J. Vet. Sci. (2005), **6**(2), 151–155

JOURNAL OF
Veterinary
Science

Identification and prevalence of *Ehrlichia chaffeensis* infection in *Haemaphysalis longicornis* ticks from Korea by PCR, sequencing and phylogenetic analysis based on 16S rRNA gene

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Genomic DNAs extracted from 1,288 *Haemaphysalis longicornis* ticks collected from grass vegetation and various animals from nine provinces of Korea were subjected to screening by genus-specific (*Ehrlichia* spp. or *Anaplasma* spp.) real-time TaqMan PCR and species-specific (*E. chaffeensis*) nested-PCR based on amplification of 16S rRNA gene fragments. In all, 611 (47.4%) ticks tested positive for genus-specific amplification of 116 bp fragment of 16S rRNA of *Ehrlichia* spp. or *Anaplasma* spp. Subsequently, 396 bp *E. chaffeensis*-specific fragment of 16S rRNA was amplified from 4.2% (26/611) tick samples. The comparison of the nucleotide sequence of 16S rRNA gene from one tick (EC-PGHL, GeneBank accession number AY35042) with the sequences of 20 *E. chaffeensis* strains available in the database showed that EC-PGHL was 100% identical or similar to the Arkansas (AF416764), the Sapulpa (U60476) and the 91HE17 (U23503) strains. The phylogenetic analysis also revealed that the *E. chaffeensis* EC-PGHL formed a single cluster with the above strains. This is the first study to report molecular detection and phylogenetic analysis of *E. chaffeensis* from *H. longicornis* ticks in Korea. The implicit significance of *E. chaffeensis* infection in *H. longicornis* ticks in Korea is discussed.

Key words: *Ehrlichia chaffeensis*, *Haemaphysalis longicornis*, prevalence, PCR

Introduction

Ehrlichia species are strict intracellular gram-negative bacteria that parasitize monocytes, granulocytes or platelets and are responsible for various vector-borne diseases in animals as well as human in different parts of the world [8,9,24]. Human monocytic ehrlichiosis (HME) caused by *Ehrlichia chaffeensis,* is an emerging tick-borne infectious disease [12,22,23] generally characterized by clinical signs of fever (100%), rash (67%), myalgia (58%), vomiting, diarrhea, and headache (25%) [2,12,26]. Diagnosis of HME is still largely based on the combined evaluation of clinical signs, laboratory and epidemiological data. Since most physicians are unfamiliar with HME, this disease is often misdiagnosed and many cases develop into more serious conditions or become carriers following improper treatment with tetracyclines or doxycyclin [2,26]. Following the first report of HME in 1987 [20], the disease has been reported in more than 30 states in the USA [29], Europe [3,19,21,25], Africa [28], the Middle East [5,17], and Asia [6,7,15,16,27]. The recent emergence and increased recognition of diseases caused by tick transmitted *Ehrlichiae* has stimulated interest of researchers in the molecular biology of these obligate intracellular bacteria [4,11,13]. In 2002, we reported the serological evidence of *E. chaffeensis* infection in human patients in Korea [14]. In addition, in our earlier studies, *E. chaffeensis* was detected from *Ixodes persulcatus* tick [18]. Majority of *Haemaphysalis longicornis* ticks were also found infected with *Ehrlichia* spp. but the species-specific identification was not attempted [18]. Recently, we have detected *E. chaffeensis* infection in horse, cattle and cats in Korea [unpublished data]. *H. longicornis* is one of the predominant tick vector prevalent in Korea. Due to the increasing reports of prevalence of *E. chaffeensis* infection in ticks and human, the present study was aimed at investigating the epidemiology of *E. chaffeensis* infection in *H. longicornis* ticks collected from different geographic regions of Korea.

Material and Methods

Tick collection and DNA

In all, 1,288 *H. longicornis* ticks including nymph and larvae were collected by dragging a flannel cloth over grass

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or by picking nymphs and adult ticks from cattle, horses, goats, dogs, cats, hedgehogs and wild rodents from 9 Korean provinces [18]. The ticks were identified and categorized with respect to developmental stages, and stored at –20°C in 1.5 ml Eppendorf tubes until required. The genomic DNA from these ticks was extracted as described previously [18].

Amplification of the 16S rRNA gene of *Ehrlichia* spp. by real-time (TaqMan) PCR

The oligonucleotide primers ESP-F (5'-agtccacgctgtaaacg atgag-3') and ESP-R (5'-ttcctttgagttttagtcttgcgac-3') complementary to the conserved regions of the 16S rRNA gene fragment (116 bp) of both *Ehrlichia* spp. and *Anaplasma* spp. were used. The composition of PCR mix, reaction conditions and the sequence of PCR probe were essentially similar to those desribed earlier [18].

Amplification of *E. chaffeensis*-specific 16S rRNA gene fragment

For the first round PCR, primer ECC (5'-agaacgaacgctggc ggcaagc-3') and ECB (5'-cgtattaccgcggctgctggca-3') targeting the conserved regions of *Ehrlichia* spp. 16S rRNA gene were used [10]. For the second round nested-PCR, primers HE1 (5'-caattgcttataacctttggttataaat-3') and HE3 (5'-tataggta ccgtcattatcttccctat-3') targeting *E. chaffeensis*-specific region of 16S rRNA gene were used [1]. The PCR mix for the first round PCR consisted of 2.5 µl of 10X PCR buffer, 2.5 µl of 25 mM $MgCl_2$, 1 µl of 2.5 mM deoxynucleoside triphosphate (dNTPs), 2.5 U of Taq-polymerase (Promega, USA), 5 pmol of each primer, EEC and ECB (Genotech, Korea), and 200 ng of template DNA in a total volume of 25 µl. The PCR conditions included an initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 2 min, extension at 72°C for 1 min 30 sec, and one cycle of extension at 72°C for 7 min. For second round nested-PCR, 5 pmol of each primer, HE1 and HE3 (Genotech, Korea) and 5 µl of first PCR product as template DNA were included in the PCR mix described for first round PCR. The reaction conditions were as follows; three cycles of denaturation at 94°C for 1 min, annealing at 55°C for 2 min, extension at 72°C for 1.5 min, followed by 37 cycles of denaturation at 92°C for 1 min, annealing at 55°C for 2 min, extension at 72°C for 1 min. PCR products were electrophoresed in a 1% (w/v) agarose gel, stained with ethidium bromide and analyzed using a still video documentation system (Gel Doc 2000; Bio- Rad, USA).

Cloning and sequence analysis

PCR amplicons were purified using a GFX PCR DNA Purification Kit (Amersham, UK) according to the manufacturer's instructions. Purified amplicons were ligated into pGEM-T easy vector (Promega, USA) as per the instructions given by the manufacturer and transformed into TOP10F' *E. coli* competent cells. The recombinant clones were verified by colony PCR amplification as described above and the recombinant plasmid DNA was purified using the Quantum Plasmid Miniprep Kit (Bio-Rad, USA) as per the manufacturer's instructions. Sequencing was performed by dideoxy termination using an ABI PRISM 3700 DNA Analyzer (Applied Biosystems, USA). Sequence data was analyzed using Chromas software version. 1.51 (Technelysium, Australia). The homology searches were made at National Center for Bio-technology Information (NCBI, USA) BLAST network service. Nucleotide sequences were aligned and phylogenetic analysis was performed using the Multiple sequence alignment program, AlinX (Vector NTI Suite version. 5.2.1.3.; InforMax, USA).

Results

Genomic DNAs extracted from 1,288 *H. longicornis* ticks collected from grass vegetation and various animals from nine provinces of Korea were subjected to screening by genus-specific (*Ehrlichia* spp. or *Anaplasma* spp.) and species-specific (*E. chaffeensis*) PCR based on amplification of 16S rRNA gene fragments. In all, 611 (47.4%) ticks tested positive for genus-specific amplification of 116 bp fragment of 16S rRNA of *Ehrlichia* spp. or *Anaplasma* spp. (Table 1). Of these more than 80% ticks collected from Gyeonggi province alone and at least one sample from each province were found PCR positive (Table 1). Subsequently, 396 bp *E. chaffeensis*-specific fragment of 16S rRNA was amplified from 4.2% (26/611) tick samples (Fig. 1). All the tick samples that tested positive to *E. chaffeensis* originated from Gyeonggi province (Table 1). The 396 bp PCR product

Table 1. PCR screening of *Haemaphysalis longicornis* ticks collected from different provinces of Korea

Province/Place*	Number of ticks	PCR positive	
		Ehrlichia and/or *Anaplasma* spp.	*E. chaffeensis*
Gangwon	10	1	0
Gyeonggi**	896	489	26
Chungbuk	40	10	0
Chungnam	10	2	0
Gyeongbuk	20	7	0
Gyeongnam	25	20	0
Jeonbuk	96	16	0
Jeonnam	32	11	0
Jeju	159	55	0
Total (%)	1,288	611 (47.4)	26 (2.0)

*Ticks were collected from grass vegetation, from cattle and horse ranches and from different animals such as cattle, horse, dogs and rodents (data not shown).

**Ticks were collected from rice fields and army training sites of Gyeonggi province.

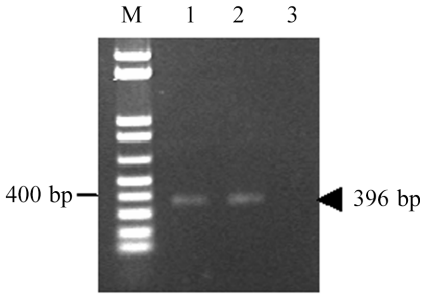

Fig. 1. Agarose gel showing *Ehrlichia chaffeensis*-specific PCR amplicon (396 bp) generated by nested-PCR using primers ECC/ECB in the primary reaction and HE1/HE3 in the nested reaction (396 bp). Lanes: 1, positive control (*E. chaffeensis* Arkansas strain); 2, DNA from the *H. longicornis*; 3, negative control (non-infected tick DNA); M, 100 bp DNA molecular mass marker (Genepia, Korea).

of *E. chaffeensis*-specific 16S rRNA gene obtained from one tick was sequenced and registered with the GenBank under the accession number AY35042 (strain EC-PGHL). The comparison of nucleotide sequence of strain EC-PGHL with the sequences of 20 representative *E. chaffeensis* strains available in the GenBank database showed that EC-PGHL was 100% identical or similar to the Arkansas (AF416764), the Sapulpa (U60476) and the 91HE17 (U23503) strains, all of these originate from the USA (Table 2). The phylogenetic analysis also revealed that the *E. chaffeensis* EC-PGHL formed a single cluster with the above strains (Fig. 2).

Discussion

Recenty, advances within molecular methods have made it possible to detect fastidious and hard-to-culture bacteria like *Ehrlichia* spp. without the need of isolation by conventional culture methods. PCR makes it possible to identify the presence of DNA of such fastidious bacteria even in culture-negative samples and directly from clinical samples collected from patients with suspected infection [14]. Competitive PCR is a standard method for this purpose as it allows the quantification of DNA and has been used successfully in a number of studies. However, this technique is labor intensive and requires that the results of multiple reactions be analyzed for each sample. In this study, we used a real-time TaqMan PCR assay as an initial screening procedure for the identification of *Ehrlichia* spp. or

Table 2. Homology comparison of the *Ehrlichia chaffeensis* 16S rRNA gene fragment (396 bp) sequences

No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1		100	100	100	98.5	97.9	97.7	97.4	97.2	96.9	96.4	96.4	96.4	96.2	96.2	96.2	95.6	94.1	92.3	92.1	91.1
2	0		100	100	98.5	97.9	97.7	97.4	97.2	96.9	96.4	96.4	96.4	96.2	96.2	96.2	95.6	94.1	92.3	92.1	91.1
3	0	0		100	98.5	97.9	97.7	97.4	97.2	96.9	96.4	96.4	96.4	96.2	96.2	96.2	95.6	94.1	92.3	92.1	91.1
4	0	0	0		98.5	97.9	97.7	97.4	97.2	96.9	96.4	96.4	96.4	96.2	96.2	96.2	95.6	94.1	92.3	92.1	91.1
5	6	6	6	6		99.5	99.2	96.7	96.4	96.2	96.4	96.4	96.4	96.7	96.1	96.7	95.9	93.6	93.1	93.1	92.0
6	8	8	8	8	2		98.2	96.2	96.4	95.9	95.6	95.6	95.6	96.4	95.4	96.7	95.1	93.1	92.5	92.6	91.8
7	9	9	9	9	3	5		96.9	96.7	96.4	96.1	96.1	96.6	95.9	95.9	95.9	96.1	94.6	92.8	93.1	92.3
8	10	10	10	10	13	13	12		99.7	99.5	95.9	95.9	95.1	96.4	95.7	96.4	94.9	96.7	91.6	91.4	92.1
9	11	11	11	11	14	14	13	1		99.7	95.7	95.7	94.9	96.2	95.4	96.2	94.6	96.4	91.9	91.7	91.9
10	12	12	12	12	15	15	14	2	1		95.4	95.4	94.6	95.9	95.1	95.9	94.4	96.2	91.1	90.9	91.6
11	13	13	13	13	14	15	14	16	17	18		100	95.4	95.7	99.7	95.7	95.6	93.1	92.1	91.9	90.6
12	13	13	13	13	14	15	14	15	17	18	0		95.4	95.7	99.7	95.7	95.6	93.1	92.1	91.9	90.6
13	13	13	13	13	14	15	14	18	20	21	18	18		94.4	95.1	94.4	99.2	92.9	91.0	91.3	92.0
14	15	15	15	15	13	15	14	14	15	14	17	17	22		95.4	100	95.2	93.9	91.4	91.4	91.1
15	15	15	15	15	14	16	15	16	17	16	1	1	28	24		95.4	95.7	92.9	91.8	91.6	90.3
16	15	15	15	15	13	13	16	14	15	16	17	17	22	0	17		95.2	93.9	91.4	91.4	91.1
17	17	17	17	17	16	17	15	20	21	22	16	16	3	19	17	19		92.9	91.3	90.5	92.1
18	21	21	21	21	25	26	20	13	14	15	27	27	28	24	28	24	27		90.4	91.6	91.4
19	30	30	30	30	27	27	27	33	32	33	31	31	35	34	31	34	34	38		99.7	91.5
20	31	31	31	31	27	27	26	34	33	32	32	32	34	34	32	34	34	38	1		91.8
21	34	34	34	34	29	28	28	30	32	31	33	33	30	31	35	35	30	31	31	30	

Percent identities between sequences of *Ehrlichia chaffeensis* 16S rRNA gene fragment is shown as the upper matrix. The lower matrix shows the number of nucleotide differences. 1, EC-PGHL Korea, AY35042; 2, *E. chaffeensis* Arkansas [USA], AF416764; 3, *E. chaffeensis* Sapulpa [USA], U60476; 4, *E. chaffeensis* 91HE17 [USA], U23503; 5, *Ehrlichia* sp. Tibet, AF414399; 6, *Ehrlichia* sp. EHt224, AF311968; 7, *Ehrlichia* sp. ERm58, AF311967; 8, *Ehrlichia* sp. HF565, AB024928; 9, *E. chaffeensis* HI-2000, AF260591; 10, *Ehrlichia* sp. Anan, AB028319; 11, *E. ovina*, AF318946; 12, *E. canis isolate* VDE, AF373613; 13, *Cowdria ruminantium,* U03776; 14, *E. ewingii* HH591-2, AY093440; 15, *Ehrlichia* sp. Germishuys, U54805; 16, *E. ewingii* 95E9-TS,U96436; 17, *Cowdria* sp. South African canine, AF325175; 18, *E. muris*, U15527; 19, *A. phagocytophilla,* AY055469; 20, *Ehrlichia* sp., AJ242785; 21, *Ehrlichia* like sp. Schotti variant, AF104680.

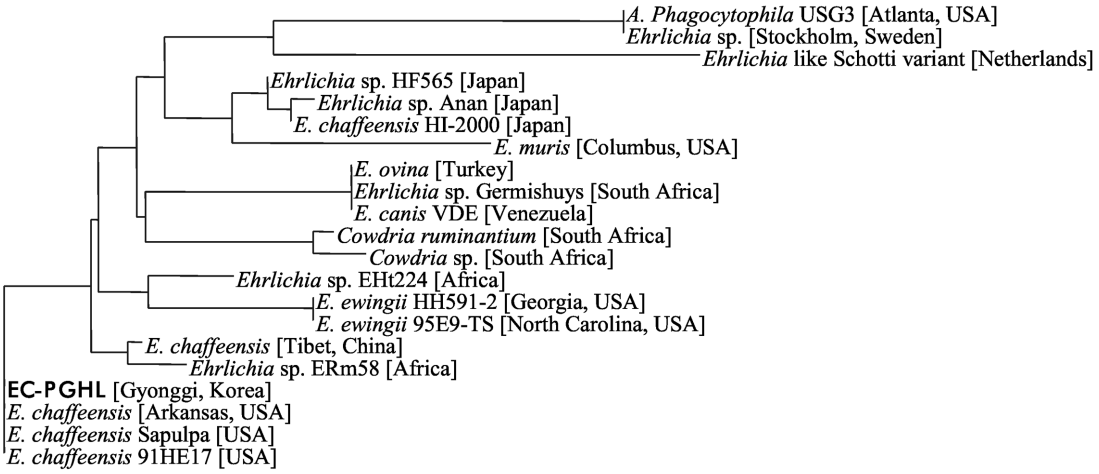

Fig. 2. Phylogenetic tree of 16S rRNA sequences of *Ehrlichia* spp. inferred using the sequence distance method and the neighbor joining algorithm. 16S rRNA gene sequences (396 bp) from 21 *Ehrlichia* isolates available in the GenBank database were included. Korean genotype of *E. chaffeensis* is shown with bold faced letter.

Anaplasma spp. DNA from tick samples. With this procedure, 611 (47.4%) out of 1,128 ticks collected from 9 provinces of Korea were identified as PCR positive. Most of the ticks (896/1,288) investigated in this study originated from the rice fields and army training sites of Gyeonggi province. Other ticks were collected from grass vegetation and cattle and horse ranches as well as from different animals such as cattle, horse, dogs and rodents (data not shown). At least one tick collected from each province was infected with *Ehrlichia* spp. and or *Anaplasma* spp. Subsequently, 611 samples that tested PCR positive in the initial screening with real-time TaqMan PCR were further subjected to species-specific detection of *E. chaffeensis* DNA by nested-PCR. Out of 611 *H. longicornis* ticks tested, 26 (4.3%) revaled PCR positive as evidenced by amplification of a unique 396 bp *E. chaffeensis*-specific PCR product. All (100%) the tick samples that tested PCR positive originated from Gyeonggi province. The higher PCR positive rates among ticks collected from Gyeonggi province may be due to the reason that maximum number of samples screened in this study originated from Gyeonggi province.

We have previously demonstrated the serological evidence of *E. chaffeensis* infection among Korean human patients [14] as well as in *I. persulcatus* ticks [18]. Although, the primary vector for *E. chaffeensis* is the lone star tick, *Amblyomma americanum,* but *A. testudinarium*, *Haemaphysalis yeni, H. flava* and *Ixodes ricinus* have also been identified as reservoirs [1,10,12,14]. In this study we detected *E. chaffensis* DNA in *H. longicornis* which is one of the predominant species of tick and often found in association with humans and animals in Korea [18]. The prevalence of *E. chaffeensis* infection in 4.3% ticks observed in this study indicate that *H. longicornis* may be predominant carrrier of *E. chaffeensis* infection in Korea and warrants further studies to investigate its possible impact on human or animal health.

Due to the geographic location of Korea, we expected that the amplified 16S rRNA gene from the tick EC-PGHL would reveal higher degrees of sequence similarity to other Asian isolates. However, the 16S rRNA sequenced from Korean *E. chaffenesis* was 100% identical or similar when compared with 16S rRNA gene sequence of the Arkansas (AF416764), the Sapulpa (U60476) and the 91HE17 (U23503) strains of *E. chaffeensis,* all of these originate from the USA. We also performed phylogenetic analysis of EC-PGHL strain in order to determine the epidemiological origin. Phylogenetic analysis also revealed that the sequence of *E. chaffeensis* EC-PGHL clustered closely on the same branch with the USA isolates. These observations suggest the possibility that *E. chaffenesis* may have migrated between USA and Korea, though such conclusion requires more evidence. To the best of our knowledge, this is the first study showing the genetic analysis of *E. chaffenesis* in *H. longicornis* ticks collected in Korea. Our findings suggests that *E. chaffeensis* may be widespread among *H. longicornis* ticks in Korea. More studies should be sought to determine its possible impact on human and animal health.

Acknowledgments

This work was supported by a Korea Research Foundation Grant (KRF-2001-041-G00090). Funding for this work was supported in part by the U.S. Department of Defense Global Emerging Infections Surveillance and Response System and Armed Forces Medical Intelligence Center, USA.

References

1. **Anderson BE, Sims KG, Olson JG, Childs JE, Piesman JF, Happ CM, Maupin GO, Johnson BJ.** *Amblyomma*

americanum: a potential vector of human ehrlichiosis. Am J Trop Med Hyg 1993, **49**, 239-244.
2. **Bakken JS, Dumler JS.** Human granulocytic ehrlichiosis. Clin Infect Dis 2000, **31**, 554-560.
3. **Baumgarten BU, Rollinghoff M, Bogdan C.** Prevalence of *Borrelia burgdorferi* and granulocytic and monocytic ehrlichiae in *Ixodes ricinus* ticks from southern Germany. J Clin Microbiol 1999, **37**, 3448-3451.
4. **Biswas B, Mukherjee D, Mattingly-Napier BL, Dutta SK.** Diagnostic application of polymerase chain reaction for detection of *Ehrlichia risticii* in equine monocytic ehrlichiosis (Potomac horse fever). J Clin Microbiol 1991, **29**, 2228-2233.
5. **Brouqui P, Dumler JS.** Serologic evidence of human monocytic and granulocytic ehrlichiosis in Israel. Emerg Infect Dis 2000, **6**, 314-315.
6. **Cao WC, Gao YM, Zhang PH, Zhang XT, Dai QH, Dumler JS, Fang LQ, Yang H.** Identification of *Ehrlichia chaffeensis* by nested PCR in ticks from southern China. J Clin Microbiol 2000, **38**, 2778-2780.
7. **Cao WC, Zhao QM, Zhang PH, Dumler JS, Zhang XT, Fang LQ, Yang H.** Granulocytic Ehrlichiae in *Ixodes persulcatus* ticks from an area in China where Lyme disease is endemic. J Clin Microbiol 2000, **38**, 4208-4210.
8. **Conrad, ME.** *Ehrlichia canis*: a tick-borne rickettsial-like infection in humans living in the southeastern United States. Am J Med Sci 1989, **297**, 35-37.
9. **Davidson DE, Dill GS, Tingpalapong M, Premabutra S, Nguen PL, Stephenson EH, Ristic M.** Canine ehrlichiosis (tropical canine pancytopenia) in Thailand. Southeast Asian J Trop Med Public Health 1975, **6**, 540-543.
10. **Dawson JE, Anderson BE, Fishbein DB, Sanchez, JL, Goldsmith CS, Wilson KH, Duntley CW.** Isolation and characterization of an *Ehrlichia* sp. from a patient diagnosed with human ehrlichiosis. J Clin Microbiol 1991, **29**, 2741-2745.
11. **Dawson JE, Biggie KL, Warner CK, Cookson K, Jenkins S, Levine JF, Olson JG.** Polymerase chain reaction evidence of *Ehrlichia chaffeensis*, an etiologic agent of human ehrlichiosis, in dogs from southeast Virginia. Am J Vet Res 1996, **57**, 1175-1179.
12. **Dumler JS, Bakken JS**. Ehrlichial diseases of humans: emerging tick-borne infections. Clin Infect Dis 1995, **20**, 1102-1110.
13. **Ewing SA, Dawson JE, Kocan AA, Barker RW, Warner CK, Panciera RJ, Fox JC, Kocan KM, Blouin EF.** Experimental transmission of *Ehrlichia chaffeensis* (Rickettsiales: Ehrlichieae) among white-tailed deer by *Amblyomma americanum* (Acari: Ixodidae). J Med Entomol 1995, **32**, 368-374.
14. **Heo EJ, Park J. Koo JR, Park MS, Park MY, Dumler JS, Chae JS.** Serologic and molecular detection of *Ehrlichia chaffeensis* and *Anaplasma phagocytophila* (human granulocytic ehrlichiosis agent) in Korean patients. J Clin Microbiol 2002, **40**, 3082-3085.
15. **Heppner DG, Wongsrichanalai C, Walsh DS, McDaniel P, Eamsila C, Hanson B, Paxton H.** Human ehrlichiosis in Thailand. Lancet 1997, **350**, 785-786.
16. **Inokuma, H, Nane G, Uechi T, Yonahara Y, Brouqui P, Okuda M, Onishi T.** Survey of tick infestation and tick-borne ehrlichial infection of dogs in Ishigaki Island, Japan. J Vet Med Sci 2001, **63**, 1225-1227.
17. **Keysary A, Amram L, Keren G, Sthoeger Z, Potasman L, Jacob A, Strenger C, Dawson JE, Waner T.** Serologic evidence of human monocytic and granulocytic ehrlichiosis in Israel. Emerg Infect Dis 1999, **5**, 775-778.
18. **Kim CM, Kim MS, Park MS, Park J, Chae JS.** Identification of *Ehrlichia chaffeensis*, *Anaplasma phagocytophila* and *A. bovis* in *Haemaphysalis longicornis* and *Ixodes persulcatus* ticks from Korea. Vector-Borne Zoon Dis 2003, **3**, 17-26.
19. **Liz JS, Anderes L, Sumner JW, Massung RF, Gern L, Rutti B, Brossard M.** PCR detection of granulocytic ehrlichiae in *Ixodes ricinus* ticks and wild small mammals in western Switzerland. J Clin Microbiol 2000, **38**, 1002-1007.
20. **Maeda K, Markowitz N, Hawley RC, Ristic M, Cox D, McDade JE.** Human infection with *Ehrlichia canis* a leukocytic rickettsia. N Engl J Med 1987, **316**, 853-857.
21. **Morais JD, Dawson JE, Greene C, Filipe AR, Galhardas LC, Bacellar, F.** First European case of ehrlichiosis. Lancet 1991, **338**, 633-634.
22. **Murphy GL, Ewing SA, Whitworth LC, Fox JC, Kocan AA.** A molecular and serologic survey of *Ehrlichia canis*, *E. chaffeensis*, and *E. ewingii* in dogs and ticks from Oklahoma. Vet Parastiol 1998, **79**, 325-339.
23. **Paddock CD, Childs JE.** *Ehrlichia chaffeensis*: a prototypical emerging pathogen. Clin Microbiol Rev 2003, **16**, 37-64.
24. **Parola P, Raoult D.** Tick-borne bacterial diseases emerging in Europe. Clin Microbiol Infect 2001, **7**, 80-83.
25. **Ravyn MD, Lamb LJ, Jemmerson R, Goodman JL, Johnson RC.** Characterization of monoclonal antibodies to an immunodominant protein of the etiologic agent of human granulocytic ehrlichiosis. Am J Trop Med Hyg 1999, **61**, 171-176.
26. **Schutze GE, Jacobs RF.** Human monocytic ehrlichiosis in children. Pediatrics. 1997, **100**, E10.
27. **Shibata SI, Kawahara M, Rikihisa Y, Fujita H, Watanabe Y, Suto C, Ito T.** New *Ehrlichia* species closely related to *Ehrlichia chaffeensis* isolated from *Ixodes ovatus* ticks in Japan. J Clin Microbiol 2000, **38**, 1331-1338.
28. **Uhaa IJ, MacLean JD, Greene CR, Fishbein DB.** A case of human ehrlichiosis acquired in Mali: clinical and laboratory findings. Am J Trop Med Hyg 1992, **46**, 161-164.
29. **Walker DH, Dumler JS.** Emergence of the ehrlichioses as human health problems. Emerg Infect Dis 1996, **2**, 18-29.