Molecular Detection and Phylogeny of Tick-Borne Pathogens in Ticks Collected from Dogs in the Republic of Korea

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Abstract: Ticks are important vectors of various pathogens that result in clinical illnesses in humans and domestic and wild animals. Information regarding tick infestations and pathogens transmitted by ticks is important for the identification and prevention of disease. This study was a large-scale investigation of ticks collected from dogs and their associated environments in the Republic of Korea (ROK). It included detecting six prevalent tick-borne pathogens (Anaplasma spp., A. platys, Borrelia spp., Babesia gibsoni, Ehrlichia canis, and E. chaffeensis). A total of 2293 ticks (1110 pools) were collected. Haemaphysalis longicornis (98.60%) was the most frequently collected tick species, followed by Ixodes nipponensis (0.96%) and H. flava (0.44%). Anaplasma spp. (24/1110 tick pools; 2.16%) and Borrelia spp. (4/1110 tick pools; 0.36%) were detected. The phylogenetetic analyses using 16S rRNA genes revealed that the Anaplasma spp. detected in this study were closely associated with A. phagocytophilum reported in humans and rodents in the ROK. Borrelia spp. showed phylogenetic relationships with B. thelilri and B. miyamotoi in ticks and humans in Mali and Russia. These results demonstrate the importance of tick-borne disease surveillance and control in dogs in the ROK.

Keywords: dog ticks; Haemaphysalis longicornis; Ixodes nipponensis; Haemaphysalis flava; anaplasmosis; Lyme borreliosis; Korea

1. Introduction

Ticks are obligate blood-feeding parasites that transmit zoonotic tick-borne pathogens, including protozoa, viruses, and bacteria, to animal and human hosts [1,2]. Approximately 10% of known tick species are vectors of pathogens of medical and veterinary importance [3]. Some tick species are known vectors of one or several tick-borne diseases (TBDs), such as borreliosis (Lyme disease and Borrelia relapsing fever and other Borrelia spp. transmitted by Ixodes ticks), babesiosis (Babesia spp. transmitted by Haemaphysalis spp., Rhipicephalus spp., and Dermacentor spp.), ehrlichiosis (Ehrlichia canis genogroup transmitted by Rhipicephalus spp., Amblyomma americanum [4–6], Ixodes persulcatus, I. ovatus, and I. silvarum), and anaplasmosis (Anaplasma phagocytophilum transmitted by I. scapularis and I. pacificus [7]). An understanding of the specific tick hosts and associated pathogens is important to identify the risks of TBDs for domestic animals and humans.
Ticks are common parasites of domestic animals, including dogs, and have a high risk of transmitting tick-borne pathogens [8–10]. Dogs are reservoirs of some tick-borne pathogens [6]. They are the most common animal bred for various purposes, including pets and military dogs. Close contact with dogs may result in the transfer of ticks and TBDs to humans. Therefore, dogs may be considered sentinel animals for TBDs impacting human health [11–14]. Identifying the prevalence of dog ticks and associated pathogens provides an understanding of the distribution of tick-borne pathogens. It raises awareness of TBDs among pet owners and other people who contact dogs [15].

In the ROK, TBDs, such as Lyme disease, anaplasmosis, ehrlichiosis, tularemia, bartonellosis, and babesiosis, are of medical importance. In particular, the number of Lyme disease cases has rapidly increased since 2012 [16–18]. Molecular and serological detection methods have revealed that over 40% of dogs in the ROK are infected with pathogens that cause TBDs, including *A. phagocytophilum*, *E. canis*, *Borrelia burgdorferi*, *Babesia gibsoni*, *Dirofilaria immitis*, and *Mycoplasma haemocanis* [19,20]. The identification of reservoir hosts and potential vectors of the pathogenic agents is of interest. A previous study using molecular detection methods reported *I. nipponensis* ticks infected with *B. garinii* in dogs in the Gyeongsangbuk province, ROK [21]. However, the prevalence of ticks on dogs and tick-borne pathogens harbored by ticks collected from dogs in the ROK remains poorly investigated.

This study was part of a large-scale tick surveillance program of domestic pets, military working dogs, and stray dogs from shelter-associated environments in the ROK. Assays to detect six common tick-borne pathogens (*A. phagocytophilum*, *A. platys*, *Borrelia spp.*, *Babesia gibsoni*, *E. canis*, and *E. chaffeensis*) were conducted. This study highlights the importance of the prevention of TBDs in dogs and humans in the ROK.

2. Results

2.1. *Distribution of Dog Ticks in the ROK*

A total of 2293 ticks categorized into 1110 tick pools were collected from 24 sites in 13 provinces or metropolitan cities in the ROK (Figure 1). Overall, 807 ticks (35.2%) were found on pet dogs, 624 (27.2%) on military dogs, 572 (24.95%) on stray dogs, and 290 (12.65%) in stray dog shelter environments (Table 1).

The highest number of ticks collected from dogs/dog shelters was observed in the northern region (1461 ticks, 63.72%), followed by the central region (767 ticks, 33.45%), and the southern region (65 ticks, 2.83%) (Table 2; Figure 1).

![Figure 1](image-url). Collection of ticks from dogs and vegetation at dog shelters in the ROK. Ticks were collected at 24 sites in 13 provinces or metropolitan areas. The number of sites per province or metropolitan area is shown in parentheses (A). The
numbers of tick pools collected from the northern, central, and southern regions are shown, with the number of collected ticks shown in parentheses (B).

Table 1. Identification of dog ticks in the ROK.

| Species                     | Stage   | Tick Pool (Number of Ticks) | Total |
|-----------------------------|---------|-----------------------------|-------|
|                             |         | Pet Dogs | Stray Dogs | Dog Shelters | Military Working Dogs |       |
| Haemaphysalis longicornis   | Larvae  | 4 (49)   | 26 (126)   | 4 (166)      | 0                      | 34 (341) |
|                             | Nymph   | 73 (606) | 51 (149)   | 9 (93)       | 15 (110)               | 148 (958) |
|                             | Adult   | 111 (129)| 282 (295)  | 29 (29)      | 478 (509)              | 900 (962) |
|                             | Sub total| 188 (784)| 359 (570)  | 42 (288)     | 493 (619)              | 1082 (2261) |
| H. flava                    | Larvae  | 0        | 0          | 0            | 0                      | 0       |
|                             | Nymph   | 1 (3)    | 0          | 1 (1)        | 1 (3)                  | 3 (7)   |
|                             | Adult   | 2 (2)    | 0          | 0            | 1 (1)                  | 3 (3)   |
|                             | Sub total| 3 (5)    | 0 (0)      | 1 (1)        | 2 (4)                  | 6 (10)  |
| Ixodes nipponensis          | Larvae  | 0        | 0          | 0            | 0                      | 0       |
|                             | Nymph   | 0        | 0          | 0            | 0                      | 0       |
|                             | Adult   | 18 (18)  | 2 (2)      | 1 (1)        | 1 (1)                  | 22 (22) |
|                             | Sub total| 18 (18)  | 2 (2)      | 1 (1)        | 1 (1)                  | 22 (22) |
| Total                       | 209 (807)| 361 (572)| 44 (290)   | 496 (624)    | 1110 (2293)            |         |

1 Ticks were collected from vegetation bordering stray dog shelter pens. Data are presented as numbers.

Table 2. Numbers of ticks collected from dogs and dog shelters by latitudinal region.

| Region   | Site             | Species                  | Total (%) |
|----------|------------------|--------------------------|-----------|
|          |                  | Haemaphysalis longicornis| H. flava  | Ixodes nipponensis |         |
|          |                  | Pet dogs                 | 552       | 4                    | 12     | 568     |
|          |                  | Stray dogs               | 189       | 0                    | 1      | 190     |
| North    | Dog shelters     | 100                      | 0         | 0                    | 0      | 100     |
| Central  | Military working dogs | 599                  | 3         | 1                    | 603    |
|          | Subtotal         | 1440 (62.80)             | 7 (0.31)  | 14 (0.61)            | 1461 (63.72) |
|          |                  | Pet dogs                 | 228       | 1                    | 6      | 235     |
|          |                  | Stray dogs               | 321       | 0                    | 1      | 322     |
|          |                  | Dog shelters             | 188       | 1                    | 1      | 190     |
|          |                  | Military working dogs    | 20        | 0                    | 0      | 20      |
|          | Subtotal         | 757 (33.01)              | 2 (0.09)  | 8 (0.35)             | 767 (33.45) |
|          |                  | Pet dogs                 | 4         | 0                    | 0      | 4       |
|          |                  | Stray dogs               | 60        | 0                    | 0      | 60      |
| South    | Dog shelters     | 0                       | 0         | 0                    | 0      |         |
|          | Military working dogs | 0                  | 1         | 0                    | 1      |
|          | Subtotal         | 64 (2.79)                | 1 (0.04)  | 0                    | 65 (2.83) |
|          | Total (%)        | 2261 (98.60)             | 10 (0.44) | 22 (0.96)            | 2293 (100) |

1 Ticks were collected from vegetation bordering stray dog shelter pens. Data are presented as numbers (percentage).

2.2. Identification of Tick Species

The most commonly collected tick species was *H. longicornis* (98.60%; 2261/2293), followed by *I. nipponensis* (0.96%; 22/2293) and *H. flava* (0.44%; 10/2293) (Table 2; Figure 2).
2.3. Detection of Tick-Borne Pathogens in Dog Ticks

Anaplasma spp. and Borrelia spp. were detected in the collected ticks using polymerase chain reaction (PCR). Detection of these pathogens was confirmed using 16S rRNA gene fragments of amplicons of 511 bp (Anaplasma spp.) and 714 bp (Borrelia spp.) (Figure 3).

Overall, 2.16% of the tick pools (24/1110 tick pools) contained Anaplasma spp. and 0.36% (4/1110 tick pools) contained Borrelia spp. Anaplasma spp. was detected in 22/1082 H. longicornis tick pools (2.03%) and 2/22 (9.09%) I. nipponensis tick pools (Table 3). Anaplasma spp. was detected in all stages (larvae, nymphs, and adults) of H. longicornis, but only in adults of I. nipponensis. Borrelia spp. was detected in 3/22 (13.64%) adult I. nipponensis tick pools and 1/1082 (0.09%) adult H. longicornis tick pools (Table 3).
Table 3. Pathogens detected by molecular (PCR).

| Scheme.          | Stage   | Number of tick pools | Anaplasma spp. | A. platys | E. canis | E. chaffeensis | Borrelia spp. | B. gibsoni |
|------------------|---------|----------------------|----------------|-----------|----------|----------------|----------------|------------|
|                  |         |                      |                |           |          |                |                |            |
| *Haemaphysalis longicornis* | Larvae  | 34                   | 3              | -         | -        | -              | -              | -          |
|                   | Nymph   | 148                  | 5              | -         | -        | -              | -              | -          |
|                   | Adult   | 900                  | 14             | -         | -        | -              | 1              | -          |
| **Subtotal**      |         | **1082**             | **22 (2.03)**  | -         | -        | -              | **1 (0.09)**   | -          |
| *H. flava*        | Larvae  | 0                    | -              | -         | -        | -              | -              | -          |
|                   | Nymph   | 3                    | -              | -         | -        | -              | -              | -          |
|                   | Adult   | 3                    | -              | -         | -        | -              | -              | -          |
| **Subtotal**      |         | **6**                | -              | -         | -        | -              | -              | -          |
| *Ixodes nipponensis* | Larvae  | 0                    | -              | -         | -        | -              | -              | -          |
|                   | Nymph   | 0                    | -              | -         | -        | -              | -              | -          |
|                   | Adult   | 22                   | 2              | -         | -        | -              | 3              | -          |
| **Subtotal**      |         | **22**               | **2 (9.09)**   | -         | -        | -              | **3 (13.64)**  | -          |
| **Total**         |         | **1110**             | **24 (2.16)**  | 0         | 0        | 0              | **4 (0.36)**   | 0          |

Data are presented as numbers (percentage).

Half of the tick pools found to be positive for *Anaplasma* spp. (12/24 tick pools) were collected from the northern region of the ROK, including 11 tick pools collected from military dogs and one tick pool collected from a pet. In the central region, eight tick pools were positive for *Anaplasma* spp., including five collected from stray dogs and three collected from pets. All four tick pools from the southern region that were positive for *Anaplasma* spp. were collected from stray dogs (Table 4).

*Borrelia* spp. was only detected in ticks collected in the northern and central regions and was predominantly detected in ticks collected from pet dogs (75%, 3/4). However, one positive pool (25%, 1/4) was collected from a stray dog (Table 4).

Table 4. Distribution of tick-borne pathogens in dog ticks collected in the ROK.

| Region    | Site          | Number of tick pools | *Anaplasma* spp. | A. platys | E. canis | E. chaffeensis | Borrelia spp. | B. gibsoni |
|-----------|---------------|----------------------|------------------|-----------|----------|----------------|----------------|------------|
|           |               |                      |                  |           |          |                |                |            |
| **Northern** | Pet dogs     | 84                   | 1 (1.19)         | -         | -        | -              | 1 (1.19)       | -          |
|           | Stray dogs    | 169                  | -                | -         | -        | -              | -              | -          |
|           | Dog shelters\(^1\) | 2                   | -                | -         | -        | -              | -              | -          |
|           | Military working dogs | 482              | 11 (2.28)       | -         | -        | -              | -              | -          |
| **Subtotal** |              | 737                  | 12 (1.63)       | -         | -        | -              | 2 (0.27)       | -          |
| **Central** | Pet dogs     | 121                  | 3 (2.48)         | -         | -        | -              | 2 (1.65)       | -          |
|           | Stray dogs    | 169                  | 5 (2.96)         | -         | -        | -              | -              | -          |
|           | Dog shelters  | 42                   | -                | -         | -        | -              | -              | -          |
|           | Military working dogs | 13               | -                | -         | -        | -              | -              | -          |
| **Subtotal** |              | 345                  | 8 (2.32)        | -         | -        | -              | 2 (0.58)       | -          |
| **Southern** | Pet dogs     | 4                    | -                | -         | -        | -              | -              | -          |
|           | Stray dogs    | 23                   | 4 (17.39)       | -         | -        | -              | -              | -          |
|           | Dog shelters  | 0                    | -                | -         | -        | -              | -              | -          |
|           | Military working dogs | 1               | -                | -         | -        | -              | -              | -          |
| **Subtotal** |              | 28                   | 4 (14.29)       | -         | -        | -              | 0              | -          |
| **Total (%)** |             | 1110                 | 24 (2.16)       | 0         | 0        | 0              | 4 (0.36)       | 0          |

\(^1\) Ticks were collected from vegetation bordering stray dog shelter pens. Data are presented as numbers (percentage).
2.4. Sequencing and Phylogenetic Analysis of Tick-Borne Pathogens

*Anaplasma* spp. detection was confirmed using sequencing analyses. The sequence of *Anaplasma* spp. detected in each of the 24 positive pools was 98.01–100% identical to previously deposited sequences of *A. phagocytophilum* in the National Center for Biotechnology Information (NCBI) GenBank database. In addition, phylogenetic analyses revealed a close relationship between the *Anaplasma* spp. detected in this study with previously reported *A. phagocytophilum* found in rodents, raccoon dogs, domestic/stray/military working dogs, and humans in the ROK, USA, Poland, Slovenia, and Norway (Figure 4).

![Figure 4](image)

**Figure 4.** The phylogenetic analysis of *Anaplasma* spp. detected in ticks collected from dogs in the ROK. The maximum-likelihood tree was created based on the nucleotide sequences of 16S RNA (511 bp) of *Anaplasma* spp. detected in this study and other countries. MEGA7 software with 1000 bootstrap replications was used to create the phylogenetic tree. The NCBI accession numbers and names of the *Anaplasma* spp. positive pools are shown in bold. The names of the province or metropolitan city and host of the detected *Anaplasma* spp. are shown in parentheses. Reference strains

- MW793432 18D115 (Gyeongsangbuk, Stray dog shelter tick)
- MW793431 18D48 (Jeju, stray dog tick)
- MW793430 18D45 (Jeju, stray dog tick)
- MW793437 18C50 (Gyeongsangbuk, companion dog tick)
- MW793429 18D41 (Jeju, stray dog tick)
- MW793428 18D40 (Jeju, stray dog tick)
- MW793436 18C03 (Gyeongsangbuk, companion dog tick)
- MW793427 17D650 (Gangwon, military dog tick)
- MW793426 17D513 (Gyeonggi, military dog tick)
- MW793425 17D746 (Daejeon, stray dog tick)
- MW793424 17D698 (Gangwon, military dog tick)
- MW793423 17D688 (Gangwon, military dog tick)
- MW793422 17D734 (Gangwon, companion dog tick)
- MW793421 17D681 (Gangwon, military dog tick)
- MW793420 17D729 (Gangwon, military dog tick)
- MW793419 17D728 (Gyeonggi, military dog tick)
- MW793418 17D727 (Gyeonggi, military dog tick)
- MW793417 17D720 (Gangwon, military dog tick)
- MW793416 17D756 (Daejeon, stray dog tick)
- MW793415 17D717 (Gangwon, military dog tick)
- MW793435 17D713 (Gangwon, military dog tick)
- MW793414 17D736 (Chungcheongbuk, companion dog tick)
- MW793434 18D341 (Daegu, stray dog tick)
- MW793433 18D167 (Daejeon, stray dog tick)

- MF351965 *A. phagocytophilum* (South Korea, rodent)
- KY458570 *A. phagocytophilum* (South Korea, Raccoon dog)
- KY319198 *A. phagocytophilum* (Italy, tick)
- KU513794 *A. phagocytophilum* (South Korea, dog)
- KR611719 *A. phagocytophilum* (South Korea, rodent)
- KP306518 *A. phagocytophilum* (South Korea, Human)
- KP276588 *A. phagocytophilum* (USA, tick)
- KP245906 *A. phagocytophilum* (Poland, tick)
- KM215233 *A. phagocytophilum* (Slovenia, tick)
- KM215231 *A. phagocytophilum* (Slovenia, tick)
- JN181070 *A. phagocytophilum* (Norway, tick)

- AF538828 *A. platys* (Japan, dog)
- FJ226454 *A. marginale* (Japan, Japanese Black cow)
- DQ343170 *A. marginale* (China, Macheng buffalo)
- AF283007 *A. centrale* (Japan, cattle)
- AB211164 *A. centrale* (Japan, wild deer)
- AB211163 *A. bovis* (Japan, wild deer)
- AB196475 *A. bovis* (Japan, wild deer)
of Anaplasma spp. with NCBI accession numbers and country of detection and host are also shown.

The sequences of the Borrelia spp. detected in four tick pools in this study were 98.62–100% identical to previously reported sequences of Borrelia spp. listed in the NCBI database. Phylogenetic analysis revealed that the Borrelia strain detected in tick pool 18D249 demonstrated a close relationship with B. theileri previously reported in ticks and cattle from Mali and Egypt, respectively. The Borrelia strains detected in the 18D12 and 18C04 tick pools were closely related to B. miyamotoi (Borrelia relapsing fever) detected in a human in Russia. The 18C01 tick pool strain was similar to B. garinii, B. tanuki, and B. bissettii strains from Russia, Japan, and the USA, respectively (Figure 5).

Figure 5. Phylogenetic analysis of Borrelia spp. detected in ticks collected from dogs in the ROK. The maximum-likelihood tree presenting the phylogenetic relationship between detected Borrelia spp. and previously reported strains was generated based on the nucleotide sequences of 16S rRNA (714 bp) of Borrelia spp. detected in this study. MEGA7 software with 1000 bootstrap replications was used. The NCBI accession numbers and names of positive Borrelia spp. tick pools are in bold. The country, province, or metropolitan city and host of the detected Borrelia spp. are shown in parentheses. Reference strains of Borrelia spp. with NCBI accession numbers and country of detection and host are also shown.

3. Discussion

This study determined the distribution of tick-borne pathogens detected in ticks collected from pet, stray, and military working dogs and dog shelter environments in the ROK. Three tick species (H. longicornis, I. nipponensis, and H. flava) were identified. H. longicornis, which is commonly associated with grass/herbaceous vegetation habitats, was the most commonly collected species. H. flava, which is commonly found in forested habitats, was notably less prevalent. I. nipponensis, which is associated with both grass/herbaceous vegetation and forested habitats, was also less prevalent [22]. As dogs are more likely to enter grass/herbaceous vegetation than forests, they are exposed to H. longicornis...
ticks more frequently in the ROK [22–24]. However, a previous study reported that only *I. nipponensis* ticks were collected from dogs in the Gyeongangbuk province [21].

While *A. phagocytophilum*, *Borrelia* spp., *Ehrlichia* spp., and *Babesia* spp. are present in the ROK, only *Anaplasma* spp. and *Borrelia* spp. were detected in this study. Few cases of *A. phagocytophilum* in humans in the ROK have been reported [25]. The composition of tick-borne pathogens in dog ticks varies worldwide. *Rickettsia* spp., *Borrelia* spp., *A. phagocytophilum*, and *Babesia* sp. have been reported in Latvia [15]. *A. phagocytophilum*, *Ehrlichia canis*, and *Babesia gibsoni* have been reported in Taiwan [26]. *E. canis*, *Hepatozoon canis*, *Rickettsia* spp., *Candidatus Neoehrlichia mikurensis* and *A. platys* have been reported in Nigeria [27]. Five genera of pathogens (*Anaplasma* spp., *Babesia* spp., *Borrelia* spp., *Ehrlichia* spp., and *Theileria cervi*) have been reported in dog ticks in Russia [28]. The pathogens detected in this study were consistent with previously reported TBDs in dogs [19,20]. These results are useful for the surveillance of TBDs present in dogs that may impact the transmission of these pathogens to dog owners or handlers throughout the ROK.

*A. phagocytophilum* has been found in various tick species worldwide [29–31]. In this study, only *H. longicornis* and *I. nipponensis* ticks were positive for *Anaplasma* spp., with a predominance in *H. longicornis* ticks, which may be due to the lower numbers of *I. nipponensis* and *H. flava* that were collected in this study. These findings are consistent with a previous study conducted in the ROK [32]. *H. longicornis* ticks carrying *Anaplasma* spp. were collected from pets, military working dogs, and stray dogs, but not from vegetation surrounding dog shelters. The phylogenetic analyses demonstrated a close relationship between the *Anaplasma* spp. detected in this study and previously reported *A. phagocytophilum* strains from dogs and humans in the ROK. Therefore, the transmission of *A. phagocytophilum* to humans may result from exposure to ticks on pets. Therefore, pet owners, dog shelter workers, and handlers of military working dogs should be educated regarding the potential of transmitting anaplasmosis.

The primary vectors of *Borrelia* spp. are *Ixodes* spp. [33,34]. In this study, *H. longicornis* ticks were also found to be vectors of *Borrelia* spp. *H. longicornis* ticks in this study may have fed on a *Borrelia*-positive animal, resulting in the detection of the pathogen. However, whether *H. longicornis* ticks are a vector of *Borrelia* spp. has not been determined. The sequence analyses demonstrated that there are at least three species or strains of *Borrelia* spp. in the ROK, including *B. theileri*, *B. miyamotoi*, and an unidentified *Borrelia* sp. Additional analyses using other genes (such as the flagellin gene and PCR-restriction fragment length polymorphism) are necessary to determine the specific phylogenetic identification of *Borrelia* spp. [35].

In this study, nationwide surveillance of dog ticks and tick-borne pathogens was conducted, and three tick species were collected. *H. longicornis* was the most prevalent tick species detected in this study, followed by *I. nipponensis* and *H. flava*. *Anaplasma* spp. and *Borrelia* spp. were detected on *H. longicornis* and *I. nipponensis* ticks only. Phylogenetic analyses suggested that at least two species of *Borrelia* (*B. theileri* and *B. miyamotoi*) were present. In contrast, a third species of *Borrelia* detected in this study remains unidentified. This study demonstrates that dogs and dog owners/handlers in the ROK have a relatively high risk of becoming infected with *Anaplasma* spp. or *Borrelia* spp. Therefore, disease screening is important not only to determine the distribution and prevalence of dog TBDs but also to understand the potential impact on veterinary and human health.

4. Materials and Methods

4.1. Collection of Ticks

Ticks were collected from pet dogs, military working dogs, stray dogs, and vegetation surrounding dog shelters in 13 provinces and metropolitan cities in the ROK from 2017 to 2018 (Figure 1). Ticks were removed using fine forceps to secure the tick mouthparts at the point of attachment and gently pulling the tick out to avoid breaking off the mouthparts. After removal, the ticks were transferred to a 50 mL conical tube and placed
in a cooler to be transferred to the Parasitic and Honeybee Disease Laboratory, Animal and Plant Quarantine Agency for species identification and pathogen detection.

4.2. Identification of Tick Species

Ticks were identified using morphological keys [36–38] then placed in 1.5 mL cryo-vials according to species, host, date, and stage of development. The samples were preserved in 70% ethanol and stored at −80°C until they were used to detect tick-borne pathogens. The tick pools each included 1–5 adult ticks, 1–30 nymphs, or 1–50 larvae.

4.3. Isolation of Tick Nucleic Acids

Total nucleic acid extraction was performed with the Maxwell RSC viral total nucleic acid purification kit (Promega, Madison, WI, USA) for each tick pool. Briefly, 330 μL of lysis buffer and six stainless steel beads with diameters of 2.381 mm (SNC, Hanam, Korea) were used to homogenize the ticks with a Precellys 24 tissue homogenizer (Bertin Instruments, Montigny-le-Bretonx, France). The tick homogenate was placed in a Maxwell RSC instrument (Promega, Madison, WI, USA) according to the manufacturer’s instructions. The purification of the total nucleic acids was conducted automatically. Finally, 50 μL of total nucleic acids were acquired from each pool and used to detect tick-borne pathogens.

4.4. Detection of Tick-Borne Pathogens

Conventional PCR was performed to detect six tick-borne pathogens: *Anaplasma* spp., *A. platys*, *E. canis*, *E. chaffeensis*, *Borrelia* spp., and *Babesia gibsoni*. Specific primers of each target agent (Table 5) and the AccuPower ProFi Taq PCR PreMix (Bioneer, Daejeon, Korea) were utilized. Each 20 μL reaction mix included 1 μL (10 pmol) of each primer, 5 μL of total nucleic acids, and 13 μL of double-distilled water (ddH2O). The PCR conditions for the detection of each pathogen are shown in Table 5.

**Table 5.** Primers used for the detection of tick-borne pathogens.

| Pathogens                | Primers | Sequences (5’-3’) | Target Gene (bp) | PCR Conditions | References |
|--------------------------|---------|-------------------|------------------|----------------|-----------|
| *Anaplasma* spp.         | PITA-F  | GTCGAACGGATTATCTTTA | 16S rRNA (511)   | 95 °C (5 min); 37 cycles of 95 °C (30 s), 50 °C (30 s), and 72 °C (40 s); 72 °C (7 min) | [39]     |
|                          | PITA-R  | TCCACCTTAACTTACCGAA |                  |                |           |
| *A. platys*              | EPLAT5  | TTTGTGCAGGCTTCATGTAT | 16S rRNA (359)   | 95 °C (5 min); 37 cycles of 95 °C (30 s), 53 °C (30 s), and 72 °C (30 s); 72 °C (7 min) | [40]     |
|                          | EPLAT3  | CTCTGTGGGTACCGTGTC |                  |                |           |
| *Ehrlichia canis*        | ECAN5   | GCAAATATTATAGCCCTGGC-TATAG | 16S rRNA (365)   | 95 °C (5 min); 37 cycles of 95 °C (30 s), 56 °C (30 s), and 72 °C (30 s); 72 °C (7 min) | [41,42] |
|                          | HE3     | TTATAGGGTAC-CCCTATTACCTTCCCTA |              |                |           |
| *E. chaffeensis*         | HE1     | ACAATATTATAGCTTATACCTATT-GTATTATA | 16S rRNA (390)   | 95 °C (5 min); 37 cycles of 95 °C (30 s), 56 °C (30 s), and 72 °C (30 s); 72 °C (7 min) | [41,42] |
|                          | HE3     | TTATAGGGTAC-CCCTATTACCTTCCCTA |              |                |           |
| *Borrelia* spp.          | B3      | GCAGCTTAAGATCTTCCGCA | 16S rRNA (714)   | 95 °C (5 min); 37 cycles of 95 °C (30 s), 58 °C (30 s), and 72 °C (1 min); 72 °C (7 min) | [43]     |
|                          | B6      | CAACCATGCACGCCTGTATAT |                  |                |           |
| *Babesia gibsoni*        | PIRO-F  | AGTCATATGCTTCGTTCATA | 18S rRNA (500)   | 95 °C (5 min); 37 cycles of 95 °C (30 s), 47 °C (30 s), and 72 °C (40 s); 72 °C (7 min) | [44]     |
|                          | PIRO-R  | CCATCATCAATTACAA |                  |                |           |
4.5. Sequencing and Phylogenetic Analysis

The products of conventional PCR were analyzed using agarose gel electrophoresis. After electrophoresis, the PCR products were purified using a QIA quick purification kit (Qiagen, Hilden, Germany) and sequenced by Macrogen (Seoul, Korea). The sequences of the Anaplasmata spp. and Borrelia spp. detected in this study were deposited in the NCBI database with accession numbers MW793414-MW793437 (Anaplasmata spp.) and MW793441-MW793444 (Borrelia spp.). The generated sequences were compared to previously reported sequences in the NCBI GenBank database. Identical sequences of the Anaplasmata spp. and Borrelia spp. were aligned using Clustal X version 2.0 [45]. Maximum-likelihood phylogenetic trees were created using the Kimura 2-parameter model, gamma distribution, and bootstrapping 1000 times with MEGA7 software [46].

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Institutional Review Board Statement: This study was approved by the Animal and Plant Ethics Committee of the Animal and Plant Quarantine Agency as complying with the Korean Legislation for Animal Protection (Law No. 15502, articles 23 and 24). The Animal Ethics Committee approval number was 2017-347.

Informed Consent Statement: All participating veterinary hospitals, shelters, and military forces provided their consent to participate in the study.

Data Availability Statement: The data presented in this study are available in the manuscript.

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References

1. de la Fuente, J.; Estrada-Pena, A.; Venzal, J.M.; Kocan, K.M.; Sonenshine, D.E. Overview: Ticks as vectors of pathogens that cause disease in humans and animals. Front. Biosci. 2008, 13, 6938–6946.
2. Ogbru, K.; Olaolu, O.S.; Ochui, S.O.; Tion, M.T. A review of some tick-borne pathogens of dogs. JASVM 2018, 3, 140–153.
3. Jongejan, F.; Uilenberg, G. The global importance of ticks. Parasitology 2004, 129 (Suppl.), 3–14.
4. Cao, W.; Zhan, L.; He, J.; Foley, J.E.; de Vlas, S.J.; Wu, X; Yang, H.; Richards, J.H.; Habbema, J.D.F. Natural Anaplasma phagocytophilum infection of ticks and rodents from a forest area of JiLin province, China. Am. J. Trop. Med. Hyg. 2006, 75, 664–668.
5. Ohashi, N.; Inayoshi, M.; Kitamura, K.; Kawamori, F.; Kawaguchi, D.; Nishimura, Y.; Naitou, H.; Hiroi, M.; Masuzawa, T. Anaplasma phagocytophilum-infected ticks, Japan. Emerg. Infect. Dis. 2005, 11, 1780–1783.
6. Shaw, S.E.; Day, M.J.; Birleson, R.J.; Breitschwerdt, E.B. Tick-borne infectious diseases of dogs. Trends Parasitol. 2001, 17, 74–80.
7. Biggs, H.M.; Behravesh, C.B.; Bradley, K.K.; Dahlgren, F.S.; Drexl, N.A.; Dumler, J.S.; Folk, S.M.; Kato, C.Y.; Lash, R.R.; Levin, M.L.; et al. Diagnosis and Management of Tickborne Rickettsial Diseases: Rocky Mountain Spotted Fever and Other Spotted Fever Group Rickettsioses, Ehrlichioses, and Anaplasmosis-United States. MMWR Recomm. Rep. 2016, 65, 1–44.
8. Abdullah, S.; Helps, C.; Tasker, S.; Newbury, H.; Wall, R. Ticks infesting domestic dogs in the UK: A large-scale surveillance programme. Parasites Vectors 2016, 9, 391.
9. Beck, S.; Schreiber, C.; Schein, E.; Krücken, J.; Baldermann, C.; Pachnicke, S.; Samson-Himmelstjerna, G.V.; Kohn, B. Tick infestation and prophylaxis of dogs in northeastern Germany: A prospective study. Ticks Tick Borne Dis. 2014, 5, 336–342.
10. Földvári, G.; Farkas, R. Isxodid tick species attaching to dogs in Hungary. Vet. Parasitol. 2005, 129, 125–131.
11. Abdullah, S.; Helps, C.; Tasker, S.; Newbury, H.; Wall, R. Prevalence and distribution of Borrelia and Babesia species in ticks feeding on dogs in the UK. Med. Vet. Entomol. 2018, 32, 14–22.
12. Duncan, A.W.; Correia, M.T.; Levine, J.F.; Breitschwerdt, E.B. The dog as a sentinel for human infection: Prevention of *Borrelia burgdorferi* C6 antibodies in dogs from southwestern and mid-Atlantic states. *Vector Borne Zoonotic Dis.* 2004, 4, 221–229.

13. Irwin, P.J.; Robertson, I.D.; Westman, M.E.; Perkins, M.; Straubinger, R.K. Searching for Lyme borreliosis in Australia: Results of a canine sentinel study. *Parasites Vectors* 2017, 10, 114.

14. Smith, F.D.; Ballantyne, R.; Morgan, E.R.; Wall, R. Estimating Lyme disease risk using pet dogs as sentinels. *Comp. Immunol. Microbiol. Infect. Dis.* 2012, 35, 163–167.

15. Namina, A.; Capligina, V.; Seleznova, M.; Krumins, R.; Aleinikova, D.; Kivrane, A.; Akopjana, S.; Lazovska, M.; Berzina, I.; Ranka, R. Tick-borne pathogens in ticks collected from dogs, Latvia, 2011–2016. *BMC Vet. Res.* 2019, 15, 398.

16. Choi, W.Y. Diagnosis and epidemiology of toxoplasmosis in Korea. *Korean J. Parasitol.* 1990, 28 (Suppl.), 41–44.

17. Im, J.H.; Baek, J.; Durey, A.; Kwon, H.Y.; Chung, M.H.; Lee, J.S. Current status of tick-borne diseases in South Korea. *Vector Borne Zoonotic Dis.* 2019, 19, 4.

18. Korea Centers for Disease Control and Prevention (KCDC). *Infectious Diseases Surveillance Yearbook 2018*; Korea Centers for Disease Control and Prevention (KCDC): Chungbuk, Korea, 2019.

19. Seo, M.G.; Kwon, O.D.; Kwak, D. Molecular detection and phylogenetic analysis of canine tick-borne pathogens from Korea. *Ticks Tick Borne Dis.* 2020, 11, 101357.

20. Suh, S.H.; Ahn, K.S.; Ahn, J.H.; Kim, H.J.; Leutenegger, C.; Shin, S. Serological and molecular prevalence of canine vector-borne diseases (CVBDs) in Korea. *Parasites Vectors* 2017, 10, 146.

21. Lee, S.H.; Goo, Y.K.; Geraldino, P.J.L.; Kwon, O.D.; Kwak, D. Molecular detection and characterization of *Borrelia garinii* (Spirochaetales: *Borreliae*) in *Ixodes nipponensis* (Ixodida: *Ixodidae*) parasitizing a dog in Korea. *Pathogens* 2019, 8, 289.

22. Johnson, J.L.; Kim, H.C.; Coburn, J.M.; Chong, S.T.; Chang, N.W.; Robbins, R.G.; Klein, T.A. Tick surveillance in two southeastern provinces, including three municipalities in the Republic of Korea during 2014. *Syst. Appl. Acarol.* 2017, 22, 271–288.

23. Kim-Jeon, M.D.; Jegal, S.; Jun, H.; Jung, H.; Park, S.H.; Ahn, S.K.; Lee, J.; Gong, Y.W.; Joo, K.; Kwon, M.J.; et al. Four year surveillance of the vector hard ticks for SFTS, Ganghwa-do, Republic of Korea. *Korean J. Parasitol.* 2019, 57, 691–698.

24. Park, J.W.; Lee, S.H.; Lee, G.S.; Seo, J.J.; Chung, J.K. Epidemiological characteristics of field tick-borne pathogens in Gwang-ju metropolitan area, South Korea, from 2014 to 2018. *Osong Public Health Res. Perspect.* 2020, 11, 177–184.

25. Kim, K.H.; Yi, J.Y.; Oh, W.S.; Kim, N.H.; Choi, S.J.; Choe, N.J.; Lee, J.K.; Oh, M.D. Human granulocytic anaplasmosis, South Korea, 2013, from 2014 to 2018. *Osong Public Health Res. Perspect.* 2015, 23, 349–353.

26. Wu, T.J.; Sun, H.J.; Wu, Y.C.; Huang, H.P. Prevalence and risk factors of canine ticks and tick-borne diseases in Taipei, Taiwan. *J. Vet. Clin. Sci.* 2009, 2, 75–78.

27. Kamani, J.; Baneth, G.; Mucumoglu, K.Y.; Waziri, N.E.; Eyal, O.; Guthmann, Y.; Harrus, S. Molecular detection and characterization of tick-borne pathogens in dogs and ticks from Nigeria. *PLoS Negl. Trop. Dis.* 2013, 7, e2108.

28. Livanova, N.N.; Fomenko, N.V.; Akimov, I.A.; Ivanov, M.J.; Tikunova, N.V.; Armstrong, R.; Konyaev, S.V. Dog survey in Russian veterinary hospitals: Tick identification and molecular detection of tick-borne pathogens. *Parasites Vectors* 2018, 11, 591.

29. Palomar, A.M.; Portillo, A.; Santibáñez, P.; Mazuelas, D.; Ronceron, L.; García-Alvarez, L.; Santibáñez, S.; Gutiérrez, O.; Oteo, J.A. Detection of tick-borne *Anaplasma* bovis, *Anaplasma phagocytophilum* and *Anaplasma centrale* in Spain. *Med. Vet. Entomol.* 2015, 29, 349–353.

30. Polin, H.; Hufnagl, P.; Haurschmid, R.; Gruber, F.; Ladurner, G. Molecular evidence of *Anaplasma phagocytophilum* in *Ixodes ricinus* ticks and wild animals in Austria. *J. Clin. Microbiol.* 2004, 42, 2285–2286.

31. Stuen, S. *Anaplasma Phagocytophilum*-the most widespread tick-borne infection in animals in Europe. *Vet. Res. Commun.* 2007, 31, 79–84.

32. Kim, C.M.; Kim, M.S.; Park, M.S.; Park, J.H.; Chae, J.S. Identification of *Ehrlichia chaffeensis*, *Anaplasma phagocytophilum*, and *A. bovis* in *Haemaphysalis longicornis* and *Ixodes persulcatus* ticks from Korea. *Vector Borne Zoonotic Dis.* 2003, 3, 17–26.

33. Fraenkel, C.J.; Garmpo, U.; Berglund, J. Determination of novel *Borrelia* genospecies in Swedish *Ixodes ricinus* ticks. *J. Clin. Microbiol.* 2002, 40, 3308–3312.

34. Kurokawa, C.; Lynn, G.E.; Pedra, J.H.F.; Pal, U.; Narasimhan, S.; Fikrig, E. Interactions between *Borrelia burgdorferi* and ticks. *Nat. Rev. Microbiol.* 2020, 18, 587–580.

35. Fukunaga, M.; Okada, K.; Nakao, M.; Konishi, T.; Sato, Y. Phylogenetic analysis of *Borrelia* species based on flagellin gene sequences and its application for molecular typing of Lyme disease borreliae. *Int. J. Syst. Bacteriol.* 1996, 46, 898–905.

36. Hoogstraal, H.; Roberts, F.H.; Kohls, G.M.; Tipton, V.J. Review of *Haemaphysalis (kaiseriana) longicornis* Neumann (resurrected) of Australia, New Zealand, New Caledonia, Fiji, Japan, Korea, and Northeastern China and USSR, and its pathogenetic and bisexual populations (*Ixodoidae, Ixodidae*). *J. Parasitol.* 1968, 54, 1197–1213.

37. Hoogstraal, H.; Wassef, H.Y. *The Haemaphysalis* ticks (*Ixodoidae: Ixodidae*) of birds. 3. *H. ornithophylus* subgen. n.: Definition, species, hosts, and distribution in the Oriental, Palearctic, Malagasy, and Ethiopian faunal regions. *J. Parasitol.* 1973, 59, 1099–1117.

38. Yamaguti, N.; Tipton, V.J.; Keegan, H.L.; Toshioka, S. Ticks of Japan, Korea, and the Ryukyu Islands. *Brigham Young Univ. Sci. Bull. Biol. Ser.* 1971, 15, 1–226.

39. Hancock, S.I.; Breitschwerdt, E.B.; Pitulle, C. Differentiation of *Ehrlichia platys* and *E. equi* infections in dogs by using 16S ribosomal DNA-based PCR. *J. Clin. Microbiol.* 2001, 39, 4577–4578.

40. Mathew, J.S.; Ewing, S.A.; Murphy, G.L.; Kocan, K.M.; Corstvet, R.E.; Fox, J.C. Characterization of a new isolate of *Ehrlichia platys* (Order Rickettsiales) using electron microscopy and polymerase chain reaction. *Vet. Parasitol.* 1997, 68, 1–10.
41. Anderson, B.E.; Sumner, J.W.; Dawson, J.E.; Tzianabos, T.; Greene, C.R.; Olson, J.G.; Fishbein, D.B.; Olsen-Rasmussen, M.; Holloway, B.P.; George, E.H. Detection of the etiologic agent of human ehrlichiosis by polymerase chain reaction. J. Clin. Microbiol. 1992, 30, 775–780.
42. Murphy, G.L.; Ewing, S.A.; Whitworth, L.C.; Fox, J.C.; Kocan, A.A. A molecular and serologic survey of Ehrlichia canis, E. chaffeensis, and E. ewingii in dogs and ticks from Oklahoma. Vet. Parasitol. 1998, 79, 325–339.
43. Kang, J.G.; Ko, S.; Smith, W.B.; Kim, H.C.; Lee, I.Y.; Chae, J.S. Prevalence of Anaplasma, Bartonella and Borrelia species in Haemaphysalis longicornis collected from goats in North Korea. J. Vet. Sci. 2016, 17, 207–216.
44. Ano, H.; Makimura, S.; Harasawa, R. Detection of Babesia species from infected dog blood by polymerase chain reaction. J. Vet. Med. Sci. 2001, 63, 111–113.
45. Larkin, M.; Blackshields, G.; Brown, N.P.; Chenna, R.; McGettigan, P.A.; McWilliam, H.; Valentin, F.; Wallace, I.M.; Wilm, A.; Lopez, R.; et al. Clustal W and Clustal X version 2.0. Bioinformatics 2007, 23, 2947–2948.
46. Kumar, S.; Stecher, G.; Tamura, K. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 2016, 33, 1870–1874.