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Detection of *Ehrlichia chaffeensis* pathogen from deer in Korea

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Abstract

Ehrlichia chaffeensis infects human and animals, and causes human monocytotropic ehrlichiosis (HME). It is emerging as a tick-borne zoonosis of concern. Although deer are important natural reservoir hosts of *E chaffeensis*, few surveys of deer in Korea for *E chaffeensis* have been conducted. Therefore, we conducted this study to confirm the occurrence of *E chaffeensis* in deer. To accomplish this study, we collected blood from total 27 deer and then polymerase chain reaction (PCR) and 16S rRNA sequence analyse to evaluate the samples for the presence of *E chaffeensis*. The results of this study indicated that 9 (33%) of the deer were infected with *E chaffeensis*. This is the first study to demonstrate that deer in Korea are infected with *E chaffeensis*, which indicates that they can act as a natural reservoir host for *E chaffeensis*.

Key words: *Ehrlichia chaffeensis*, Tick-borne zoonosis, PCR, 16S rRNA sequence, Deer.

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Introduction

Ehrlichia chaffeensis, which is primarily transmitted to humans by ticks from infected animals, is an obligatory intracellular bacterium that infects the monocyte-macrophage and causes human monocytic ehrlichiosis (HME). *E chaffeensis* infection also cause an acute febrile systemic illness that can be fatal when treatment is delayed due to misdiagnosis or when an immuno-compromised patient is infected[1-3]. Confirmation of *E chaffeensis* infection is based on the demon-

stration of typical inclusions in peripheral blood monocytes, which is increasingly being accomplished by polymerase chain reaction (PCR) using primers specific for *E chaffeensis*.

In Korea, *E chaffeensis* infection have been observed in human, small mammals, dogs and ticks using PCR assay[4-8]. Therefore, it is emerging as a tick-borne zoonosis of concern in Korea.

It has been reported that deer are important natural reservoirs of *E chaffeensis* in many countries[9-11], and infection of livestock by tick-borne diseases including *E chaffeensis* has caused economic losses[12].

Currently, approximately 140,000 deer are bred as livestock in Korea, and 10,000 of these are in Jeonbuk. Although deer are important livestock animals in Korea, few studies evaluating the infection of deer with *E chaffeensis* have been conducted. Therefore, we conducted this study to evaluate the presence of *E chaffeensis* in farmed deer in Jeonbuk, Korea, using nested PCR analysis for their blood.

Materials and methods

Collection of blood samples

Blood was collected from the antlers of 27 Korean spotted deer (*Cervus nippon*) that were bred on farms in Jeonbuk. It was contained into an ethylenediaminetetraacetic acid (EDTA) anticoagulant tube. The DNA from the samples was then extracted and analyzed for the presence of *E chaffeensis* using PCR as described below.

DNA extraction

Genomic DNA was extracted from the blood samples using a GENE ALL™ Blood Total DNA Purification kit (General bio system, Korea) according to the manufacturer's instructions.

Nested PCR for the detection of *E chaffeensis*

Initially, for amplification of *Ehrlichia* spp. 16S rRNA gene, ECC and ECB primers (Table 1)[13] were used with a total PCR reaction volume of 20 ㎕. The PCR mixture contained 2 ㎕ of template DNA in 1 ㎕ of 10 mM deoxynucleoside triphosphate (dNTPs), 2 ㎕ of 10× PCR buffer (w/20 mM $MgCl_2$), 0.3 ㎕ of 10 pmol ECC and ECB primers and 0.3 ㎕ of 5 U/mL *Taq* DNA polymerase (iNtRon Biotechnology, Korea).

The amplification conditions for PCR consisted of initial denaturation at 94℃ for 3 min, followed by 30 cycles of denaturation at 94℃ for 1 min, annealing at 65℃ for 2 min and extension at 72℃for 2 min, with one cycle of final extension at 72℃ for 10 min.

Nested amplification targeting the *E chaffeensis*-specific region of the 16S rRNA gene was conducted using 2 ㎕ of the primary PCR product as a template DNA in a second 20 ㎕ reaction mixture that contained the specific primers, HE1 and HE 3 (Table 1)[1]. The remainder of the mixture was identical to that used for amplification of *Ehrlichia* spp. The nested PCR was conducted in two stages. The first stage consisted of 2 cycles of denaturation at 94℃ for 1 min, annealing at 55℃

for 2 min and extension at 72℃ for 1.5 min, and the second consisted of 36 cycles of denaturation at 92℃ for 1.5 min, annealing at 55℃ for 2 min, extension at 72℃ for 1.5 min and one cycle of final extension at 72℃ for 10 min.

Table 1. Oligonucleotide primers for the detection of *E chaffeensis* pathogen

Target species	Step	Oligonucleotide primers	Sequence(5'-3')	Annealing Temp(℃)	Target gene
E chaffeensis	First	ECC	AGAACGAACGCTGGCGGCAAGC	65	16S rRNA
		ECB	CGTATTACCGCGGCTGCTGGCA		
	Second	HE1	CAATTGCTTATAACCTTTTGGTTATAAAT	55	16S rRNA
		HE3	TATAGGTACCGTCATTATCTTCCCTAT		

Cloning and sequence analysis

For PCR analysis of *E chaffeensis*, amplified genomic DNA was prepared for sequencing using the GFX™ PCR DNA purification kit (Amersham Biosciences, UK) according to the manufacturer's instructions. Purified amplicons were then ligated into T&A cloning vector kit (RBC, Taiwan) and transformed into DH5α *E coli* competent cells (RBC, Taiwan). The recombinant clones were verified using one-step plasmid preparation, and the recombinant plasmid DNA was then purified using the Wiazrd® plus SV minipreps (Promega, USA). Sequencing was performed using dideoxy termination method with an automatic sequencer (ABI PRISM® 3700 DNA Analyzer, USA). The sequence data were then collected using ABI Prism data collection software (ver. 2.1) and analyzed using ABI Prism sequence analysis software (ver. 2.1.1) and chromas software (ver. 2.3) (Technelysium Pty, Ltd, Australia). Sequence homology searches were conducted using the National Center for Bio-technology Information (National Institute of Health, USA) BLAST network service. The nucleotide sequences were then aligned and compared using Mult-Alin software (ver. 5.4.1) (http://bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html) and an alignment program (http://xylian.igh.cnrs.fr/bin/align-guess.cgi).

Identification of amplified DNA

Each PCR product was separated by 1.5% agarose gel electrophoresis, stained with ethidium bromide (EtBr) and photographed using a Gel-Doc 2000 system (Bio-USA).

Results

Blood samples collected from 9(33.3%) deer in Jeonbuk, Korea, were positive for *E chaffeensis*, as indicated by the presence of an approximately 390 bp band (Fig 1). No bands for PCR product was observed in control sample that included uninfected deer blood (lane N in Fig 1) and water (lanes W in Fig 1).

A phylogenetic analysis of two (deer-E3 and E12) of nine samples that were positive for *E chaffeensis* revealed that E3 and E12 were 98.7% homologous.

In addition, the sequence of sample deer-E3 was 99.7 % homologous to that of samples obtained from the USA (accession No. AF416764), Korea (accession No. AY350424) and China (accession No. AF147752). The sequence of sample deer-E3 was also found to be 99.2% homologous with *E chaffeensis* from whole blood obtained from dog (EF621763) in Korea. And the sequence of sample deer-E 12 were also found to be 98.5% homologous to that of samples from the USA (accession No. AF416764), Korea (accession No. AY350424) and China (accession No. AF147752).

Taken together, these results indicate that *E chaffeensis* obtained from deer in Korea is highly similar to the sequence obtained from *H lognicornis* tick (accession No. AY350424) and dog (EF621763), which suggests that the DNA of *E chaffeensis* from animals and ticks in Korea are nearly identical. Sequences obtained from deer-E 3 and E 12 of this study were deposited into Genbank of the National Center for Bio-Information (National Institute of Health) BLAST network service as accession No. EU-682762 and EU682763, respectively.

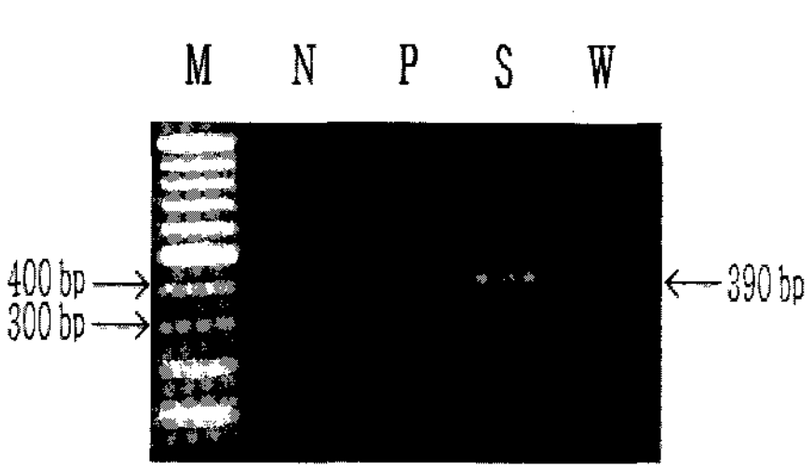

Fig 1. 1.5% Agarose gel electrophoresis of the PCR amplified *E chaffeensis* gene reveals the presence of a 390 bp DNA fragment.

Lane: (M) DNA marker (1 kbp DNA ladder), (N) control consisting of normal deer blood, (P) positive control (cattle blood infected with *E chaffeensis*), (S) deer sample, (W) water control to ensure that no contamination of primers occurred.

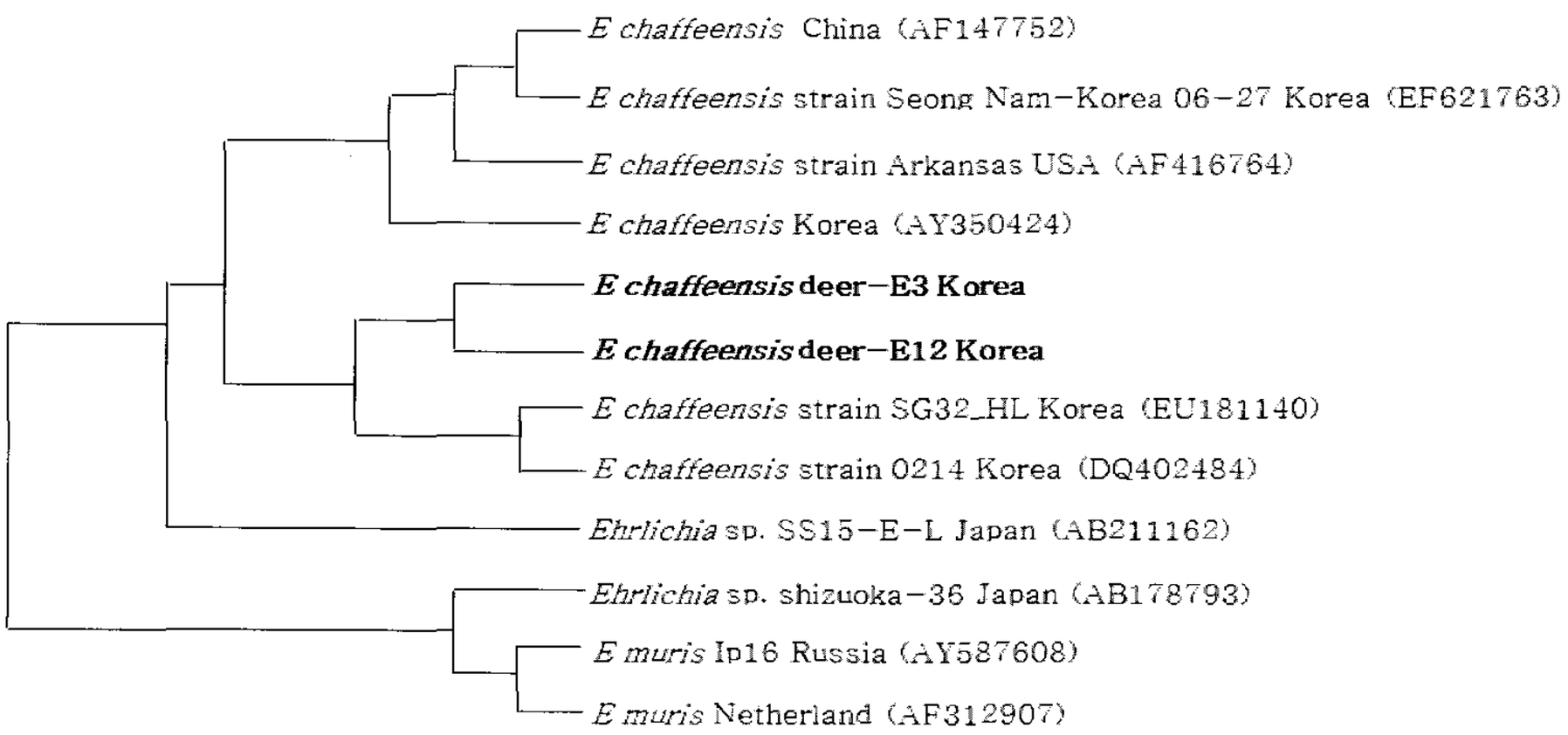

Fig 2. Phylogenetic tree of the *E chaffeensis* pathogen in deer in Korea.

The homologies of two samples (deer-E3 and E12) that were positive for *E chaffeensis* were evaluated and found to be 98.7% homologous for E3 and E12. The sequences of two samples are highly similar to that of *E chaffeensis* found in Korea.

Discussion

This study was conducted to detect *E chaffeensis* infection in deer in Jeonbuk, Korea. *E chaffeensis* is emerging as a tick-borne zoonosis and the presence of this pathogen has been investigated in deer in many countries[9-11,13-15]. In addition, Heo et al (2002) identified antibodies against *E chaffeensis* in Korean patients[4]. Furthermore, *E chaffeensis* has been identified in *H longicornis* and *Ixodes persulcatus* ticks, as well as in dogs and small mammals in Korea[5-8].

Varela et al[14] reported that all deer that were experimentally subjected to primary and secondary infection with *E chaffeensis* were positive when PCR was conducted on their whole blood. The positive PCR results following secondary infection indicate that primary infection of deer with *E chaffeensis* does not protect against subsequent exposure. Therefore, failure to protect deer from infection with *E chaffeensis* in deer can adversely impact the production of deer.

PCR amplification of *E chaffeensis* on whole blood from white-tailed deer indicates that they are capable of supporting an *E chaffeensis* infection that results in rickettsemia for at least 2 weeks, and that they are capable of becoming persistently infected with *E chaffeensis*[9,13].

In 1997, Lockhart et al verified that *E chaffeensis* is primarily maintained in nature via a tick vector-vertebrate reservoir system that consists of lone star ticks and white-tailed deer[15]. This is similar to the results of our study, which suggest that the detection of *E chaffeensis* in the whole blood of deer could occur as a result of rickettsemia due to repeated infection by ticks in wild.

In 2006, Eum et al[16] conducted a study of deer infected with tick-borne diseases, including *Theileria orientalis*, *Babesia odocoilei*, *Anaplasma phagocytophilum* and *E chaffeensis*, in Jeonbuk. The results of their study indicated that *T orientalis* was only detected in all tested deer. However, we found that 33.3% of the deer evaluated in our study were infected with *E chaffeensis*.

Phylogenetic analysis of *E chaffeensis* was conducted using the two PCR product (deer-E3 and E12) of amplified nine samples. The results revealed that the sequences of these products were 99.7% and 98.5% homologous to that of *E chaffeensis* (accession No. AY350424) obtained from *H longicornis* tick in Korea. This tick species is overwhelmingly numerous among tick species collected from grazing animals in Korea[5-6]. Therefore, we should collect ticks from deer infected with *E chaffeensis* and investigate for tick species. Also, it will be allowed the survey for the homology of *E chaffeensis* found in blood samples and ticks in deer.

This study is the first to confirm the presence of *E chaffeensis* in deer in Korea, which suggests that deer in Korea are important reservoirs of *E chaffeensis*. Although only a small number of deer were tested, we observed a 33.3% infection rate. However, a larger study should be conducted to obtain a better estimate of the infection rate of *E chaffeensis* in deer in Korea. Furthermore, additional studies evaluating the presence

of other tick-borne pathogens, such as *Theileria*, *Babesia* and *Anaplasma*, in deer in Korea should also be conducted.

In conclusion, this study indicates that *E chaffeensis* may result in economic losses to deer farming operations.

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