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Epidemiological and genetic analyses of emerging tick-borne pathogens in bovine, caprine and canine

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Abstract

The Republic of Korea (ROK) has a diverse and widespread distribution of tick-borne pathogens (TBPs); however, the scarcity of published research limits the exhaustively understanding of their epidemiology. Therefore, the present study aimed to identify the prevalence of TBPs circulating among cattle, goats and dogs using molecular, serological and phylogenetic techniques, thus providing essential information that may assist in elucidating the epidemiology of these infectious agents in the country.

A total of 1,116 whole blood samples (384 cattle, 302 goats and 430 dogs) were collected in different provinces and metropolitan cities of the ROK, with which three independent studies were conducted:

The first one focused on evaluating the role that Korean cattle and goats may play in the maintenance of *Anaplasma* infection. Polymerase chain reaction (PCR) was performed to investigate the prevalence and genetic diversity of *Anaplasma* spp. from 686 whole blood samples (384 cattle and 302 goats). This study revealed that *Anaplasma* infection in cattle was mostly caused by *A. phagocytophilum* (21.1%), while in goats was mainly due to *A. bovis* (7.3%). Cases of co-infection were also found, being for cattle the type involving *A. bovis* and *A. phagocytophilum* (16.7%), while for goats was *A. bovis* and *A. capra* (1.0%). A special case of triple co-infection caused by the pathogens *A. bovis*, *A. phagocytophilum* and *A. capra*, was identified in only one cow (0.3%). Phylogenetic analysis revealed new variants that are circulating among Korean cattle, including five novel strains of the *A. phagocytophilum 16S rRNA* gene and one from *A. capra gltA* gene. Moreover, this research contributes to the ratification of cattle as a potential reservoir of *A. capra* and demonstrates *Anaplasma* co-infection types that may be found in Korean domestic ruminants. As anaplasmosis is a zoonotic disease, this study could be crucial in making important decisions for public health policy.

The second study had as its main objective the evaluation of the clinical presentation of canine babesiosis caused by the hemo-parasite *Babesia gibsoni*, based on the anamnesis, and from the comparison of hematological and biochemical test results. For this study, a total of 400 whole blood sam-

ples of dogs suspected for babesiosis were analyzed by PCR and by a rapid diagnostic test kit (VetAll Laboratories®) for the detection of antibodies against to this hemo-parasite. Out of the 400 dogs, 36 (9.0%) of them resulted to be *B. gibsoni*-infected, while 24 (6.0%) were found to be seroreactive animals. The evaluation of the clinical symptomatology of each canine patient revealed that the three courses of canine babesiosis are present in the country, being the acute course (55.8%) the most predominant, that tend to consist of inappetence, lethargy, pyrexia, gastrointestinal symptoms and, occasionally, hematuria; along with common hematological abnormalities, such as thrombocytopenia (93.3%) and anemia (86.7%), and in lower degree of presentation the biochemical abnormalities, such as hyperbilirubinemia (53.3%), hypoalbuminemia (40.0%) and increased aspartate aminotransferase (AST) enzyme activity (26.7%). This research has shown that *B. gibsoni* is an endemic hemo-parasite in the ROK, capable of producing a variable clinical manifestation in infected dogs, in which for its accurate diagnosis, a descriptive history of the clinical signs, hematology and biochemical profile, along with the performance of the PCR assay are of essential importance. The results of this study not only provide a better understanding of the clinical occurrence of canine babesiosis, but will also help to plan pragmatic preventive strategies against this potent threat in the ROK.

The third research aimed to contribute to the epidemiological surveillance of canine tick-transmitted infections with zoonotic risk in the ROK by investigating the seroprevalence of the pathogens *Anaplasma* spp., *Borrelia burgdorferi* and *Ehrlichia canis*. A total of 430 whole blood samples were tested with immunochromatographic kits (VetAll Laboratories®) for rapid

diagnosis of antibodies against the abovementioned TBPs. The seroprevalence rates found were as follows: 9.8% for *Anaplasma* spp., 2.8% for *B. burgdorferi* and 1.4% for *E. canis*. There was no statistical significance in any of the risk factors evaluated in this study, such as sex, age and history of tick exposure, that could be associated with the development of humoral immune response in the seroreactive dogs. However, there was only one exception for dogs seroreactive to *Anaplasma* spp., where the risk factor "tick exposure" resulted statistically significant (p=0.047). This serosurvey reveals the widespread presence of *Anaplasma* spp., *B. burgdorferi* and *E. canis* throughout the ROK, and suggests that dogs may play a key role as reservoirs and sentinel animals for multiple zoonotic infectious agents in the country.

Through these epidemiological analyses it can be generally concluded that cattle, goats and dogs in the ROK act as carriers or host animals of different TBPs, which in turn are frequently co-exposed to multiple combinations of infectious agents with zoonotic potential, leading to a negative impact on public health. Therefore, the elucidation of the geographic distribution along with the infection rates of these pathogens contributes positively to the implementation of new prevention and control strategies in high-risk areas of this country.

Keywords: *Anaplasma* spp., *Babesia gibsoni*, *Borrelia burgdorferi*, *Ehrlichia canis*, PCR, phylogenetic analysis, serology

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16S rRNA	16S ribosomal RNA
ALT	Alanine aminotransferase
***ank*A**	Ankyrin-related protein
AST	Aspartate aminotransferase
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
BUN	Blood urea nitrogen
CGA	Canine granulocytic anaplasmosis
CME	Canine monocytic ehrlichiosis
CVBDs	Canine vector-borne diseases
gltA	Citrate synthase
CBC	Complete blood count
DIC	Disseminated intravascular coagulation
DNPs	Double nucleotide polymorphisms
ELISA	Enzyme-linked immunosorbent assay
groEL	Heat shock protein
HGA	Human granulocytic anaplasmosis
HME	Human monocytic ehrlichiosis
IMHA	Immune-mediated hemolytic anemia

IFA	Indirect fluorescence immunoassay
ICCT	Infectious canine cyclic thrombocytopenia
KCDC	Korean Centers for Disease Control and Prevention
LB	Lyme borreliosis-associated group
msp2	Major surface protein 2
msp4	Major surface protein 4
msp5	Major surface protein 5
MODS	Multiple organ dysfunction syndrome
NCBI	National Center for Biotechnology Information
nPCR	Nested polymerase chain reaction
PCR	Polymerase chain reaction
RF	Relapsing fever-associated group
SFTS	Severe fever with thrombocytopenia syndrome
SFTSV	Severe fever with thrombocytopenia syndrome virus
SNPs	Single nucleotide polymorphisms
ROK	Republic of Korea
TBD	Tick-borne disease
TBDs	Tick-borne diseases
TBE	Tick-borne encephalitis
TBPs	Tick-borne pathogens
VBDs	Vector-borne diseases

GENERAL INTRODUCTION

Ticks and the pathogens they can transmit have represented a major health concern for many centuries and even in the current years, where the emergence of new TBDs as well as new vertebrate hosts, along with the steady increase in the abundance and spatial distribution of many important tick species, have been part of the key factors in triggering a remarkable global impact on veterinary and public health (Léger et al., 2013).

Today, ticks inhabit almost every continent, with the number of species worldwide topping 900. The fossil records suggest that ticks originated 65-146 million years ago. They are efficient vectors of multiple pathogens (protozoa, bacteria, rickettsiae or viruses) due to their potential interactions with several different vertebrate hosts during their life cycle (Pfäffle et al., 2013). These TBPs are well known to be maintained in enzootic cycles involving ticks and wild animal hosts, with epizootic spread to other mammals, including livestock and humans. Many aspects of the transmission cycle of TBDs are strongly influenced by multifactorial conditions including pathogens, vectors, animal reservoirs, the environment and human behavior, and these might allow for emergence or introduction in a suitable setting (Yamaji et al., 2018).

Asia, the largest continent in the world, is experiencing a rapid social, demographic and economic transformation, thereby placing this region as an ever-growing economic powerhouse in the years to come. Sustained economic growth in this continent has resulted in increased demand for products and services, and substantial urbanization. These factors have triggered

a series of human mediated environmental alterations, such as deforestation and encroachment of humans into natural ecosystems, that now link previously isolated ecologic niches and give pathogens new opportunities to thrive. During the past century, Asia has been in the limelight for emergence and pathogenicity of a large number of infectious diseases that have taken a substantial toll on the health of millions of people (Colella et al., 2020). A striking example in terms of TBDs is the emergence of severe fever with thrombocytopenia syndrome (SFTS), a zoonotic tick-borne disease (TBD) caused by a novel bunyavirus that was identified for the first time in 2010 in rural areas of China, with a fatality rate of 12 to 47%. However, following its first identification there have been subsequent reports of SFTS cases in the ROK and in Japan (Hwang et al., 2017).

In the ROK, the epidemiology of TBPs and their vectors still remains relatively uninvestigated. To date it is known that the tick *Haemaphysalis longicornis* is the most dominant species in this country (Im et al., 2019), and has been identified as a vector of several infectious agents, such as *Ehrlichia chaffeensis*, *Ehrlichia canis*, *Anaplasma phagocytophilum*, *Anaplasma bovis*, *Rickettsia rickettsii*, *Borrelia afzelii*, *Babesia microti* and severe fever with thrombocytopenia syndrome virus (SFTSV) (Kim et al., 2003; Hwang et al., 2017). In addition, in the neighboring countries, such as North Korea and Japan, *H. longicornis* as well has been acting as a vector of *Bartonella* spp., *Borrelia* spp., and *B. gibsoni* (Kang et al., 2016; Iwakami et al., 2014).

Several TBDs have been described in the ROK largely affecting humans, livestock and companion animals. According to a research performed

in 2019 about the current status of TBDs in the ROK, there are at least ten confirmed TBDs occurring in this country. Among the bacterial are Lyme disease, coxiellosis (Q fever), spotted fever group rickettsiae, anaplasmosis, ehrlichiosis, tularemia, and bartonellosis. On the other hand, viral TBDs in the ROK include the newly SFTS and the tick-borne encephalitis (TBE). And lastly but not less, the protozoal TBD named babesiosis (Im et al., 2019). The infectious agents of these pathologies are mostly zoonotic, representing a great challenge for Korean public health since their control and prevention is often exceedingly difficult because it requires the disruption of a complex chain of transmission, involving a great variety of vertebrates, where in most cases pets and livestock have been identified as sentinels and as the major reservoirs for human infections, respectively (Boulanger et al., 2019). Fortunately, new molecular tools and analytical techniques such as gene sequencing and analysis have enabled scientists to gain insights into tick biology and have resulted in a better understanding of TBDs (Dantas et al., 2012).

Therefore, this study used molecular, phylogenetic and serological techniques to investigate in depth and better understand the infection status and geographical distribution of some of the major TBPs present in the ROK among cattle, goats and dogs, with the main purpose of becoming a key source of information for the implementation of control and preventive measures for both human and animal health. This epidemiological survey has been divided in 3 chapters, including the identification of co-infection cases with *Anaplasma* spp. in cattle and goats, which has a molecular and genetic study orientation, followed by the molecular and serological identi-

fication of *B. gibsoni* in dogs, which also included a clinic analysis of the disease canine babesiosis, and the last chapter that contains the serological survey of antibodies against *Anaplasma* spp., *B. burgdorferi* and *E. canis*, also performed in companion dogs.

LITERATURE REVIEW

1. Ticks

Ticks are obligate bloodsucking arthropods that parasitize all vertebrates worldwide; approximately 900 different species of ticks exist and have been classified into three families. The two primary families are Ixodidae (hard ticks) and Argasidae (soft ticks). Ixodidae family is composed of approximately 700 species and 13 genera. The main genera of this family are *Amblyomma*, *Dermacentor*, *Haemaphysalis*, *Rhipicephalus*, *Hyalomma*, and *Ixodes*. While Argasidae family is composed of approximately 193 species and 5 genera, where *Argas* and *Ornithodoros* are the most important (Amsden et al., 2005; Boulanger et al., 2019). The third family, Nuttalliellidae, has only been identified in Africa. This family is composed of a single species named *Nuttalliella namaqua* (Parola et al., 2001). Hard-bodied ticks (Ixodidae) are the most common vector for TBDs in several countries. Only 28 species out of 878 from these families have been recognized to transmit human pathogens, which include organisms such as viruses, protozoa and bacteria (Rodríguez et al., 2018).

1.1. Morphology

Ticks are large acarines (several millimeters long) with a non-segmented body or idiosome (merged prosoma [or thorax] and opisthosoma [or abdomen]), four pairs of legs in nymphs and adults, and three pairs of legs in larvae. Hard ticks have a hard dorsal scutum covering one third of their body, except for males as their entire body is covered by the scutum. This is why

male hard ticks ingest small quantities of blood or no blood at all. Soft ticks do not have any scutellum. Their tegument looks like crumpled leather. The feeding structure of hard ticks is located in front of the head; it is quite large for *Ixodes* ticks and smaller for other hard ticks. Soft ticks have a very small and barely visible feeding structure located on the underside of the body or sub-terminal part of their body (Boulanger et al., 2019).

1.2. Development stages

Ticks go through four development stages: eggs, larvae, nymphs, and male/female adults. Blood must be ingested in-between each stage to induce molting. Ticks are thus hematophagous at all stages (Boulanger et al., 2019).

Female hard ticks drop off their vertebrate host after the blood meal, to lay thousands of eggs in the vegetation and die. *Ixodes* male ticks (Prostriata) do not feed, but they can be found on a host when they need to breed. Males of other hard ticks (Metastriata) can feed, but only very small quantities of blood (twice their weight). Most of these ticks feed on three different hosts and drop off on the ground between each stage to molt (three-host cycle, most ticks). For two-host cycles, larvae and nymphs stay on the same host and for one host cycles, the whole life cycle of the tick occurs on one animal (Apanaskevich et al., 2013).

Soft ticks have several nymphal stages (between two and eight), and feed on blood between each of these stages. Both males and females feed, and females lay a few hundred eggs after each blood meal. A blood meal lasts approximately 15 to 30 minutes, except for larvae as they can feed for up to several days. This is why soft ticks are very rarely observed on pa-

tients, but rather in their habitats (Apanaskevich et al., 2013).

1.3. The blood meal

Ticks are all strictly hematophagous. Therefore, the search for hosts to feed on is essential for their survival. They have developed a highly complex detection system (sensory bristles, pedipalps, Haller's organ on front legs) to detect hosts, mainly wild animals. The blood meal is long in hard ticks, i.e., between 3 and 10 hours depending on the development stage, and tick bites rather occur during the day. Female adults may ingest blood amounts equivalent to up to 100 times their weight. However, later-stage soft ticks (nymphs and adults) only feed for a few minutes and bites rather occur at night (Boulanger et al., 2019).

1.4. Tick bite and saliva

The tick bite mechanism is particularly sophisticated. After finding a warm and humid spot on the skin, ticks pierce the epidermis with their sharp blades (chelicerates) and settle with their denticulated harpoon (hypostome). Ticks then secrete a cement-like substance that keeps them firmly attached to the skin and prevents premature dropping off. An inoculation compartment is formed around the feeding structures, followed by a hemorrhage. The role of tick saliva has been extensively studied and most of the molecules involved have been identified, mainly in hard ticks due to the long duration of their blood meals. Saliva has a pharmacological action on the host (coagulation and pain inhibition, etc.) but it also inhibits the host's local immune responses through immunosuppressive properties targeting various

skin cells (Bonnet et al., 2017). Tick saliva may cause allergic reactions in some hosts, either by inducing an immediate reaction at the bite site, that should be distinguished from erythema migrans observed with Lyme borreliosis, or leading to ascending paralysis mainly observed with *Ixodes* tick saliva (Hall-Mendelin et al., 2011). Saliva of *Argas* soft ticks is also known to induce an anaphylactic shock in susceptible individuals (Hilger et al., 2005).

1.5. Tick species found in the Republic of Korea

The most recent tick survey conducted nationwide by the Korean Centers for Disease Control and Prevention (KCDC) infectious disease reporting system (http://is.cdc.go.kr) was performed in 2019 with a total of 107,863 ticks, where *Haemaphysalis longicornis* resulted to be the more prevalent tick species with a percentage of 97.9% (105,577/107,863). Successively, *Haemaphysalis flava* ranked second with an extremely low percentage of 1.9% (2,081/107,863), followed by the species *Ixodes nipponensis* (0.2%, 163/107,863), *Amblyomma testudinarium* (<0.1%, 32/107,863), and lastly *Haemaphysalis japonica* (<0.1%, 10/107,863) (Noh et al., 2019) (Figure 1).

1.5.1. *Haemaphysalis longicornis*

The life cycle of *H. longicornis* comprises an egg stage, deposited by the adult on pasture once it has fully engorged and detached from the host. A six-legged larva hatches, feeds when it has found a host and detaches once engorged, undergoing further development on pasture, and eventually molting to the eight-legged nymph. This stage, attains a host, feeds and detaches

on engorgement then molts to the adult stage (Heath, 2016) (Figure 2).

This tick is native to eastern Asia (Figure 3a) and known as a well-established exotic species in Australia, New Zealand and several Pacific islands (Zhao et al., 2020). In August 2017, it was first identified on a sheep in New Jersey, USA (Rainey et al., 2018) and was subsequently detected on domestic and wild animals, humans and vegetation in several eastern states (Beard et al., 2018).

At least fifteen *H. longicornis*-associated agents have been detected in mainland China of which ten are known to be pathogenic to humans, including *B. microti*, *Rickettsia raoultii*, *E. chaffeensis*, *A. phagocytophilum*, and *Borrelia garinii* (Table 1). *H. longicornis* has been documented as a competent vector to transmit SFTSV, a virus closely related to Heartland virus in the USA. It can also transmit *Theileria orientalis* complex, which can cause severe anemia in livestock (Zhao et al., 2020).

In the ROK, *H. longicornis* has been recorded in 14 of 17 provinces and has been shown to be the most abundant tick species in the western and southern coastal areas of the country. In addition, approximately 21 host species of *H. longicornis* have been identified, of which 15 of them are different species of domestic and wild animals (Figure 4). Some host species that have been only identified in this country include *Naemorhedus goral* (goral), *Capreolus pygargus* (Siberian roe deer), *Oryctolagus cuniculus* (European rabbit), *Meles leucurus* (Asian badger), and five species of migratory birds (Zhao et al., 2020).

In the ROK, SFTSV and TBE virus have been identified in *H. longicornis* throughout the country, while *R. rickettsii*, *A. phagocytophilum*, *E.*

chaffeensis, *B. afzelii*, and *B. microti* have been carried by *H. longicornis* in diverse but specific areas (Figure 3b). However, further studies have also identified *H. longicornis* as a vector of *E. canis* and *A. bovis* (Kim et al., 2003).

According to epidemiological and geographical distribution studies of this species of tick, it has been identified that the most suitable habitats for *H. longicornis* is in easter Asia, involving central and eastern China, Japan, the ROK, and North Korea (Zhao et al., 2020).

2. Tick-borne pathogens under study

2.1. Bacterial

2.1.1. *Anaplasma*

The genus *Anaplasma*, discovered in 1910, belongs to the family Anaplasmataceae, in the order Rickettsiales, and comprises six confirmed species: *A. marginale*, *A. centrale*, *A. ovis*, *A. phagocytophilum*, *A. bovis* and *A. platys* (Table 2). This classification has been valid since 2001, when Dumler and colleagues significantly reorganized the order Rickettsiales based on phylogenetic analyses of 16S ribosomal RNA (*16S rRNA*) and the *groESL* genes (Battilani et al., 2017). However, in 2015 a new species also belonging to the genus *Anaplasma*, tentatively named *A. capra*, was discovered in China (Li et al., 2015).

Anaplasma spp. are causative agents of TBDs (i.e., anaplasmosis) with a remarkable impact on human and animal health. The effects of anaplasmo-

sis on the health and productivity of domestic animals have been known for over a century, but even today it is still considered a major cause of economic losses in livestock farming, in tropical and temperate regions. Conversely, recognition of *Anaplasma* as a genus of public health significance is more recent and has contributed to rising interest about these bacteria, resulting in greater information about their molecular biology, genetics and pathobiology (Woldehiwet, 2010).

2.1.1.1. *Anaplasma phagocytophilum*

A. phagocytophilum is an obligate intragranulocytic parasite of remarkable importance in human and veterinary health. The prototype of *A. phagocytophilum* was first described in 1940 (Gordon et al., 1940).

This bacterium is largely distributed across Europe, the USA and Asia; moreover, it has been detected in South America and Africa. Its host range is wide, including humans, carnivores, ruminants, rodents, insectivores, birds and reptiles (Stuen et al., 2013).

Transmission of *A. phagocytophilum* involves ticks belonging to the *Ixodes* genus: *I. ricinus* in Europe, *I. scapularis* in Eastern USA, *I. pacificus* and *I. spinipalpis* in Western USA, and *I. persulcatus* in Asia and Russia (Woldehiwet et al., 2010). Moreover, DNA of *A. phagocytophilum* has been detected in other tick species, including *Dermacentor* spp., *Haemaphysalis* spp. and *Amblyomma* spp., but their vector competence and their role in the epidemiological cycle of *A. phagocytophilum* are still unclear (Paulauskas et al., 2012).

Once in a receptive host, *A. phagocytophilum* infects neutrophils and

eosinophils, forming mulberry-like colonies called “morulae” (Woldehiwet et al., 2010). The percentage of infected phagocytic cells can vary greatly depending on the phase of bacteremia, host susceptibility and bacterial strain involved. Infected hosts develop leukopenia, neutropenia, and reduction in neutrophil function, with a consequent state of immunosuppression that may promote the occurrence of opportunistic infections (Battilani et al., 2017).

In human beings, *A. phagocytophilum* causes human granulocytic anaplasmosis (HGA), a disease first described in 1994 in the USA (Chen et al., 1994). Since that first case, detection of HGA has constantly increased worldwide, mostly in the USA, where 2,782 cases were described in 2013 (Adams et al., 2015).

A. phagocytophilum is also the causative agent of tick-borne fever in sheep and goats, and pasture fever in cattle. Granulocytic anaplasmosis in ruminants is characterized by fever, weakness, anorexia and abortion, and a sudden drop in milk production is a common clinical sign in dairy cattle. Moreover, *A. phagocytophilum* has immunosuppressive effects, resulting in increased incidence of secondary infections such as tick pyemia due to *Staphylococcus aureus* (Woldehiwet, 2006).

In dogs, *A. phagocytophilum* can cause both asymptomatic and clinically evident infections. The most common symptoms of canine granulocytic anaplasmosis are fever, lethargy and anorexia, but also lameness, vomiting, diarrhea, splenomegaly, reluctance to move, pale mucous membranes, epistaxis, polyuria/polydipsia, and lymphadenomegaly have been described (Nair et al., 2016).

2.1.1.2. *Anaplasma bovis*

A. bovis is an obligate parasite of monocytes, described for the first time in 1936 in cattle (Donatien and Lestoquard, 1936). *A. bovis* is mainly distributed in Africa, Asia and South America, but it has also been reported in the USA and southern Europe (Battilani et al., 2017).

Cattle and buffalo are considered the main hosts of *A. bovis*; however, the infection has also been detected in goats, dogs, roe deer, red deer, sika deer, Korean water deer (*Hydropotes inermis argyropus*), marsh deer (*Blastocerus dichotomus*), Brazilian brown brocket deer (*Mazama gouazoubira*), Mongolian gazelle, cotton-tail rabbits, leopard cats (*Prionailurus bengalensis*), raccoons, and eastern rock sengi (*Elephantulus myurus*) (Uilenberg, 1993; Atif, 2016).

Several tick species are suspected to be involved in *A. bovis* transmission, including *Hyalomma* spp., *Haemaphysalis* spp., *Amblyomma variegatum*, *Rhipicephalus appendiculatus* and *Rhipicephalus sanguineus* (Battilani et al., 2017).

Infected cattle and buffalo generally develop a mild clinical illness, but severe diseases have also been described. Hyperthermia, weakness, weight loss, pale mucous membranes, prescapular lymph node inflammation and occasionally death can characterize these latter forms (Battilani et al., 2017).

2.1.1.3. *Anaplasma ovis*

A. ovis is one of the three intraerythrocytic *Anaplasma* species; it was first described in sheep in 1912 by Bevan (Bevan, 1912; Dumler et al., 2001). *A. ovis* has been found in Africa, Asia, Europe and the USA. In addi-

tion to sheep, this bacterium has also been detected in goats and wild ruminants. Recently, a variant of *A. ovis* was detected in a human patient, suggesting a zoonotic potential of this organism (Chochlakis et al., 2010).

The ticks *Rhipicephalus bursa* and *Dermacentor andersoni* are considered the main vector of infection, however *Rhipicephalus sanguineus* and sheep keds (*Melophagus ovinus*) can also act as vectors. Biting flies may also be involved in transmission of *A. ovis*. Sheep and goats infected by *A. ovis* generally developed a mild disease; however, in the presence of co-infections or stress factors these animals can develop severe clinical illness characterized by fever, anorexia, depression, weakness, pale mucous membranes, lower milk production, coughing, dyspnea, rumen atony, abortion and death (Battilani et al., 2017).

2.1.1.4. *Anaplasma capra*

A. capra was first recognized in goats (*Capra aegagrus hircus*) in northeastern China. However, *16S rRNA* sequences of *A. capra* have also been detected in sheep and dogs from China, in deer and serows from Japan, and in goats, cattle and water deer from the ROK, suggesting that this pathogen may affect a wide range of mammals, especially small ruminants. However, it is unclear at present whether *A. capra* can infect other mammalian species, therefore further research is needed to determine the full range *A. capra* reservoir hosts.

In addition, there is also significant difference in several biological features between *A. capra* and other well-recognized *Anaplasma* species. *Anaplasma* species organisms usually infect bone marrow-derived cells in blood,

however, *A. capra* may infect endothelial cells in vivo. Consequently, microscopic examination of peripheral blood smears is not sensitive enough for diagnosis of *A. capra* infection in both animals and humans (Yang et al., 2017).

A. capra has been identified in *I. persulcatus* and *H. longicornis* ticks in China. However, pathogen detection in arthropods is not sufficient to validate its vector competence. To date, there is very little information of the natural cycles of *A. capra* (Yang et al., 2016).

Twenty-eight human cases caused by *A. capra* have been reported in Heilongjiang province in northeast China. The disease caused by *A. capra* presents non-specific symptoms with fever, headache, malaise, rash, eschar and chills; these common signs are very difficult to distinguish clinically from other TBDs (Yang et al., 2017).

2.1.2. *Ehrlichia*

Ehrlichia are small, obligately intracellular, gram-negative, tick-transmitted bacteria responsible for life-threatening emerging human zoonoses and diseases of veterinary importance. Organisms in the genus *Ehrlichia* were first described in the early 20th century (Paddock and Childs, 2003). Recently, the members of the genus were reclassified using contemporary molecular methods. Hence, the new taxonomy has resulted in a smaller *Ehrlichia* genus consisting of five members (*E. canis*, *E. chaffeensis*, *E. muris*, *E. ruminantium* and *E. ewingii*) (Table 3) following the reassignment of six previously recognized members to the genera, *Anaplasma* (*E. phagocytophila*, *E. equi*, *E. platys* and *E. bovis*) and *Neorickettsia* (*E. sennetsu* and *E.*

risticii), and acquisition of one new member from the genus *Cowdria* (*C. ruminantium*) (Dumler et al., 2001).

E. chaffeensis and *E. ewingii* are recognized as human pathogens as well as pathogens of veterinary importance that include *E. canis* and *E. ruminantium*. The genus *Ehrlichia* is now part of a newly created family Anaplasmataceae, which also includes the genera *Anaplasma*, *Wolbachia*, and *Neorickettsia*, but remains in the order Rickettsiales (Dumler et al., 2001).

2.1.2.1. *Ehrlichia canis*

E. canis is the type species for the genus *Ehrlichia* and is the primary etiologic agent of canine monocytic ehrlichiosis (CME), a serious and sometimes fatal, globally distributed disease of dogs (Keefe et al., 1982). In 1935, *E. canis* infection was recognized as a disease of dogs by veterinarians working at the Pasteur Institute in Algeria (Donatien and Lestoquard, 1935). The disease gained attention during the Vietnam conflict in the late 1960s because >200 dogs belonging to the US military forces died as a result of an epizootic of highly fatal hemorrhagic disease known as tropical canine pancytopenia (Walker et al., 1970). Development of the disease was associated with heavy infestations of *Rhipicephalus sanguineus* (the brown dog tick), which is the primary vector of *E. canis*. The age, breed and immune competence of dogs are believed to influence the severity of infection, and some affected dogs may not have clinical signs of illness.

Ehrlichiosis in dogs has three distinct phases. During the acute phase, which generally lasts one to three weeks, dogs have nonspecific signs; these include fever, anorexia, weight loss, signs of depression, splenomegaly and

lymphadenopathy. Anemia and thrombocytopenia may be detected. If affected dogs are not treated, a subclinical phase develops in which dogs appear to return to normal but remain seropositive; mild thrombocytopenia may be detected. In certain dogs, chronic infection may develop, which can be severe and life threatening in immunocompromised dogs or certain breeds (e.g., German Shepherd). In the severe form of disease, marked weight loss and emaciation, lymphadenopathy, pyrexia and hemorrhagic disease are commonly observed. Severe pancytopenia with nonregenerative anemia may also be detected, death results from extensive hemorrhage or secondary infection (McQuiston et al., 2003).

Cases of human infection with *E. canis* have been reported in the medical literature. In 1996, *E. canis* was isolated from the blood of a healthy adult in Venezuela who reported close contact with an *E. canis*-infected dog. Although morulae were observed on cytologic examination of a blood smear from this individual, the infection did not result in clinical signs. However, in 2006 in the same country, from a total of 20 human patients with clinical signs compatible with human monocytic ehrlichiosis (HME), 6 of them were positive for *E. canis 16S rRNA* gene sequences. The six patients had the same base mutation as the sequence of the *E. canis* Venezuelan human *Ehrlichia* (VHE) strain previously isolated from the asymptomatic human (Perez et al., 2006).

In addition to infection with *E. canis*, a granulocytic form of ehrlichiosis has been observed in dogs infected with *E. ewingii* and *A. phagocytophilum*. Common clinical signs associated with these infections include fever, lethargy, lymphopenia and thrombocytopenia (McQuiston et al., 2003).

2.1.3. *Borrelia*

The spirochete genus *Borrelia*, was first described in 1907 by Swellengreb, with *Borrelia anserina* designated as the type species (Sherman et al., 1989). The genus currently comprises 42 named species including 21 species within the relapsing fever-associated group (RF), 20 species within the Lyme borreliosis-associated group (LB), and one species (*Borrelia turcica*) within the novel reptile-associated group. However, there is a wide diversity of novel *Borrelia* species that do not yet have an official name (Margos et al., 2020).

Lyme disease (also called Lyme borreliosis or LB) is caused by a group of closely related *Borrelia* species. This group is collectively called *Borrelia burgdorferi* sensu lato (in a broad sense) (Table 4), whereas *Borrelia burgdorferi* sensu stricto (in a strict sense) refers to the originally isolated species (Norris, 2009).

2.1.3.1. *Borrelia burgdorferi*

B. burgdorferi is a highly motile, helix-shaped organism that can be visualized under dark-field microscopy and is grouped with two other pathogenic spirochete genera, *Leptospira* and *Treponema* (Madison-Antenucci et al., 2020).

Canine borreliosis was first described in the USA in the 1980s, and in nearly all European countries in the recent years. Reports on canine infections by *B. burgdorferi* have come from Germany, the Netherlands, Belgium, France, the United Kingdom, Slovakia and Spain (Skotarczak et al., 2003).

Ticks of the genus *Ixodes*, which are vectors of *B. burgdorferi*, carry

the bacteria to many vertebrates, as the spirochaete does not exhibit any particular specificity towards the host species. In natural conditions, the clinical form of borreliosis is found only in species from outside the forest biotope, i.e., humans, dogs, cats, horses and cows; however, most often it affects dogs and humans (Appel et al., 1993; Magnarelli et al., 2000).

When a tick sucks blood, the spirochaetes *B. burgdorferi* first enter the skin of the host and then spread to adjacent tissues where the infection stabilizes, not being eliminated by the host immune system. The processes that initiate and sustain the disease have not been well defined; however, it is known that *B. burgdorferi* occurs in the inflamed and chronically infected tissues of the host. Each year in Europe, North America and Asia, a large number of people and animals are infected; however, not all infected individuals develop clinical symptoms of the disease (Skotarczak et al., 2003).

Although human Lyme disease has been described in numerous papers, the canine borreliosis has not been thoroughly studied, despite great similarities. In dogs, Lyme disease develops most often in arthritic form, with inflammation of limb, usually carpal or tarsal joints; one or both joints swell, and groin and prescapular lymph nodes enlarge. These symptoms are accompanied by malaise (which is manifested by fever, lack of appetite and fatigue), and lameness after a few days. Myocarditis rarely develops in canine borreliosis; however, in older dogs, renal form and neurological dysfunctions appear (Chang et al., 2001).

Many authors have reported that incidence of *B. burgdorferi* infections in humans and dogs depends on the occurrence of ticks, depending in turn on geographical location and biotope, which represent a risk indicator for

both humans and dogs. Dogs that live in areas infested with ticks, where human Lyme disease cases are recorded, develop antibodies against *B. burgdorferi* (Skotarczak et al., 2003).

2.2. Protozoal

2.2.1. *Babesia*

Babesia are tick-transmitted protozoan hemoparasites, of great economic, veterinary and medical impact worldwide. They are considered to be the second most commonly found parasites in the blood of mammals after trypanosomes, and they have also been described infecting birds. In their vertebrate hosts they reproduce asexually inside erythrocytes, and together with *Theileria* spp. they are referred to as piroplasm or piroplasmids. The sexual phase of *Babesia* life cycle typically takes place in Ixodid ticks, which acquire and transmit the parasites during their blood meals (Schnittger et al., 2012).

Victor Babes (1888) was the first to discover microorganisms inside bovine erythrocytes of Romanian cattle that presented hemoglobinuria; and he later observed a similar organism in sheep blood. Five years later in the USA, Smith and Kilbour described that the presence of an intraerythrocytic parasite was the cause of tick-transmitted Texas Cattle Fever, a disease that had long stricken cattle ranchers in the Southern US states. This turned out to the first description of an arthropod-transmitted pathogen of vertebrates. The parasites described by Babes, and Smith and Kilbour were later named *Babesia bovis*, *B. ovis*, and *B. bigemina*, respectively. Soon afterwards, ba-

besias parasitizing the blood of other domestic animals were observed, such as those that eventually became known as *B. canis* and *B. caballi*, described by Piana and Galli-Valerio (1895) and by Koch (1904), in dog and horse erythrocytes, respectively. Since these early findings, more than 100 different *Babesia* species have been discovered (Table 5), and thanks to the advances in microscopy, cell biology and molecular biology techniques the knowledge of the *Babesia* world is rapidly expanding (Schnittger et al., 2012).

2.2.1.1. *Babesia gibsoni*

B. gibsoni has recently been recognized as an important pathogen that affects dogs in the Middle East, Africa, Asia, Europe and many areas of the USA (Boozer et al., 2005). The specific vector of *B. gibsoni* infection in the USA is not well established but is suspected to be *R. sanguineus*, the brown dog tick. In other countries, *Haemaphysalis* ticks, such as *H. longicornis* and *H. bispinosa* are also known to transmit *B. gibsoni* infection (Aysul et al., 2013).

Dogs become infected when ticks feed for 2 to 3 days and release sporozoites into the circulation. Inside the host, the organisms attach to the red cell membrane and are engulfed by endocytosis. In the cytoplasm, binary fission occurs, resulting in merozoites. Ticks become infected with merozoites during feeding and may remain infective for many generations through transstadial and transovarial transmission. In experimental infections derived from naturally infected dogs in Oklahoma, parasites were detected 1 to 5 weeks after inoculation. Parasitemia peaked at 1.9% to 6% by 4 to 6

weeks after infection. Easily detectable parasitemia (>0.1%) was present for 3 to 4 weeks. The severity of clinical signs was highly variable and developed approximately 1 to 2 weeks after infection. After initial parasitemia, the immune system does not totally eradicate the infection, and a chronic carrier state remains (Meinkoth et al., 2002). *B. gibsoni* can cause hyperacute, acute and chronic infections. Hyperacute infections are rare and primarily occur in puppies, resulting in rapid death. These infections are presumed to be maternally acquired. Acute *B. gibsoni* infections are typically associated with fever, lethargy, thrombocytopenia and anemia. Chronic infections may be completely asymptomatic or may be characterized by intermittent fever, lethargy and weight loss (Boozer et al., 2005).

In addition to tick-borne transmission vertical transmission is also suspected, e.g., *B. gibsoni* infections have been identified in a dam and her 3-day-old puppies. Transmission can also occur through transfusion of infected blood. Interestingly, there have been several reports of infections in dogs that have been attacked by Pitbull Terriers. Retrospective evaluation of dogs other than American Pitbull Terriers diagnosed with *B. gibsoni* infection found that six of 10 dogs had a history of being bitten by a Pitbull Terrier. Although unknown, the mechanism that seems most likely is direct blood–blood contact during fighting. Possible transmission through nursing, breeding, saliva or swallowing blood has not been evaluated (Birkenheuer et al., 2003).

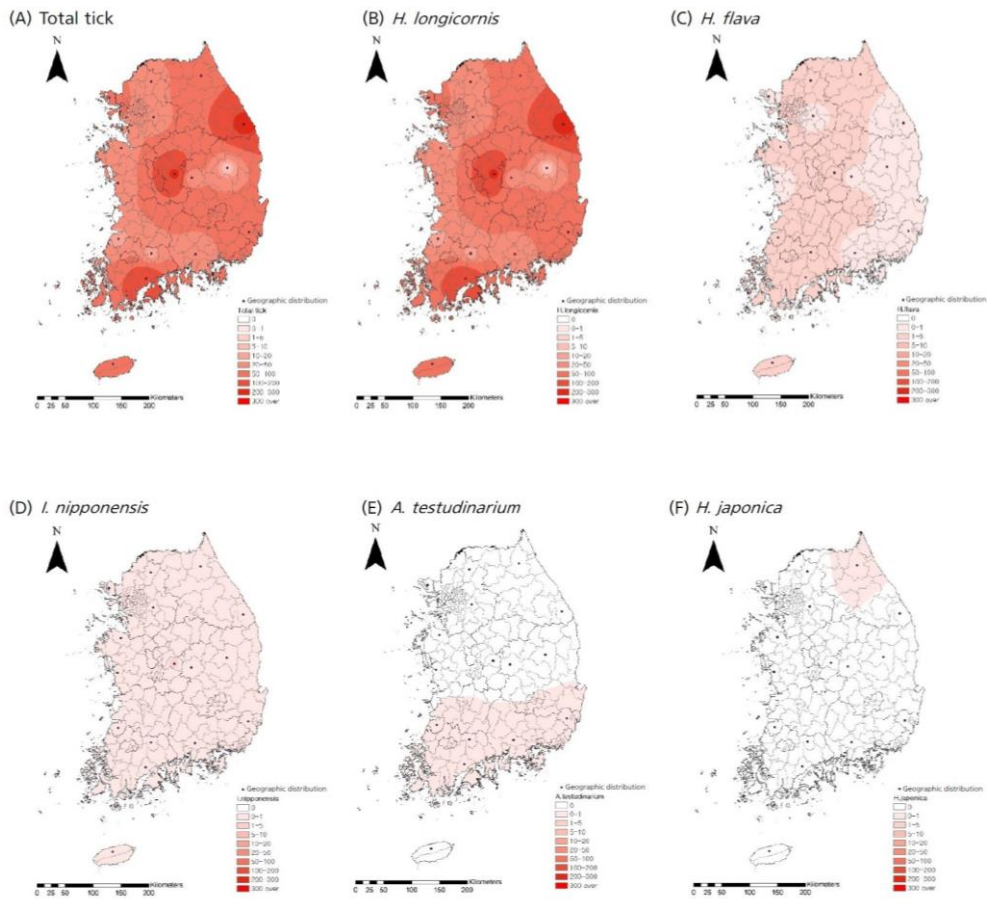


Figure 1. Surveillance of tick density in the Republic of Korea. Adapted from Noh et al. (2019).

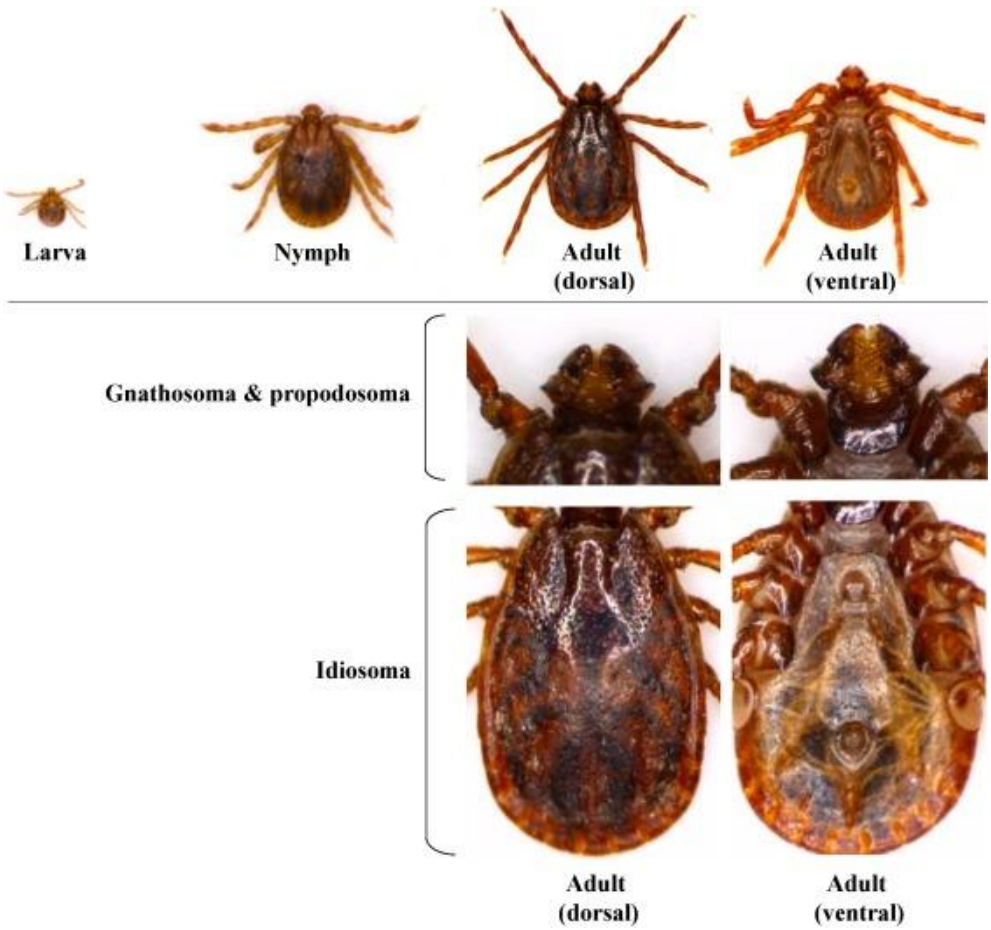
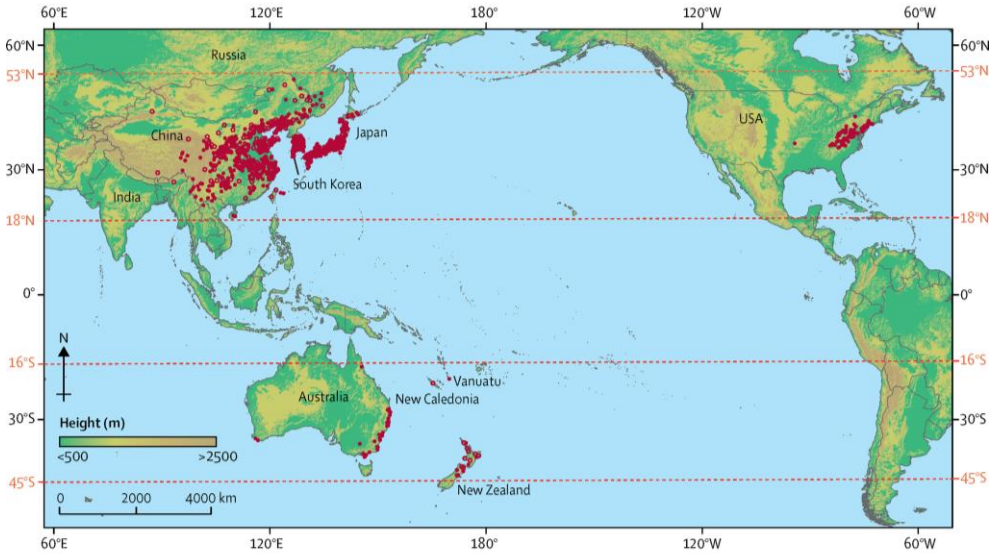


Figure 2. Morphological characterization of larva, nymph, and dorsal and ventral of adult *Haemaphysalis longicornis*. Adapted from Lee et al. (2019).

(**a**)



(**b**)

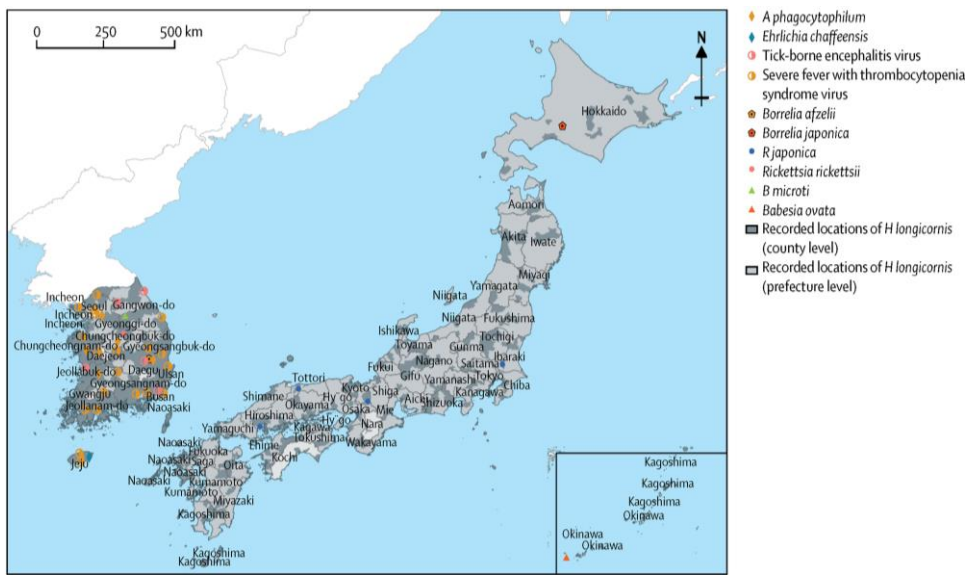


Figure 3. Geographical distribution of *Haemaphysalis longicornis*. (**a**) Recorded locations of *H. longicornis* worldwide. (**b**) *H. longicornis*-associated pathogens. Three human pathogens were reported in Japan and seven in the ROK. Adapted from Zhao et al. (2020).

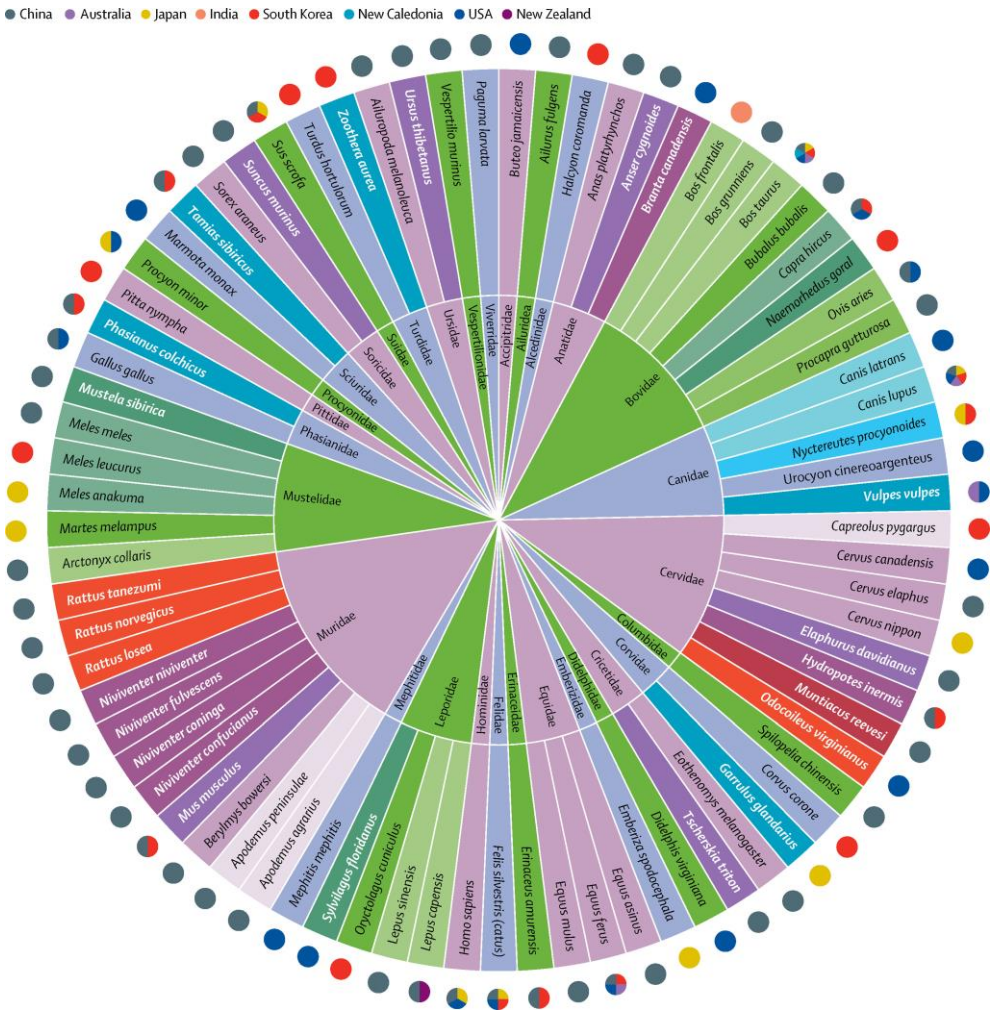


Figure 4. Hosts of *Haemaphysalis longicornis* in different countries. The inner circle represents the families of host animals. The outer circle represents the species of host animals. Under the same family, species belonging to one genus are shown in the same color. Small circles outside represent the countries where the hosts of *H. longicornis* were recorded. In the eight countries, a total of 77 species of animals were identified as hosts of *H. longicornis*, which belong to 61 genera and 30 families. Adapted from Zhao et al. (2020).

Table 1. Worldwide prevalence of *Haemaphysalis longicornis*-associated pathogens

	Positive rate (95% CI)	No. of studies
Spotted fever group rickettsiae		
Rickettsia japonica	0.0536 (0.0103–0.1246)	8
Rickettsia raoultii	0.0245 (0–0.1014)	3
Rickettsia rickettsii	0.0112	1
Rickettsia heilongjiangensis	0.0136 (0.0066–0.0226)	3
Candidatus Rickettsia tarasevichiae	0.0666 (0.0058–0.1795)	2
Candidatus Rickettsia jingxinensis	0.2224 (0–0.8358)	2
Rickettsia canadensis	0.0012	1
Anaplasmataceae		
Anaplasma capra	0.0081 (0.0046–0.0125)	2
Anaplasma phagocytophilum	0.0297 (0.0141–0.0502)	18
Anaplasma platys	0.0196 (0–0.1596)	2
Anaplasma ovis	0.0735	1
Ehrlichia canis	0.0074 (0–0.0699)	2
Ehrlichia chaffeensis	0.0153 (0–0.0504)	6
Ehrlichia ewingii	0.0012	1
***Borrelia burgdorferi* sensu lato**		
Borrelia garinii	0.0696 (0–0.3444)	3
Borrelia afzelii	0.0044 (0–0.0269)	3
Borrelia japonica	0.0769	1
Borrelia miyamotoi	0.0123	1
Babesia		
Babesia microti	0.0088 (0.0053–0.0130)	3
Babesia ovata	0.1029	1

Table 1. Worldwide prevalence of *Haemaphysalis longicornis*-associated pathogens (Continued)

	Positive rate (95% CI)	No. of studies
Francisella		
Francisella tularensis	0.0089 (0.0013–0.0212)	2
Bartonella		
Bartonella henselae	0.0500	1
Coxiella		
Coxiella burnetii	0.0057 (0–0.0451)	2
Toxoplasma		
Toxoplasma gondii	0.0924	1
Viruses		
Severe fever with thrombocytopenia syndrome virus	0.0121 (0.0068–0.0185)	39
Jingmen tick virus	0.0053	1
Bocavirus	0.3200	1
Nairobi sheep disease virus	0.0044 (0.0002–0.0133)	3
Lymphocytic choriomeningitis virus	0.0984	1
Tick-borne encephalitis virus	0.0006 (0.0001–0.0014)	3

If there was only one study included for a certain species of *H. longicornis*-associated pathogen, the positive rate was calculated by positive ticks divided by total ticks and without a 95% CI. If the number of studies was two or more, the combined positive rate and 95% CI were estimated by meta-analysis. Adapted from Zhao et al. (2020).

Table 2. Characteristics of *Anaplasma* spp., main hosts and diseases

Species	Disease(s)	Main hosts	Host cells	Primary vectors	Distribution	Old name(s)
A. bovis	Bovine anaplasmosis	Cattle, buffaloes	Monocytes	*Amblyomma* spp. *Rhipicephalus* spp. *Hyalomma* spp. *Haemaphysalis* spp.	Africa, USA, Europe, South America, Asia	*Ehrlichia bovis*
A. centrale	Mild anaplasmosis in cattle (Vaccine strain)	Cattle	Erythrocytes	*Rhipicephalus simus*	Worldwide in tropical and subtropical regions	*Anaplasma centrale*
A. marginale	Bovine anaplasmosis	Cattle, wild ruminants	Erythrocytes	*Ixodes* spp. *Dermacentor* spp. *Rhipicephalus* spp.	Worldwide in tropical and subtropical regions	*Anaplasma marginale*
A. ovis	Ovine anaplasmosis	Sheep, goats, wild ruminants	Erythrocytes	*Dermacentor* spp. *Rhipicephalus* spp. *Melophagus ovinus*	Africa, Asia, Europe, USA	*Anaplasma ovis*

Table 2. Characteristics of *Anaplasma* spp., main hosts and diseases (Continued)

Species	Disease(s)	Main hosts	Host cells	Primary vectors	Distribution	Old name(s)
A. phagocytophilum	HGA, equine anaplasmosis, tick-borne fever of ruminants, anaplasmosis of dogs and cats	Humans, horse, ruminants, rodents, carnivores, insectivores	Granulocytes, endothelial cells	*Ixodes* spp.	Worldwide	*Ehrlichia* (Cytoecetes) *phagocytophila*, HGE agent, *E. equi*
A. platys	Cyclic thrombocytopenia in dogs	Dogs	Platelets	*Rhipicephalus sanguineus*	Worldwide	*Ehrlichia platys*

HGA, human granulocytic anaplasmosis; HGE, human granulocytic ehrlichiosis. Adapted from Battilani et al. (2017).

Table 3. *Ehrlichia* species, their host, diseases and vectors

***Ehrlichia* species**	**Vertebrate host**	**Disease(s)**	**Vector**	
			Natural	**Experimental**
E. canis	Canines	CME	*R. sanguineus*	*R. sanguineus* *D. variabilis*
E. chaffeensis	Humans, dogs, cattle, goats, deer, sheep	HME	*A. americanum*	*A. americanum*
E. ewingii	Canines		*A. americanum*	
E. muris	Murine		*H. flava* *I. persulcatus*	
E. ruminantium	Cattle, goats, sheep, wild ungulates	Heartwater (cowdriosis)	*A. variegatum* *A. hebraeum* *A. pomposum* *A. gemma* *A. lepidum* *A. sparsum* *A. astrion*	*A. variegatum* *A. hebraeum* *A. pomposum* *A. gemma* *A. lepidum* *A. tholloni* *A. sparsum* *A. astrion* *A. cohaerens* *A. marmoreum* *A. maculatum* *A. cajennense* *A. disimile*

CME, canine monocytic ehrlichiosis; HME, human monocytic ehrlichiosis. Adapted from Esemu et al. (2011).

Table 4. Currently known *Borrelia burgdorferi* sensu lato species/genomospecies

***Borrelia* species**	**Vector**	**Hosts/reservoirs**	**Geographical distribution**
B. afzelii	*I. ricinus, I. persulcatus*	Rodents	Europe, Asia
B. andersoni	*I. dentatus*	Cotton tail rabbit	United States
B. bissettii	*I. ricinus, I. scapularis, I. pacificus, I. minor*	Rodents	Europe, United States
B. burgdorferi sensu stricto	*I. ricinus, I. scapularis, I. pacificus*	Rodents, birds, lizards, big mammals	North America, Europe
B. californiensis	*I. pacificus, I. jellisonii, I. spinipalpis*	Kangaroo rat, mule deer	United States
B. carolinensis	*I. minor*	Rodents, birds	United States
B. garinii	*I. ricinus, I. persulcatus, I. hexagonus, I. nipponensis*	Birds, lizards, rodents	Europe, Asia
B. japonica	*I. ovatus*	Rodents	Japan
B. lusitaniae	*I. ricinus*	Rodents, lizards	Europe, North Africa
B. sinica	*I. ovatus*	Rodents	China
B. tanukii	*I. tanuki*	Unknown (possible dogs and cats)	Japan
B. turdi	*I. turdus*	Birds	Japan
B. spielmanii	*I. ricinus*	Rodents	Europe
B. valaisiana	*I. ricinus, I. granulatus*	Birds, lizards	Europe, Asia
Genomospecies 1	*I. pacificus*	Unknown	United States
Genomospecies 2	*I. pacificus*	Unknown	United States

Adapted from Liu (2011).

Table 5. *Babesia* spp. parasites of domestic animals

Host(s)	Species	Distribution
Cattle	*B. bovis*	Africa, America, Asia, Australia, Europe
	B. bigemina	Africa, America, Asia, Australia, Europe
	B. major	Asia, Europe
	B. occultans	Africa
	B. ovata	Asia
	B. divergens	Europe
	B. sp. *Kashi*	China
Water buffalo	*B. orientalis*	Asia
	B. bovis	America, Asia
	B. bigemina	America, Asia
Horse	*T. equi*	Europe, America
Horse, donkey, mule	*B. caballi*	Africa, America, Asia, Europe
Pig	*B. trautmanni*	Africa, Europe
Sheep, goat	*B. crassa*	Asia
	B. ovis	Africa, Asia, Europe
	B. motasi	Africa, Asia, Europe
Sheep	*B.* sp. *Xinjiang*	China
Dog	*B. vogeli*	Africa, America, Asia, Australia, Europe
	B. conradae	America (USA)
	B. gibsoni	Asia, Africa, America, Europe
	B. vitalii	America (Brazil)
	Babesia sp.	America (USA)
	B. rossi	South Africa
	T. annae	Spain, Portugal
	B. canis	Europe
Cat	*B. felis*	South Africa
	B. (canis) presentii	Asia (Israel)

Adapted from Schnittger et al. (2012).

CHAPTER I

Co-infection with *Anaplasma* species and novel genetic variants detected in cattle and goats

Abstract

Anaplasmosis, a TBD with multiple reservoirs, has been evolving in its pathogenesis, increasing domestic ruminants susceptibility to simultaneous infections with multiple pathogens. However, there is limited information regarding anaplasmosis in domestic ruminants in the ROK. Therefore, the aim of this study was to evaluate the role of Korean cattle and goats in *Anaplasma* infection maintenance. PCR was performed to investigate the prevalence and genetic diversity of *Anaplasma* spp. from 686 whole blood samples collected from different ROK provinces. *Anaplasma* infection was mostly caused by *A. phagocytophilum* (21.1%) in cattle, and *A. bovis* (7.3%) in goats. Co-infection cases were found in cattle: *A. bovis* and *A. phagocytophilum* (16.7%), and in goats: *A. bovis* and *A. capra* (1.0%). Notably, a triple co-infection with *A. bovis*, *A. phagocytophilum*, and *A. capra* was found in one cow. Phylogenetic analysis revealed novel variants of the *A. phagocytophilum 16S rRNA* and *A. capra gltA* genes. This investigation

contributes to the ratification of cattle as a potential reservoir of *A. capra* and demonstrates *Anaplasma* co-infection types in Korean domestic ruminants. As anaplasmosis is a zoonotic disease, this study could be crucial in making important decisions for public policy.

Keywords: *Anaplasma phagocytophilum*, *Anaplasma bovis*, *Anaplasma capra*, cattle, goat, co-infection, Republic of Korea

1. Introduction

Anaplasmosis, an infectious but non-contagious TBD, classically related as a disease of ruminants, is caused by obligate intra-erythrocytic bacteria of the genus *Anaplasma* (Aubry et al., 2011) discovered in 1910 by Theiler (Theiler, 1910). This genus belongs to the Anaplasmataceae family and is composed of six confirmed species based on host cell tropism: *A. phagocytophilum*, *A. bovis*, and *A. platys*, which infect neutrophils (Woldehiwet, 2010), monocytes (Sreekumar et al., 1996), and platelets (Uilenberg et al., 1979), respectively, whereas *A. marginale*, *A. centrale*, and *A. ovis* infect erythrocytes (Munderloh et al., 2004). Furthermore, the newly recognized *Anaplasma* species, *A. capra* might infect endothelial cells (Li et al., 2015).

Anaplasmosis in domestic ruminants is caused by two main etiological agents: *A. marginale* in cattle (de la Fuente et al., 2005) and *A. ovis* in sheep and goats (Underwood et al., 2015). However, additional studies on the genus *Anaplasma* have detected other *Anaplasma* spp. (Ybañez et al., 2016) that may act as a causative agent of anaplasmosis in ruminants. In the ROK, *A. phagocytophilum* (Han et al., 2018) and *A. bovis* (Park et al., 2018) infections have been recently detected in Holstein cattle. Moreover, a recent study that was conducted in this country suggests that cattle may serve as potential reservoirs of *A. capra* (Seo et al., 2018). Currently, *A. centrale* has not been detected in the ROK (Seo et al., 2018), but this does not rule it out as a causative agent of bovine anaplasmosis, since a clinical case associated with this species has been reported in the European continent (Carelli et al., 2008). Regarding caprine anaplasmosis, in addition to *A. ovis* infection, *A. phagocytophilum* and *A. bovis* infections have been detected in goats from

Central and Southern China (Liu et al., 2012) and *A. capra* infection in Korean native goats (*Capra aegagrus hircus*) (Seo et al., 2019).

Additionally, co-infection cases with *Anaplasma* species have been identified in Chinese domestic ruminants using PCR, such as *A. ovis* plus *A. bovis*, *A. ovis* plus *A. phagocytophilum*, *A. bovis* plus *A. phagocytophilum* in goats (Liu et al., 2012), *A. bovis* plus *A. phagocytophilum* in cattle, and *A. ovis* plus *A. phagocytophilum* in sheep (Yang et al., 2015). In the ROK, co-infection with *A. phagocytophilum* and *A. phagocytophilum*-like *Anaplasma* spp. has been detected in cattle (Seo et al., 2018).

Tick-borne zoonoses have been observed since the second half of the 19th century (Hoogstraal et al., 1967). To date, there are three *Anaplasma* species that have been recognized as zoonotic pathogens: *A. phagocytophilum* (Kim et al., 2014), *A. ovis* (Chochlakis et al., 2010), and *A. capra*, recently isolated from 28 human patients during acute phase illness in Heilongjiang, China (Li et al., 2015). In the ROK, serological evidence of *A. phagocytophilum* infection in human subjects with acute fever was first described in 2002, with a seropositivity rate of 1.8%. These results were also confirmed by western blotting and TaqMan real-time PCR (Heo et al., 2002). Moreover, a recent study in the ROK reported a clinical case of human granulocytic anaplasmosis in a patient with a history of tick bite, which was confirmed by seroconversion, PCR, and sequence analysis of *A. phagocytophilum* (Kim et al., 2014).

Anaplasma spp. are mainly transmitted to humans and animals by specific species of ticks belonging to the Ixodidae family (Battilani et al., 2017). In the ROK, *A. phagocytophilum* and *A. bovis* infections have been identi-

fied not only in ticks of the *Haemaphysalis* genus, such as: *H. longicornis*, and *H. flava*, but also in ticks belonging to the *Ixodes* genus, such as: *I. persulcatus*, and *I. nipponensis* (Kang et al., 2016). *H. longicornis* has been recognized as the most common tick species that infests Korean native goats (Seo et al., 2020) and Korean grazing cattle (Kang et al., 2013). This arthropod vector is considered the most prevalent tick species associated with TBPs, followed by *Ixodes* spp., throughout the ROK (Oh et al., 2009).

Considering that ticks transmit more pathogenic species that any other group of blood-feeding arthropods worldwide (Durden et al., 2006), and given the increase in the human population along with the emergence of new animal species acting as reservoir hosts, it is expected that over the years, humans will become one of the most common blood sources for ticks (Baneth et al., 2014), thus representing an impact on public health and a disadvantage to livestock farming due to the economic losses. The recognition of *Anaplasma* as a genus of public health significance has promoted an interest in these bacteria, translating into remarkable information about their molecular biology, genetics, and pathobiology (Battilani et al., 2017). However, in the ROK there is limited information regarding *Anaplasma* infection in domestic ruminants. Therefore, this study aimed to investigate the prevalence and genetic variability of *Anaplasma* species circulating in cattle and goats according to geographic distribution to provide epidemiological information that could be crucial in making important decisions for animal and public health.

2. Materials and Methods

2.1. Sample collection

A total of 686 whole blood samples from domestic ruminants (384 cattle and 302 black goats) were collected from different provinces of the ROK, which were randomly selected between August 2015 and June 2020. These samples were collected in sterile 10 mL tubes containing EDTA anticoagulant and transported to the laboratory in an icepack container. Goat blood samples were collected in Jeollabuk-do (*n*=37), Gwangju metropolitan city (*n*=41), and Jeollanam-do (*n*=224) province, while cattle blood samples were collected in Gyeongsangbuk-do (*n*=90), Gyeongsangnam-do (*n*=65), Jeollanam-do (*n*=65), Gyeonggi-do (*n*=50), Chungcheongnam-do (*n*=60), and Chungcheongbuk-do (*n*=54) provinces (Figure 5).

2.2. DNA extraction

Genomic DNA was extracted from 200 μL whole blood samples using a commercial LaboPass DNA Purification Kit (Cosmo Genetech, Seoul, ROK) according to the manufacturer's instructions. The extracted DNA was stored at -20°C until further analysis. The quantity and purity of the extracted DNA were calculated using a NanoDrop spectrophotometer (Implen NanoPhotometer Classic, Munich, Germany).

2.3. PCR amplification

DNA samples were subjected to nested PCR (nPCR) to amplify the *16S rRNA* gene fragments of *A. phagocytophilum* and *A. bovis*. The first round

was performed using the primer pair AE1-F/AE1-R, which amplifies the *16S rRNA* gene shared by all *Anaplasma* spp. (Table 6). In the second round, PCR-positive samples were reamplified by employing species-specific primer sets EE3/EE4 and ABKf/AB1r for *A. phagocytophilum* (Barlough et al., 1996) and *A. bovis* (Kang et al., 2011), respectively. For multilocus genotyping, *A. phagocytophilum* major surface protein 2 (*msp2*) (Lin et al., 2004) and ankyrin-related protein (*ank*A) (Massung et al., 2000) gene fragments were amplified using nPCR, and *A. bovis* heat shock protein (*groEL*) gene using semi-nested PCR (Guo et al., 2019). For *A. capra*, the *16S rRNA* (Li et al., 2015), citrate synthase (*glt*A) (Yang et al., 2016), *groEL*, and major surface protein 4 (*msp4*) (Yang et al., 2017) genes were amplified using the primer sets described in Table 6. The *A. ovis msp4* gene was amplified using single-step PCR with the primer pair MSP45/MSP43 (de la Fuente et al., 2007).

These reactions were performed in a total volume of 30 μL, containing 10 pmol of each primer, 15 μL of 2x Taq PCR Pre-mix (BioFACT, Daejeon, ROK), 50 to 100 ng of genomic DNA samples for the first round of PCR, and 1 μL of the first PCR product was used as template DNA for the nPCR. Each reaction was conducted in a SimpliAmp Thermal Cycler (Thermo Fisher Scientific, Foster City, CA) under optimal cycling conditions (Table 6). The PCR products were visualized under UV light after 1.5% agarose gel electrophoresis, using a 100 base pairs (bp) ladder (SiZer-100 DNA Marker Solution, iNtRON Biotechnology, Gyeonggi-do, ROK) as a DNA size marker.

2.4. DNA sequencing and phylogenetic analysis

The PCR products were purified using the DNA Gel Extraction S & V Kit (BIONICS, Daejeon, ROK) and were directly sequenced using an Applied Biosystems 3730 DNA Analyzer (Thermo Fisher Scientific, Foster City, CA). The obtained sequences were evaluated with Chromas software, aligned using the multiple sequence alignment program ClustalX 2.1, compared with reference sequences searched in the National Center for Biotechnology Information (NCBI), analyzed using the Basic Local Alignment Search Tool (BLAST) to determine the identity percentage between them, and finally examined with a similarity matrix. Relationships between individuals were assessed by the neighbor-joining method with nucleotide distance (*p* distance) for 1,000 replications with a bootstrap analysis. A phylogenetic tree was constructed based on nucleotide alignment using MEGA 6.06 software.

2.5. Nucleotide sequence accession numbers

The sequences obtained in this study have been deposited in the GenBank database with the following accession numbers: *A. phagocytophilum 16S rRNA* (MT754291 to MT754365), *A. bovis 16S rRNA* (MT754858 to MT754934), *A. bovis groEL* (MW122296 to MW122372), *A. capra 16S rRNA* (MT798599 to MT798604), *A. capra msp4* (MT721148 to MT721149), *A. capra gltA* (MT721142 to MT721147), and *A. capra groEL* (MT721150).

3. Results

3.1. Distribution of *Anaplasma* infection

Anaplasma infection in domestic ruminants was detected in five out of the seven tested provinces and one metropolitan city. The highest infection rate by causative agents of bovine anaplasmosis was found in the Gyeongsangbuk-do province (92.0%), where 83 out of 90 cattle were carriers of one or more *Anaplasma* species. In particular, the cattle tested in this province presented severe tick infestation, which were visible on different body parts of the animal (Figure 6). Chungcheongbuk-do was reported to have an infection rate of 3.7%, followed by Chungcheongnam-do and Jeollanam-do, which were provinces with a lower prevalence of *Anaplasma* species at 1.7% and 1.5%, respectively. In the Gyeongsangnam-do and Gyeonggi-do provinces, neither single infection nor co-infection cases were detected. In goats, the highest prevalence was observed in Jeollabuk-do (27.0%), followed by Gwangju metropolitan city (7.3%) and Jeollanam-do province (4.6%), which had the lowest infection rates (Figure 5).

3.2. *Anaplasma* species prevalence: single infection and co-infection

As described in Table 7, sixteen out of 384 cattle blood samples tested positive for *A. phagocytophilum* (4.2%), which was the dominant single infection among the analyzed *Anaplasma* species, followed by *A. bovis* with 1.3% (5/384). Single infection with *A. capra* occurred in only one cow (0.3%) from the Chungcheongbuk-do province. *A. ovis* infection was not found in any of the tested cattle blood samples. Interestingly, co-infection

with two pathogens (*A. bovis* and *A. phagocytophilum*) was found in 64 cattle (16.7%). Additionally, a special case of triple infection caused by the pathogens *A. bovis*, *A. phagocytophilum*, and *A. capra*, was identified in only one cow (0.3%) from Gyeongsangbuk-do province.

On the other hand, goats were infected with *A. bovis* and *A. capra*. The dominant species in all the single infection cases was *A. bovis* with 6.3% (19/302), followed by *A. capra*, which was present in 0.3% of the total number of goats analyzed. Neither *A. phagocytophilum* nor *A. ovis* was detected in the analyzed goat blood samples. In contrast with the cattle results, co-infection cases in goats were caused by *A. bovis* and *A. capra*, reaching an infection rate of 1.0% (3/302). These findings indicate that the overall infection rate per host was 22.7% (87/384) in cattle and 8.0% (23/302) in goats, as shown in Table 7.

3.3. Total number of animals infected per *Anaplasma* species analyzed

Infection rates of *Anaplasma* spp. were also calculated for each etiological agent. In the present study, 81 (21.1%) cattle were positive for *A. phagocytophilum*, 70 (18.2%) for *A. bovis*, and 2 (0.5%) for *A. capra*. On the other hand, 22 (7.3%) goats were positive for *A. bovis* and four (1.3%) for *A. capra* (Table 8).

3.4. PCR and molecular identification

PCR amplification of the *16S rRNA* gene was successfully achieved for the identification of *A. phagocytophilum*, *A. bovis*, and *A. capra*, generating fragments of 925 bp, 547 bp, and 1,499 bp, respectively (Figure 7). For mul-

tilocus genotyping of *A. phagocytophilum*, no samples were positive for *ank*A and *msp2* gene fragments. Amplification of the *A. bovis groEL* gene was successfully achieved with a fragment length of 845 bp (Figure 8). Regarding *A. capra*, two cattle and four goats were positive for its *16S rRNA* gene (1,499 bp) as well as for the *gltA* gene (594 bp). However, only one goat was positive for *groEL* gene (874 bp), and two goats were positive for the *msp4* gene fragment with a length of 656 bp (Figure 9).

3.5. Sequences and phylogenetic analysis

To investigate the genetic variability of *A. phagocytophilum*, the 75 gene sequences obtained from cattle were analyzed and compared with sequences downloaded from the NCBI. Analysis based on the *A. phagocytophilum 16S rRNA* gene fragments (925 bp) revealed the presence of five distinct strains, named variant type (VT): VT1 (*n*=44), VT2 (*n*=5), VT3 (*n*=16), VT4 (*n*=9) and VT5 (*n*=1) (Figure 10). The phylogenetic analysis demonstrated that these strains were classified into the first clade together with sequences previously isolated from North Korean *H. longicornis* ticks (KC422267) and Japanese wild deer (AB196721), sharing an identity percentage range of 98.0–99.6% and 96.3–99.6%, respectively (Figure 11).

Further analyses of *A. phagocytophilum 16S rRNA* gene sequences revealed that the strains obtained in this study shared a high degree of identity (99.5–99.6%) with North Korean tick isolates (Table 9); however, they presented single and double nucleotide polymorphisms, in which one or two consecutive nucleotides were altered compared with the reference sequence (KC422267; Figure 12), suggesting that all of them are novel variants of the

A. phagocytophilum 16S rRNA gene.

The phylogenetic analysis of *A. bovis 16S rRNA* gene enabled classify-cation of this *Anaplasma* species into two different clades. Clade I included isolates from China, Australia, Malaysia, and Japan, while clade II contained the 63 cattle sequences and the two different strains found in goat blood samples tested in this study [BG346 (*n*=8) and BG348 (*n*=6)] (Figure 13), along with those from Chinese goats (MH255939; Figure 11), which were highly related, sharing an identity percentage of 100% with cattle isolates, 99.8% with variant BG346, and 100% with the variant BG348 (Table 10). The variant BG346 exhibited the highest sequence identity (100%) with *H. longicornis* tick isolates (GU064901) from the ROK.

A. capra 16S rRNA sequences (Figure 14) obtained from cattle and goats showed 100% identity with the sequences detected in *Rhipicephalus microplus* ticks (MH762077) and cattle (MG869510) from China and Korean water deer (LC432114; Table 11). Although only two goat sequences were generated through amplification of the *A. capra msp4* gene (Figure 15), the results of the phylogenetic classification were identical to those shown by amplification of the *16S rRNA* gene (Figure 11). In other words, all *A. capra* sequences of these two genes were classified into an independent clade clearly distinct from other members of *Anaplasma* species. The *msp4* sequences showed 100% identity with the sequences isolated from Korean water deer (LC432231), dogs (MK838607), and humans (KM206277) from China (Figure 16, Table 12).

In contrast, phylogenetic analysis of *A. capra* based on *gltA* and *groEL* genes classified the sequences into two major clades (Figure 17). Sequence

analysis of the *A. capra gltA* gene revealed two different variants (Figure 18). Five isolates (MT721147, MT721145, MT721144, MT721143, and MT721142) were found to be 100% identical to those obtained from Chinese *R. microplus* ticks (MH716413), while the isolate MT721146 obtained from a cattle showed an identity of 99.8% (with one substitution, G/A at position 460) with Chinese tick isolates (MH716413), and 99.6% with the rest of the clustered sequences, which were isolated from Chinese goats, dogs, and sheep (Figure 17a, Table 13), suggesting that this is a novel variant of the *A. capra gltA* gene, which has never been reported before (Figure 19).

For the *A. capra groEL* gene, one sequence was acquired from a goat (MT721150) (Figure 20). This sequence shared 100% identity with the isolates from Chinese cattle (MG932131) and Korean water deer (LC432184) belonging to clade I, together with samples isolated from sheep (99.6%, MG869385), human being (99.6%, KM206275), *R. microplus* ticks (99.6%, MG869481), and goats (99.2%, MH174929) from China (Figure 17b, Table 14).

For *A. bovis groEL* gene sequences, three different variants were obtained: variant I (represented by the isolates C2 [cattle] and BG346 [goat]), variant II (represented by the isolate MC3 [cattle]), and variant III (represented by the isolates MC9 [cattle] and BG348 [goat]) (Figure 21). Variant I shared 100% identity with *H. longicornis* ticks (MK340768), while variant II was 100% identical to *R. microplus* ticks (MK340785). Both variants belonged to clade I together with Chinese isolates. Finally, variant III showed 100% identity with the sequences from Chinese *H. longicornis* ticks

(MK340767), thus representing the second clade (Figure 17b, Table 15).

4. Discussion

In recent years, environmental factors such as global warming and deforestation have favored the increase in tick populations due to changes in their seasonal activity. However, the introduction of new animal species acting as a potential reservoir of one or even multiple pathogen species has led to a rapid distribution and expansion in the number of ticks. This phenomenon can be exemplified by anaplasmosis, a TBD distributed worldwide that has evolved in its pathogenesis over time. Despite recent reports of new species belonging to the genus *Anaplasma* and the considerable increase in their zoonotic potential, few studies on *Anaplasma* infection have been carried out in the ROK. The present study was mainly focused on investigating the genetic variability and prevalence of *Anaplasma* species as single and multiple infection in cattle and goats from different provinces of the ROK.

This investigation revealed that the Korean cattle were mainly infected with *A. phagocytophilum* (21.1%, 81/384) instead of *A. bovis*, whose main hosts are supposed to be cows and buffalo, since its first identification in cattle in 1936 (Battilani et al., 2017). Of the 81 cattle infected with *A. phagocytophilum*, 80 were from Gyeongsangbuk-do province. Conversely, a study carried out in 2016 in the same province identified *A. phagocytophilum* with a relatively low infection rate (4.7%) (Seo et al., 2018) compared with the results obtained in this investigation (88.9%). *A. bovis* was the second dominant pathogen (18.2%, 70/384) identified in the present study. Based on the geographic distribution, Gyeongsangbuk-do was also the prov-

ince with a higher number of carriers of *A. bovis* (74.4%). These findings differed from those of previous molecular studies conducted in the ROK, which reported a comparatively low prevalence of *A. bovis* in cattle: 4.2% (3/71) in Jeju Island (Park et al., 2018), 1.9% (11/581) in Gyeongsangnam-do, and 0.2% (1/638) in Gyeongsangbuk-do province (Seo et al., 2018). This sudden increase in *Anaplasma* spp. prevalence rates may be largely associated with climate change. It is known that the Korean peninsula is gradually transitioning to a subtropical climate (Seo et al., 2018), where a hot summer season and changes in rainfall patterns can favor the abundance and spread of ticks to different localities.

In addition, the present study also identified *A. capra* infection in cattle. In 2018, *A. capra* was described for the first time in Korean cattle from Gyeongsangnam-do with a low infection rate (0.4%) (Seo et al., 2018). These results are similar to those obtained in this study (0.5%). An important point to consider is that one cow positive for *A. capra* was infected with *A. phagocytophilum* and *A. bovis*, which is the first detection of triple infection with *Anaplasma* species in the ROK. It should also be noted that this is the first molecular study of co-infection cases caused by several *Anaplasma* species in cattle, in which triple and doble infection cases were detected, with the latter type involving *A. phagocytophilum* and *A. bovis*. The findings of the present investigation, along with previous studies performed in the ROK (Seo et al., 2018; Seo et al., 2018), demonstrates that the cattle residing in provinces located at a lower latitude, such as Gyeongsangbuk-do and Gyeongsangnam-do, are naturally infected with *Anaplasma* spp., thus suggesting that cattle may play an important role in the enzootic maintenance of

Anaplasma infection.

On the other hand, goats were found to be carriers of two *Anaplasma* species: *A. bovis* (7.3%) and *A. capra* (1.3%). The results obtained in the current study coincide with those previously described in Ulsan metropolitan city, ROK, which reported infection rates of 8.6% and 2.2% for *A. bovis* and *A. capra*, respectively (Seo et al., 2019). Interestingly, this investigation revealed three co-infection cases due to *A. bovis* and *A. capra*, two of which were in Jeollabuk-do and the other in Jeollanam-do province. Thus, the results of this study shed light on co-infection types that may be found among Korean goats. Although *A. phagocytophilum* and *A. ovis* were not identified in this investigation, we cannot rule them out as causative agents of caprine anaplasmosis, since a study conducted in China demonstrated the prevalence of these species, even reporting cases of triple infection with *A. ovis*, *A. bovis*, and *A. phagocytophilum* (Liu et al., 2012). It is also worth mentioning that a serological study conducted in the ROK detected antibodies against the major surface protein 5 (*msp5*) of *A. marginale*, *A. centrale*, and *A. ovis*, using a commercial competitive enzyme-linked immunosorbent assay (ELISA). The seroprevalence rate reported in this study was 6.6% (36/544) in native Korean goats (Lee et al., 2015). However, identification of a species among *A. marginale*, *A. centrale*, and *A. ovis* was not performed by PCR; these findings compared with the data obtained in the present study suggest that *Anaplasma* infection in goats could be caused by other *Anaplasma* species, in addition to *A. bovis* and *A. capra* pathogens. Additional studies are necessary to corroborate this finding, taking into consideration a large sample size that involves the different Korean provinces.

Despite the fact that the genes that are most often targeted to investigate the genetic diversity of *A. phagocytophilum* involve the *16S rRNA* locus, *groESL* operon, major surface protein coding genes (*msp2* and *msp4*), and *ank*A gene (Battilani et al., 2017), PCR amplification of the *msp2* and *ank*A genes was unsuccessful in the present study. This could be due to the complex epidemiological cycles of *A. phagocytophilum*, which involves different genetic variants, vectors, and host species (Battilani et al., 2017). However, according to the results obtained by the amplification of the *A. phagocytophilum 16S rRNA* gene and its phylogenetic analysis, this investigation revealed that five novel strains are circulating among Korean cattle. In addition, the multilocus genotyping of *A. capra* not only favored the ratification of cattle as a potential reservoir of this *Anaplasma* species but also contributed to the identification of a new variant of the *A. capra gltA* gene, thus aiding in elucidation of the genetic variability of *A. phagocytophilum* and *A. capra*, two species with zoonotic importance.

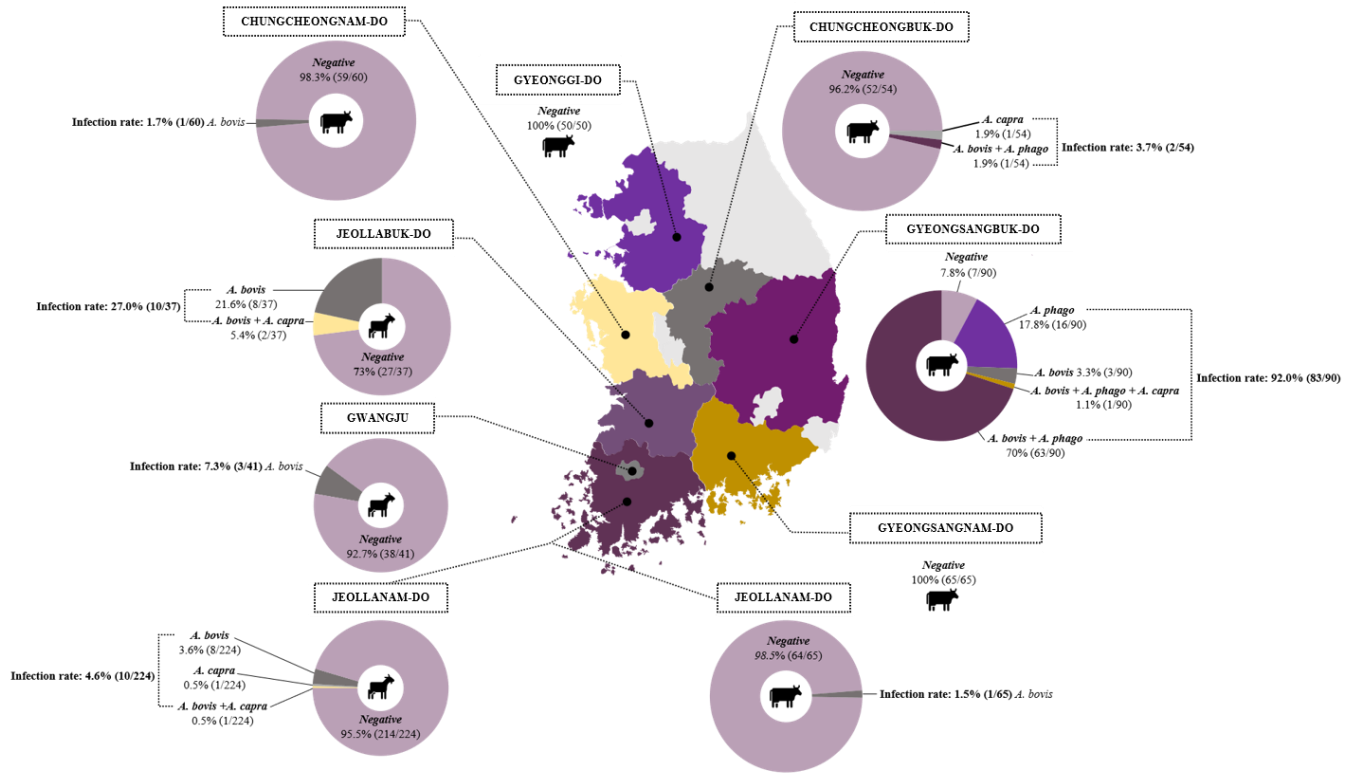


Figure 5. Geographical distribution of *Anaplasma* species found in cattle and goat whole blood samples in 2015-2020, the Republic of Korea.

Pie charts represent *Anaplasma* spp. infection rates for single infection and co-infection cases identified in each tested province. The cattle and goat icons indicate the sampled sites for respective animal species.

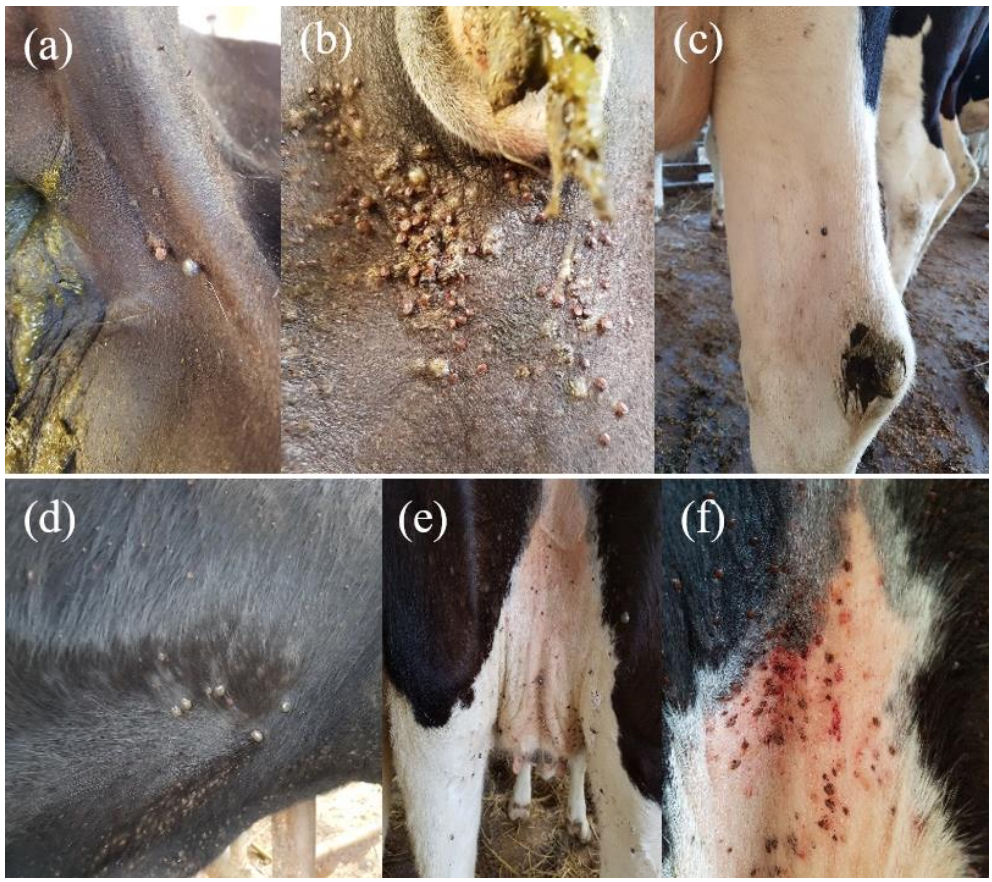


Figure 6. Cattle from the Gyeongsangbuk-do province with the highest *Anaplasma* spp. infection rates. (**a**) Ticks were attached behind the tail, (**b**) around the anus, where there was an accumulated number of ticks feeding on the host. (**c**) Ticks were also attached to the legs as well to (**d**) the dewlap; lesions caused by ticks were perceptible in this region. (**e**) Teats and (**f**) rear udder were the areas that presented the heaviest infestation, where different tick stages were evident. Additionally, local redness (red spots) and rashes were noticeable in those sites.

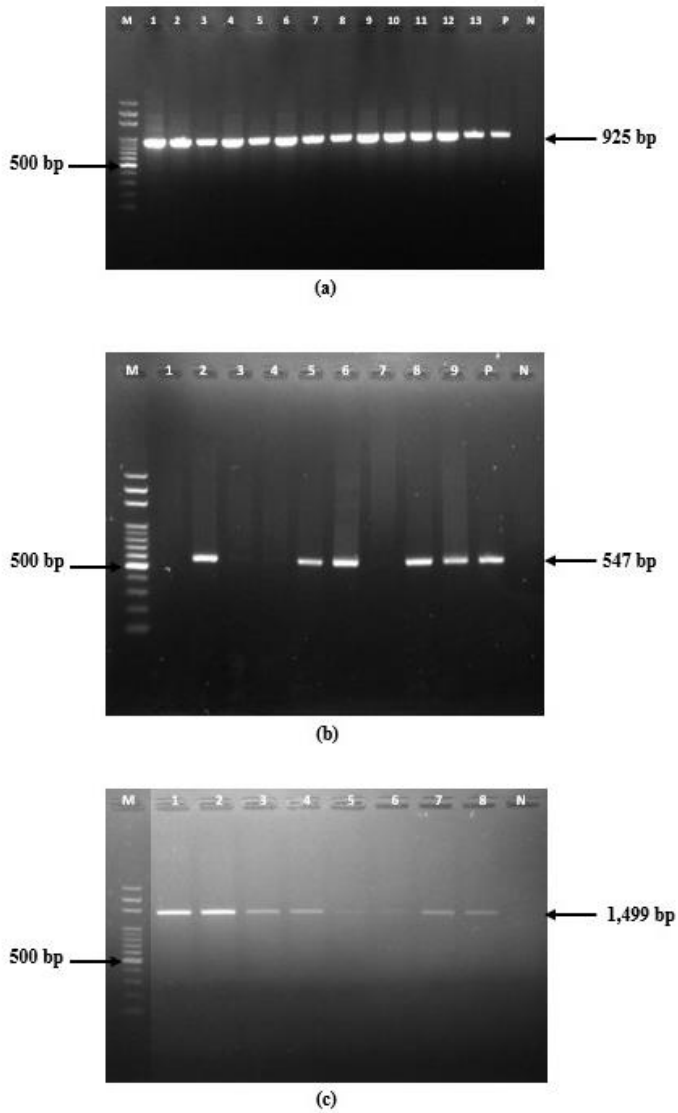


Figure 7. Representative results of agarose gel electrophoresis based on the amplification of the *Anaplasma* species *16S rRNA* gene found in cattle and goat whole blood samples. (**a**) PCR products of *A. phagocytophilum* from cattle samples with a fragment length of 925 bp. (**b**) Lines 2, 5, 6, 8 and 9 show PCR products of *A. bovis* from cattle and goat samples with a fragment length of 547 bp. (**c**) PCR products of *A. capra* from cattle and goat samples with a fragment length of 1,499 bp. M, DNA size marker (100 bp ladder); P, positive control; N, negative control (TE buffer).

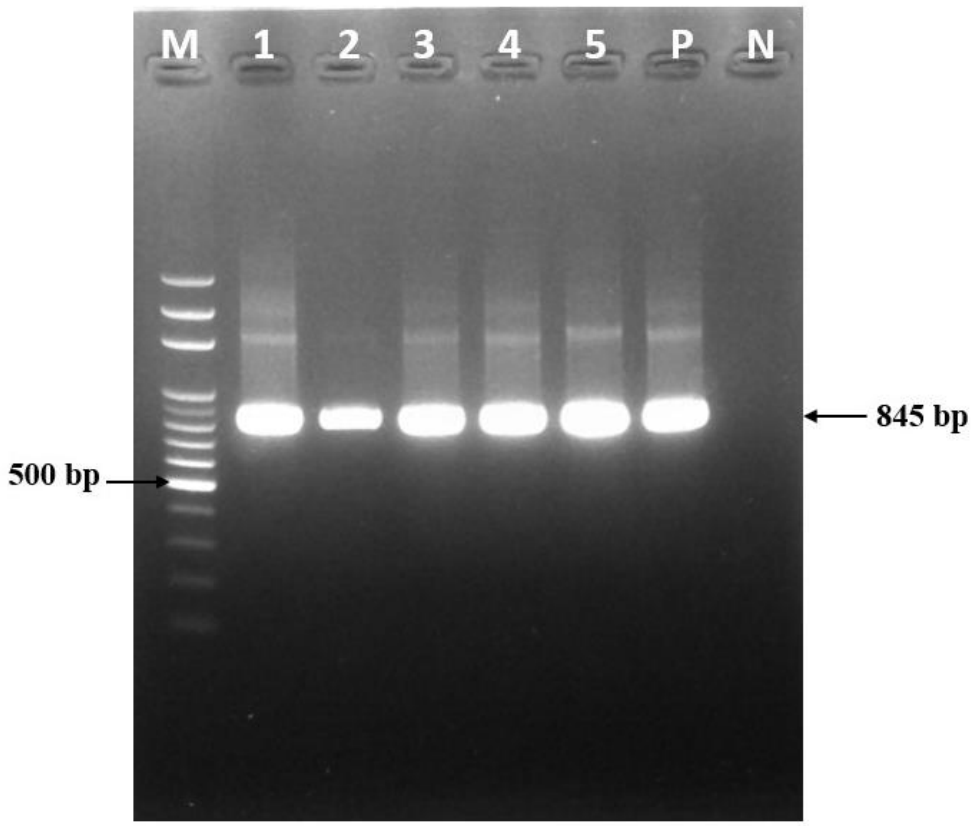


Figure 8. Representative results of agarose gel electrophoresis based on the amplification of the *Anaplasma bovis groEL* gene found in cattle and goat whole blood samples. Lines 1 to 5 show the PCR products of *A. bovis groEL* gene with a fragment length of 845 bp. M, indicates DNA size marker (100 bp ladder); P, the positive control and N, the negative control (TE buffer).

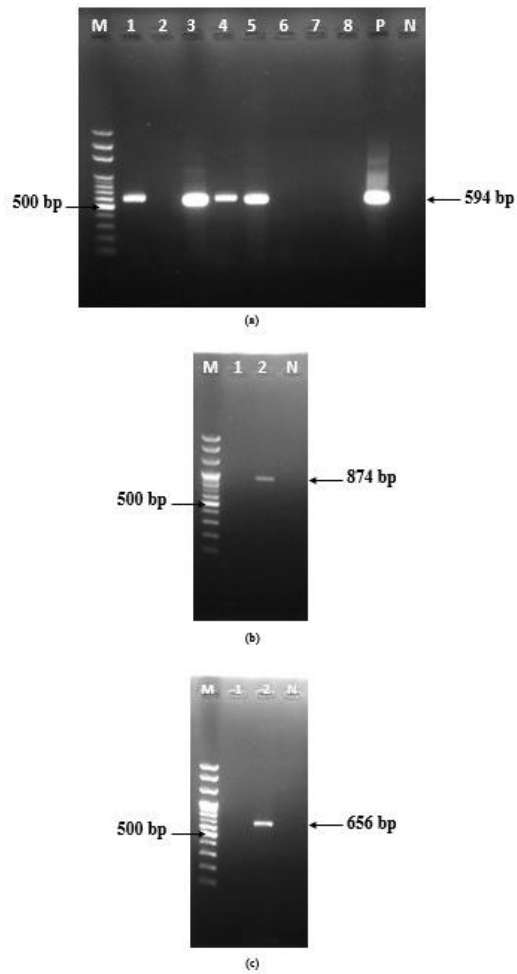


Figure 9. Representative results of agarose gel electrophoresis based on the amplification of the *Anaplasma capra gltA, groEL* and *msp4* genes found in cattle and goat whole blood samples. (**a**) Lines 1, 3-5 show PCR products of *A. capra gltA* gene found in cattle and goat samples with a fragment length of 594 bp. Lines 2, 6-8 show negative samples. (**b**) Line 2 shows the PCR product of *A. capra groEL* gene found in a goat sample with a fragment length of 874 bp, and line 1 a negative sample. (**c**) Line 2 indicates the PCR product of *A. capra msp4* gene found in a goat sample with a fragment length of 656 bp, while line 1 shows a negative sample. M, DNA size marker (100 bp ladder); P, positive control; N, negative control (TE buffer).

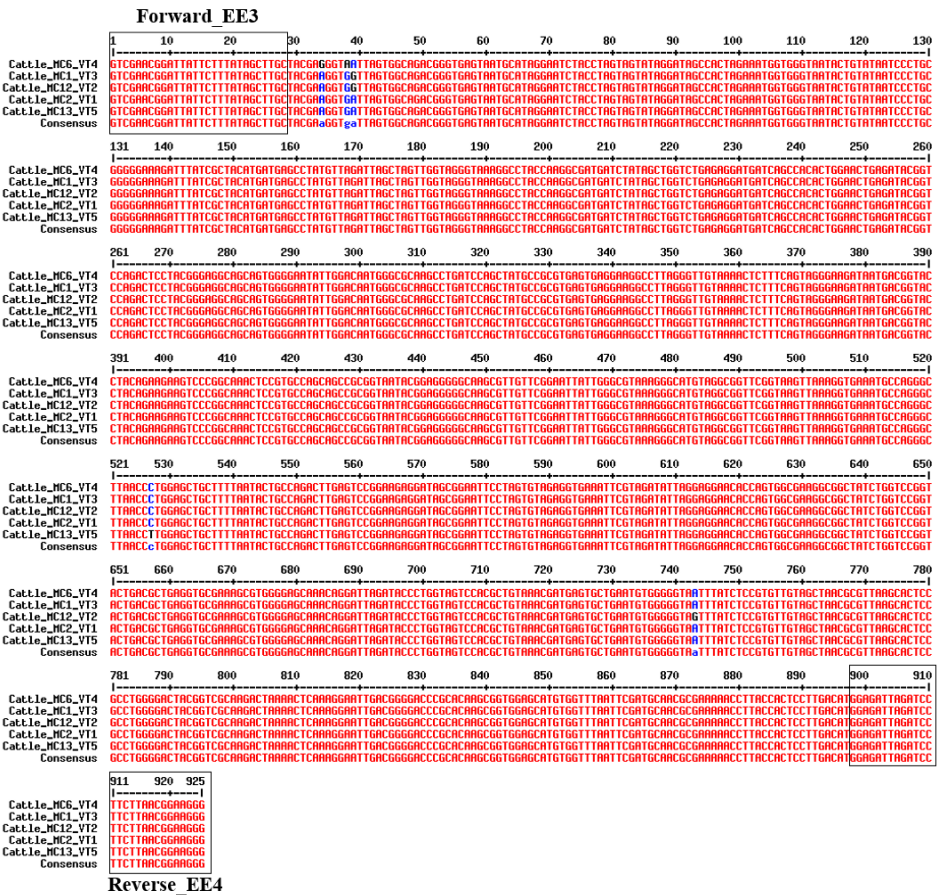


Figure 10. Multiple sequence alignment of the five novel *Anaplasma phagocytophilum 16S rRNA* gene variants found in cattle whole blood samples. Boxes indicate the forward and reverse primer regions. A residue that is highly conserved appears in high-consensus color (red) and as an uppercase letter in the consensus line. A residue that is weakly conserved appears in low-consensus color (blue) and as a lowercase letter in the consensus line. Other residues appear in neutral color (black). Fragments length, 925 bp.

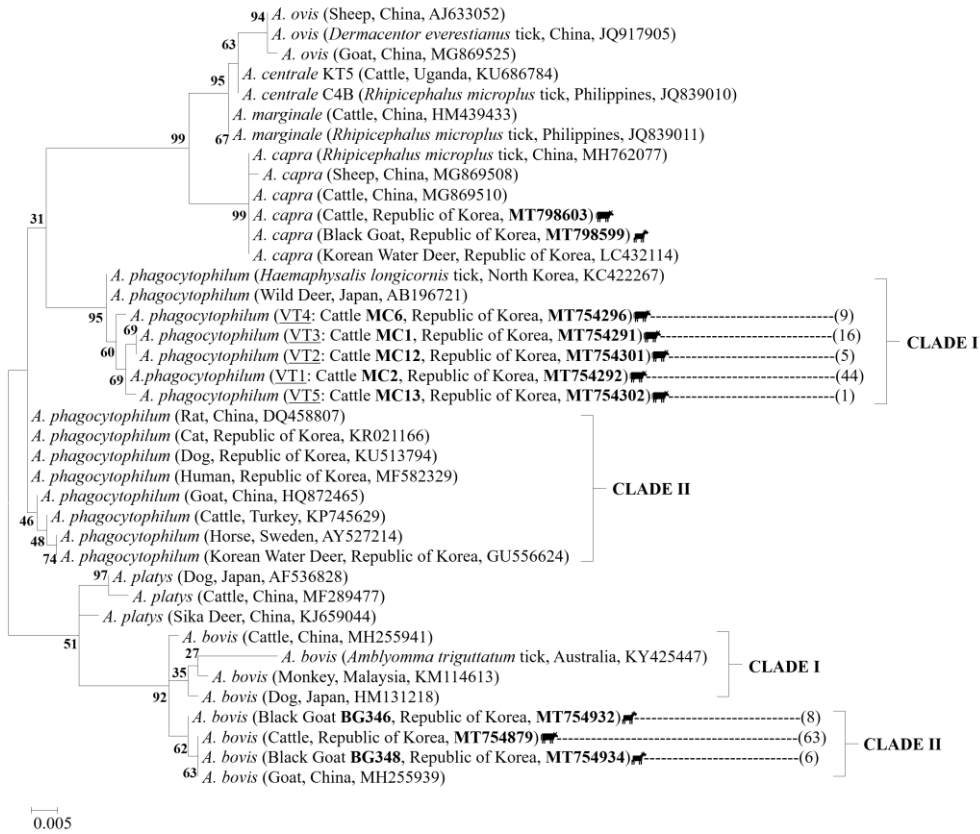


Figure 11. Phylogenetic tree based on the *16S rRNA* gene fragments (547 bp) of the *Anaplasma* species. The sequence alignments were performed among the *A. phagocytophilum, A. bovis* and *A. capra* sequences obtained in this study and other members of the family Anaplasmataceae. The phylogenetic tree was constructed using the neighbor-joining method with 1,000 replicates of the alignment (MEGA 6.06 software). Cattle and goat icons indicate the sequences found in this study for the respective animal species. Isolate, country, and GenBank accession numbers are shown in parentheses. Novel variants of *A. phagocytophilum* are indicated by the abbreviation VT (variant type). The numbers in brackets represent the total number of sequences that are identical to the representative sequence. The scale bar represents the number of nucleotide substitutions between sequences. Clades are denoted by roman numbers. Numbers on the branches indicate percent support for each clade.

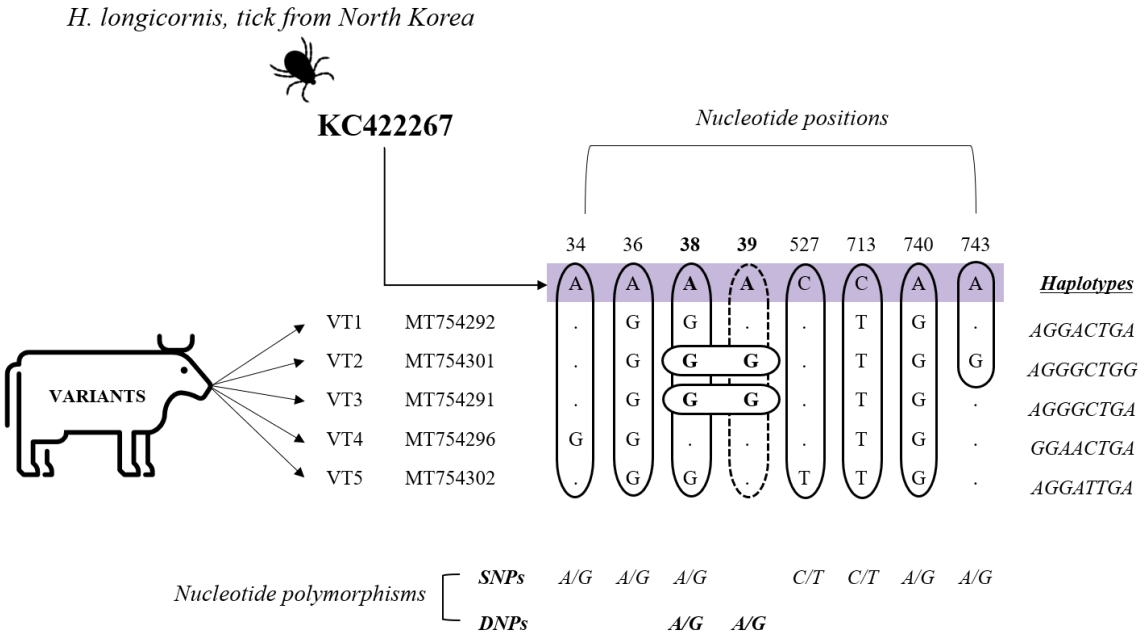


Figure 12. Novel variants of *Anaplasma phagocytophilum 16S rRNA* gene found in cattle. KC422267: reference sequence. Through the comparison between the reference sequence and the five variants identified in this study, single nucleotide polymorphisms (SNPs) were identified at positions 34, 36, 38, 740, and 743 with variation A/G; and positions 527 and 713 with variation C/T. Double nucleotide polymorphisms (DNPs) were found at positions 38 and 39 variation A/G, which are indicated by bold letters. The haplotypes generated due to the DNA variations found along the sequences are shown.

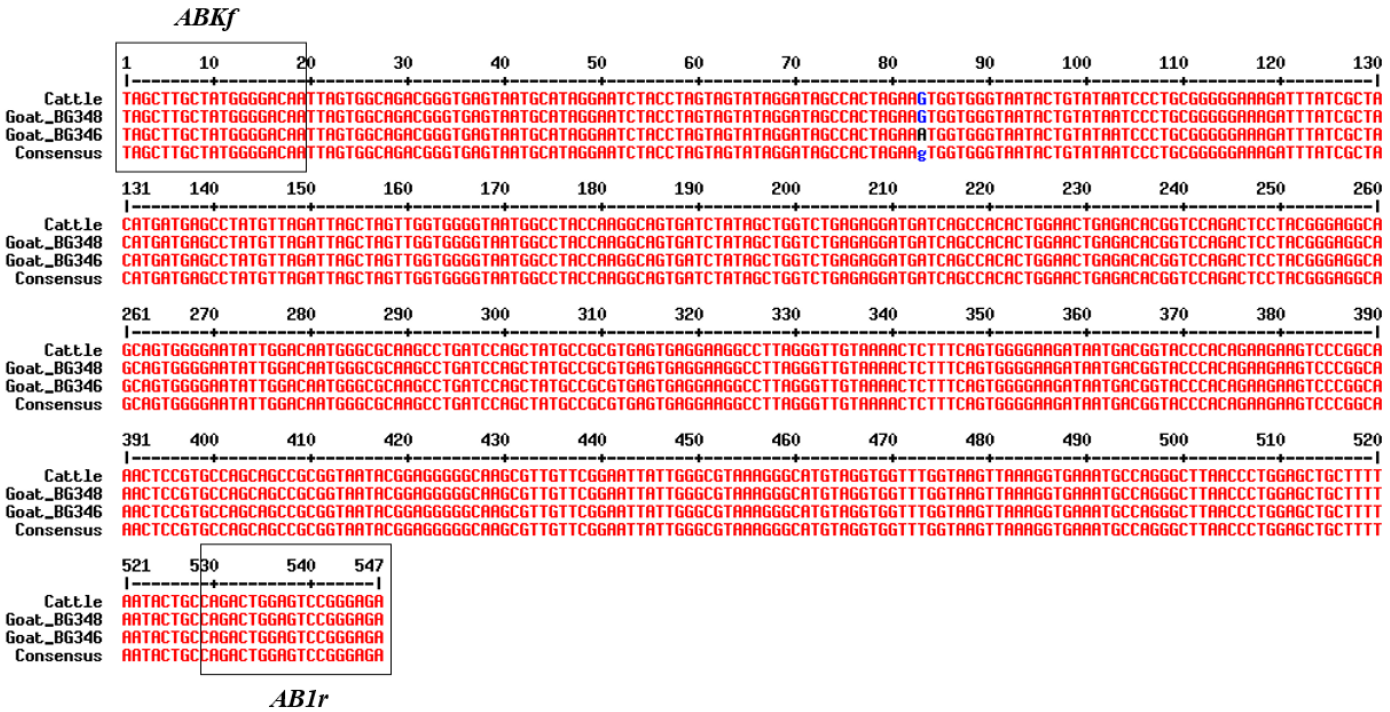


Figure 13. Multiple alignment of *Anaplasma bovis 16S rRNA* gene sequences found in cattle and goat whole blood samples. Boxes indicate the forward (*ABKf*) and reverse (*AB1r*) primer regions. A single sequence was found in cattle whole blood samples [Cattle, representative sequence]. This sequence was 100% identical to one of the two sequences found in goat [100% identical to Goat_BG348, as shown in the alignment]. Whereas the sequence Goat_BG346 showed a point mutation at position 83, variation G/A, as indicated in black color. Fragments length, 547 bp.

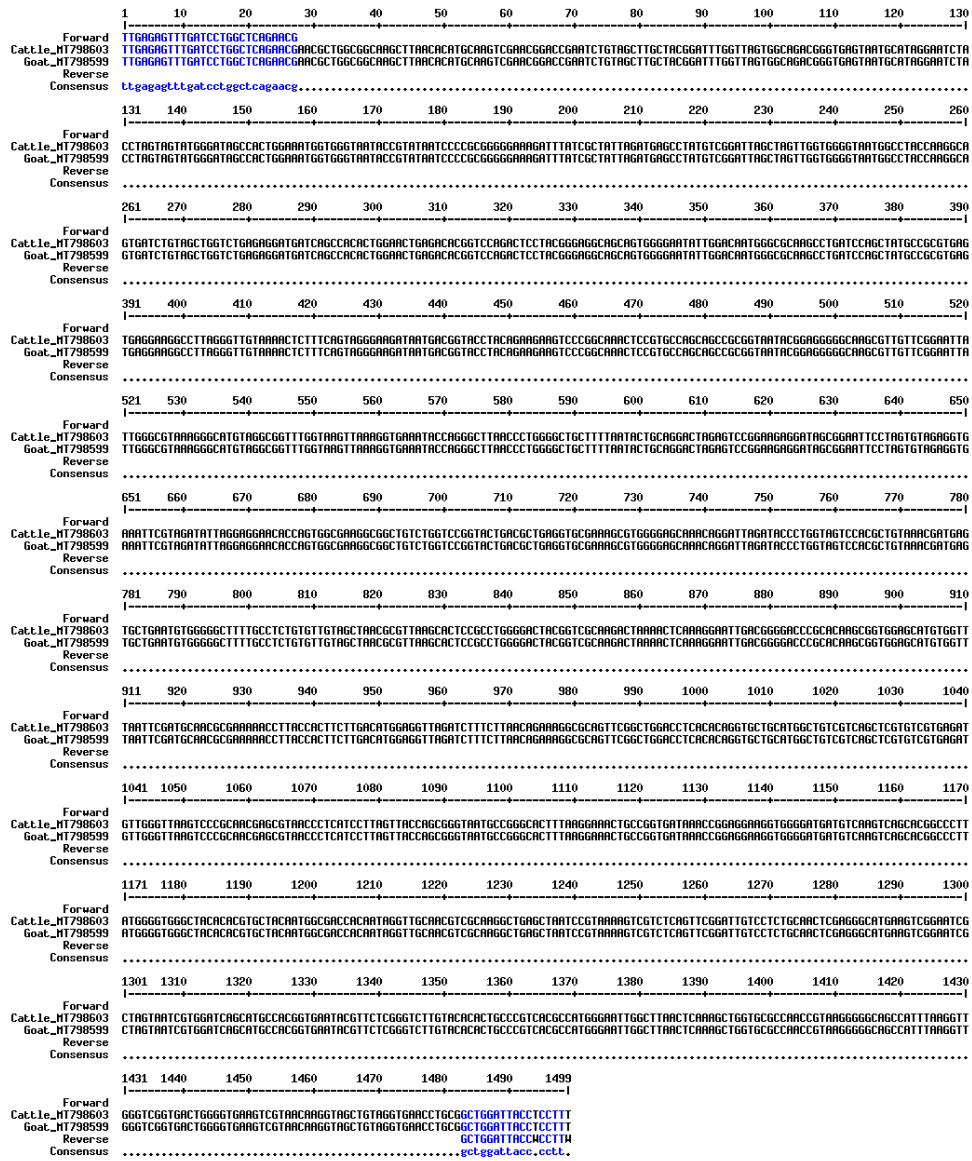


Figure 14. Multiple alignment of *Anaplasma capra 16S rRNA* gene sequences found in cattle and goat whole blood samples. Primers forward and reverse are shown in blue color. The cattle and goat sequences found in this study were 100% identical to each other. MT798603, representative sequence for cattle; MT798599, representative sequence for goats. Fragments length, 1,499 bp.

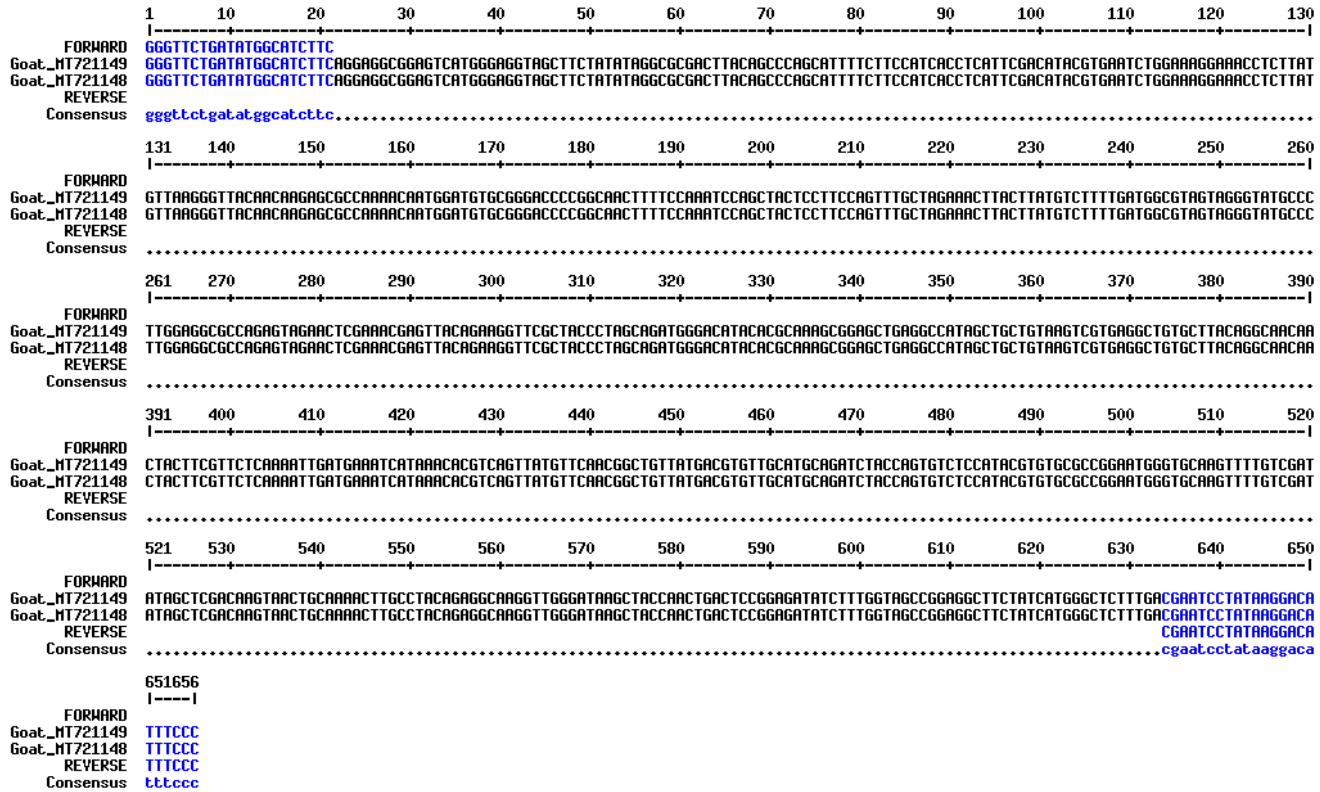


Figure 15. Multiple alignment of *Anaplasma capra msp4* gene sequences found in cattle and goat whole blood samples. Primers forward and reverse are shown in blue color. The two sequences generated in this study were from goat blood samples and were found to be 100% identical to each other, with a fragment length of 656 bp.

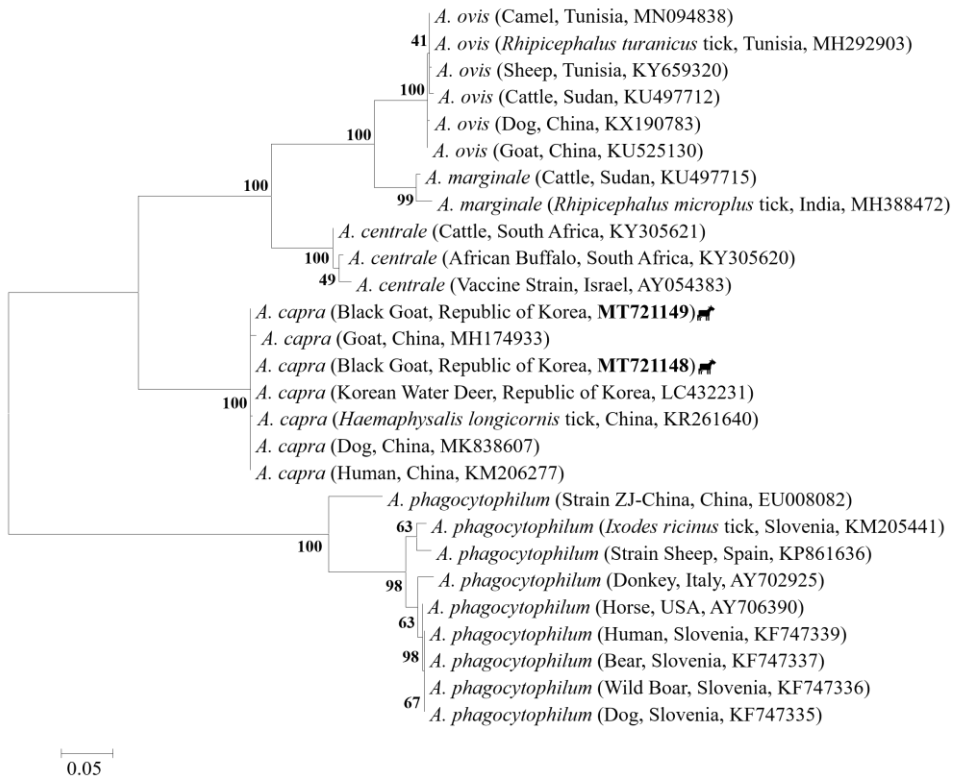


Figure 16. Phylogenetic tree based on the *msp4* gene fragments (527 bp) of the *Anaplasma* species. The sequence alignments were performed among the *A. capra* sequences obtained in this study and other members of the family Anaplasmataceae. The phylogenetic tree was constructed using the neighbor-joining method with 1,000 replicates of the alignment (MEGA 6.06 software). Goat icons indicate the sequences found in this study. Isolate, country, and GenBank accession numbers are shown in parentheses. The scale bar represents the number of nucleotide substitutions between sequences. Numbers on the branches indicate percent support for each clade.

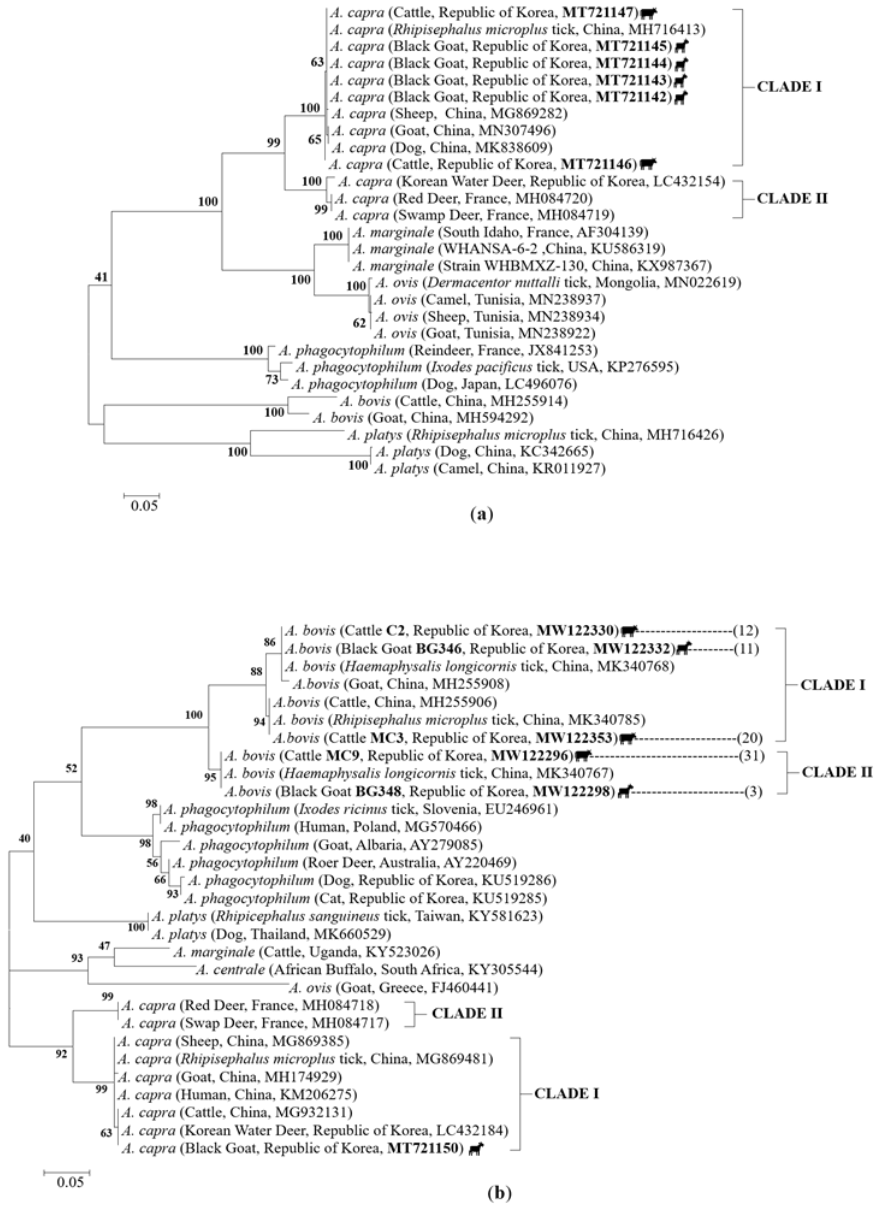


Figure 17. Phylogenetic tree based on the *gltA* and *groEL* gene fragments of the *Anaplasma* species. The numbers of nucleotides were 480 bp and 238 bp in the final alignment for (**a**) *gltA* and (**b**) *groEL* genes, respectively. Cattle and goat icons indicate the sequences found in this study. The numbers in brackets represent the total number of sequences that are identical to the representative sequence. Isolate, country, and GenBank accession numbers are shown in parentheses. The scale bar represents the number of nucleotide substitutions between sequences. Clades are denoted by roman numbers. Numbers on the branches indicate percent support for each clade.

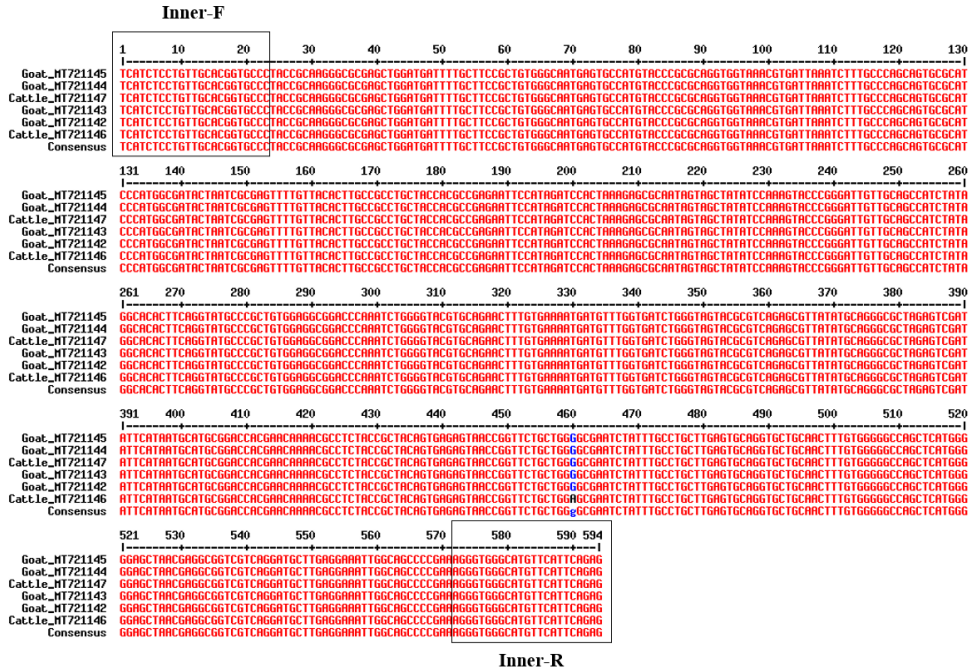
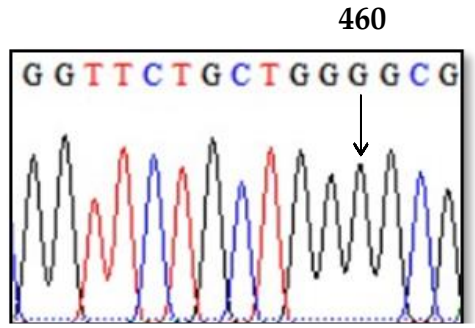
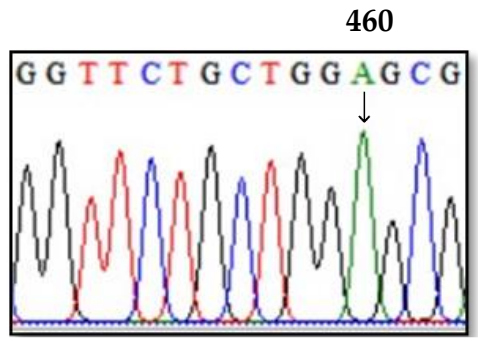


Figure 18. Multiple sequence alignment between the representative sequences of the *Anaplasma capra gltA* gene and the novel variant found in cattle whole blood samples. Boxes indicate the forward (*Inner-F*) and reverse (*Inner-R*) primer regions. The sequences under the GenBank accession numbers MT721145, MT721144, MT721147, MT721143, and MT721142 were 100% identical to each other, while the sequence MT721146 found in cattle shared an identity of 99.8%, showing single nucleotide polymorphism at position 460, as indicated in black color. Fragments length, 594 bp.



(**a**)



(**b**)

Figure 19. DNA sequencing electropherogram of *Anaplasma capra gltA* gene sequences found in cattle whole blood samples. Arrows indicate the point mutation found in the obtained *A. capra gltA* sequences, which was variation G/A at position 460. (**a**) Indicates the sequences obtained from cattle and goat isolates MT721147, MT721145, MT721144, MT721143, and MT721142. (**b**) Represents the novel variant found in cattle whole blood samples (MT721146).

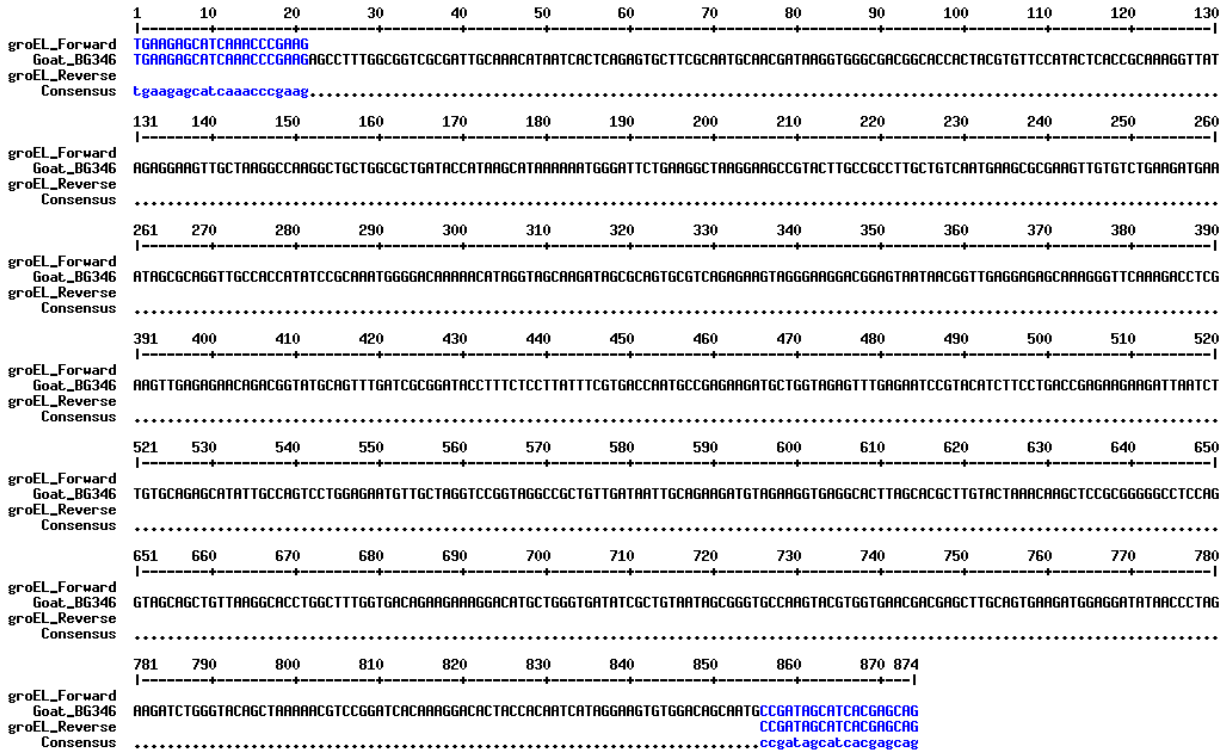


Figure 20. Alignment of the *Anaplasma capra groEL* gene sequence found in goat whole blood samples. A single sequence of *A. capra groEL* gene was obtained in this study from goat whole blood samples. Blue color represents primer regions. Fragments length, 874 bp.

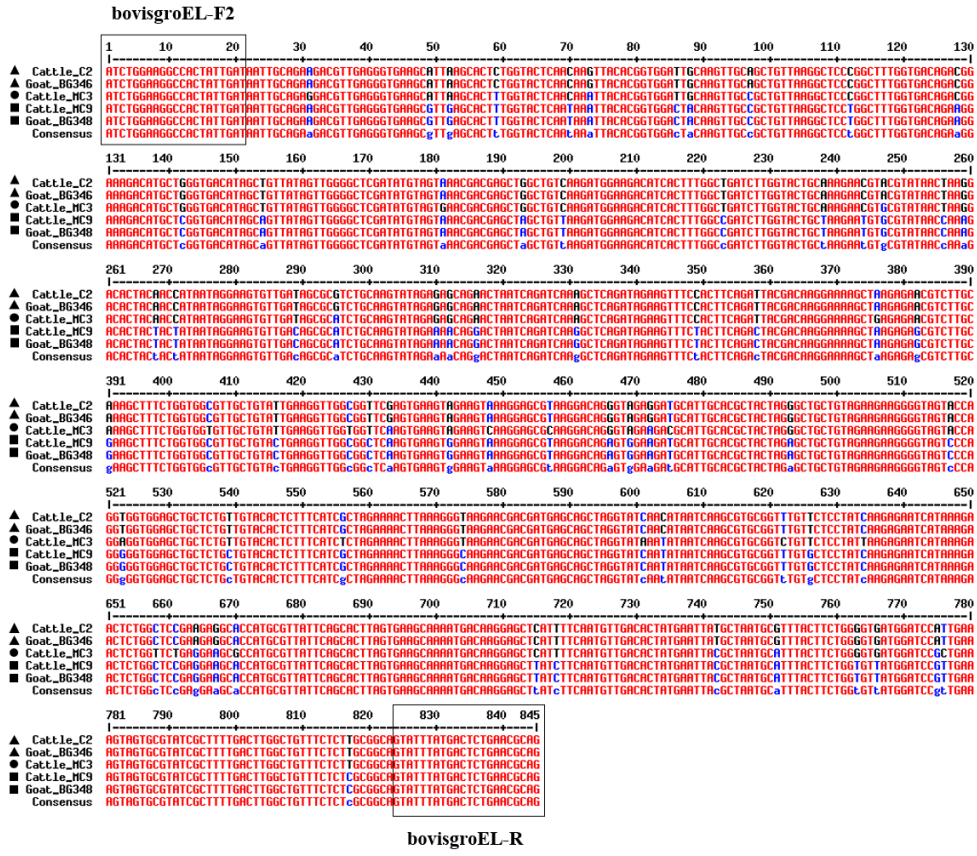


Figure 21. Multiple sequence alignment of the *Anaplasma bovis groEL* gene variants found in cattle and goat whole blood samples. Boxes indicate the forward and reverse primer regions. A residue that is highly conserved appears in high-consensus color (red) and as an uppercase letter in the consensus line. A residue that is weakly conserved appears in low-consensus color (blue) and as a lowercase letter in the consensus line. Other residues appear in neutral color (black). Variant I, represented by triangles (isolates C2 [cattle] and BG346 [goat]); variant II, represented by a circle (isolate MC3 [cattle]); and variant III, represented by squares (isolates MC9 [cattle] and BG348 [goat]). Fragments length, 845 bp.

Table 6. Oligonucleotide primers and polymerase chain reaction (PCR) conditions used for the amplification of *Anaplasma* species from cattle and goat whole blood samples

Species	Target gene	Primer name and PCR conditions	5′-Nucleotide sequences-3′			Cycles	Amplicon size (bp)	References
			Denaturation (°C /min)	Annealing (°C /min)	Extension (°C /min)			
Anaplasma spp.	*16S rRNA* [1]	*AE1-F*	AAGCTTAACACATGCAAGTCGAA			35	1,406	Oh et al. (2009)
		AE1-R	AGTCACTGACCCAACCTTAAATG					
		Conditions	94/1	56/1	72/1.5			
A. phagocytophilum	*16S rRNA* [2]	*EE3*	GTCGAACGGATTATTCTTTATAGCTTGC			25	926	Barlough et al. (1996)
		EE4	CCCTTCCGTTAAGAAGGATCTAATCTCC					
		Conditions	94/0.50	56/0.50	72/0.75			
	msp2	*msp2fullF*	TCAGAAAGATACACGTGCGCCC			35	1,079	Lin et al. (2004)
		msp2fullR	TTATGATTAGGCCTTTGGGCATG					
		Conditions	94/1	54/1	72/1			
		msp2F	GGTTACATAAGGGCCGCAAAGGTG			25	467	
		msp2R	CCGGCGCATGTGTAAGGTGAAA					
		Conditions	94/0.5	57/0.5	72/0.5			
	*ank*A	*U7*	GCGTCTGTAAGGCAGATTGTG			35	1,696	Massung et al. (2000)
		1R1	TATACACCTGGAGTAGGAAC					
		Conditions	94/1	57/1	72/1.5			
		U8	TAAGATAGGTTTAGTAAGACG			25	460	
		1R7	TGCATCGTCATTACGCACAAGGTC					
		Conditions	94/0.75	57/0.75	72/0.75			
A. bovis	*16S rRNA* [3]	*ABKf*	TAGCTTGCTATGGGGACAA			25	547	Kang et al. (2011)
		AB1r	TCTCCCGGACTCCAGTCTG					
		Conditions	94/0.5	59/0.5	72/0.5			
	groEL	*bovis-groEL-F1*	GTTCGCAGTATTTTGCCAGT			30	845	Guo et al. (2019)
		bovisgroEL-R	CTGCRTTCAGAGTCATAAATAC					
		bovis-groEL-F2	ATCTGGAAGRCCACTATTGAT					
		Conditions	94/0.7	56/0.7	72/1			

Table 6. Oligonucleotide primers and polymerase chain reaction (PCR) conditions used for the amplification of *Anaplasma* species from cattle and goat whole blood samples (Continued)

Species	Target gene	Primer name and PCR conditions	5′-Nucleotide sequences-3′			Cycles	Amplicon size (bp)	References
			Denaturation (°C /min)	Annealing (°C /min)	Extension (°C /min)			
A. capra	*16S rRNA*	*Forward*	TTGAGAGTTTGATCCTGGCTCAGAACG					
		Reverse	WAAGGWGGTAATCCAGC			30	1,499	
		Conditions	94/0.75	57/0.75	72/0.75			Li et al. (2015)
	gltA	*Outer-f*	GCGATTTTAGAGTGYGGAGATTG					
		Outer-r	TACAATACCGGAGTAAAAGTCAA			30	1,031	
		Conditions	94/0.75	55/0.75	72/0.75			
		Inner-f	TCATCTCCTGTTGCACGGTGCCC					
		Inner-r	CTCTGAATGAACATGCCCACCCT			30	594	Yang et al. (2016)
		Conditions	94/0.75	60/0.75	72/0.75			
	groEL	*Forward*	TGAAGAGCATCAAACCCGAAG					
		Reverse	CTGCTCGTGATGCTATCGG			30	874	
		Conditions	94/0.75	55/0.75	72/0.75			Yang et al. (2017)
	msp4	*Forward*	GGGTTCTGATATGGCATCTTC					
		Reverse	GGGAAATGTCCTTATAGGATTCG			30	656	
		Conditions	94/0.75	53/0.75	72/0.75			
A. ovis	*msp4*	*MSP45*	GGGAGCTCCTATGAATTACAGAGAATTGTTTAC					
		MSP43	CCGGATCCTTAGCTGAACAGGAATCTTGC			35	852	De la Fuente et al. (2007)
		Conditions	94/0.5	60/0.5	68/1			

[1] Primer pair used in the first round for the amplification of the *16S rRNA* gene shared by all *Anaplasma* spp.; [2] species-specific primer sets used in the second round for the amplification of the *16S rRNA* gene of *A. phagocytophilum*; [3] species-specific primer sets used in the second round for the amplification of the *16S rRNA* gene of *A. bovis*.

Table 7. Prevalence of single infection and co-infection cases with *Anaplasma* species detected in cattle and goat blood samples in 2015-2020, the Republic of Korea

Animal species	No. tested	No. of positive (IR, %)	Type of infection identified in tested blood samples							Not infected (%)
			Single infection				Double infection		Triple infection	
			A. phago (%)	*A. bovis* (%)	*A. capra* (%)	*A. ovis* (%)	*A. bovis* + *A. phago* (%)	*A. bovis* + *A. capra* (%)	*A. phago* + *A. bovis* + *A. capra* (%)	
Cattle	384	87 (22.7)	16 (4.2)	5 (1.3)	1 (0.3)	0 (0)	64 (16.7)	0 (0)	1 (0.3)	297 (77.3)
Goat	302	23 (8.0)	0 (0)	19 (6.3)	1 (0.3)	0 (0)	0 (0)	3 (1.0)	0 (0)	279 (94.4)
Total	**686**	**110 (16.0)**	**16 (2.3)**	**24 (3.5)**	**2 (0.3)**	**0 (0)**	**64 (9.3)**	**3 (0.4)**	**1 (0.1)**	**576 (83.9)**

IR, infection rate; *A. phago*, *Anaplasma phagocytophilum*.

Table 8. Prevalence rates per *Anaplasma* species analyzed in cattle and goats in 2015-2020, the Republic of Korea

Host	Collected province	No. tested	*A. phago* (%)	*A. bovis* (%)	*A. capra* (%)	*A. ovis* (%)
Cattle	Gyeongsangbuk-do	90	80 (88.9)	67 (74.4)	1 (1.1)	0 (0)
	Gyeongsangnam-do	65	0 (0)	0 (0)	0 (0)	0 (0)
	Jeollanam-do	65	0 (0)	1 (1.5)	0 (0)	0 (0)
	Gyonggi-do	50	0 (0)	0 (0)	0 (0)	0 (0)
	Chungcheongbuk-do	54	1 (1.9)	1 (1.9)	1 (1.9)	0 (0)
	Chungcheongnam-do	60	0 (0)	1 (1.7)	0 (0)	0 (0)
	Subtotal	384	81 (21.1)	70 (18.2)	2 (0.5)	0 (0)
Goat	Jeollabuk-do	37	0 (0)	10 (27.0)	2 (5.4)	0 (0)
	Gwangju metropolitan city	41	0 (0)	3 (7.3)	0 (0)	0 (0)
	Jeollanam-do	224	0 (0)	9 (4.0)	2 (1.0)	0 (0)
	Subtotal	302	0 (0)	22 (7.3)	4 (1.3)	0 (0)
Grand total		**686**	**81 (11.8)**	**92 (13.4)**	**6 (0.9)**	**0 (0)**

A. phago, Anaplasma phagocytophilum.

Table 9. Genetic identity matrix based on the *16S rRNA* gene fragments of *A. phagocytophilum* (547 bp) from cattle

No.	Isolate	Country	GenBank accession number	No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	Cattle [1]	ROK	MT754296, MT754301	**1**	**100**	99.6	99.6	99.6	99.5	99.5	98.5	98.5	98.5	98.5	98.4	98.2	98.0	98.0
2	Cattle [1]	ROK	MT754292	**2**	2	**100**	99.6	99.6	99.8	99.5	98.4	98.4	98.4	98.4	98.0	98.2	98.0	98.0
3	*H. longicornis* tick	NK	KC422267	**3**	2	2	**100**	100	99.5	99.5	98.5	98.5	98.5	98.5	98.4	98.5	98.4	98.4
4	Wild deer	Japan	AB196721	**4**	2	2	0	**100**	99.5	99.5	98.5	98.5	98.5	98.5	98.4	98.5	98.4	98.4
5	Cattle [1]	ROK	MT754291	**5**	3	1	3	3	**100**	99.6	98.2	98.2	98.2	98.2	97.8	98.0	98.2	98.2
6	Cattle [1]	ROK	MT754302	**6**	3	1	3	3	2	**100**	98.2	98.2	98.2	98.2	97.8	98.0	97.8	97.8
7	Rat	China	DQ458807	**7**	8	9	8	8	10	10	**100**	100	100	100	99.6	99.5	99.3	99.3
8	Cat	ROK	KR021166	**8**	8	9	8	8	10	10	0	**100**	100	100	99.6	99.5	99.3	99.3
9	Dog	ROK	KU513794	**9**	8	9	8	8	10	10	0	0	**100**	100	99.6	99.5	99.3	99.3
10	Human	ROK	MF582329	**10**	8	9	8	8	10	10	0	0	0	**100**	99.6	99.5	99.3	99.3
11	Goat	China	HQ872465	**11**	9	11	9	9	12	12	2	2	2	2	**100**	99.8	99.6	99.6
12	Cattle	Turkey	KP745629	**12**	10	10	8	8	11	11	3	3	3	3	1	**100**	99.8	99.8
13	Horse	Sweden	AY527214	**13**	11	11	9	9	10	12	4	4	4	4	2	1	**100**	100
14	Water deer	ROK	GU556624	**14**	11	11	9	9	10	12	4	4	4	4	2	1	0	**100**

Isolate, country, and GenBank accession numbers of each sequence are specified in the left site of the matrix. White boxes of the matrix indicate the percent identity between sequences and gray boxes shown the nucleotide differences between them. [1] Representative sequences of *A. phagocytophilum 16S rRNA* gene obtained in this study. ROK, Republic of Korea; NK, North Korea.

Table 10. Genetic identity matrix based on the *16S rRNA* gene fragments of *A. bovis* (547 bp) from cattle and goat

No.	Isolate	Country	GenBank accession number	No.	1	2	3	4	5	6	7	8
1	Black goat [1]	ROK	MT754932	**1**	**100**	99.8	99.8	99.8	99.5	99.1	98.9	98.0
2	Cattle [1]	ROK	MT754879	**2**	1	**100**	100	100	99.3	99.3	99.1	98.2
3	Black goat [1]	ROK	MT754934	**3**	1	0	**100**	100	99.3	99.3	99.1	98.2
4	Goat	China	MH255939	**4**	1	0	0	**100**	99.3	99.3	99.1	98.2
5	Cattle	China	MH255941	**5**	3	4	4	4	**100**	99.3	99.1	98.2
6	Dog	Japan	HM131218	**6**	5	4	4	4	4	**100**	99.5	98.2
7	Monkey	Malaysia	KM114613	**7**	6	5	5	5	5	3	**100**	98.4
8	*A. triguttatum* tick	Australia	KY425447	**8**	11	10	10	10	10	10	9	**100**

Isolate, country, and GenBank accession numbers of each sequence are specified in the left site of the matrix. White boxes of the matrix indicate the percent identity between sequences and gray boxes shown the nucleotide differences between them. [1] Representative sequences of *A. bovis 16S rRNA* gene obtained in this study. ROK, Republic of Korea.

Table 11. Genetic identity matrix based on the *16S rRNA* gene fragments of *A. capra* (547 bp) from cattle and goat

No.	Isolate	Country	GenBank accession number	No.	1	2	3	4	5	6
1	Black goat [1]	ROK	MT798599	**1**	**100**	100	100	100	100	99.8
2	Cattle [1]	ROK	MT798603	**2**	0	**100**	100	100	100	99.8
3	*R. microplus* tick	China	MH762077	**3**	0	0	**100**	100	100	99.8
4	Cattle	China	MG869510	**4**	0	0	0	**100**	100	99.8
5	Water deer	ROK	LC432114	**5**	0	0	0	0	**100**	99.8
6	Sheep	China	MG869508	**6**	1	1	1	1	1	**100**

Isolate, country, and GenBank accession numbers of each sequence are specified in the left site of the matrix. White boxes of the matrix indicate the percent identity between sequences and gray boxes shown the nucleotide differences between them. [1] Representative sequences of *A. capra 16S rRNA* gene obtained in this study. ROK, Republic of Korea.

Table 12. Genetic identity matrix based on the *msp4* gene fragments of *A. capra* (527 bp) from goat

No.	Isolate	Country	GenBank accession number	No.	1	2	3	4	5	6
1	Black goat [1]	ROK	MT721149, MT721148	**1**	**100**	100	100	100	99.8	99.4
2	Water deer	ROK	LC432231	**2**	0	**100**	100	100	99.8	99.4
3	Dog	China	MK838607	**3**	0	0	**100**	100	99.8	99.4
4	Human	China	KM206277	**4**	0	0	0	**100**	99.8	99.4
5	*H. longicornis* tick	China	KR261640	**5**	1	1	1	1	**100**	99.2
6	Goat	China	MH174933	**6**	3	3	3	3	4	**100**

Isolate, country, and GenBank accession numbers of each sequence are specified in the left site of the matrix. White boxes of the matrix indicate the percent identity between sequences and gray boxes shown the nucleotide differences between them. [1] Representative sequences of *A. capra msp4* gene obtained in this study. ROK, Republic of Korea.

Table 13. Genetic identity matrix based on the *gltA* gene fragments of *A. capra* (480 bp) from cattle and goat

No.	Isolate	Country	GenBank accession number	No.	1	2	3	4	5	6	7	8	9	10
1	Black goat [1]	ROK	MT721145	**1**	**100**	100	100	99.8	99.8	99.8	99.8	88.1	88.1	87.9
2	Cattle [1]	ROK	MT721147	**2**	0	**100**	100	99.8	99.8	99.8	99.8	88.1	88.1	87.9
3	*R. microplus* tick	China	MH716413	**3**	0	0	**100**	99.8	99.8	99.8	99.8	88.1	88.1	87.9
4	Cattle [1]	ROK	MT721146	**4**	1	1	1	**100**	99.6	99.6	99.6	88.3	88.3	88.1
5	Sheep	China	MG869282	**5**	1	1	1	2	**100**	99.6	99.6	87.9	87.9	87.7
6	Goat	China	MN307496	**6**	1	1	1	2	2	**100**	100	87.9	87.9	87.7
7	Dog	China	MK838609	**7**	1	1	1	2	2	0	**100**	87.9	87.9	87.7
8	Red deer	France	MH084720	**8**	57	57	57	56	58	58	58	**100**	100	98.1
9	Swap deer	France	MH084719	**9**	57	57	57	56	58	58	58	0	**100**	98.1
10	Water deer	ROK	LC432154	**10**	58	58	58	57	59	59	59	9	9	**100**

Isolate, country, and GenBank accession numbers of each sequence are specified in the left site of the matrix. White boxes of the matrix indicate the percent identity between sequences and gray boxes shown the nucleotide differences between them. [1] Representative sequences of *A. capra gltA* gene obtained in this study. ROK, Republic of Korea.

Table 14. Genetic identity matrix based on the *groEL* gene fragments of *A. capra* (238 bp) from goat

No.	Isolate	Country	GenBank accession number	No.	1	2	3	4	5	6	7	8	9
1	Black goat [1]	ROK	MT721150	**1**	**100**	100	100	99.6	99.6	99.6	99.2	91.2	91.2
2	Cattle	China	MG932131	**2**	0	**100**	100	99.6	99.6	99.6	99.2	91.2	91.2
3	Water deer	ROK	LC432184	**3**	0	0	**100**	99.6	99.6	99.6	99.2	91.2	91.2
4	Human	China	KM206275	**4**	1	1	1	**100**	100	100	99.6	91.2	91.2
5	Sheep	China	MG869385	**5**	1	1	1	0	**100**	100	99.6	91.2	91.2
6	*R. microplus* tick	China	MG869481	**6**	1	1	1	0	0	**100**	99.6	91.2	91.2
7	Goat	China	MH174929	**7**	2	2	2	1	1	1	**100**	90.8	90.8
8	Red deer	France	MH084718	**8**	21	21	21	21	21	21	22	**100**	100
9	Swap deer	France	MH084717	**9**	21	21	21	21	21	21	22	0	**100**

Isolate, country, and GenBank accession numbers of each sequence are specified in the left site of the matrix. White boxes of the matrix indicate the percent identity between sequences and gray boxes shown the nucleotide differences between them. [1] Representative sequences of *A. capra groEL* gene obtained in this study. ROK, Republic of Korea.

Table 15. Genetic identity matrix based on the *groEL* gene fragments of *A. bovis* (238 bp) from cattle and goat

No.	Isolate	Country	GenBank accession number	No.	1	2	3	4	5	6	7	8	9
1	Cattle [1]	ROK	MW122330	**1**	100	100	100	99.2	98.0	98.0	98.0	91.2	91.2
2	Black goat [1]	ROK	MW122332	**2**	0	100	100	99.2	99.8	98.0	98.0	91.2	91.2
3	*H. longicornis* tick	China	MK340768	**3**	0	0	100	99.2	99.8	98.0	98.0	91.2	91.2
4	Goat	China	MH255908	**4**	2	2	2	100	97.1	97.1	97.1	90.3	90.3
5	Cattle	China	MH255906	**5**	5	5	5	7	100	100	100	92.4	92.4
6	*R. microplus* tick	China	MK340785	**6**	5	5	5	7	0	100	100	92.4	92.4
7	Cattle [1]	ROK	MW122353	**7**	5	5	5	7	0	0	100	92.4	92.4
8	Cattle [1]	ROK	MW122296	**8**	21	21	21	23	18	18	18	100	100
9	Tick	China	MK340767	**9**	21	21	21	23	18	18	18	0	100
10	Black goat [1]	ROK	MW122298	**10**	21	21	21	23	18	18	18	0	0

Isolate, country, and GenBank accession numbers of each sequence are specified in the left site of the matrix. White boxes of the matrix indicate the percent identity between sequences and gray boxes shown the nucleotide differences between them. [1] Representative sequences of *A. bovis groEL* gene obtained in this study. ROK, Republic of Korea.

CHAPTER II

Clinical and subclinical cases of canine babesiosis caused by *Babesia gibsoni*

Abstract

Canine babesiosis has been scarcely investigated in the ROK. It is well known that *B. gibsoni* is its primary causative agent; however, its clinical presentation has not been completely clarified in the country. Consequently, the aim of this study was focused on evaluating the clinical appearance of this parasitic infection based on the anamnesis, and from the comparison of the hematological and biochemical test results. Four hundred whole blood samples from patients with presumptive diagnosis of tick-borne disease were analyzed by PCR for the amplification of the *Babesia* spp. *18S rRNA* gene and by a rapid diagnostic test kit (VetAll Laboratories®) for the detection of *B. gibsoni* seroreactive animals. Out of the 400 dogs suspected for babesiosis, 36 (9.0%) resulted to be *B. gibsoni*-infected, while 24 (6.0%) were found to be seroreactive animals. The current investigation revealed that all the courses of the disease are present in the ROK, being the acute course the most predominant, that tends to consist of inappetence, lethargy, pyrexia, gastrointestinal symptoms and, occasionally, hematuria; along with common hematological abnormalities, such as thrombocytopenia and ane-

mia, and in lower degree of presentation the biochemical abnormalities, such as hyperbilirubinemia, hypoalbuminemia and elevated liver enzymes. This research has shown that *B. gibsoni* is an endemic hemo-parasite that produces a variable clinical manifestation in dogs, in which for its accurate diagnosis, a descriptive history of the clinical signs, hematology and biochemical profile, along with the performance of the PCR assay are of essential importance. These findings, in turn, will also help in planning pragmatic preventive strategies against this potent threat in the ROK.

Keywords: Canine babesiosis, *Babesia gibsoni*, Asian genotype, dogs, PCR, rapid diagnostic test kit, Republic of Korea

1. Introduction

Canine babesiosis (piroplasmosis) is an emerging tick-borne hemolytic disease, caused by intraerythrocytic protozoan parasites of the genus *Babesia* and *Theileria* (He et al., 2017). To date, there are eight species belonging to the aforementioned genera that might infect canines. These species have been classified based on the piroplasm length. The four large piroplasms (3.0–5.0 µm) (Carret et al., 1999) detected in dogs are: *B. canis vogeli*, *B. canis canis* (Irwin et al., 2004), *B. canis rossi* (Schoeman et al., 2007), and the novel *Babesia* sp. identified in North America (Birkenheuer et al., 2004). On the other hand, small canine piroplasm classification (1.5–2.5 µm) (Carret et al., 1999) is composed of *Theileria annae* (*Babesia microti*-like sp.) (Yisachar-Mekuzas et al., 2013), *B. gibsoni*, *B. conradae,* and *B. vulpes* (Kjemtrup et al., 2006; Baneth et al., 2015). Furthermore, species such as *Theileria annulata*, *Theileria equi*, *Theileria* sp., and *B. caballi*, have been recently detected by molecular diagnostic techniques in clinically and subclinically infected dogs (Terao et al., 2015; Rosa et al., 2014). Companion dogs (*Canis familiaris*) are mainly infected by *B. canis* and *B. gibsoni* (Wozniak et al., 1997), two species worldwide distributed with clinical significance in veterinary medicine due to the tremendous variation not only in their geographical distribution, but also in their tick vectors, clinical manifestation, and host's response to the infection, which varies depending on the age and the immune status of the infected animals (Boozer et al., 2003).

B. gibsoni was recognized for the first time in 1910 in India (Groves et al., 1968) and since, it has been reported in the Middle East, Africa, Asia, Europe, United States, and in some Latin American and Caribbean countries

(Boozer et al., 2005; Panti-May et al., 2020). Dogs become infected with this pathogen when ticks feed and release sporozoites into the red blood cells (erythrocytes), a process which is usually completed within 2–3 days (Taboada et al., 1991). The clinical manifestation of the disease typically occurs after an incubation period of 10 to 28 days (Köster et al., 2015) and it varies widely from hyper-acute, acute to chronic or even subclinical infections (Schoeman et al., 2009). The most common course of *B. gibsoni* infection is the acute state, which is characterized by fever, lethargy, thrombocytopenia, lymphadenopathy, splenomegaly, and hemolytic anemia (Beck et al., 2009). The hyper-acute course is presumed to be maternally acquired, resulting in the rapid death of puppies (Booze et al., 2005). However, further studies revealed that this is a common disease state characterized by shock and extensive tissue damage in American Pitbull Terriers and Staffordshire Bull Terriers, thus is also presumed to be transmitted by dog bites (Schoeman et al., 2009). Regarding chronic *B. gibsoni* infection and subclinical disease course, the extremely useful tool for their identification is by PCR assay, due to the non-specific symptoms (intermittent fever, lethargy, and weight loss) and completely asymptomatic infections, respectively (Boozer et al., 2005; Tuttle et al., 2003).

Recent analyzes based on the *18S rRNA* gene fragment of *B. gibsoni* revealed that there are at least three different *B. gibsoni* genotypes in circulation: the Asian genotype, originally found in India, the North American genotype, found in USA, California, and the European genotype, formerly known as *Babesia microti*-like organism (Hartelt et al., 2007). From these three known *B. gibsoni* genotypes, its Asian genotype, has been a perennial

challenge for clinical veterinarians (Lin et al., 2012) due to the variability in the efficacity of the treatment used. After been trying several drugs for years, finally the Atovaquone-Azithromycin drug combination has been demonstrated to be an effective therapeutic strategy for chronically infected dogs, as well been considered as the only treatment that has resulted in either the complete elimination of infection or the suppression of parasitemia below the limit of detection (Birkenheuer et al., 2004). *Babesia* species are mainly transmitted by tick vectors, however, dog bites, blood transfusions, and transplacental transmission are also alternative routes of transmission (He et al., 2017). The four genera of ticks that have been reported as vectors of *Babesia* spp. are: *Rhipicephalus*, *Ixodes*, *Haemaphysalis*, and *Hyalomma*, all of them belonging to the Ixodidae family (Hamšíková et al., 2016). In the ROK, the most commonly infected tick species with *Babesia* spp. are the *Haemaphysalis longicornis* and *Haemaphysalis flava* ticks, that were collected from grass, vegetation, and human patients (Hong et al., 2019). According to a tick surveillance *H. longicornis*, *H. flava*, *Ixodes nipponensis*, and *Rhipicephalus sanguineus* lato were found to be the tick's species that most frequently infested Korean domestic dogs (Choe et al., 2011), however, the main tick vector of *B. gibsoni* has not yet been elucidated in the country. There are few surveys of *B. gibsoni* infection in canines in the ROK. The first molecular detection was performed in German Shepherds dogs from Gangwon-do and Gyeonggi-do provinces and it was identified an infection rate of 1.8% (9/501) (Song et al., 2004). Subsequently, in 2009, it was reported an infection rate of 24.8% (29/117) (Lee et al., 2009), being this the last molecular survey performed in the country. Currently, there is only one

serological evidence of *B. gibsoni* in Korean domestic dogs, using ELISA and indirect fluorescence immunoassay (IFA) kits, in which a seroprevalence of 1.7% (38/2,215) was identified (Lee et al., 2020).

In this direction, the present study primarily aimed at investigating the molecular and serological prevalence of *B. gibsoni* in Korean domestic dogs as well as understanding the clinical occurrence of the infection through the classification and evaluation of the patients according to the different disease courses, thus providing new findings that may help in the elucidation of the common clinical presentation, geographical distribution, and risk factors associated with canine babesiosis in the country.

2. Materials and Methods

2.1. Sampling methodology and patient data collection

A total of 400 whole blood samples were collected from companion dogs at different veterinary hospitals and clinical centers located in 7 metropolitan cities and 9 provinces that make up the ROK (Figure 22), which were then referred to the Laboratory of Veterinary Internal Medicine, Seoul National University, ROK, between April 2019 and December 2020. The referred samples were collected in sterile blood collection tubes containing EDTA anticoagulant and submitted to the laboratory in an icepack container. The criteria for referring the sample to the laboratory was based on the presumptive diagnosis of tick-borne disease given by the veterinarian based on the clinical symptomatology along with the hematological and biochemical abnormalities presented by the patient on the day of his visit to the

veterinary hospital or clinical center. Along with the whole blood sample, the patient data, its clinical records, and the hematological and blood chemistry results were provided to the laboratory team for further analysis and interpretation.

2.2. DNA extraction and PCR amplification

The genomic DNA was extracted from 200 µL whole blood samples using a commercial LaboPass DNA Purification Kit (Cosmo Genetech, Seoul, ROK) according to the manufacturer's instructions. The extracted DNA was stored at -20°C until further analysis. For the detection of *Babesia* species, a single PCR assay was carried out to amplify its *18S rRNA* gene using the primer pair *BabgenF* 5'-GAAACTGCGAATGGCTCATTA-3' and *Babesiarev1* 5'-CCATGCTGAAGTATTCAAGAC-3' (Santos et al., 2009). These reactions were performed in a total volume of 30 µL, containing 10 pmol of each primer, 15 µL of 2x Taq PCR Pre-mix (BioFACT, Daejeon, ROK), and 50 to 100 ng of genomic DNA samples, and then conducted in a SimpliAmp Thermal Cycler (Thermo Fisher Scientific, Foster City, CA) under optimal cycling conditions. The cycling conditions used in this study for the successful amplification consisted of 30 cycles, each of 1 min at 94°C for denaturation, followed by annealing for 1 min at 55°C, and extension for 1 min at 72°C. The PCR products were visualized under UV light after 1.5% agarose gel electrophoresis, using a 100 bp ladder (SiZer-100 DNA Marker Solution, iNtRON Biotechnology, Gyeonggi-do, ROK) as a DNA size marker.

2.3. Rapid antibody test for *B. gibsoni*

SensPERT™ Canine *Babesia gibsoni* Ab Test Kit (sensitivity of 91.8% and specificity of 93.5% *vs*. IFA) (VetAll Laboratories®, Goyang-si, ROK) was used for the detection of seroreactive dogs. Using a dropper as a pipette, 1 drop (10 µL) of whole blood sample was dispensed into the specimen well and waited for it to be completely absorbed. Subsequently, 2 drops (80 µL) of buffer were dispensed on it, and finally the reading of the test results, which was performed after 10 min according to the manufacturer's instructions. The test result can appear on Control (C) and Test (T) lines where the principles of immunochromatography are used. The interpretation of the results was carried out as follows: negative result, when only the control line appeared, and positive result, when both test and control lines appeared.

2.4. DNA sequencing and phylogenetic analysis

Positive PCR products were purified using the DNA Gel Extraction S & V Kit (BIONICS, Daejeon, ROK) and sequenced using an Applied Biosystems 3730xl capillary DNA Analyzer (Thermo Fisher Scientific, Foster City, CA). Nucleotide sequences were evaluated and confirmed by Chromas software. They were then compared with reference sequences downloaded from the NCBI to determine the accuracy of the PCR method. The multiple sequence alignment was performed with the program ClustalX 2.1, and corrected with BioEdit software (v 7.2.5). Finally, a similarity matrix was constructed to determine the percentage of identity between sequences. Phylogenetic analysis was performed based on the maximum likelihood method in MEGA 6.06 software. The stability of the tree obtained was estimated by

bootstrap analysis for 1,000 replicates.

2.5. Nucleotide sequence accession numbers

The representative sequences obtained in this study of the *B. gibsoni 18S rRNA* gene have been submitted and deposited in the GenBank database under accession numbers MW485704 to MW485725.

2.6. Statistical analysis

The statical analysis was performed using the analytical software package GraphPad Prism (v 5.04; GraphPad Software, Inc., La Jolla, CA). To analyze the significant differences that might exist between regions, genders, age groups and breeds of dogs, the Chi-square test or Fisher's exact test was performed. Differences were considered statistically significant if the *p* value was ≤ 0.05.

3. Results

3.1. Molecular detection

Single PCR amplification of the *Babesia* spp. *18S rRNA* gene revealed that thirty-six (9.0%) out of 400 companion dogs were positive to *Babesia* infection. According to the geographic distribution of the sampling sites, *Babesia* spp. infection was detected in three metropolitan cities, named Busan (40.0%, 6/15), Incheon (12.5%, 1/8) and Seoul (11.5%, 15/131), and in four provinces, including Jeju-do (100%, 1/1), Gyeongsangnam-do (14.3%, 2/14), Chungcheongnam-do (10.0%, 1/10) and Gyeonggi-do (8.0%, 10/125).

There was no statistical significance despite the difference observed between the infection rates (p=0.9693) (Table 16).

Infection rates obtained according to the sex of the dogs were closely similar, 8.9% (17/192) and 8.5% (15/176), for male and female, respectively. Thus, the difference was not significant (p=0.5852) (Table 16). Based on the age groups, the highest infection rates of *Babesia* spp. were detected in dogs between 2 and 4 years old (12.9%, 15/116), and dogs between 8 and 10 years old (10.5%, 6/57), followed by the lowest infection rates reported in dogs within the age range of 5 to 7 years old (8.9%, 8/90), less than or equal to 1 year old (3.9%, 2/51), and greater than or equal to 11 years old (2.0%, 1/49) (Table 16). From the 44 dog breeds analyzed in this study, 17 were positive by PCR amplification. Some of the breeds positive to *Babesia* spp. were Irish Terrier, Italian Greyhound, Beagle, American Pitbull Terrier, Pompitz and the rest that have been listed in Table 17. Further statistical analysis showed that there is no significant difference between breeds (p=0.1375) (Table 17).

3.2. Seroprevalence: Rapid antibody test results

From the 400 dogs under study, 24 (6.0%) of them were reactive for antibodies against *B. gibsoni*. Busan, Incheon, and Seoul metropolitan cities were not only PCR positive, but also seropositive, with a seroprevalence rate of 13.3% (2/15), 12.5% (1/8), and 7.6% (10/131), respectively. Contrary, Ulsan (12.5%, 1/8) and Daegu (6.5%, 2/31) metropolitan cities were only seropositive. Regarding seroprevalence by province tested, antibodies to *B. gibsoni* were found in dogs from Gyeongsangnam-do (13.3%, 2/14) and

Gyeonggi-do (4.8%, 6/125), provinces where also was detected the antigen (Table 16). There were no significant differences among seroprevalence rates reported in the sampled sites (p=0.6922) (Table 16).

In contrast to PCR results, female dogs (6.8%, 12/176) showed a seemingly higher seroprevalence than male dogs (5.2%, 10/192), and the dogs within the age range of 5 to 7 years old were found to be the most seroreactive (8.9%, 8/90), followed by dogs ranging in age from 8 to 10 years old (8.8%, 5/57). The p value for sex classification was 0.6607, and for age groups was 0.4628, both were not statistically significant (Table 16).

According to breed classification, eleven dog breeds were seroreactive to *B. gibsoni*, including American Pitbull Terrier (50.0%, 1/2), Beagle (50.0%, 2/4), Pompitz (50.0%, 1/2), Miniature Pinscher (16.7%, 1/6), Bichon Frise (12.5%, 2/16), Poodle (11.5%, 7/61), Golden Retriever (8.3%, 1/12), Maltese (5.6%, 4/71), Jindo (5.6%, 1/18), Mixed (4.7%, 2/43), and Pomeranian (3.8%, 1/26). However, the difference observed between breeds was not significant (p=0.4312) (Table 17).

3.3. Sequencing and phylogenetic analysis

The alignment of the *Babesia* spp. *18S rRNA* nucleotide sequences generated in the present study revealed that all sequences had a fragment length of 641 bp and were a 100% identical to each other (Figure 23). Through the BLAST and the phylogenetic analysis based on the *18S rRNA* gene fragment of *Babesia* species, it was confirmed the genetic relationship between the sequences of this study and the references sequences downloaded from the GenBank database, which were 100% identical to previous-

ly sequenced *B. gibsoni* as shown in Figure 24.

Nucleotide sequences of this study (MW485704–MW485725) shared an identity of 100% with sequences previous detected not only in dogs from countries of the Asian continent, such as Bangladesh (LC008285), India (KJ142323), China (KP666166), ROK (AB478322), Taiwan (FJ769386), and Japan (LC012808), but also to the sequences isolated from dogs of the European continent, including the countries Slovakia (KP737862), Hungary (JX110651), Spain (AY278443), Italy (FJ554534), and Serbia (KJ696717) (Figure 24, Table 18). However, all these European isolates belong to the *B. gibsoni* Asian genotype.

Sequences isolated from other animal species but that also shared 100% identity with the sequences identified in this study were those obtained from masked pal civets (Taiwan, JQ710685) and cats (China, KY073362) (Figure 24, Table 18). Additionally, in the same cluster, low identity percentages were shared with the isolates obtained from Thai dholes (99.1%, MK144331), Pakistani dogs (97.2%, KU168833), and Chinese wild boars (91.9%, JX962780) (Figure 24, Table 18).

3.4. Clinical signs

For the evaluation of the common clinical presentation of the *B. gibsoni* infection, 34 clinical records out of the 36 *B. gibsoni* PCR-positive dogs were provided and classified into three different groups according to their clinical signs, as follows: dogs manifesting symptoms of the complicated form of canine babesiosis (Group I), dogs with mild to non-specific symptoms (Group II), and dogs apparently healthy (Group III) (Table 19).

The physical examination revealed that the most common clinical signs present in the canine patients were inappetence (61.8%, 21/34), lethargy (50.0%, 17/34), pyrexia (47.1%, 16/34), gastrointestinal symptoms, including diarrhea and vomiting (29.4%, 10/34), and hematuria (23.5%, 8/34). Fewer dogs had pale mucous membranes (14.7%, 5/34), weight loss (5.9%, 2/34), depression (5.9%, 2/34), jaundice (5.9%, 2/34), eye-edema (2.9% 1/34), and respiratory symptoms (2.9% 1/34). Diagnostic imaging tests revealed that 17.6% (6/34) of the *B. gibsoni* infected dogs had splenomegaly and only 2.9% (1/34) presented hepatomegaly. Other clinical complications apart from the symptoms already described above were immune-mediated hemolytic anemia (IMHA) (11.8%, 4/34), pancreatitis (5.9%, 2/34), compromised liver function (2.9%, 1/34), colitis (2.9%, 1/34), cholangiohepatitis plus urolithiasis (2.9%, 1/34), and ovarian cysts plus chronic bronchitis (2.9%, 1/34). In terms of tick exposure, 22 dogs (64.7%) had a history of tick bites, 9 dogs (26.5%) had an unknown history, and 3 dogs (8.8%) had a history of never having been bitten or infested by ticks (Table 19). Based on the classification according to the acute, chronic, or subclinical courses of canine babesiosis, 19 out of the 34 dogs (55.8 %) presented the complicated form of the disease (Figure 25), 11 (32.4%) the chronic course, and 4 (11.8%) resulted to be completely asymptomatic, which were considered as subclinical carriers of *B. gibsoni* (Table 19).

3.5. Hematologic and biochemical test results

Complete blood count (CBC) and biochemistry test results were available for 26 of the 36 *B. gibsoni* PCR-positive dogs, including 15 dogs acute-

ly infected, 8 chronically infected, and 3 subclinically infected. Hematological abnormalities found in hematocrit and hemoglobin levels revealed that 21/26 (80.8%) dogs were anemic (PCV <37.1%; Hb <12.9 g/dL). Red blood cell count showed that 20/26 (76.9%) dogs had erythrocytopenia (RBC <5.7 10x12/L). In addition, it was found that the distribution of red blood cells was also altered with low levels (RDW <11.9%) in 2/26 (7.7%) patients, and high levels (RDW >14.5%) in 6/26 (23.1%) patients. Platelet blood count revealed thrombocytopenia (PLT <148 10x19/L) in 23/26 (88.5%) dogs, and thrombocytosis (PLT >484 10x19/L) in only 1/26 (3.8%) dog. Some white blood cell disorders found in analyzed samples were neutropenia (NEUT <3.9 10x19/L) in 4/26 (15.4%) dogs, neutrophilia (NEUT >8 10x19/L) in 3/26 (11.5%) dogs, lymphocytopenia (LYMPH <1.3 10x19/L) in 4/26 dogs, lymphocytosis (LYMPH >4.1 10x19/L) in 6/26 (23.1%) dogs, monocytepenia (MONO <0.2 10x19/L) in 2/26 (7.7%) dogs, monocytosis (MONO >1.1 10x29/L) in 3/26 (11.5%) dogs, eosinopenia (EOS <0.06 10x19/L) in 4/26 (15.4%) dogs, eosinophilia (EOS >1.23 10x19/L) in 1/26 (3.8%) dog, basopenia (BASO <0 10x9/L) in 1/26 (3.8%) dog, and basophilia (BASO >0.1 10x9/L) in 1/26 (3.8%) dog (Table 20).

Regarding serum biochemical abnormalities, liver enzymes values revealed that 3/26 (11.5%) dogs had increased alanine aminotransferase (ALT) enzyme activity (ALT >100 U/L), and 5/26 (19.2%) dogs had an elevated aspartate aminotransferase (AST) enzyme activity (AST >42.5 U/L). Azotemia (BUN >30 mg/dL), and high serum creatinine concentration (>1.6 mg /dL) were observed in only one patient (3.8%). Other abnormalities included hyperbilirubinemia (>0.7 mg/dL) in 10/26 (38.5%) dogs, hypoalbuminemia

(<2.3 g/dL) in 8/26 (30.8%) dogs, hypoproteinemia (<4.9 g/dL) in 2/26 (7.7%) dogs, and hyperproteinemia (>7.2 g/dL) in 2/26 (7.7%) dogs (Table 20).

When comparing the hematology and blood chemistry test results between the 3 different presentations of the disease, it was identified that the most common abnormalities in the acute infection were thrombocytopenia (93.3%, 14/15), anemia (86.7%, 13/15), hyperbilirubinemia (53.3%, 8/15), hypoalbuminemia (40.0%, 6/15), increased AST enzyme activity (26.7%, 4/15), neutropenia (26.7%, 4/15), lymphocytopenia (26.7%, 4/15), and lymphocytosis (26.7%, 4/15). While for the case of chronic and subclinical states, primarily thrombocytopenia and anemia were found (Table 20).

4. Discussion

Vector-borne diseases (VBDs), considered as the leading cause of morbidity and mortality in dogs worldwide (Xu et al., 2015), have represented a significant challenge in veterinary medicine. Such is the case of babesiosis, a persistent endemic disease caused by one of the most common hemoparasites (*Babesia*) in the whole world second only to trypanosomes (Yabsley et al., 2013). Unsurprisingly, one of the numerous targets of the *Babesia* parasite is the domestic dogs (Solano-Gallego et al., 2016), causing in them a serious hemolytic disease called canine babesiosis. This canine TBD has been thoroughly investigated in several countries, however, in the ROK there is a lack of studies regarding its clinical presentation. Hence, the present study was carried out not only to determine the molecular and serological prevalence of *B. gibsoni*, but also to identify the clinical and hematolo-

gical abnormalities that may be commonly found in dogs with clinical and subclinical infection.

In Asia, previous molecular studies regarding *B. gibsoni* infection in canines have reported infection rates that vary greatly according to the geographical distribution of the countries under study, being China (47.2%) (Guo et al., 2020), Bangladesh (30.0%) (Terao et al., 2015), and Taiwan (23.2%) (Lee et al., 2010), the countries with the higher molecular prevalence, and Japan (8.8%) (Inokuma et al., 2004), India (7.9%) (Singh et al., 2016), Malaysia (3.3%) (Mokhtar et al., 2013), and the ROK, the countries with lowest rates of infection. The first case of *B. gibsoni* infection in the ROK was described in 1989 in an acutely infected dog (Chae et al., 1989). Molecular surveys conducted on a large scale in the 21st century in this country, specifically between the years 2003 and 2009, revealed an exponential growth of *B. gibsoni* infection, from 1.8% (9/501) (Song et al., 2004) to 24.8% (29/117) (Lee et al., 2009). However, in the current investigation it was observed a decrease in its infection rate, being only of 9.0% (36/400).

The present study involved all metropolitan cities and provinces of the ROK as sample sites, nevertheless, the total number of samples collected by each site differed markedly, therefore in this study was impossible to conclude which region had the highest prevalence of dogs infected by the *B. gibsoni* parasite. Even so, a trend of *B. gibsoni* infection was observed in the Seoul metropolitan city (11.5%, 15/131) and its surroundings, which include Gyeonggi-do province (8%, 10/125), and Incheon metropolitan city (12.5%, 1/8). This could be due to the fact that Gyeonggi-do is the most populous province in the ROK, consequently its pet ownership rate (specifically dogs)

is also the highest of all provinces, followed by Seoul and Incheon, as reported by the Ministry of Agriculture, Food and Rural Affairs of the ROK in 2019, thus also explaining why the highest number of whole blood samples were referred from veterinary hospitals and clinical centers in Gyeonggi-do province.

Serological studies of *B. gibsoni* in domestic canines performed in Malaysia, China, and Japan have reported seroprevalence of 17.7% (Rajamanickam et al., 1985), 9.23% (Cao et al., 2015), and 2.4% (Kubo et al., 2015), respectively, which were relatively high compared to that reported in the ROK in 2020 (1.7%, 38/2215) (Lee et al., 2020). However, the seroprevalence identified in the present study was 6.0% (24/400), indicating an increase in the number of animals reactive to antibodies against *B. gibsoni*. According to the regions sampled in the current investigation along with the ones sampled in the aforementioned serological survey, it can be assumed that *B. gibsoni* is distributed nationwide, since seropositive dogs to this parasite have been reported in the northern, central, and southern regions of the ROK.

When comparing the molecular and serological results obtained in this study, there was a notable difference between the total of antigen-positive animals and the total of antibody-positive animals [*n*=36 (9.0%) *vs*. *n*=24 (6.0%)]. This pattern of results is consistent with previous studies, which suggested that it is perfectly feasible for a dog to be seronegative but positive to *Babesia* infection, as infections by species such as *B. canis* and *B. gibsoni* manifest acutely (Solano-Gallego et al., 2016). Therefore, these results strongly imply that most of the antigen-positive animals in this study

had the acute state of the disease, which means that the babesial organisms had already established their intraerythrocytic infection, thus the parasitemia was higher, being the babesial antigen easily detectable by PCR. This idea was further supported by the findings obtained by analyzing each clinical history of the antigen-positive dogs, which indeed revealed that a large part of the animals had the complicated course of the disease. Although the antibody response to babesial infection usually takes 8 to 10 days to develop (Boozer et al., 2005), some immunosuppressed patients, puppies, or clinically affected dogs with per-acute or acute infection are initially seronegative. Thus, in these cases where false negatives may occur, seroconversion is strongly recommended as a serologic technique to confirm *Babesia* infection (Solano-Gallego et al., 2016). The same situation of false-negative dogs may occur when attempting to make an early diagnosis of the initial phase of the disease, for example when serum is sampled before the production of antibodies. Consequently, the diagnosis of canine babesiosis should be always confirmed by PCR, which is an extremely useful tool in identifying the DNA of the causative agent infecting the dog, detecting low levels of parasitemia, recognizing subclinical infections, and monitoring response to therapy (Boozer et al., 2005).

Regarding host susceptibility, in addition to immunocompetence, age is one of the characteristics that may predispose them to infection and its severity, with young dogs being the most prone to severe babesiosis (Ayoob et al., 2010). The findings of the present study highlighted that theory. It was found that juveniles, specifically within the age range of 2 to 4 years old, were the most affected by *B. gibsoni* antigen infection, while mature ani-

mals (8 to 10 years old) were the most seroreactive. Poor humoral response and maternal antibody protection are suspected to directly influence the lower seroprevalence observed in immature patients (Ayoob et al., 2010). Although the differences observed in the infection rates among the breeds tested were not statistically significant, a breed predisposition has been suggested in the USA (Boozer et al., 2005), citing the vulnerability of the American Pitbull Terrier breed for the development of *B. gibsoni* infection. The same results of predisposition of this breed were observed in a study previously conducted in the ROK (Lee et al., 2009). Despite the fact that several breed predilection factors have been considered, such as vertical transmission, dog-fighting, genetic susceptibility, and poor parasite control, these have not yet been fully elucidated. No significant sex predisposition to the disease was observed in the present study, however, the proportion of male dogs infected with *B. gibsoni* was relatively high compared to female dogs. This pattern of results is consistent with those obtained in the studies performed by Song et al. (2004) and Lee et al. (2009), where they suggested that male dogs are more likely to be kept outdoors as guard dogs compared to females, and are also more likely to be used as fighting dogs. This explains well why there is a higher risk of tick exposure for male dogs in the ROK (Song et al., 2004; Lee et al., 2009).

The phylogenetic analysis of the *Babesia* spp. *18S rRNA* gene confirmed that the sequences identified in the current investigation were 100% identical to *B. gibsoni* (Asian genotype). The existence of two additional genotypes, North American and European, is well known; however, subsequent studies based on the *18S rRNA* gene revealed that these were more

closely related to the genus *Theileria* (Kjemtrup et al., 2000). Recently, the names *B. conradae* and *Theileria annae* have been proposed for the North American and European genotypes, respectively (Kjemtrup et al., 2000; Camacho et al., 2001).

In addition, this study demonstrated that the clinical expression of canine babesiosis, caused by *B. gibsoni*, in the ROK range from subclinical to severe, the latter being the most common, thus differing from the results reported in the USA, where they found that the subclinical state was the predominant (Birkenheuer et al., 2005). In the present investigation, the acute state was characterized by pyrexia, pale gums, lethargy, depression, inappetence, weight loss, vomiting, diarrhea, hematuria, jaundice, splenomegaly, and hepatomegaly. Regarding jaundice, in this study there were only two (5.9%) icteric patients, thus supporting the evidence that clinical icterus is rare to be found in *Babesia* infection (Boozer et al., 2003). Interestingly, 17.6% of the *B. gibsoni*-infected dogs presented splenomegaly, being this abnormality considered as a risk factor for the development of natural and potentially fatal babesiosis (Solano-Gallego et al., 2011). Other organ dysfunctions found in the patients acutely infected were pancreatitis, liver failure, and chronic bronchitis. Goris and colleagues considered that the multiple organ dysfunction syndrome (MODS) develops as a consequence of dysregulation of proinflammatory and anti-inflammatory mechanisms resulting in overwhelming auto-destructive inflammation (Goris et al., 1985). In addition, another study has also demonstrated a correlation between the number of organs affected and mortality (Welzl et al., 2001).

With respect to the clinical presentation of the chronic state, in this

study it varied greatly from mild symptoms, such as pyrexia, lethargy, inappetence, diarrhea or vomiting, to completely asymptomatic patients. In general, subclinical carrier dogs were observed to be apparently healthy, however, it is important to keep in mind that there is the possibility that an individual can remain as an infected carried for many months, possibly even for life (Schoeman et al., 2009), this being a potential risk for the rapid spread of the disease.

The most common hematological abnormalities found in the present study were anemia (80.8%) and thrombocytopenia (88.5%). The pathogenesis of the anemia in canine babesiosis is not dependent on the intensity of parasitism. Onishi and colleagues demonstrated the presence of hemolytic factors in the serum of *B. gibsoni*-infected dogs and the correlation between the activity and the anemia in the infection (Onishi et al., 1990), therefore it seems that the hemolytic factors in the serum of *B. gibsoni*-infected dogs play an important role in the development of anemia in the infection. Thrombocytopenia is considered as the hallmark symptom associated with canine babesiosis, which may result from immune-mediated platelet destruction, platelet sequestration in the spleen, elevated body temperatures or disseminated intravascular coagulation (DIC) (Barić Rafaj et al., 2013). The severity and rapid recovery of the platelet counts have led to the suggestion that immune-mediated mechanisms are involved (Bilić et al., 2018).

Patients of this study also presented alterations in the white blood cell count, with values above the normal range as well as below the normal range. These findings were further supported by a previous research that suggested that leukocyte count is extremely variable in dogs with babesiosis

and can range from leukopenic to leukemoid (Meinkoth et al., 2002).

Biochemistry abnormalities revealed that hepatic enzyme activities, including ALT and AST, and bile acids and albumin levels could be altered in a patient infected with *B. gibsoni*. This pattern of results is consistent with previous studies (Birkenheuer et al., 1999; Myburgh et al., 2009). Consequently, it is of crucial importance to assess liver biofunction while measuring cholinesterase activity in cases of canine babesiosis (Shabani et al., 2020). Blood urea nitrogen (BUN) and creatine levels were elevated in only one patient. A possible reason for this elevated level in some dogs may be due to the non-renal accumulation of ammonia in serum as a result of hemolytic anemia (Mittal et al., 2019). Azotemia appears to be a common complication of *B. microti*-like piroplasm (*T. annae*) infection, however findings of this study along with those previously reported in the USA (Ullal et al., 2018) suggest the possibility of also finding cases of canine babesiosis due to *B. gibsoni* Asian genotype with azotemia and proteinuria.

There were potential limitations concerning the results of this study. The sample size, by metropolitan city and province analyzed, was insufficient to generate a statistically valid conclusion regarding the prevalence of *B. gibsoni*. In addition, not all veterinarians provided complete medical histories specifying the age, sex, and breed of the patients under study, which affected the statistical significance of the predisposing factors of the disease. CBC and chemistry tests results were also not provided in their entirety. However, despite these limitations, the study has provided clear support and evidence regarding clinical signs and hematological abnormalities commonly found in clinically and subclinically infected dogs, as well as a general

knowledge of the molecular and serological prevalence of the *B. gibsoni* parasite in the ROK.

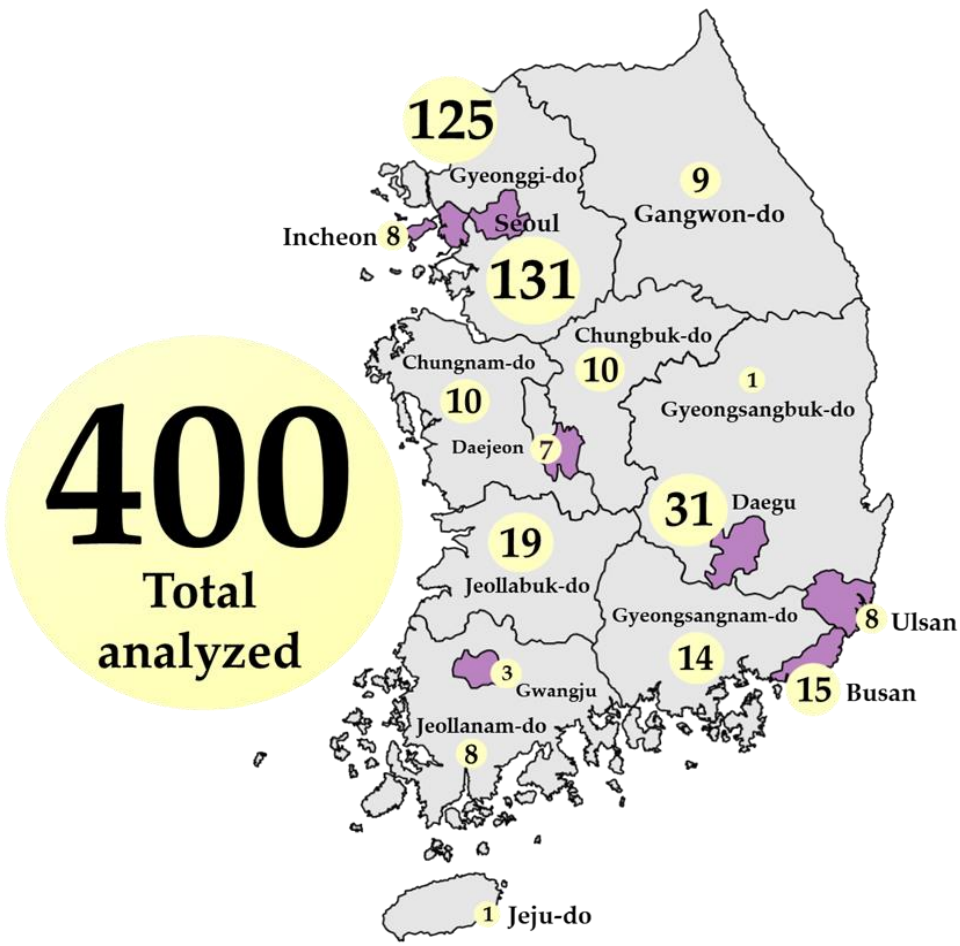


Figure 22. Map of the Republic of Korea showing the sampled sites for the detection of *Babesia gibsoni* infection in companion dogs, 2019-2020. Gray color indicates sampled provinces, while purple color represents tested metropolitan cities. The number of samples analyzed per site are shown in circles.

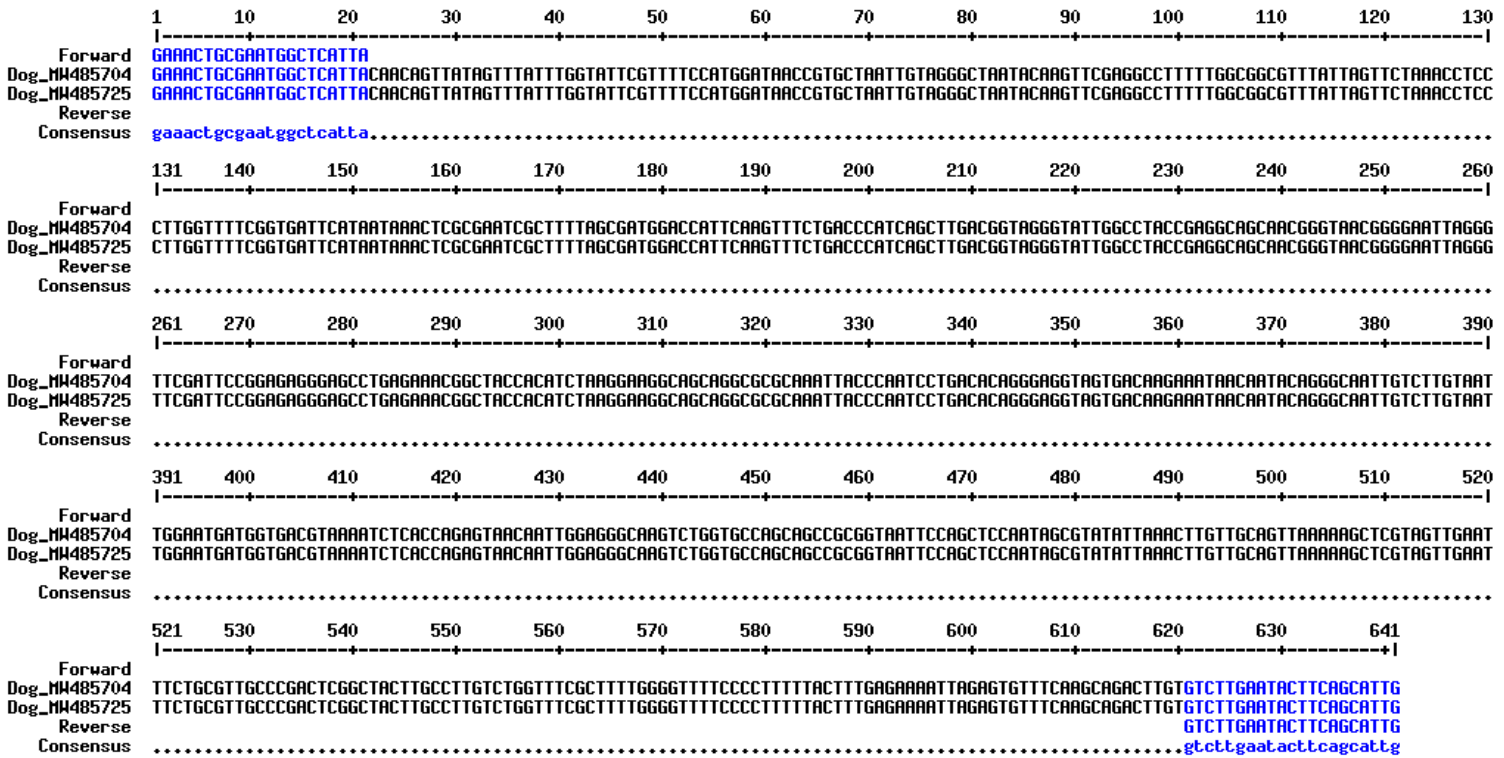


Figure 23. Alignment of the *Babesia* spp. *18S rRNA* gene sequences found in dog whole blood samples. The sequences obtained in this study from dog whole blood samples were 100% identical to each other. Blue color represents primer regions. Fragments length, 641 bp.

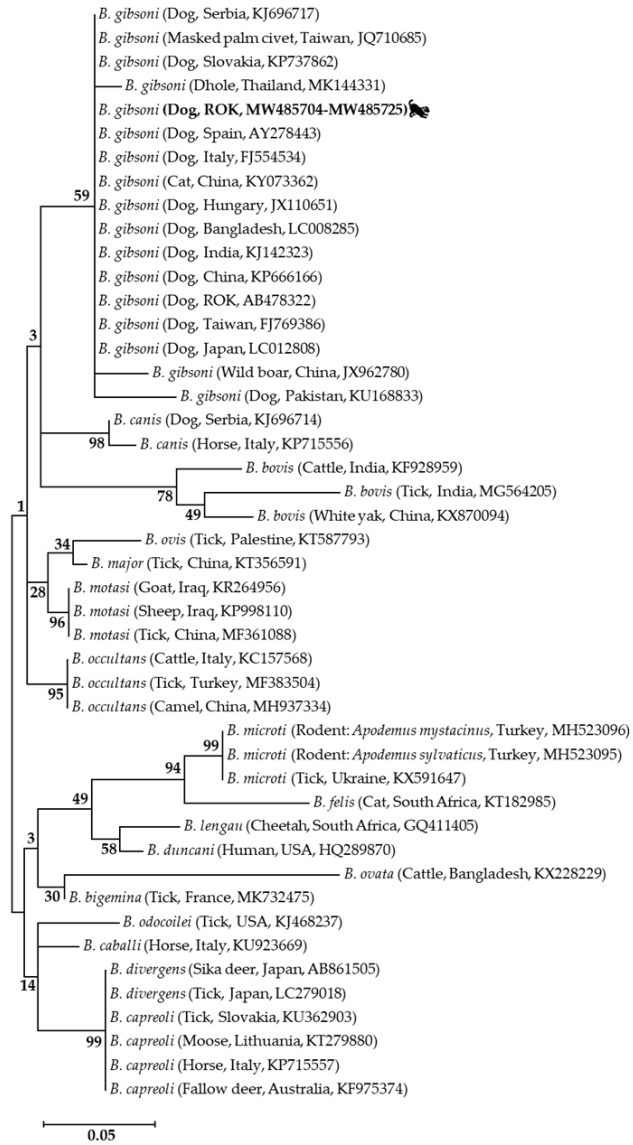


Figure 24. Phylogenetic relationship based on the partial nucleotide sequences of the *Babesia* spp. *18S rRNA* gene fragment (314 bp). Multiple sequence alignments were generated with ClustalX program (version 2.1). The neighbor-joining method with 1,000 bootstraps was used to perform the phylogenetic tree. The scale bar indicates the number of substitutions per nucleotide. Sequences generated in this study are indicated by a dog icon. Isolate, country of origin, and GenBank accession number are shown in parentheses.

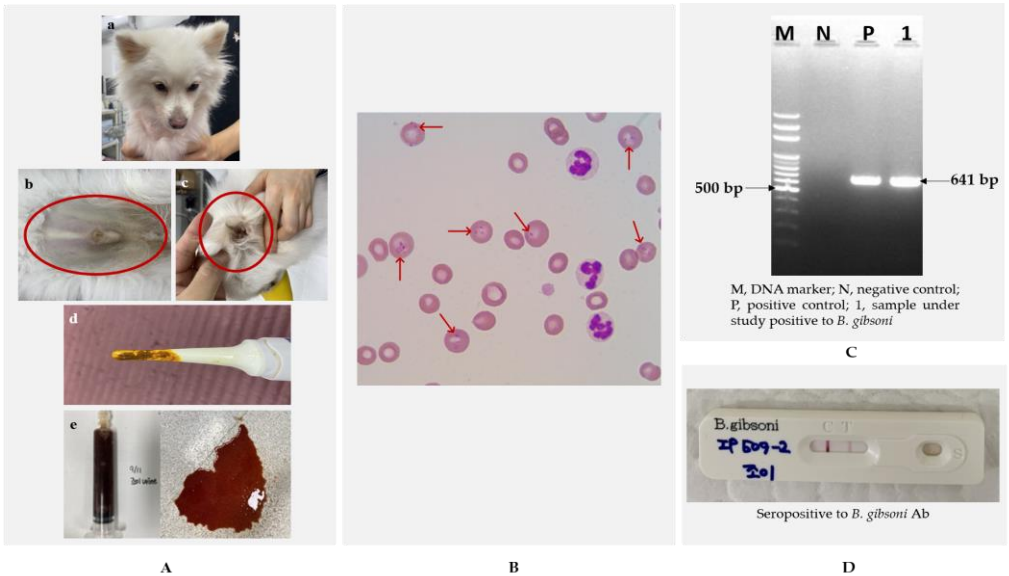


Figure 25. Case of complicated canine babesiosis in the Republic of Korea. (**A**) A 3-year-old (a) Pompitz (Pomeranian plus Japanese spitz) male dog was presented at the Gyeongsang National University Veterinary Teaching Hospital, with the history of pyrexia, jaundice in the (b) skin of the abdomen and at the base of the (c) ears, inappetence, (d) gastrointestinal symptoms, such as diarrhea and vomiting, (e) hematuria, pancreatitis, and with a history of tick exposure. The hemogram findings were normocytic normochromic regenerative moderate anemia, leukopenia (neutropenia 2.78 10^9/L, RR: 3.9–8), and thrombocytopenia (17 10^9/L, RR: 148–484). Chemistry results indicated hyperbilirubinemia (1.0 mg/dL, RR: 0.1–0.7). (**B**) The microscopic examination of the blood smear revealed the pleomorphic intraerythrocytic *B. gibsoni* piroplasms. Clinical record and whole blood sample were submitted to the Laboratory of Veterinary Internal Medicine of Seoul National University for further analysis. The patient resulted (**C**) PCR-positive to *B. gibsoni*, and (**D**) seroreactive to *B. gibsoni* antibodies (SensPERT Canine *Babesia gibsoni* Ab Test Kit, VetAll Laboratories®). The patient was urgently hospitalized under the treatment of blood transfusion, and maintained on oral Atovaquone (13.4 mg/kg every 8 hours) and Azithromycin (10 mg/kg every 24 hours) for 10 days. Response to treatment was monitored by hematocrit and CBC tests along with repeated PCR testing for *B. gibsoni* at 30 and 60 days.

Table 16. Comparison between antigen and antibody prevalence of *Babesia gibsoni* in companion dogs in 2019-2020, the Republic of Korea

Risk Factor	Classification	No. tested	PCR positive			Seropositive		
			No.	IR (%)	*p* value	No.	IR (%)	*p* value
Region	***Metropolitan cities***							
	Seoul	131	15	11.5		10	7.6	
	Incheon	8	1	12.5		1	12.5	
	Daejeon	7	0	0		0	0	
	Daegu	31	0	0		2	6.5	
	Ulsan	8	0	0		1	12.5	
	Busan	15	6	40.0		2	13.3	
	Gwangju	3	0	0		0	0	
	Provinces							
	Gyeonggi-do	125	10	8.0	0.9693	6	4.8	0.6922
	Gangwon-do	9	0	0		0	0	
	Chungcheongbuk-do	10	0	0		0	0	
	Chungcheongnam-do	10	1	10.0		0	0	
	Gyeongsangbuk-do	1	0	0		0	0	
	Gyeongsangnam-do	14	2	14.3		2	13.3	
	Jeollabuk-do	19	0	0		0	0	
	Jeollanam-do	8	0	0		0	0	
	Jeju-do	1	1	100		0	0	
	Total	**400**	**36**	**9.0**		**24**	**6.0**	
Sex	Male	192	17	8.9	0.5852	10	5.2	0.6607
	Female	176	15	8.5		12	6.8	
	NS	32	4	12.5		2	6.3	
	Total	**400**	**36**	**9.0**		**24**	**6.0**	
Age	≤ 1 year old	51	2	3.9	0.1375	2	3.9	0.4628
	2~4 years old	116	15	12.9		7	6.0	
	5~7 years old	90	8	8.9		8	8.9	
	8~10 years old	57	6	10.5		5	8.8	
	≥ 11 years old	49	1	2.0		1	2.0	
	NS	37	4	2.7		1	2.7	
	Total	**400**	**36**	**9.0**		**24**	**6.0**	

IR, Infection Rate; *p* value, $p \leq 0.05$ statistically significant test result; NS, Not Specified (sex and age not specified in the clinical record).

Table 17. *Babesia gibsoni* antigen and antibody detection results by companion dog breed

Breed	No. tested	PCR positive			Seropositive		
		No.	IR (%)	*p* value	No.	IR (%)	*p* value
Schnauzer	6	1	16.7		0	0	
Pomeranian	26	4	15.4		1	3.8	
Poodle	61	6	9.8		7	11.5	
Italian Greyhound	2	1	50.0		0	0	
Spitz	7	0	0		0	0	
Maltese	71	2	2.8		4	5.6	
Border Collie	4	0	0		0	0	
Jindo	18	2	11.1		1	5.6	
Dachshund	6	1	16.7		0	0	
Mixed	43	6	14.0		2	4.7	
Golden Retriever	12	0	0		1	8.3	
Pungsan	6	0	0		0	0	
Boston Terrier	2	0	0		0	0	
Pekines	2	0	0		0	0	
Yorkshire Terrier	8	2	25.0		0	0	
Papillon	2	0	0		0	0	
Bichon Frise	16	3	18.8		2	12.5	
Chihuahua	4	0	0		0	0	
Shih Tzu	15	0	0		0	0	
Shetland Sheepdog	3	0	0		0	0	
Old English Sheepdog	2	0	0		0	0	
Cocker Spaniel	7	0	0	0.3406	0	0	0.4312
Welsh Corgi	6	0	0		0	0	
Beagle	4	2	50.0		2	50.0	
Doberman	2	0	0		0	0	
French Bulldog	2	0	0		0	0	
Akita	1	0	0		0	0	
Labrador Retriever	5	0	0		0	0	
Great Pyrenees	3	1	33.3		0	0	
Sapsali	2	0	0		0	0	
Cane Corso	1	0	0		0	0	
German Wirehaired Pointer	1	0	0		0	0	
Bernese Mountain	1	0	0		0	0	
Parson Russell Terrier	1	0	0		0	0	
Pug	2	0	0		0	0	
Cavalier King Charles Spaniel	2	0	0		0	0	
Shiba Inu	3	0	0		0	0	
German Shepherd	1	0	0		0	0	
Borzoi	1	0	0		0	0	
American Pitbull terrier	2	1	50.0		1	50.0	
Coton de Tulear	1	0	0		0	0	
Miniature Pinscher	6	1	16.7		1	16.7	
Irish Terrier	1	1	100		0	0	
Pompitz	2	1	50.0		1	50.0	
NS	27	1	3.7		1	3.7	
Total	**400**	**36**	**9.0**		**24**	**6.0**	

IR, Infection Rate; *p* value, $p \leq 0.05$ statistically significant test result; NS, Not Specified (breed not specified in the clinical record).

Table 18. Genetic identity matrix based on the *18S rRNA* gene fragments of *Babesia gibsoni* (314 bp) from dogs

No.	Isolate	Country	GenBank accession number	No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	Dog [1]	ROK	MW485704-MW485725	**1**	**100**	100	100	100	100	100	100	100	100	100	100	100	100	100	99.1	97.2	91.9
2	Dog	ROK	AB478322	**2**	0	**100**	100	100	100	100	100	100	100	100	100	100	100	100	99.1	97.2	91.9
3	Dog	China	KP666166	**3**	0	0	**100**	100	100	100	100	100	100	100	100	100	100	100	99.1	97.2	91.9
4	Dog	Serbia	KJ696717	**4**	0	0	0	**100**	100	100	100	100	100	100	100	100	100	100	99.1	97.2	91.9
5	Dog	Taiwan	FJ769386	**5**	0	0	0	0	**100**	100	100	100	100	100	100	100	100	100	99.1	97.2	91.9
6	Dog	Japan	LC012808	**6**	0	0	0	0	0	**100**	100	100	100	100	100	100	100	100	99.1	97.2	91.9
7	Dog	India	KJ142323	**7**	0	0	0	0	0	0	**100**	100	100	100	100	100	100	100	99.1	97.2	91.9
8	Dog	Bangladesh	LC008285	**8**	0	0	0	0	0	0	0	**100**	100	100	100	100	100	100	99.1	97.2	91.9
9	Dog	Hungary	JX110651	**9**	0	0	0	0	0	0	0	0	**100**	100	100	100	100	100	99.1	97.2	91.9
10	Cat	China	KY073362	**10**	0	0	0	0	0	0	0	0	0	**100**	100	100	100	100	99.1	97.2	91.9
11	Dog	Italy	FJ554534	**11**	0	0	0	0	0	0	0	0	0	0	**100**	100	100	100	99.1	97.2	91.9
12	Dog	Spain	AY278443	**12**	0	0	0	0	0	0	0	0	0	0	0	**100**	100	100	99.1	97.2	91.9
13	MPC	Taiwan	JQ710685	**13**	0	0	0	0	0	0	0	0	0	0	0	0	**100**	100	99.1	97.2	91.9
14	Dog	Slovakia	KP737862	**14**	0	0	0	0	0	0	0	0	0	0	0	0	0	**100**	99.1	97.2	91.9
15	Dhole	Thailand	MK144331	**15**	2	2	2	2	2	2	2	2	2	2	2	2	2	2	**100**	96.2	90.9
16	Dog	Pakistan	KU168833	**16**	6	6	6	6	6	6	6	6	6	6	6	6	6	6	8	**100**	89.0
17	WB	China	JX962780	**17**	17	17	17	17	17	17	17	17	17	17	17	17	17	17	19	23	**100**

Isolate, country, and GenBank accession numbers of each sequence are specified in the left site of the matrix. White boxes of the matrix indicate the percent identity between sequences and gray boxes shown the nucleotide differences between them. [1] Representative sequences of *B. gibsoni 18S rRNA* gene obtained in this study. MPC, Masked palm civet; WB, Wild boar; ROK, Republic of Korea.

Table 19. Clinical findings observed in companion dogs infected with *Babesia gibsoni*

No.	Patient data			Tick exposure	Anamnesis	Other complications
	Breed	Age	Sex			
Group I. Acute infection (*n*=19)						
1	Yorkshire Terrier	4y	F	Yes	Pale gums, lethargy, hematuria	None
2	Yorkshire Terrier	NS	F	Yes	Inappetence, hematuria	None
3	Bichon	2y	M	No	Pyrexia, lethargy, inappetence	IMHA
4	Pomeranian	NS	M	-	Lethargy, inappetence, splenomegaly	None
5	Great Pyrenees	10y	F	Yes	Pyrexia, lethargy, inappetence	None
6	Poodle	8y	M	Yes	Pyrexia, lethargy, inappetence, GS	None
7	Maltese	6y	F	-	Lethargy, inappetence, hematuria	None
8	Poodle	10y	NS	-	Pale gums, lethargy, inappetence, weight loss, depression, hematuria	Pancreatitis, hepatic compromise
9	Dachshund	5y	M	Yes	Pyrexia, GS, splenomegaly, hepatomegaly	Colitis
10	Poodle	10y	F	No	Eye edema, jaundice, hematuria	None
11	Mixed	6y	M	-	Pyrexia, lethargy, inappetence, hematuria, splenomegaly	Cholangiohepatitis, urolithiasis
12	Mixed	13y	M	Yes	Pyrexia, inappetence, weight loss, GS	None
13	Maltese	8y	M	Yes	Pyrexia, lethargy, inappetence, GS	None
14	Irish Terrier	3y	M	Yes	Pyrexia, pale gums, lethargy, GS	IMHA
15	Pompitz	3y	M	Yes	Pyrexia, pale gums, inappetence, GS, jaundice, hematuria, splenomegaly	Pancreatitis
16	Poodle	3y	M	Yes	Pyrexia, inappetence, hematuria	None
17	Bichon Frise	6m	M	-	Lethargy, inappetence, splenomegaly	None
18	Mixed	6y	F	-	Pyrexia, pale gums, lethargy, GS, RS, splenomegaly	Ovarian cysts, chronic bronchitis
19	Miniature Pinscher	3y	F	Yes	Pyrexia, inappetence, depression	None
Group II. Chronic infection (*n*=11)						
20	Beagle	3y	F	Yes	Lethargy, inappetence	None
21	Mixed	6y	M	Yes	Pyrexia	IMHA
22	Beagle	10y	F	Yes	Lethargy, inappetence	None
23	Bichon	1y	M	Yes	Lethargy, inappetence	None
24	Jindo	2y	NS	Yes	Pyrexia, inappetence	None
25	Italian Greyhound	4y	M	Yes	Pyrexia	None
26	Poodle	6y	F	-	Inappetence, GS	None
27	Pomeranian	5y	F	Yes	Lethargy, inappetence	None
28	Mixed	2y	M	Yes	GS	None
29	Pomeranian	4y	M	Yes	Lethargy, inappetence	None
30	Poodle	4y	M	Yes	Pyrexia, GS	IMHA
Group III. Subclinical infection (*n*=4)						
31	Mixed	3y	F	No	Apparently healthy	None
32	Schnauzer	7y	F	-	Apparently healthy	None
33	Jindo	2y	F	-	Apparently healthy	None
34	American Pitbull Terrier	2y	F	Yes	Apparently healthy	None

NS, Not Specified (age and sex not specified in the clinical record); GS, Gastrointestinal Symptoms; RS, Respiratory Symptoms; IMHA, Immune-mediated hemolytic anemia; y, years old; m, months; F, Female; M, Male; Dash, unknown data.

Table 20. Comparison of hematologic and biochemical test results of *Babesia gibsoni* PCR-positive dogs in 2019-2020, the Republic of Korea

Blood test	Parameters		Reference values		Results											
					Low				Normal				High			
					Disease course			Total	Disease course			Total	Disease course			Total
	Name	Unit	Min	Max	A	C	S	No. (%)	A	C	S	No. (%)	A	C	S	No. (%)
Hemogram (*n*=26)	PCV	%	37.1	57	13	6	2	21 (80.8)	2	2	1	5 (19.2)	0	0	0	0 (0)
	RBC	10x12/L	5.7	8.8	11	6	3	20 (76.9)	4	2	0	6 (23.1)	0	0	0	0 (0)
	Hb	g/dL	12.9	18.4	13	6	2	21 (80.8)	2	2	1	5 (19.2)	0	0	0	0 (0)
	RDW	%	11.9	14.5	1	1	0	2 (7.7)	11	5	2	18 (69.2)	3	2	1	6 (23.1)
	PLT	10x9/L	148	484	14	7	2	23 (88.5)	1	0	1	2 (7.7)	0	1	0	1 (3.8)
	WBC	10x9/L	5.2	13.9	2	2	0	4 (15.4)	10	5	3	18 (69.2)	3	1	0	4 (15.4)
	NEUT	10x9/L	3.9	8	4	0	0	4 (15.4)	10	7	2	19 (73.1)	1	1	1	3 (11.5)
	LYMPH	10x9/L	1.3	4.1	4	0	0	4 (15.4)	7	6	3	16 (61.5)	4	2	0	6 (23.1)
	MONO	10x9/L	0.2	1.1	2	0	0	2 (7.7)	12	6	3	21 (80.8)	1	2	0	3 (11.5)
	EOS	10x9/L	0.06	1.23	2	2	0	4 (15.4)	13	5	3	21 (80.8)	0	1	0	1 (3.8)
	BASO	10x9/L	0	0.1	0	1	0	1 (3.8)	15	6	3	24 (92.3)	0	1	0	1 (3.8)
Biochemistry (*n*=26)	ALT	U/L	10	100	0	0	0	0 (0)	13	8	2	23 (88.5)	2	0	1	3 (11.5)
	AST	U/L	11.7	42.5	0	0	0	0 (0)	11	7	3	21 (80.8)	4	1	0	5 (19.2)
	BUN	mg/dL	5	30	0	0	0	0 (0)	14	8	3	25 (96.2)	1	0	0	1 (3.8)
	Creatinine	mg/dL	0.4	1.6	0	0	0	0 (0)	14	8	3	25 (96.2)	1	0	0	1 (3.8)
	Total bilirubin	mg/dL	0.1	0.7	0	0	0	0 (0)	7	6	3	16 (61.5)	8	2	0	10 (38.5)
	Albumin	g/dL	2.3	3.9	6	1	1	8 (30.8)	9	7	2	18 (69.2)	0	0	0	0 (0)
	Total protein	g/dL	4.9	7.2	2	0	0	2 (7.7)	12	7	3	22 (84.6)	1	1	0	2 (7.7)

A, Acute (*n*=15); C, Chronic (*n*=8); S, Subclinical (*n*=3); PCV, Packed-cell volume; RBC, Red Blood Cell Count; Hb, Hemoglobin; RDW, Red Cell Distribution Width; PLT, Platelet Count; WBC, White Blood Cell Count; NEUT, Neutrophils; LYMPH, Lymphocytes; MONO, Monocytes; EOS, Eosinophils; BASO, Basophils; ALT, Alanine Aminotransferase; AST, Aspartate Aminotransferase; BUN, Blood Urea Nitrogen.

CHAPTER III

Serological evidence of *Anaplasma* spp., *Borrelia burgdorferi* and *Ehrlichia canis* in dogs by rapid diagnostic test kits

Abstract

Emergent and re-emergent tick-borne infections in dogs, such as *Anaplasma* spp., *Borrelia* spp., and *Ehrlichia* spp., are gaining increasing attention worldwide. The rise in pet ownership and the close relationship between dogs and their owners are some of the most concerning factors, as dogs may act as competent reservoirs for human tick-transmitted infectious agents. Consequently, the aim of the present study was to contribute to the epidemiological surveillance of canine tick-transmitted infections with zoonotic risk in the ROK by investigating the seroprevalence of the pathogens *Anaplasma* spp., *Borrelia burgdorferi* and *Ehrlichia canis*. In this study, a total of 430 whole blood samples from domestic dogs were tested using SensPERT Ab Test Kits (VetAll Laboratories®) for the detection of seroreactive animals. The seroprevalence rates identified were 9.8% (42/430) for *Anaplasma* spp., 2.8% (12/430) for *B. burgdorferi*, and 1.4% (6/430) for *E. canis*. There was no statistical significance in any of the risk factors eval-

uated in this study, such as sex, age, and history of tick exposure, that could be associated with the development of humoral immune response in the seroreactive dogs. There was only one exception for dogs seroreactive to *Anaplasma* spp., where the risk factor “tick exposure” resulted statistically significant (p=0.047). This serological survey exhibited the widespread presence of *Anaplasma* spp., *B. burgdorferi* and *E. canis* throughout the ROK, and in turn suggests that dogs may play a key role as sentinel animals of multiple zoonotic infectious agents in the country.

Keywords: *Anaplasma* spp., *Borrelia burgdorferi*, *Ehrlichia canis*, seroprevalence, dog, Republic of Korea

1. Introduction

Canine vector-borne diseases (CVBDs), recognized as emerging infectious threat, not only to dogs but also to human being, have been increasing globally over the years, specifically in terms of incidence and geographical distribution (Potkonjak et al., 2020; Dantas-Torres et al., 2016). Ticks, as the main vector of pathogen transmission to animals, have been associated with the dissemination of numerous bacterial, parasitic, and viral diseases (Sharifah et al., 2020). Among the bacterial pathogens that these hematophagous arthropods can transmit to dogs, the Anaplasmataceae, Spirochaetaceae, and Rickettsiaceae families are of primary medical and veterinary concern (Chomel et al., 2011).

One of the widely distributed species of the Anaplasmataceae family is *A. phagocytophilum*, a bacterium that primarily infects neutrophils, although occasionally it can also be found in eosinophilic granulocytes (Chirek et al., 2018), and is transmitted by *Ixodes* ticks, which are also competent vectors for *Borrelia burgdorferi* (Liu et al., 2018). *A. phagocytophilum* is the causative agent of canine granulocytic anaplasmosis (CGA), a disease which its clinical manifestation ranges from self-limiting to severe. Commonly, lethargy, inappetence, and fever are observed, however lameness, pale mucous membranes, tense abdomen, diarrhea, surface bleeding, vomiting, tachypnea, enlarged lymph nodes, and splenomegaly may also occur (Silaghi et al., 2011; Lee et al., 2017). On the other hand, *A. platys*, the bacterium responsible for causing infectious canine cyclic thrombocytopenia (ICCT) (Harrus et al., 1997), infects platelets and it is assumed to be transmitted by *Rhipicephalus sanguineus* ticks, a vector of multiple pathogens, including *Ehrlichia*

canis (Volgina et al., 2013). In North America, *A. platys* is considered less pathogenic than *A. phagocytophilum*; nevertheless, reported clinical abnormalities include fever, anorexia, bleeding disorders, and anterior uveitis (Gaunt et al., 2010). Interestingly, among the Anaplasmataceae, *E. canis* was the first agent of monocytic ehrlichiosis to be identified in dogs (Chomel et al., 2011). Experimentally, infection with *E. canis* results in acute, subclinical, and chronic disease states with dogs exhibiting a variety of clinical signs and laboratory abnormalities such as fever, lethargy, lameness, oculonasal discharge, thrombocytopenia, non-regenerative anemia, proteinuria, leukopenia, and hyperglobulinemia during various stages of infection (Gaunt et al., 2010; Murphy et al., 1998). The infection with the spirochete *B. burgdorferi*, the causative agent of Lyme borreliosis, its known to be less frequent in dogs than in humans, causing in them milder clinical signs. Only about 5% to 10% of dogs exposed to infected ticks develop clinical borreliosis, which is characterized by the presentation of fever, inappetence, malaise, limb/joint disorders, or neurologic abnormalities (Fritz et al., 2009; Magnarelli et al., 1987).

Since the bacteria *Anaplasma* spp., *E. canis* and *B. burgdorferi* are capable of infecting not only dogs but also humans, this research aims to be a key contribution to public health surveillance of TBDs in the ROK, by investigating the seroprevalence rates and by identifying the geographical distribution of seroreactive animals to the above-mentioned pathogens.

2. Materials and Methods

2.1. Study area and sample collection

The study was conducted in 7 metropolitan cities and 9 provinces of the ROK (Table 21). A total of 430 whole blood samples were collected at different veterinary hospitals and clinics and then referred to the Laboratory of Veterinary Internal Medicine of Seoul National University, ROK, between April 2019 to December 2020. All animals tested in this study were dogs with a history of tick bites or clinical signs suggesting a presumptive diagnosis of TBD. Whole blood samples were collected in capillary blood collection tubes containing EDTA anticoagulant and then were transported under cold chain to the laboratory for further processing.

2.2. Serologic analysis

Serological testing was performed using SensPERT rapid assay test kits (VetAll Laboratories®, Goyang-si, ROK) for the detection of antibodies against *Anaplasma* spp. (*A. phagocytophilum*/*A. platys*) (sensitivity and specificity of 100% *vs*. IFA), *B. burgdorferi* (sensitivity and specificity of 100% *vs*. IFA), and *E. canis* (sensitivity of 97.7% and specificity of 100% *vs*. IFA). The serological test procedure and the interpretation of test results were carried out according to the manufacturer's instructions.

2.3. Statistical analysis

The Chi-squared test was used to analyze the different risk factors that may be associated with the humoral immune response. This test was per-

formed with the GraphPad Prism software package (v 5.04; GraphPad Software, Inc., La Jolla, CA). The results were considered statistically significant when the *p* value was ≤ 0.05.

3. Results

From the three TBPs under study, a large number of dogs resulted seroreactive to *Anaplasma* spp. (*A. phagocytophilum*/*A. platys*). The overall seroprevalence rate identified for this bacterium was 9.8% (42/430). Based on the sample sites, seropositive provinces to *Anaplasma* spp. were Gyeongsangbuk-do (100%, 2/2), Jeollanam-do (50.0%, 4/8), Jeollabuk-do (36.8%, 7/19), Gyeongsangnam-do (26.7%, 4/15), Chungcheongnam-do (20.0%, 2/10), Gangwon-do (14.3, 1/7) and Gyeonggi-do (5.7%, 8/140), along with Ulsan (33.3%, 3/9), Daejeon (28.6%, 2/7), Busan (6.7%, 1/15), and Seoul (5.5%, 8/146) metropolitan cities (Figure 26).

Secondly, *B. burgdorferi*, which was identified in 12/430 dogs (2.8%). The *B. burgdorferi* seropositive dogs were from Gyeongsangnam-do (20.0%, 3/15), Jeollabuk-do (5.3%, 1/19), and Gyeonggi-do (1.4%, 2/140) provinces, and from the metropolitan cities of Busan (6.7%, 1/15) and Seoul (3.4%, 5/146) (Figure 26).

Lastly, with a lower seroprevalence rate, *E. canis*, which was identified in only 6/430 (1.4%) dogs. Gyeongsangnam-do, and Gyeonggi-do were the seropositive provinces, along with Busan, and Seoul metropolitan cities, with a seroprevalence rate of 6.7%, 0.7%, 13.3%, and 1.4%, respectively (Figure 26).

Moreover, of the 430 dogs, 7 (1.6%) were co-detected with double and triple antibodies. The type of co-infection found were *Anaplasma* spp. plus *B. burgdorferi* in 3 dogs (0.7%), *Anaplasma* spp. plus *E. canis* in 1 dog (0.2%), *B. burgdorferi* plus *E. canis* in 1 dog (0.2%), and triple co-infection with *Anaplasma* spp., *B. burgdorferi*, and *E. canis* in 2 dogs (0.5%).

According to the gender classification, male dogs had a relatively high seroprevalence compared to the one identified in female dogs, being these 10.4% *vs*. 8.5% for *Anaplasma* spp., 3.3% *vs.* 2.4% for *B. burgdorferi*, and 1.6% *vs*. 0.5% for *E. canis*. However, the Chi-squared analysis showed no significant relationship between gender and the capacity to produce antibodies (Table 22). Based on the age groups, the highest seroprevalence of *Anaplasma* spp. was identified in dogs between 8 and 10 years old (10.6%, 7/66), while dogs between 5 and 7 years old were found to be the most seroreactive to *B. burgdorferi* and *E. canis*, with rates of 4.1% (4/97) and 2.1% (2/97), respectively. As shown in Table 22, these differences were not statistically significant. However, when evaluating the risk factor "tick exposure" linked to seroprevalence, a significant difference was observed only for *Anaplasma* spp. (*A. phagocytophilum*/*A. platys*) with a *p* value of 0.047 (Table 22).

4. Discussion

There is a global concern regarding the growing spectrum of TBDs affecting animals and humans. Since the past century, Asia has been in the limelight for emergence and pathogenicity of a large number of infectious diseases that have taken a substantial toll on the health not only of animals

but also of millions of people (Colella et al., 2020). This phenomenon can be clearly represented by the current situation of the Korean population, which are dealing with many important zoonotic TBDs, such as anaplasmosis, Lyme borreliosis, ehrlichiosis, babesiosis, and the emerging SFTS. Despite the fact that the ROK is a relatively small country, there has been a steady increase in the number of dog and cat owners over the years. For the year 2018, an average population of 9.32 to 9.97 million dogs and cats was estimated, which is expected to increase to, approximately, 11.55~16.49 million by 2027 (Lee et al., 2020). Since companion animals can act as competent reservoirs and sentinels for a variety of zoonotic diseases, the ROK has been making a constant effort to investigate and keep up to date the occurrence of tick-borne infections in pets and their owners.

Regarding *Anaplasma* species infecting Korean domestic dogs, to date only *A. phagocytophilum* has been identified by PCR with an extremely low prevalence, ranging from 0.1% to 2.3% (Lee et al., 2016; Suh et al., 2017). Serological techniques, such as ELISA and IFA, have revealed a seroprevalence of *A. phagocytophilum* varying from 15.1% to 26.4% (Lee et al., 2020; Suh et al., 2017; Bell et al., 2012; Lim et al., 2010), these rates being slightly higher than those obtained in the present study with the use of the SensPERT Ab Test Kit (9.8%). An interesting fact to highlight is that a different pattern of *Anaplasma* infections has been seen in China, where in addition to *A. phagocytophilum*, dogs have also been infected by *A. ovis*, *A. bovis* (Cui et al., 2017), and *A. capra* (Shi et al., 2019) species. Consequently, continued epidemiological surveillance of these pathogens in the ROK is recommended, as different genetic variants of zoonotic *A. capra* have re-

cently been identified circulating in Korean cattle (Miranda et al., 2021).

B. burgdorferi in the ROK has only been identified by serological techniques (ELISA and IFA), despite having also been evaluated by real-time PCR (Suh et al., 2017). The seroprevalence rates reported over the years have been 2.2% (5/229) in 2010, 1.1% (2/182) in 2012, and 1.1% (6/532) in 2017 (Suh et al., 2017; Bell et al., 2012; Lim et al., 2010). The current study revealed a seroprevalence of 2.8% (12/430), which is quite similar to the one identified in 2010 (Lim et al., 2010). However, a serosurvey conducted in 2020, with a larger study population, identified a seroprevalence of 6.4% (142/2,215), this being the highest seroprevalence of *B. burgdorferi* reported to date in the ROK (Lee et al., 2020). China and Japan, the two-close neighboring countries of the ROK, have also identified seroreactive dogs to this spirochete, been Japan the country with the highest seroprevalence rate, ranging from 10.2% to 27.3% (Zhang et al., 2017; Uesaka et al., 2016).

Concerning *Ehrlichia* species, two causative agents of canine ehrlichiosis have been identified in the ROK, *E. chaffeensis* and *E. canis*. In 2008, there were two clinical cases of canine ehrlichiosis caused by *E. chaffeensis*, where one of the two dogs was also infected with *Babesia gibsoni*, another emerging tick-borne pathogen in the ROK (Yu et al., 2008). Moreover, in 2020 was identified the seroprevalence of *E. chaffeensis*, this being 2.3% (Lee et al., 2020). On the other hand, seroprevalence of *E. canis*, in this country, has being ranging from 0 to 22.5% between the years 2010 to 2017 (Suh et al., 2017; Bell et al., 2012; Lim et al., 2010; Jung et al., 2012). This pattern of results is consistent with the seroprevalence identified in the present study (1.4%), which is within the above-mentioned range. In China and

Japan, the seroprevalence of *Ehrlichia* spp. as well has been low, just of 1.3% and 1.5%, respectively (Zhang et., 2017; Kubo et al., 2015).

Infection with multiple tick-transmitted pathogens or with multiple genotypes of the same pathogenic species can also occur in an individual animal following heavy exposure to ticks (Shaw et al., 2001). In the current study different types of co-infection were identified being the predominant the combination of *Anaplasma* spp. plus *B. burgdorferi*. One study performed in the region of Minnesota, USA, found an association between concurrent *A. phagocytophilum* and *B. burgdorferi* seroreactivity and clinical illness in dogs. Dogs testing positive for antibodies to both pathogens, *A. phagocytophilum* and *B. burgdorferi*, were nearly twice as likely to have clinical signs consistent with anaplasmosis and/or borreliosis when compared to dogs that were seroreactive to only one of these organisms (Beall et al., 2008). Therefore, since the present investigation already demonstrated that co-infection with *Anaplasma* spp. and *B. burgdorferi* it is also occurring in the ROK, further studies will be useful to clarify the relationship between seroreactivity and the clinical presentation in this country.

There was no statistical significance in any of the risk factors evaluated in this study, such as sex, age, and history of tick exposure, that could be associated with the presence of antibodies against *Anaplasma* spp., *B. burgdorferi* and *E. canis*. There was only one exception for the case of *Anaplasma* spp., where the risk factor “tick exposure” was statistically significant (p=0.047). In the ROK, *Haemaphysalis longicornis*, *Ixodes persulcatus* and *Ixodes turdus* ticks have been identified as potential vectors of *A. phagocytophilum* (Kim et al., 2003). Moreover, according to a tick surveillance con-

ducted in 2011, *H. longicornis* (48.9%), *H. flava* (17.3%), *Ixodes nipponensis* (1.7%), and *Rhipicephalus sanguineus* sensu lato (0.5%) were found to be the most prevalent tick's species infesting Korean dogs (Choe et al., 2011). Among the tick species mentioned above *H. longicornis* is the one that is widely distributed throughout the ROK (Chong et al., 2013). This tick is the main vector for SFTSV, a new tick-viral zoonoses in the East Asian countries, including China, Japan, and the ROK (Jo et al., 2019). The most recent seroprevalence of SFTSV identified in Korean dogs has been 21.4%, however in humans after the identification of the first clinical case in 2003, 335 cases (73 deaths; case fatality rate 21.8%) were reported during 2013 to 2016 (Kang et al., 2019). These findings together with the results of the current investigation strengthens the importance of the epidemiological surveillance of TBPs in the ROK since many of them have zoonotic potential. Furthermore, the serological survey shed new light not only on the geographic distribution and seroprevalence rates of *Anaplasma* spp., *B. burgdorferi* and *E. canis* in dogs, but also in the use of the SensPERT One-rapid test kit (Vet-All Laboratories®, Goyang-si, ROK) as a useful tool for the identification of seroreactive animals.

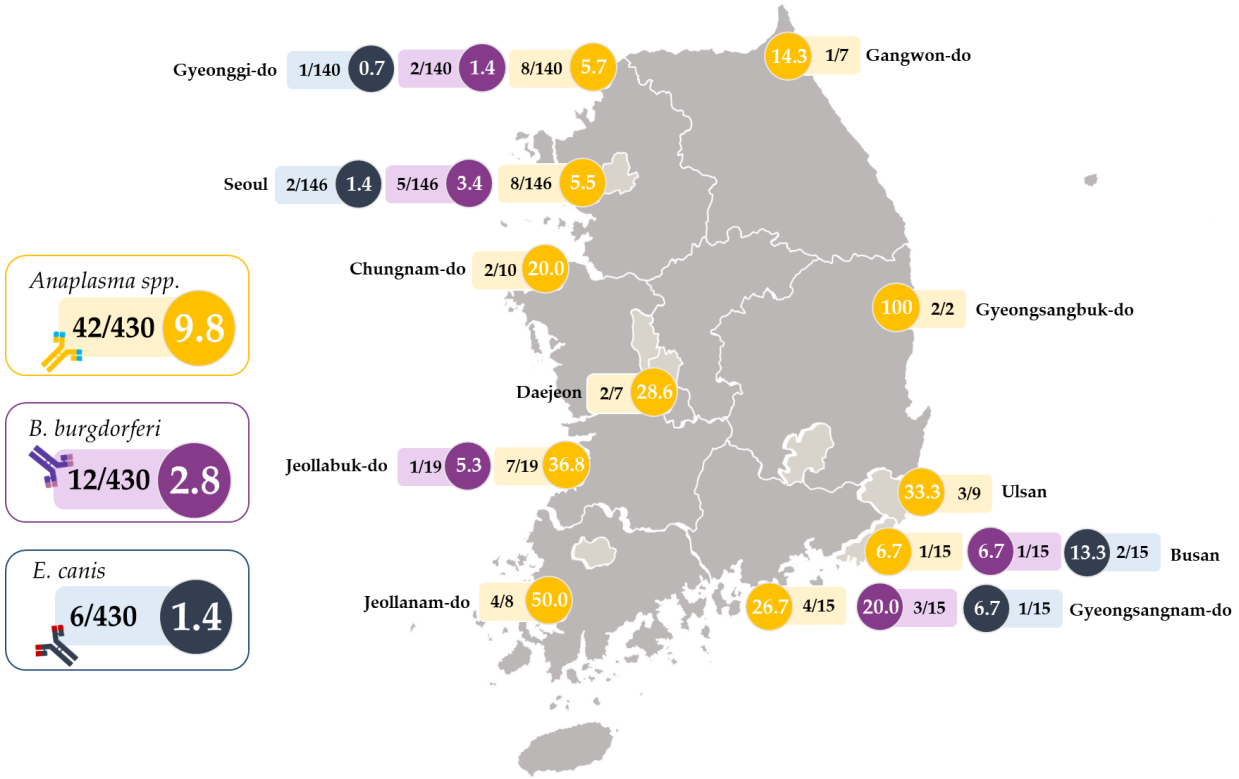


Figure 26. Map illustrating the distribution of seropositive dogs to *Anaplasma* spp., *B. burgdorferi* and *E. canis* in 2019-2020, the Republic of Korea. Infection rates are shown in circles: yellow color for *Anaplasma* spp., purple for *B. burgdorferi*, and blue for *E. canis*.

Table 21. Geographic location of the veterinary hospitals and clinics where whole blood samples were collected from dog patients between 2019-2020, the Republic of Korea

Location	Name	No. of collected samples
Metropolitan cities (*n*=7)	Seoul	146
	Incheon	8
	Daejeon	7
	Daegu	32
	Ulsan	9
	Busan	15
	Gwangju	1
Provinces (*n*=9)	Gyeonggi-do	140
	Gangwon-do	7
	Chungcheongbuk-do	10
	Chungcheongnam-do	10
	Gyeongsangbuk-do	2
	Gyeongsangnam-do	15
	Jeollabuk-do	19
	Jeollanam-do	8
	Jeju-do	1
Total		**430**

Table 22. Evaluation of the risk factors that may be associated with the presence of antibodies against *Anaplasma* spp., *B. burgdorferi* and *E. canis* in companion dogs from the Republic of Korea in 2019-2020

Risk factor	No. tested	*Anaplasma* spp.			*B. burgdorferi*			*E. canis*		
		No.	IR (%)	*p* value	No.	IR (%)	*p* value	No.	IR (%)	*p* value
Sex										
Male	182	19	10.4		6	3.3		3	1.6	
Female	211	18	8.5	0.540	5	2.4	0.579	1	0.5	0.247
Unknown	37	5	13.5		1	2.7		2	5.4	
Total	**430**	**42**	**9.8**		**12**	**2.8**		**6**	**1.4**	
Age										
≤ 1 year old	57	4	7		1	1.8		1	1.8	
2-4 years old	119	12	10.1		6	5		1	1	
5-7 years old	97	10	10.3		4	4.1		2	2.1	
8-10 years old	66	7	10.6	0.832	0	0	0.334	1	1.5	0.834
≥ 11 years old	52	3	5.8		1	1.9		0	0	
Unknown	39	6	15.4		0	0		1	2.6	
Total	**430**	**42**	**9.8**		**12**	**2.8**		**6**	**1.4**	
Tick exposure										
Yes	222	24	10.8		8	3.6		5	2.3	
No	68	2	2.94	0.047*	0	0	0.112	0	0	0.140
Unknown	140	16	11.4		4	2.9		1	0.7	
Total	**430**	**42**	**9.8**		**12**	**2.8**		**6**	**1.4**	

IR, Infection Rate; *p* value, $p \leq 0.05$* statistically significant test result.

GENERAL CONCLUSION

TBDs have been on the rise since the beginning of the 20th century due to the increased human activities impacting forest ecosystems, wildlife, and domestic animals. Although several tools have been developed to monitor the risks of TBDs, their research has been neglected compared with mosquito-borne diseases. Therefore, the present investigation, through the epidemiological analyses conducted for the identification of different emerging TBPs in cattle, goats, and dogs, demonstrates novel and key information for the better understanding of the occurrence of TBDs in the ROK. In summary, the analysis of the results obtained in the present investigation leads to the following conclusions:

1.1. In the ROK, cattle are mainly infected with *A. phagocytophilum*, while goats act mainly as carriers of *A. bovis*. Since *A. phagocytophilum* is a zoonotic pathogen, human contact with livestock can be considered as one of the main sources of risk for triggering *A. phagocytophilum* transmission to humans, especially for farmers and associated occupational groups in rural areas. Thus, continuous surveillance of this zoonotic agent in different livestock farms in the country is of essential importance.

1.2. The study contributed to the ratification of cattle as a potential reservoir of the emerging zoonotic *A. capra*. This pathogen recently discovered in clinically infected humans in China, may also be considered as a zoonotic infectious agent threatening the Korean population.

1.3. Since this is the first study in the ROK investigating the molecular identification of *A. ovis*, further large-scale studies are needed to corroborate whether or not *A. ovis* infection occurs among domestic ruminants in the country.

1.4. This study is the first molecular report of co-infection types that may be found among Korean cattle and goats, which in turn, by multilocus genotyping and phylogenetic analysis of the nucleotide sequences, contributed to the identification of five novel strains of the *A. phagocytophilum 16S rRNA* gene, and a new variant of the *A. capra gltA* gene circulating among Korean cattle.

2.1. Based on the results of the molecular and serological surveillance of *B. gibsoni* infection in dogs, it can be concluded that this hemo-parasite is endemic in the ROK, since antigen-positive and seroreactive animals have been identified across whole country.

2.2. The investigation revealed that the clinical presentation of canine babesiosis, caused by *B. gibsoni*, in the ROK occurs in its three presentations or courses, i.e., acute, chronic, and subclinical, being its acute course the most predominant, thus representing a great challenge for veterinary clinicians due to the severity of clinical signs.

2.3. The classification of patients according to the clinical presentation of the disease, canine babesiosis, favored the identification of the pattern of

symptoms and hematological and biochemical abnormalities to be found in acutely, chronically and subclinically infected animals.

2.4. Since subclinical presentation of *B. gibsoni* infection is also present in the ROK, continued surveillance of this hemo-parasite in the country using molecular techniques is of vital importance for its identification. Moreover, the detection of these dogs may help to prevent the spread of *B. gibsoni* as they may remain asymptomatic carriers of the disease for several months or even for life without treatment.

2.5. The sample size was insufficient to generate a statistically valid conclusion regarding the geographic distribution and the risks factors associated with *B. gibsoni* infection. Despite these limitations, this research can be seen as a first step towards integrating two line of research, molecular and serological identification of *B. gibsoni* along with the evaluation of the clinical presentation of the infection, that to the best of our knowledge, have not been directly linked in the ROK.

3.1. The serological survey for the detection of antibodies against *Anaplasma* spp., *B. burgdorferi* and *E. canis* suggests that dogs in the ROK are simultaneously exposed to a wide range of TBPs, since some dogs were co-detected with double and triple antibodies.

3.2. The serological survey presents understandable and objective information on the spatial patterns of the occurrence of TBPs throughout the ROK

which in turn can help the inhabitants of each province to avoid high-risk areas.

3.3. The findings shed new light in the use of the SensPERT One-rapid test kit (VetAll Laboratories®) as a useful tool for the identification of seroreactive animals.

In general, it can be concluded that cattle, goats and dogs in the ROK act as carriers or host animals of different TBPs, which in turn are frequently co-exposed to multiple combinations of infectious agents with zoonotic potential, leading to a negative impact on public health. Therefore, the elucidation of the geographic distribution along with the infection rates of these pathogens contributes positively to the implementation of new prevention and control strategies in high-risk areas of this country.

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국 문 초 록

소, 염소 그리고 개에서 참진드기 매개 병원체의 역학 및 유전학적 분석

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국내에는 참진드기매개병원체(tick-borne pathogens, TBPs)가 다양하고 광범위하게 분포되어 있다. 그러나 연구자료가 드물어 이 질병의 역학적 깊은 이해에는 한계가 있다. 따라서, 본 연구는 TBPs의 발생을 규명하고, 분자, 혈청학 및 계통유전학 기술들을 통해 소, 염소, 개에서 순환하는 병원체 및 종의 유형을 확인하는 것을 목표로 하였다.

국내 각 지방과 수도권에서 총 1,116마리의 전혈 샘플(소 384마리, 염소 302마리, 개 430마리)을 학보하였다. 동물의 진드기 매개 병원체의 식별을 위해 3가지의 독립적인 연구를 수행하였다.

첫 번째는 소와 염소의 *Anaplasma* spp.을 평가하는 데 초점을 맞추었다. *Anaplasma* spp.의 유병률 및 유전적 다양성을 조사하기 위해 686 마리의 전혈 샘플(소 384마리, 염소 302마리)에서 중합효소 연쇄 반응(polymerase chain reaction, PCR)이 수행되었다. 이 연구에서 소의 경우 *Anaplasma* 감염은 주로 *A. phagocytophilum*(21.1%)에 기인했고, 염소의 경우, 주로 *A. bovis*(7.3%)에 기인했다는 것을 밝혔다. 혼합감염의 경우도 발견되었는데, 소의 경우, *A. bovis*와 *A. phagocytophilum*의 혼합감염은 16.7%였으며, 염소의 경우, *A. bovis*와 *A. capra*의 혼합감염은 1.0%로 나타났다. 참진드기매개병원체인 *A. bovis, A. phagocytophilum, A. capra*의 3가지 혼합감염의 경우는 특이하게 소 한 마리에서만 확인되었다(0.3% 감염률). 이들 병원체의 유전자 염기서열을 토대로 한 계통발생학적 분석으로 볼 때 소와 염소 사이에서 *A. phagocytophilum 16S rRNA* 유전자 5개의 유전자와 *A. capra gltA* 유전자에서 1개를 포함한 새로운 변종이 있음을 밝혔다. 또한 이 연구는 소가 *A. capra*의 잠재적 보균동물이라는 것을 알 수 있었으며, 국내 반추 동물에서 발견될 수 있는 *Anaplasma* 혼합 감염 유형을 보여준다. 아나플라마증은 인수공통감염병으로서, 이 연구 결과는 공중보건 정책을 수립을 하는데 있어서 매우 중요할 수 있다.

두 번째로 *Babesia gibsoni*가 원인체인 개 바베시아증의 임상적

사례에 대한 연구로서 혈액학적 및 생화학적 검사 결과와 주혈기생충에 의해 유발된 바베시아증의 임상증상의 평가를 주요 목표로 하였다. 이 연구를 위해 바베시아증으로 의심되는 개 총 400마리의 전혈 샘플에서 PCR 검사를 수행하였으며, 혈청에서 항체 검사를 위하여 신속진단키트(VetAll Laboratories® 제공)를 이용하여 분석하였다. 개 400마리 중 36마리(9.0%)가 *B. gibsoni* 항원에 감염되었으며, 24마리(6.0%)에서는 혈청 항체가 검출되었다. 각 개 환자의 임상 증상에 대한 평가에 따르면, 한국에 바베시아증의 세 가지 단계가 존재하며, 급성이(55.8%) 가장 많게 나타났고, 식욕 부진, 혼수, 발열, 위장 증상, 때때로 혈뇨 등으로 임상증상이 있었다. 혈소판감소증(93.3%)과 같은 일반적인 혈액학적 이상과 함께 빈혈(86.7%), 고빌리루빈혈증(53.3%), 저알부민혈증(40.0%), aspartate aminotransferase(AST) 증가(26.7%) 등의 생화학적 이상이 나타났다. 이 연구결과 감염된 개에서 다양한 임상증상이 나타났으며, 한국에서는 *B. gibsoni*가 풍토성 주혈원충이며, 정확한 진단을 위하여 혈액학적, 생화학적 검사 및 PCR 분석이 중요하였다. 이 연구의 결과는 개 바베시아증의 발생을 이해하는데 중요한 정보를 제공할 뿐만 아니라 보다 효과적인 예방 전략을 계획하는 데에 도움이 될 것이다.

세 번째 연구는 *Anaplasma* spp., *Borrelia burgdorferi*, *Ehrlichia canis*의 혈청 항체 조사를 통해 참진드기매개병원체의 위험으로부터 개 진드기 감염의 역학 감시에 기여하는 것을 목표로 했다. 총 430마리의 전혈 샘플에서 위에서 언급한 참진드기매개병원체에 대한 항

체의 신속한 진단을 위한 신속항원검사키트(VetAll Laboratories®)로 실험하였다. 분석된 병원체 별 혈청 양성률은 *Anaplasma* spp.가 9.8%, *B. burgdorferi* 2.8%, *E. canis* 1.4%였다. 이 연구에서 평가된 위험요인인 성별, 나이, 참진드기 노출경력에서 통계적 유의성은 없었으나, *Anaplasma* spp.의 혈청 항체에 대한 양성률에서는 위험요인인 진드기 노출이 통계적으로 유의성있는 결과를 나타내었다 (p=0.047). 이 혈청학적 조사는 개가 이 지역에서 참진드기에 의한 여러 동물 감염의 신속한 전파와 전염에 핵심적인 역할을 할 수 있음을 시사하며 *Anaplasma* spp., *B. burgdorferi*, 그리고 *E. canis*가 한국 전역에 광범위하게 존재하고 있음을 확인하였다.

이러한 역학적 분석을 통해 일반적으로 소, 염소 및 개가 다른 참진드기매개병원체들의 보균 또는 숙주 동물로 작용하며, 이는 동물 질병을 발생시키거나 감염원으로 노출되어 공중보건에 부정적인 영향을 미친다고 결론 내릴 수 있다. 따라서, 이러한 병원균의 감염률과 함께 지리적 분포의 명확화는 대한민국의 고위험 지역에서 새로운 예방 및 통제 전략의 구현에 긍정적인 기여를 할 것이다.

키워드: *Anaplasma* spp., *Babesia gibsoni*, *Borrelia burgdorferi*, *Ehrlichia canis*, 중합효소연쇄반응(유전자 증폭), 계통발생학적 분석, 혈청학

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