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Ehrlichia and *Borrelia* spp. Infection in German Shepherd Dogs in Korea

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Abstract : The presence of the tick-borne pathogens *Ehrlichia* and *Borrelia* in German Shepherd dogs in Korea was determined by enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR). A total of 291 dogs were randomly selected from five Korean provinces from October 2005 through September 2006. The seroprevalence of antibodies to canine *Ehrlichia* and *Borrelia* agents detected by ELISA (Snap® 3Dx® Test, IDEXX Laboratories) was 7.56% (22 dogs) and 1.72% (5 dogs) respectively, throughout the country. Positive antibodies against both pathogens were detected in two dogs (0.69%). The provincial distribution of seroprevalence against *Ehrlichia* was1.28% (1 of 78) in Gyeonggi-do, 12.64% (11 of 87) in Gangwon-do, 9.76% (4 of 41) in Chungcheong-do, 8.93% (5 of 56) in Gyeongsang-do, and 3.45% (1 of 29) in Jeolla-do. According to PCR analysis, *Ehrlichia chaffeensis* target DNA was amplified in 3.09% (9 of 291 dogs) of blood samples, 2.41% (7 of 291) from Gangwon-do and 0.69% (2 of 291) from Chungcheong-do. The oligonucleotide sequences (SNU-EC3 and SNU-EC5) from the PCR fragment examined in Korea were closely related to *E. chaffeensis* isolated from the tick *Haemaphysalis longicornis*, in China and the state of Arkansas in the US. Based on these results, the presence of *E. chaffeensis* infection was identified in German Shepherds being bred in Korea. These results bring to light the importance of paying close attention to tick-borne infections such as Lyme disease during clinical diagnosis. This infectious disease should be included as a differential diagnosis for patients who participate in outdoor activity from spring to fall or who have thrombocytopenia or leucopenia.

***Key words* :** tick-borne pathogens, borreliosis, ehrlichiosis, *Ehrlichia chaffeensis*, German Shepherd.

Introduction

Ehrlichia and *Borrelia* are tick-borne pathogenic agents that are known to infect humans and animals throughout the world (15,18). Dogs can be infected by several species of *Ehrlichia* and *Borrelia* agents, which cause clinical diseases resulting in mild or no symptoms to severe clinical manifestation.

Ehrlichia canis and *Ehrlichia ewingii* are thought to be strictly canine parasites (2,20). *E. canis* infects monocytes and is the causative agent of classical canine ehrlichiosis (formally called tropical canine pancytopenia) (14). Dogs in the acute stage of *E. canis* infection respond well to treatment; however, the bacteria may be difficult to eliminate and dogs can become chronically infected, serving as reservoirs for the organism. When recrudescence occurs, dogs may experience severe disease. *E. ewingii* infects canine granulocytes and is responsible for a mild acute disease, canine granulocytic ehrlichiosis (CGE), which may lead to polyarthritis in chronically infected dogs (2,17,45). Several other *Ehrlichia* spp. may infect or cause diseases in dogs but they are usually associated with other hosts. *Ehrlichia chaffeensis* is the causative agent of human monocytic ehrlichiosis but following experimental injections in dogs, positive signs of infection were detected (11,13).

The only known vectors of *ehrlichieae* are ixodid ticks (40,41). Vectors for *E. canis* include the brown dog tick *Rhipicephalus sanguineus* (22) and possibly the American dog tick *Dermacentor variabilis* (25). The lone star tick, *Amblyomma americanum*, has been experimentally proven to transmit both *E. chaffeensis* (16) and *E. ewingii* (5). *D. variabilis* has also been implicated as a potential vector for *E. chaffeensis* (4).

Canine ehrlichiosis was first recognized in North America in 1962 in dogs from the state of Oklahoma (17). At that time, *E. canis* was the only species of *Ehrlichia* of concern for canines. Although classical canine ehrlichiosis and CGE have been recognized as diseases in Oklahoma, since their original descriptions, little information is available on the prevalence of these diseases or their causative agents. In a previous study of Oklahoma dogs, an indirect fluorescent antibody (IFA) test was used to examine antibody prevalence to *E. canis* within sera (42). The study revealed a seroprevalence of 53% for *E. canis*. At that time, *E. ewingii* and *E. chaffeensis* had not yet been identified as separate species capable of infecting dogs. More recently, in a US study, in the southeastern portion of the state of Virginia, *E. chaffeensis* and *E. ewingii* were detected by polymerase chain reaction (PCR) and IFA in dogs, and that study implicated dogs as reservoirs for *E. chaffeensis*

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(12). Another recent US report revealed that granulocytic ehrlichiosis occurred in dogs from the states of Minnesota and Wisconsin and is likely a zoonotic disease caused by an agent closely related to *Anaplasma phagocytophilum* but distinct from *E. ewingii* (21).

Although no cases of human granulocytic ehrlichiosis (HGA) and human monocytic ehrlichiosis (HME) have been reported, seroepidemiological findings suggest the presence of HME and HGA agents in Korea (23,36). In 2000, the first suspected case of *E. chaffeensis* was reported in an active duty soldier stationed in Korea (43). Subsequently, Heo *et al.*, (23) identified antibodies against *E. chaffeensis* and *A. phagocytophilum* among serum samples from patients with febrile illness of otherwise unknown etiology in Korea by IFA and Western immunoblot tests. These pathogens have been identified in ticks collected from wild animals and grass vegetation in Korea with molecular evidence of *E. chaffeensis* and *A. phagocytophilum* based on genus-specific TaqMan® PCR and species-specific PCR (26). In 2005, Lee *et al.* identified *E. chaffeensis* in *Haemaphysalis longicornis* ticks from Korea by PCR.

Lyme borreliosis was first reported in a dog in 1984 (33). The pathogenic role of the tick for transmitting the spirochete *Borrelia burgdorferi* in humans is well established. Few human cases of borreliosis and detection of the agent from *Ixodes* ticks exist in Korea. The purpose of this study was to survey the prevalence of canine *Ehrlichia* and *Borrelia* infections in outdoor German Shepherd dogs in Korea.

Materials and Methods

Blood sample collection

Blood samples were collected from 291 dogs from five provinces, Gangwon-do (n = 87), Chungcheong-do (n = 41), Gyeongsang-do (n = 56), Jeolla-do (n = 29), Seoul and Gyeonggi-do (n = 78) in Korea between October 2005 and September 2006. Dogs sampled were from annual training programs at working dog training centers. Selection was random and based on handler participation. Whole blood was collected by venipuncture from the cephalic vein for complete blood cell count (CBC), serology, and PCR.

Complete blood cell count

Blood cells were counted using an MS9-5 Hematology Analyzer (MELET SCHLOESING Laboratories, France). Thrombocytopenia was classified into three levels as follows: < 100,000 platelets/μl of blood = severe thrombocytopenia; 100,000~200,000 platelets/μl of blood = moderate thrombocytopenia; and 200,000~500,000 platelets/μl = normal.

Serologic test

The Snap® 3Dx® assay for Heartworm/Borreliosis/Ehrlichiosis (IDEXX) was performed according to the manufacturer's recommendations. The sensitivity of this test for detecting ehrlichiosis and borreliosis is 98.9% and 95.0%, and the specificity is 98.2% and 99.9% respectively, according to information provided by the manufacturer.

PCR analysis

DNA extraction was performed with a Blood Total DNA Purification Kit (GENE ALL™, General Biosystem, Korea) using whole blood (200 μl) according to the manufacturer's instructions.

Detection of *E. chaffeensis*, *E. ewingii* and *E. canis*

Primers ECC and ECB amplify all *Ehrlichia* species (Table 1) (12). For identification of *Ehrlichia* species, the primary PCR product was used as the template in a second reaction mixture with each species specific primers. Primers HE1 and HE3 were used for *E. chaffeensis*-specific amplification (Table 1) (4), primers EE52 and HE3 were used for *E. ewingii*-specific amplification, and primers ECAN5 and HE3 were used for *E. canis*-specific amplification (Table 1) (35). The primer sequences used aligned with the corresponding regions of the *Ehrlichia* species 16S rRNA genes and with primers used for *E. chaffeensis* and *E. canis* PCR (12,35).

Positive control DNA was purified from *E. chaffeensis*-infected DH82 cells supplied by Dr. William Nicholson from the Center for Disease Control and Prevention, National Center for Infectious Disease, Atlanta, GA, USA. *B. burgdorferi* DNA was provided by Dr. Janet Foley at the Center for Vector-borne Diseases, University of California at Davis, CA, USA.

Table 1. Oligonucleotide primers used for the detection of tick-borne pathogens in Korea

Agents	Primers	Nucleotide sequences (5'-3')	Expected size	References
Ehrlichia spp.	ECC ECB	AGAACGAACGCTGGCGGCAAGC CGTATTACCGCGGCTGCTGGCA	450 bp	Dawson *et al.* (1994)
E. chaffeensis	HE1 HE3	CAATTGCTTATAACCTTTTGGTTATAAAT TATAGGTACCGTCATTATCTTCCCTAT	390 bp	Murphy *et al.* (1998)
E. canis	ECAN5 HE3	CAATTATTTATAGCCTCTGGCTATAGGA TATAGGTACCGTCATTATCTTCCCTAT	365 bp	Murphy *et al.* (1998)
E. ewingii	EE52 HE3	CGAACAATTCCTAAATAGTCTCTGAC TATAGGTACCGTCATTATCTTCCCTAT	350 bp	Murphy *et al.* (1998)
Borrelia burgdorferi	BBOSPF BBOSPR	AAAGAATACATTAAGTGCGATATT GGGCTTGTAAGCTCTTTAACTG	597 bp	Wang *et al.* (1999)

PCR reactions (20 μl) consisted of 50~100 ng of template DNA, 1 pmol of each primer, 200 μM dNTPs, PCR buffer (Super Bio, Korea), and 1 U of Super Taq DNA Polymerase (Super Bio, Korea). The enzyme was added to the reaction after an initial 3 min denaturation step at 94°C. Reactions with species-specific primers consisted of two-stages. The first stage entailed 3 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 2 min, and extension at 72°C for 1.5 min. The second stage involved 37 cycles of denaturation at 92°C for 1 min, annealing at 55°C for 2 min, and extension at 72°C for 1.5 min. Products of amplification reactions were separated on 1.0% agarose gels and visualized with ethidium bromide under an ultraviolet (UV) transilluminator.

Detection of *B. burgdorferi*

Identification of *B. burgdorferi* was performed using PCR with each species-specific primer set (Table 1) as described previously (19,39,46). The *osp*C gene was selected for the identification of *B. burgdorferi*. The temperature cycling profile for *B. burgdorferi* PCR was 1 cycle of 1 min at 96°C, followed by 20 cycles of 40 sec at 95°C, 35 sec at 54°C, and 2 min at 72°C.

The PCR reaction was performed using 50~100 ng of template DNA using species-specific primer sets and a PCR mixture as described previously. PCR products were electrophoresed in 1% agarose gel, stained with ethidium bromide, and photographed using a still video documentation system (Digital Gel Documentation System, UVT-260D, USA).

Cloning, nucleotide sequencing, and phylogenetic analysis

To confirm the identification of *Ehrlichia* species-specific PCR products with target-specific genes, the PCR products were purified by a Wizard Plus DNA Purification System (Promega, USA), ligated with a pGEM-T Easy Vector System (Promega), transformed into *E. coli* strain DH5α competent cells, and grown overnight at 37°C on Luria Bertani (LB) plates containing 100 ng/ml of ampicillin. The recombinant clones were verified by the colony PCR of the respective clones. Three clones of each isolate were arbitrarily chosen for sequencing the forward and reverse strands. Plasmid DNA for sequencing was prepared with SV Minipreps DNA Purification System (Promega) according to the manufacturer's instructions.

Amplified and purified DNA was prepared for direct sequencing using a QIAquick Gel Extraction Kit (QIAGEN Inc., Germany) and sequenced by dideoxy termination with an automatic sequencer (ABI 3100 Genetic Analyzer, Bionics, Korea). The sequence data were collected using ABI Prism Data Collection software (ver.2.1), and analyzed by ABI Prism Sequence analysis software (ver. 2.1.1) and Chromas software (ver.1.51) (Technelysium Pty., Ltd., Queensland, Australia).

Sequence homology searches were made via the National Center for Biotechnology Information (National Institute of Health) BLAST network service. The sequences were translated to amino acid sequences and aligned initially using the ClustalX (1.60) program. Sequences from the 16S rRNA gene of *E. chaffeensis* were used for phylogenetic analysis. Aligned sequences were determined by the neighbor-joining (NJ) method with nucleotide distances (p-distance) with 100 replications by the bootstrap test. Phylogenetic analysis based on the obtained sequences was conducted using maximum-likelihood estimation (PAUP* 4.0b for Macintosh™).

Statistical analysis

Statistical analysis was performed using the SPSS (ver. 12.0) statistical computer program. Chi-square (χ^2) tests and Fisher exact tests were used to compare each of the prevalence values among age, sex, and geographical distribution. $P < 0.05$ was considered statistically significant.

Results

There was a significant difference in the thrombocyte counts among non-infected, seropositive, and PCR positive dogs. Thrombocytopenia was found in 63.6% (14 of 22) of seropositive dogs and 77.8% (7 of 9) of PCR positive dogs (Table 2).

The prevalence of ELISA antibody to canine ehrlichial agents identified with the Snap® 3Dx® Test Kit was 7.56% (22 of 291 dogs) throughout the country and seroprevalence of canine borreliosis was 1.72% (5 of 291 dogs). Seropositive cases of both ehrlichiosis and borreliosis were found in 2 of 291 canine sera samples (0.69%) (Table 3).

Table 3. Serological test results of German Shepherd dogs (n = 291) in Korea

Antibodies	Number of positive	Positive rates (%)
Ehrlichiosis	22	7.56
Borreliosis	5	1.72
Both ehrlichiosis and borreliosis	2	0.69

Table 2. Thrombocytopenia in German Shepherd dogs (n = 291) in Korea

Classification	Number of seropositive / Number of tested (%)	Number of PCR positive / Number of tested (%)
Severe thrombocytopenia	8/22 (36.4)	5/9 (55.6)
Moderate thrombocytopenia	6/22 (27.3)	2/9 (22.2)
Normal range	7/22 (31.8)	2/9 (22.2)
Thrombocytopenia rate	14/22 (63.6)	7/9 (77.8)

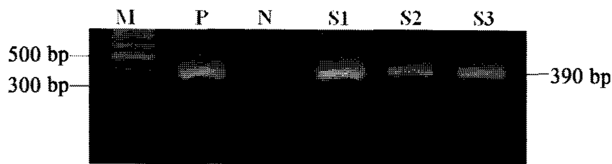

Fig 1. Nested PCR amplification of 390 bp products for *Ehrlichia chaffeensis* in German Shepherd dogs in Korea. Lane M, 100 bp DNA size marker; P, positive control; N, negative control; S1~S3, positive samples.

Table 4. Detection of tick-borne pathogens in German Shepherd dogs (n = 291) in Korea by species-specific nested PCR

Agents	Number of PCR positive	Infection rates (%)
E. chaffeensis	9	3.09
E. canis	0	0
E. ewingii	0	0
B. burgdorferi	0	0

Specific DNA of *E. chaffeensis* was amplified from 9 of 291 (3.09%) canine blood samples by species-specific nested PCR. Nested PCR results were negative for *E. canis* and *E. ewingii* (Fig 1 and Table 4). *B. burgdorferi* DNA was not amplified.

As presented in Fig 2, the phylogenetic tree represents the position of *E. chaffeensis* SNU-EC3 (GenBank accession number, GAN EU564809) and SNU-EC5 (GAN EU564810) from German Shepherd dogs in Gangwon-do, Korea. The tree was constructed after aligning the 16S rRNA gene fragment sequence (390 bp) obtained from GenBank and sequenced during this study with the ClustalX program. The scale represents a number of substitutions per base and indicates horizontal distance. The numbers at the nodes of the tree represent the number of bootstrap replicates of 400 which displayed the indicated sequence groupings. Phylogenetic analysis based on nucleotide sequence comparison also revealed that Korean strains of *E. chaffeensis,* SNU-EC3 and SNU-EC5, clustered closely to those of China and the United States.

The age distribution of the dogs ranged from 4-9 years. Positive Snap®3Dx® serologic ELISA results were identified in 14 of 130 (10.77%) 4-6 year olds and 8 of 78 (10.26%) 7-9 year olds (Table 5), while PCR analysis was positive in 3 of 130 (2.31%) 4-6 year olds and 6 of 78 (7.69%) 7-9 year olds (Table 5).

In terms of sex distribution, there was no significant difference in the prevalence rate, which was 6.30% (8 of 127) in female and 8.54% (14 of 164) in male dogs based on the sero-

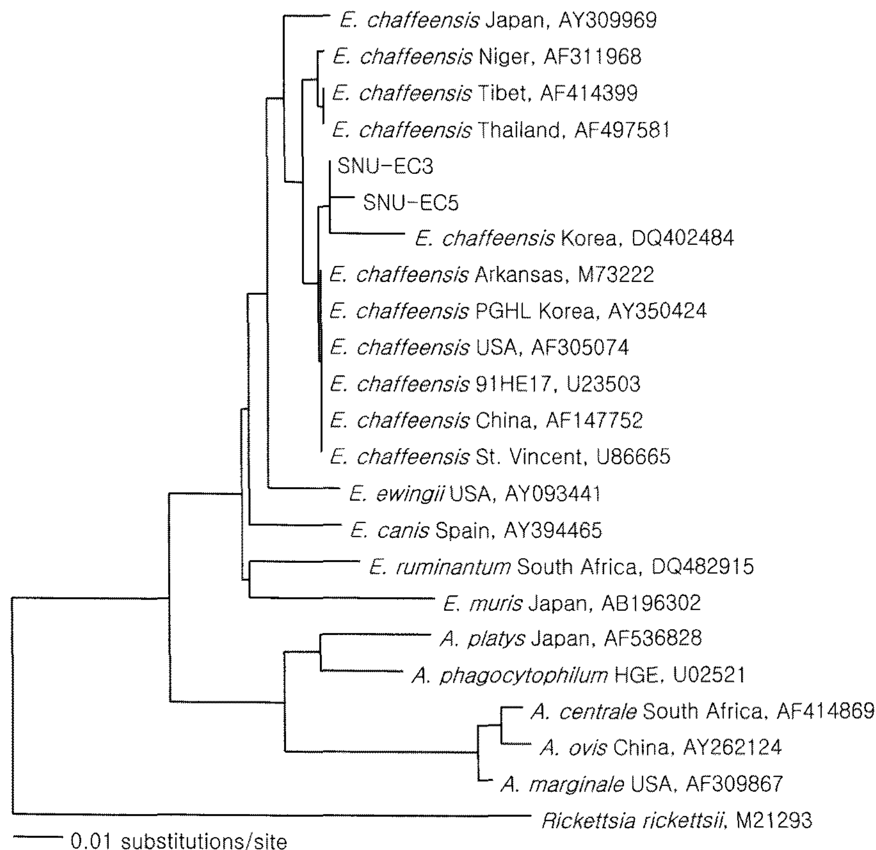

Fig 2. Phylogenetic tree generated using maximum-likelihood estimation (PAUP* 4.0b for Macintosh™) of 16S rRNA gene sequences from 23 representative *E. chaffeensis* SNU-EC3 and SNUE-C5 sequences amplified from German Shepherd dogs in Gangwon-do, Korea.

Table 5. Prevalence of ehrlichial agent by age of German Shepherd dogs in Korea

Age	Number of dogs	Seropositive (%)	PCR positive (%)
< 1 year	12	0	0
1-3 years	70	0	0
4-6 years	130	14 (10.77)	3 (2.31)
7-9 years	78	8 (10.26)	6 (7.69)
> 10 years	1	0	0
Total	291	22 (7.56)	9 (3.09)

Table 6. Sex-based differences in seroprevalence of ehrlichial agents in German Shepherd dogs in Korea

Sex	Number of dogs	Number of seropositive (%)	Number of PCR positive (%)
Female	127	8 (6.30)	3 (2.36)
Male	164	14 (8.54)	6 (3.66)
Total	291	22 (7.56)	9 (3.09)

Table 7. Geographical prevalence of canine ehrlichial agent in German Shepherd dogs in Korea

Provinces	Number of dogs	Number seropositive (%)	Number PCR positive (%)
Seoul, Gyeonggi-do	78	1 (1.28)	0
Gangwon-do	87	11 (12.64)	7 (8.05)
Chungcheong-do	41	4 (9.76)	2 (4.88)
Gyeongsang-do	56	5 (8.93)	0
Jeolla-do	29	1 (3.45)	0
Total	291	22 (7.56)	9 (3.09)

logic ELISA test and 2.36% (3 of 127) in female and 3.66% (6 of 164) in male dogs based on the PCR analysis (Table 6).

The regional distribution of seroprevalence was 1 of 78 (1.28%) in Seoul and Gyeonggi-do Province, 11 of 87 (12.64%) in Gangwon-do, 4 of 41 (9.76%) in Chungcheong-do, 5 of 56 (8.93%) in Gyeongsang-do, and 1 of 29 (3.45%) in Jeolla-do. Of the 22 positive sera, the highest prevalence was observed in Gangwon-do (Table 7).

Discussion

Since human granulocytic and monocytic ehrlichiosis were first reported in 1994 and 1987 respectively, they have been found in many countries through molecular and serologic testing (8,10,34). Most *Ehrlichia* and *Anaplasma* species were found in *Ixodes* ticks in the US and Europe (1,30,44). In Asia, *Ehrlichia* spp. were identified from *Haemaphysalis* spp., as well as *Ixodes* ticks (24,26). *Haemaphysalis longicornis* is generally the most abundant species in Korea, especially around pastures for grazing cattle. Recently, several papers have documented the existence of *Ehrlichia* and *Anaplasma* agents within vectors, domestic/wild animals and humans in Korea (9,27,28).

E. chaffeensis sequences were compared with other isolates of *E. chaffeensis* available in the GenBank database. The sequence identity of *E. chaffeensis* SNU-EC3 and SNU-EC5 (present study) were 99.7% and 99.2% compared to a Korean strain from tick (AY350424), USA isolate (AF416764), and Chinese isolate (AF147752), respectively. Comparative analysis of nucleotide sequences of Korean strains determined in this study with the 16S rRNA gene fragment sequences of 22 known *Ehrlichia* species were available in the GenBank database. The phylogenetic analysis showed that *E. chaffeensis* SNU-EC3 and SNU-EC5 were clustered together with those of *E. chaffeensis*-Korea, -USA, and -Chinese strains. This result indicates the possibility of an epidemiological link between Korea and China. It will be interesting to characterize these pathogens further on a molecular level to identify their chain of transmission and possible human health hazard.

Specific DNA of *B. burgdorferi* was not amplified in this study. The first detection of *Borrelia* sp. was from *Ixodes* ticks in Korea in 1992 (38). In 1993, Park *et al.* (37) isolated *B. burgdorferi* from *Ixodes persulcatus* and *Apodemus agrarius* in Korea. However, few cases of human Lyme borreliosis have been reported in Korea (29,31,32) and as of yet canine Lyme borreliosis has not been documented in Korea. We suspected the possibility of canine Lyme borreliosis infections in dogs, but did not find any amplification of *B. burgdorferi* DNA in this study; however, we did confirm the presence of antibodies against *B. burgdorferi* in dogs in Korea. Consequently, we presume that the canine *Borrelia* agent could be present in ticks and possibly reservoir animals in Korea.

Ixodes and *Haemaphysalis* ticks play an important role as reservoirs of latent infections of various tick-borne pathogens (3,6,7). In 2003, three members of the family *Anaplasmataceae* including *E. chaffeensis*, *A. phagocytophilum,* and *Anaplasma bovis* were initially described in Korea (26). This study has enabled us to provide further information on the epidemiology of tick-associated bacteria in Korea, where little information on this subject exists. Further studies are required for a detailed understanding of these newly emerging tick-borne diseases in Korea.

Veterinarians need to check for additional tick-borne pathogens in recreational, outdoor, and working dogs that travel to areas where ticks exist in high numbers. Korean veterinarians also need to check for *Babesia gibsoni* and *Anaplasma platys* and moreover, we recommend veterinarians check for tick-borne diseases, which include *E. chaffeensis* and *B. burgdorferi.*

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독일 셰퍼드 개에서 *Ehrlichia*와 *Borrelia* spp.의 감염

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요 약 : 국내 독일 셰퍼드로부터 에를리히아증과 라임병에 대한 항체가 조사를 위하여 효소면역측정법을 사용하였으며, 중합효소연쇄반응을 이용하여 진드기매개성 병원체를 조사하였다. 2005년 10월부터 2006년 9월까지 국내 5개 지방으로부터 291마리 독일 셰퍼드를 선정하였다. 개 에를리히아 항체 조사를 위한 ELISA (Snap® 3Dx® test kit, IDEXX) 검사결과 전국적으로 7.56%(22마리)의 에를리히아 양성 항체 보균개와 1.72%(5마리)의 보렐리아 양성 항체 보균개로 조사되었다. 이 중 2마리에서는 두 가지 병원체 모두에 대한 양성 항체가 검출되었다. 에를리히아에 대한 양성항체를 지방별로 보면 경기도는 1.28%(78마리 중 1마리), 강원도는 12.64%(87마리 중 11마리), 충청도는 9.76%(41마리 중 4마리), 경상도는 8.93%(56마리 중 5마리), 전라도는 3.45%(56마리 중 5마리)로 조사되었다. *Ehrlichia chaffeensis* 유전자를 검출한 결과 전국 평균이 3.09%(291마리 중 9마리), 강원도는 2.41%(291마리 중 7마리) 그리고 충청도는 0.69%(291마리중 2마리)로 조사되었다. 중합효소연쇄반응으로 증폭된 유전자(SNU-EC3과 SNU-EC5)에 대한 유전자염기서열분석결과 한국(*Haemaphysalis longicornis* 진드기)과 중국 그리고 Arkansas 분리주인 *E. chaffeensis*와 매우 유사하였다. 이 연구의 결과 국내에서 사육되는 독일셰퍼드에서 에를리히아증의 병원체와 라임병에 대한 항체가 검출된 것으로 볼 때 임상 진단에 있어서 진드기매개전염병에 대한 중요성을 인식해야 할 것으로 판단된다. 따라서 혈소판감소증이나 백혈구감소증이 있는 경우 그리고 봄부터 가을까지 야외활동을 한 경력이 있는 환자에 대한 감별진단 항목에 반드시 포함시켜야 할 것을 제안한다.

주요어 : 진드기매개병원체, 보렐리아증, 에를리히아증, 에를리히아 샤펜시스, 독일셰퍼드