehrlichia phagocytophila*. Int. J. Syst. Evol. Microbiol. **51:**2145–2165.
10. **Dumler, J. S., K. M. Asanovich, J. S. Bakken, P. Richter, R. Kimsey, and J. E. Madigan.** 1995. Serologic cross-reactions among *Ehrlichia equi, Ehrlichia phagocytophila,* and human granulocytic ehrlichia. J. Clin. Microbiol. **33:**1098–1103.
11. **Felek, S., A. Unver, R. W. Stich, and Y. Rikihisa.** 2001. Sensitive detection of *Ehrlichia chaffeensis* in cell culture, blood, and tick specimens by reverse transcription-PCR. J. Clin. Microbiol. **39:**460–463.
12. **Goodman, J. L., C. Nelson, B. Vitale, J. E. Madigan, J. S. Dumler, T. J. Kurtti, and U. G. Munderloh.** 1996. Direct cultivation of the causative agent of human granulocytic ehrlichiosis. N. Engl. J. Med. **334:**209–215.
13. **Ijdo, J. W., Y. Zhang, E. Hodzic, L. A. Magnarelli, M. L. Wilson, S. R. Telford III, S. W. Barthold, and E. Fikrig.** 1997. The early humoral response in human granulocytic ehrlichiosis. J. Infect. Dis. **176:**687–692.
14. **Keysary, A., L. Amram, G. Keren, Z. Sthoeger, I. Potasman, A. Jacob, C. Strenger, J. E. Cawson, and T. Waner.** 1999. Serologic evidence of human monocytic and granulocytic ehrlichiosis in Israel. Emerg. Infect. Dis. **5:**775–778.

15. **Morais, J. D., J. E. Dawson, C. Greene, A. R. Filipe, L. C. Galhardas, and F. Bacellar.** 1991. First European case of ehrlichiosis. Lancet **338:**633–634.
16. **Pusterla. N., J. B. Huder, C. M. Leutenegger, U. Braun, J. E. Madigan, and H. Lutz.** 1999. Quantitative real-time PCR for detection of members of the *Ehrlichia phagocytophila* genogroup in host animals and *Ixodes ricinus* ticks. J. Clin. Microbiol. **37:**1329–1331.
17. **Ravyn, M. D., J. L. Goodman, C. B. Kodner, D. K. Westad, L. A. Coleman, S. M. Engstrom, C. M. Nelson, and R. C. Johnson.** 1998. Immunodiagnosis of human granulocytic ehrlichiosis by using culture-derived human isolates. J. Clin. Microbiol. **36:**1480–1488.
18. **Ree, H. I., T. E. Kim, I. Y. Lee, S. H. Jeon, U. W. Hwang, and W. H. Chang.** 2001. Determination and geographical distribution of *Orientia tsutsugamushi* serotypes in Korea by nested polymerase chain reaction. Am. J. Trop. Med. Hyg. **65:**528–534.
19. **Shibata, S.-I., M. Kawahara, Y. Rikihisa, H. Fujita, Y. Watanabe, C. Suto, and T. Ito.** 2000. New *Ehrlichia* species closely related to *Ehrlichia chaffeensis* isolated from *Ixodes ovatus* ticks in Japan. J. Clin. Microbiol. **38:**1331–1338.
20. **Uhaa, I. J., J. D. MacLean, C. R. Greene, and D. B. Fishbein.** 1992. A case of human ehrlichiosis acquired in Mali: clinical and laboratory findings. Am. J. Trop. Med. Hyg. **46:**161–164.
21. **Walls, J. J., M. Aguero-Rosenfeld, J. S. Bakken, J. L. Goodman, D. Hossain, R. C. Johnson, and J. S. Dumler.** 1999. Inter- and intralaboratory comparison of *Ehrlichia equi* and human granulocytic ehrlichiosis (HGE) agent strains for serodiagnosis of HGE by the immunofluorescent-antibody test. J. Clin. Microbiol. **37:**2968–2973.
22. **Wong, S. J., G. S. Brady, and J. S. Dumler.** 1997. Serologic responses to *Ehrlichia equi, Ehrlichia chaffeensis,* and *Borrelia burgdorferi* in patients from New York State. J. Clin. Microbiol. **35:**2198–2205.