Molecular Identification of Pathogens in Ticks Collected in South Korea

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Abbreviation

TBPs---------- Tick-Borne Pathogens

NCBI---------- National Center for Biotechnology Information

SFGR---------- Spotted Fever Group Rickettsia

U.S----------United States

SFG----------Spotted fever group

TG---------- Typhus group

KCDC---------- Korea Centers for Disease Control and Prevention

CLB----------Coxiella Like Bacteria

KOI----------Intraerythrocytic parasite

TBRF----------Tick Borne Relapsing Fever

LB---------- Lyme Borreliosis

BLAST ---------- Basic Local Alignment Search Tool

PCR ---------- Polymerase Chain Reaction

UV----------Ultraviolet

SFTS----------Sever Fever Thrombocytopenia Syndrome

RNase----------Ribonuclease

qPCR ----------quantitative PCR

cDNA----------complementary DNA

H. longicornis----------*Haemaphysalis longicornis*

H . flava----------*Haemaphysalis flava*

Abstract

Introduction: Ticks are considered the main arthropod vectors for infectious disease agents. They are related to veterinary and medical health issues. Studying the regional difference of pathogen-detection rate in ticks is an essential factor in developing and initiating prevention strategies**.**

Methods: This study focuses on investigation of tick-borne pathogens from the ticks collected in vegetation by flagging. The morphological examination was performed for tick species and their developmental stages (*Haemaphysalis longicornis* and *Haemaphysalis flava*). For bacteria and protozoa detection, 109 tick samples from Wonju, Gunsan, and Yangsan were collected during August 2014-2016, undergone DNA extraction. Detection for specific bacteria and protozoa using pathogen-specific PCR methods has been done mostly; *Rickettsia* spp. was identified using gltA and ompA primers. For severe fever with thrombocytopenia syndrome (SFTS) virus detection, RNA of 50 tick samples from Samcheok (April 2020) was extracted to perform reverse transcription-PCR using the virus-specific primer. The amplicon product has been sequenced, and the results were compared with the Basic Local Alignment Searching Tool (BLAST) using NCBI database for identification.

Results: Among 109 ticks examined, the detection rate for bacterial and protozoal has been recorded as 32(29.35%). The detection rate for the specific pathogens was 19.26% for *Rickettsia japonica*, 1.83% for *Coxiella burnetii*, and 8.25% for *Theilleria luwansheni*, respectively. From total samples which were examined, one mixed infection was observed between *Rickettsia japonica* and *Theilleria luwansheni* from the collection site of Wonju, while Anaplasmataceae, *Borrelia* spp., and *Bartonella* spp. were not identified. The viral pathogen for SFTS were examined in 50 tick samples from the Samcheok site, but all the results of their RNA extraction were negative.

Conclusion: *H. longicornis* had a higher pathogen detection rate than *H. flava*. *Rickettsia japonica, Coxiella burnetii, Theilleria lunwenshuni* were detected among the selected pathogens. *T. lunwenshuni* were detected in ticks collected in Wonju among the target protozoa. In *H. longicornis, Rickettsia japonica* was the most frequently detected in Wonju, Gunsan and Yangsan among other targeted pathogens (Anaplasmataceae, *Borrelia* spp, *Bartonella* spp, and, SFTS v). The information in this study may provide important information to inform the nearby community, human and animal's health sectors in order to activate surveillance and monitoring against tick-borne pathogen about the possible emergence should be cautiously monitored. Further research and routine surveillance would be important for mitigation the disease prevalence and controlling the transmission in all stages.

Keywords: Tick-borne diseases, Anaplasmataceae, Rickettsiaceae, *Coxiella* spp, *Borrelia* spp, *Bartonella* spp, and Piroplasmidea, SFTS v, South Korea.

CHAPTER ONE

INTRODUCTION

1.1 Background:

Ticks are compulsory hematophagous ectoparasites that transmit organisms, and cause diseases in humans, domestic and wild animals (1). They are presently viewed to be second to mosquitoes as vectors of human infectious sickness around the world. Two households of ticks are of scientific significance: Ixodidae (hard ticks) and Argasidae (soft ticks). Hard ticks are the fundamental ticks appearing as vectors of human disease. However soft ticks are additionally acknowledged to transmit human infectious diseases, which are frequently ignored. Argasid ticks include 4 genera and about 185 species, together with three genera represented through a massive variety of species: *Carios* (87 species), *Argas* (57 species), and *Ornithodoros* (36 species). The fourth genus (*Otobius*) is represented with the aid of three species (2) .Ticks transmit micro-organisms during their feeding procedure on animals and the human hosts. Ticks' ability to transmit the disease to its contact through a particular route is determined by its an ecological, physiological, and behavioral characteristic of survival (3). During their feed on the host's blood, ticks have been found to inoculate various microorganisms that are pathogenic and non-pathogenic. Pathogenic organisms that have been found to be transmitted by ticks include protozoa (*Theilleria* and *Babesia* spp.), viruses(severe fever with thrombocytopenia virus (SFTSV), tick-borne encephalitis virus (TBEV), Powassan virus (POWV), Omsk hemorrhagic fever virus (OHFV), Langat virus (LGTV)) and bacteria (*Ehrlichia* and *Anaplasma* spp., *Bartonella* spp., and *Borrelia* spp.). In contrast, while the non-pathogenic microorganisms are found is genera of *Rickettsia, Francisella,* and *Coxiella* which exhibit alternative lifestyles as mutualistic tick symbionts. However, non-pathogenic microorganisms have been observed to play a role in transmitting tick-borne pathogens in both humans and animals (4).

Numerous diseases are caused by pathogens found in ticks depending on the type of micro-organism inoculated into the host. All the micro-organisms cause intra-cellular destruction of the host cells, causing typical symptoms of tick-related illnesses like fever/chills; all tickborne disease patients can experience a fever at varying degrees. Other symptoms that have been found include headache, fatigue, and muscle aches. The severity and time of onset of these symptoms depend on the disease and the patient's immune system(CDC, USA). Unique clinical features such as jaundice have been found in severe fever with thrombocytopenia syndrome (SFTS), long-lasting, or permanent neuropsychiatric sequelae patients with tick-borne encephalitis (5). Tick-borne disease is responsible worldwide for significant economic losses and potential health threats in humans and animals. However, the clinical diagnosis is easy to overlook because no specific clinical signs are associated with these diseases (6).There are limited studies on the prevalence of tick and TBP. However, the global hazard of TBPs is continuing to increase and is raising public health concern. This calls for constant identification of new diagnostic methods. Screening of ticks for such pathogens through molecular epidemiological methods might also disclose the prevalence of tick-borne pathogens in unique geographic environments. Some of these agents, such as *Rickettsia prowazekii* (typhus fever), *Yersinia pestis* (plague), *Francisella tularensis* (tularemia), *Coxiella burnetii* (Q fever), West Nile virus, Rift Valley fever virus, and hantavirus, are now recognized as significant emerging vector-borne infections with the potential of being used for bioterrorism worldwide. Recently, ehrlichial and rickettsial infections have been mentioned to exist in a broad band throughout Europe, Asia, Africa, and the America. Other tick-borne organisms, such as some *Borrelia* and *Bartonella* spp., have additionally been proven to cause diseases in animals and human beings. In the United States and South Korea, rodents (e.g., the white- footed mouse (*Peromyscus leucopus*) and white-tailed deer (*Odocoileus virginianus*) are reservoirs of *Ehrlichia* and *Anaplasma* spp. In Europe, various rodent species are implicated as natural reservoirs for

Ehrlichia and *Anaplasma* spp. Additionally, *Ehrlichia* spp. have been isolated from wild mice in Japan (7).

Studies were done in 2007 and 2015 found the existence of numerous tick species in Asian countries like Iran. From the studies, *Boophilus, Dermacentor, Haemaphysalis, Hyalomma, Ixodes*, and *Rhipicephalus* were isolated from approximately 1,500 sheep, 1,200 goats, and 500 cattle of 12 herds(8). Besides, 25,566 ticks from 9 species of domesticated animals and 1,385 ticks from 20 animal species were collected in Sri Lanka (9). Another study done in North Korea on 292 of *H. longicornis* using the 16S ribosomal RNA and ITS pathogen-specific nested polymerase chain reaction in 2016 found 26.4% *Anaplasma bovis*, 5.1% *Bartonella grahamii*, 4.1% *Anaplasma phagocytophilum*, 3.4% *Bartonella henselae* 3.4, and 1.0% *Borrelia* spp. (10). Also, another study on 21,158 ticks belonging to 3 genera and 6 species collected at 6 provinces and four metropolitan areas in South Korea from March to October 2014, 3317 *H. flava*, 249 *Ixodes nipponensis*, 11 *Amblyomma testudinarium* , 8 *H. phasiana* ,and 3 *Ixodes turdus* were identified(11).

In South Korea, zoonotic pathogens affecting human health, including spotted fever group (SFG) rickettsia, *Ehrlichia*, and *Anaplasma* spp., *Bartonella spp.*, *Borrelia* spp., and tick-borne encephalitis virus have been isolated from ticks. However, dragging or sweeping vegetation for collecting ticks amongst a range of habitats offers an estimate of their geographical, habitat, seasonal, and lifestyles stage distributions and health-related risks related to the military, agriculture, and leisure activities. These facts serve to supply facts that are integral to improving tick-borne disease threat assessments(12). In South Korea, reviews on vector-borne illnesses and its pathogens are ubiquitous, which consist of anaplasmosis in human, *B. burgdorferi* in human, *Bartonella* spp. in Korean water deer, *C. burnetii* in raw milk, *Hepatozoon* spp. in leopard cat, and *Theileria* spp. in Chinese water deer. Climate change due to global warming has engendered more splendid subtropical climate, which may increase the

hazard of vector-borne illnesses nationally. The warm summer season, in particular, grants perfect surroundings for vectors all through the country (13).

1.2 Problem Statement

To date, several species of tick-borne pathogens have been documented in South Korea. Tick-borne disease is responsible for significant economic losses and potential health threats in humans and animals globally. However, the clinical diagnosis is easy to overlook because there is no specific clinical sign associated with these diseases. Recent studies show that PCR is an effective and highly sensitive method for the detection of these pathogens.

1.3 Aim

The study aimed at molecular identification of pathogens in ticks collected in South Korea.

1.4 Specific Objectives

1. To determine the detection rate of tick-borne pathogens in collected samples.
2. To determine local tick species' ecology, pathogens, and their distribution among the selected study sites.
3. To indicate the sources of the emerging tick-borne pathogens for prevention and control strategies for human and animals' health.

CHAPTER TWO
LITERATURE REVIEW

2.1. Historical reviews of ticks in Korea:

In Korea, concerns related to ticks and tick-borne pathogens have been increasing, and many studies on tick population and tick-borne diseases have been conducted, primarily tick surveillance studies related to host species (small mammals, migratory birds, wild boars) (15)(16), or provinces(7)(12). Tick-borne pathogens, including *Bartonella* spp, *Anaplasma* spp, Rickettsiaceae, *Coxiella* spp, *Borrelia* spp, Piroplasmidea, and a newly emerging pathogen called severe fever with thrombocytopenia syndrome virus (SFTSv), were conducted (17)(18). Given that the current climate is continually changing in Korea and in other countries, there may be changes in the habitats of existing organisms and the influx of new species (19)(20).

Ticks have been implicated as vectors of a lot of human and animal pathogens in Korea. There are 22 ticks and 18 tick-borne pathogens (some of which are zoonoses, infectious retailers transmissible beneath herbal prerequisites from wild or home vertebrate animals to humans) recognized in South Korea. Many urban Koreans spend weekends trekking in mountainous areas, where the trails and paths are commonly nicely worn and free of tick harborage (vegetation and leaf litter). However, when hikers go off the crushed direction into forested areas covered with leaf litter and grass patches, the conceivable for human-tick interplay dramatically increases. Still, urbanization seems to have decreased the typical publicity of the Korean population to ticks in South Korea, small wild mammals (e.g., rodents and insectivores) and their related ticks are hosts to a range of zoonotic pathogens: Spotted Fever Group Rickettsia, *Ehrlichia*, and *Anaplasma* spp., *Bartonella* spp., *B. burgdorferi*, and tick-borne encephalitis virus. Humans are an accidental host due to meeting ticks during leisure activities, out of doors development and maintenance, agricultural, and military training (5).

2.2. Types of *Haemaphysalis* Ticks

2.2.1. *Haemaphysalis longicornis*

It is native to East Asia (Japan, China, the former Union of Soviet Socialist Republic, Korea) ,however, has grown to be a major invasive pest of cattle in New Zealand, parts of Australia, and various Pacific islands (New Caledonia, Fiji, Western Samoa, Tonga, Vanuatu). *H. longicornis* is additionally recognized to parasitize humans, with reports often describing numerous cases from Australia, China, Japan, New Zealand, Russia, and South Korea. Populations in their native range survive in cold winters (e.g., in northeast China, mean monthly temperatures of −5°C in December and January), while invasive populations show up notably in hotter areas. Susceptibility to dry climates may put additional regional limitations on this species' range. While the most critical hazard posed via this species is excessive infestations of cattle that can lead to weak points and, in some cases, exsanguination and death. It has also been implicated in transmitting of various diseases of clinical and veterinary concern, including *Rickettsia japonica*, the agent of Oriental spotted fever, *Theileria orientalis*, the agent of cattle theileriosis, and a newly described bunyavirus inflicting Severe Fever with Thrombocytopenia Syndrome (SFTS). Additionally, field populations of ticks found infected with *Anaplasma*, *Ehrlichia*, and *Borrelia* spp. in China and Korea, consist of a family of species recognized to occur in New Jersey in the U.S. (e.g., *A. phagocytophilum*, *E. chaffeensis*) (14). Ticks can withstand a broad range of temperature, from their developmental threshold of ∼12°C to almost 40°C at their deadly limit. However, their tolerance of dehydration is less comprehensive, especially in the larva and adult stage, the former especially being the stage that generally determines suitable biotopes for the tick and its present distributional limits. The importance of *H. longicornis* to the New Zealand livestock enterprise has recently improved through the establishment and the unfolding of *T. orientalis* Ikeda among dairy and beef cattle, though the tick has continually posed production-limiting problems for cattle, deer, and to a lesser extent, sheep. The tick's function as a vector of theileriosis and how elements of the tick's

biology influence the spread and protection of this ailment are discussed. It is proposed that, of available natural world hosts, the brown hare with its wide-ranging habits, is a vital disseminator of ticks (15). *H. longicornis* was gathered specifically in habitats consisting of grasses and different herbaceous vegetation and is associated with the giant wild and home mammals that use these habitats. In South Korea, *H. longicornis* is the tick mostly collected from grasses and herbaceous vegetation habitats, and these have been shown to have high tick-borne infection rates (16).

2.2.2. *Haemaphysalis flava:*

A blood-sucking challenging tick and is extensively allotted in Asian international locations such as India, Sri Lanka, Vietnam, Japan, and China. Hosts of *H. flava* include the horse, hog, dog, sheep, cattle, hedgehog, and panda. *H. flava* transmits many pathogenic bacteria, such as *Ehrlichia*, *Rickettsia*, and *Bartonella* spp. (17), which are most regularly amassed from conifer and blended wooded area habitats, where there is a greater density of small to medium-sized mammals and migratory and indigenous birds (18). The challenging tick *H. flava* is extensively distributed in East Asia, and feeds on different types of mammals and birds. It has high scientific and veterinary importance due to its remarkable ability to transmit several pathogens. Among the hard ticks, this tick is one of the most important vectors of tick-borne diseases. Encephalitis and Lyme borreliosis, are two primary tick-borne diseases transmitted by *H. flava* species in Korea. There is also evidence that this species also transmits spotted fever in Japan. Thus, high-quality tick control is integral to guard the health of humans and animals. Strategies for tick control based only on chemical acaricides has become less efficient due to the fact that the conventional method has certain implicit drawbacks, such as polluting surroundings and increasing tick resistance to acaricides (19).

2.3. Anaplasmataceae:

It consists of the genera *Anaplasma*, *Ehrlichia*, *Neorickettsia*, and *Wolbachia*. Except for *Wolbachia spp*., the other Anaplasmataceae pathogenic for humans live within endosomes in professional phagocytic cells. Bacteria from the genera *Anaplasma* and *Ehrlichia* are obligate intracellular bacteria transmitted by arthropods, mainly ticks, from one vertebrate host to another. Transmission usually occurs transstadially, although transovarial transmission has been reported. In the vertebrate host, the bacteria infect hematopoietic cells. *Anaplasma* and *Ehrlichia* can cause persistent infection in vertebrate hosts, which allows these hosts to be reservoirs(20). The family Anaplasmataceae is known as a source of important emerging tick-borne diseases in domestic animals, and humans. Infections may be caused by a single specie or several species concurrently. In the United States (U.S), rodents, particularly the white-footed mouse (*Peromyscus leucopus*) and the white-tailed deer (*Odocoileus virginianus*) are potential reservoirs. In Europe, several rodent species are implicated as natural reservoirs for *Ehrlichia* and *Anaplasma* spp. Also, *Ehrlichia* isolated from wild mice was reported in Japan. Human ehrlichiosis is an emergent disease in South Korea, and the impact of this disease on human populations is unknown, as most cases are unreported. There was one suspected case of ehrlichiosis in a patient at the 121 General Hospital, Yongsan. To date, several species of *Ehrlichia* spp. , and *Anaplasma* spp. have been documented in South Korea. Tick-borne disease is responsible for significant economic losses and potential health threats in humans and animals globally. However, the clinical diagnosis is easy to overlook because there is no specific clinical sign associated with these diseases. Recent studies show that PCR is an effective and highly sensitive method for detecting of *Ehrlichia* and *Anaplasma* species (21).

2.4. Rickettsiaceae:

The organisms covered in these family are Gram-negative, obligate, intracellular bacteria that grow in the cytoplasm or vacuoles inside the host cell(s) and are vectored by arthropods and trematodes. The first rickettsiosis described was once epidemic typhus, referred to only as 'typhus fever'; and the first tick-borne rickettsiosis reported in the United States used to be Rocky Mountain spotted fever, the causative agent of which is *Rickettsia rickettsii* . Since then, disease(s) resulting from infections with micro-organisms in the genera Rickettsia and Orientia have been reported around the world. Many contributors of *Rickettsia* are viewed to be emerging pathogens worldwide and consist of bacteria such as *Rickettsia japonica*, *Rickettsia africae*, *Rickettsia honei*, *Rickettsia felis,* and *Rickettsia slovaca* (22). Members of *Rickettsia* spp. are obligate intracellular micro-organisms first described with the aid of Ricketts in 1909. The genus Rickettsia individuals are historically characterized into two predominant groups, the spotted fever group (SFG) and the typhus group(TG), with most of the regarded species belonging to the SFG. Two species, *R. typhi* and *R. prowazekii*, make up the TG. Classification into the two groups used to be primarily based on their physiological traits of intracellular localization, optimal growth temperature, and cross-reaction of serum from a contaminated affected person with somatic antigens of three traces of Proteus (22). Most SFG rickettsia is are harbored through ixodid ticks and are transmitted through their bites at some stage in feeding (saliva).TG rickettsiae can cause infection via inhalation through the aerosolization or contamination of dust particles floating in the air. An uncommon infection route is via the conjunctivae, through the exposure of contaminated tick hemolymph on fingers from crushed ticks(23). The (SFG) of diseases is associated with lice, fleas, mites, and ticks. Most of these diseases begin with nonspecific symptoms, such as fever, headache, myalgia, and nausea, at 2–14 days after a bite by the culpable vector. The appearance of a rash and other specific symptoms varies depending on the species. The Mediterranean spotted fever (*Rickettsia conorii*

infection) is characterized by a febrile rash with eschar, Japanese spotted fever (*Rickettsia japonica* infection) is associated with relatively small and shallow eschar formation, and Rocky Mountain spotted fever (*Rickettsia rickettsii* infection) is rarely associated with eschar formation and can have relatively high mortality. Siberian tick typhus (*Rickettsia sibirica* infection) causes mild disease with lymphangitis expanding from the inoculation eschar to the draining lymph node (24).

2.5. *Bartonella* spp:

The genus *Bartonella* represents a prototypical instance for zoonotic pathogens as *Bartonella* species are infectious marketers for human beings and animals. Bartonella's bacterial genus comprises gram-negative, sluggish developing, and facultative intracellular pathogens that infect broadly speaking mammalian hosts and are regularly transferred by way of blood-sucking arthropod vectors. *Bartonella* infections of people and animals are frequently characterized utilizing intraerythrocytic bacteremia. At least thirteen species of *Bartonella* have been recognized as pathogenic to human beings, with three species accountable for most of the clinically applicable infections in humans: *B. bacilliformis, B. quintana* and *B.henselae* (25). *B. henselae* is properly regarded international as a zoonotic agent infecting each cat and their fleas and has additionally been observed in ticks (26). Cats are the foremost reservoir host for the species *B. henselae, B. clarridgeiae* (both of which can motive cat scratch disease), and *B. koehlerae* (a causative agent of endocarditis in humans). Infected cats are frequently clinically asymptomatic though they go through relapsing bacteremia over a long period. Of the three most extensive human pathogenic *Bartonella* species *B. henselae* is the most frequent symptomatic infection inflicting agent recognized in the advanced medical setting (25). According to one study, *Bartonella* DNA was isolated from *H. longicornis, H. flava, I. persulcatus*, and *I. nipponensis*, and *B. elizabethae* was identified in the spleen of striped field mouse(27). In other studies, *B. grahamii* and *B. schoenbuchensis*-related species isolation from

Korean water deer have been evidenced (28). In South Korea, human infections of *B. henselae* have also been described (29). However, among 300 healthy Korean individuals, a 15% seroprevalence of *B. henselae* was detected (30).

2.6. *Borrelia* spp:

In Africa, tick- borne relapsing fever Borrelia spp. consisting of neglected vector-borne pathogens are accountable for several febrile signs are most often suspected in malaria-like signs. Tick-borne relapsing fever has been identified as a fundamental essential reason for ailment and demise in numerous areas of Africa . Two marketers of tick-borne relapsing fever have been detected in North Africa, specifically *B. crocidurae* and *B. hispanica*. An uncultured bacterium, "*B. merionesi*," has additionally been detected in Ornithodoros ticks in Morocco, and "*Candidatus Borrelia algerica*" has been suggested in febrile sufferers in Algeria(2). In Germany, different genospecies of the *B. burgdorferi* (*sensu lato*) complex, *B. miyamotoi, A. phagocytophilum*, and *Rickettsia* spp. are amongst the most important bacterial pathogens transmitted using *I. ricinus*. These pathogens are the cause of a variety of illnesses in humans and animals. *B. burgdorferi* (s.l.) is the causative agent of Lyme borreliosis (LB), while *B. miyamotoi* causes febrile sickness and has been related to meningoencephalitis in immunocompromised sufferers in Europe(31).

A developing public health problem, Lyme disease is the most often stated vector-borne sickness in the U.S. and Europe, inflicting a tremendous public fitness burden. The variety of annual instances of Lyme disease in the U.S. has climbed to over 300,000 and is predicted to be upward . Human contamination with Lyme disease's etiologic agent *B. burgdorferi*, results in sickness with multifarious signs and symptoms. The tick presents herbal surroundings for *B. burgdorferi* such that intact spirochetes may additionally be obtained and observed (32). According to the KCDC infectious disease reporting system, the number of patients (including suspected cases) has increased from 2012 to 2017. Increases in physicians' awareness of Lyme

disease and climate changes and the popularity of outdoor activities, are considered the major causes of the increased Lyme disease reports. Lyme disease is distributed all over South Korea and mainly occurs from May to November . The mortality rate of Lyme diseases was 1.04% from 2011 to 2017(33).

2.7. *Coxiella* spp:

Coxiella genus includes *C. burnetii*, *C. cheraxi*, and unclassified CLB. *C. burnetii* is a zoonotic pathogen that causes acute or chronic illness and flu-like symptoms in humans, and abortion in animals. *C. burnetii* is shed in milk, feces, and urine from infected animals and can be transmitted by inhalation of aerosolized microorganisms. Due to its environmental resistance, route of transmission, and difficulty to diagnose, *C. burnetii* is designated as a category B potential biological weapon by the United States (13). *C. burnetii* related pathogens in the order Legionellales, had long been viewed as an obligate intracellular pathogen, until the improvement of a gadget that enabled axenic culturing in synthetic media7. The reservoir hosts of *C. burnetii* are massive animals, which encompass various species of cattle and wildlife. Human infections usually take place following the inhalation of contaminated aerosols. After uptake into goal phagocytes, *C. burnetii* initiates the formation of a phagolysosome-like compartment, termed the *Coxiella*-containing vacuole, which is notably acidified (34) .*C. burnetii* is the etiological agent of human Q fever, a zoonotic sickness reported worldwide and inflicting an ailment with signs including fever, hepatitis, and respiratory issues. Ticks play a vital role in the circulation of *C. burnetii* in herbal foci and are accountable for disseminating the contamination amongst animals. The presence of *C. burnetii* was earlier isolated from *I. ricinus, D. reticulatus, D. marginatus*, *H. concinna*, and *H. inermis* ticks in Slovakia(35).

2.8. Piroplasmidea:

Piroplasmidea an order of parasites in the phylum Apicomplexa. They multiply by binary fission, and as sporozoan parasites, they possess sexual and asexual phases(sexual reproduction occurs in the tick gut).They include the tick parasites *Babesia* spp. and *Theileria* spp.

2.8.1. *Babesia* spp:

Babesiosis is a global rising tick-borne ailment that is growing in frequency and geographic range. It imposes an enormous health burden, specifically on immunocompromised and those who are infected through blood transfusion (36). *B. microti* is the fundamental etiologic agent of human babesiosis and is endemic in the northeastern and the higher Midwestern United States. The geographic enlargement of babesiosis has accompanied Lyme disease; however, it has remained too restricted. The emergence of human babesiosis poses a sever health risk in most endemic areas. Fever is the salient sign of babesiosis and is regularly accompanied by a collection of non-specific symptoms, explaining why diagnosis may be delayed or missed. The diagnosis is established by using the identification of babesia organisms on Giemsa stained blood smears, detecting babesia DNA with the aid of PCR, or a four-fold upward push in anti-*Babesia* antibody titers in acute and convalescent sera. The sickness might also be extreme or fatal, especially in sufferers who are older than 50 years of age, and those who are immunocompromised regardless of age. Most sufferers have complete healing following a general 7 to a 10-day round of antimicrobial therapy (37). Two important *Babesia* parasites, namely, *B. microti* (so- called small Babesia) and *B. divergens* (large *Babesia*, or the genus *Babesia* sensu stricto) have been acknowledged to be concerned in human infections in the United States and Europe, respectively. In East Asia, instances of human babesiosis have been reported in Taiwan and Japan. Both have been precipitated *B. microti*-like parasites, which frequently show up asymptomatically. Although surveys of wild rodents and cattle reported *Babesia* parasites in South Korea, the first case of human babesiosis without overseas travel

was reported in Gurae (Jeonnam province) in 2008. The intraerythrocytic parasite (KO1) in the patient's blood was typically seen as paired pyriformis and ring forms; however, Maltese cross types had no longer been seen, and the parasite confirmed morphological aspects like those of the genus *B.sensu stricto*. The sequence of the 18S rRNA gene of KO1 was closely associated with that of *Babesia* spp. Isolated from sheep in China (similarity, 98%). This study offered the first proof of the presence of a hitherto unknown new type of Babesia parasite capable of infecting humans (38).

2.8.2. *Theileria* spp:

They can infrequently be differentiated morphologically. The reachable proof shows that they signify parasites that merge into one another by using gradation in virulence, determination of transmitter, manufacturing of signs and lesions, and improvement of immunity (39).The genus *Theileria* includes tick-transmitted protozoa characterized by schizonts in lymphoid cells, and piroplasm's in the vertebrate host's red blood cells. Transmission of *T. orientalis* occurs through the feeding of contaminated ticks of the Haemaphysalis genus. New Zealand has solely one livestock-infesting tick present, *H. longicornis*, and this is viewed as the vector of *T. orientalis* in this country. Ticks are infected while feeding on a contaminated host whose erythrocytes incorporate *Theileria* spp. piroplasms. (40).

Bovine theileriosis is a tick-borne haemoprotozoan disorder brought on by using parasites of the genus Theileria in bovines, which is categorized into two groups of lymphoproliferative *Theileria* spp. (*T. parva* and *T. annulata*) and non-lymphoproliferative *Theileria* spp. Lymphoproliferative *Theileria* spp. cause excessive mortality and morbidity due to uncontrolled lymphocyte proliferation in tropical and subtropical areas of the world. Although non-lymphoproliferative *Theileria* spp *(T. orientalis*) is believed to have solely moderate or no pathogenicity in cattle, recent outbreaks of oriental theileriosis in the Asia-Pacific region have caused significant concerns in the cattle industry due to sever problems such as reduced growth

and production losses in infected cattle. However, in Korea, *T. orientalis* is thought to be the causative agent of bovine theileriosis with a high prevalence of *H. longicornis*, a primary biological vector of *T. orientalis* (41).

2.9. Severe fever with thrombocytopenia syndrome (SFTS):

During 2009 a rising infectious disease with an unknown agent was noted in China; it was characterized by extreme fever, thrombocytopenia, leukocytopenia, gastrointestinal symptoms, and a high case-fatality rate. The sickness was named severe fever with thrombocytopenia syndrome (SFTS). A novel phlebovirus was remoted from acute patients, ticks, and domestic animals as the disease's causal agents. Recently, demonstrated SFTS instances had been suggested from Japan and South Korea. Another recorded case in the USA from a febrile patient with thrombocytopenia and leukocytopenia in 2010 (42). From 2010 to 2016, they had a suggested typical case fatality rate of 5.3~32%. Studies have shown that about 10% of SFTSV infections are due to bites of *H. longicornis*, the implicated vector of this viral infection. Moreover, the current reviews of human-to-human SFTSV transmission in China and Korea have raised public health concerns(43).

KCDC observed that SFTSv used to be detected in samples from *H. longicornis* ticks amassed throughout 2011–2012 in South Korea. Seroconversion and viremia of SFTSv have been established in domesticated animals such as goats, sheep, cattle, pigs, and dogs; these animals have been implicated as intermediate hosts in SFTSv-endemic areas. They demonstrated a case of SFTS in South Korea in 2012 by using isolation of SFTSV from saved blood samples gathered quickly earlier than the patient's death. The affected person had a history of an insect bite while working on a crop farm in Hwacheon-gun, Gangwon Province, the northern most part of South Korea. Phylogenetic evaluation of the RNA-dependent RNA polymerase gene showed that the virus isolate was closely related to SFTSV strains isolated in China and Japan. As of July 5, 2013, KCDC had verified 13 cases of SFTS through RT-PCR; of these patients,

eight died, and five recovered (44). The range of SFTS cases has increased, notably in China, from 511 in 2011 to almost 1,500 cases in 2012. The initial case fatality rate stated was once up to 30%; however, current estimates range between 10% and 12% except Japan and South Korea, where the mortality rate is as high as 32–47%. The sickness is ordinarily considered amongst farmers engaged in agricultural or forest workers. Most of the cases (86%) were detected in subjects 50 years of age or older, with the fatality rate increasing with age. The SFTS case incidence was observed to be comparable for females and males (45). In South Korea, the case-fatality rate is mostly higher than that in China or Japan. This situation has motivated lookup to understand the epidemiology of the SFTS virus and the biology of its vectors. However, statistics concerning the ecology of vector species and environmental factors affecting the abundance and distribution of the vectors in South Korea remains limited. A survey of the seasonal abundance of three hard tick species in the Gyeonggi-do place reported the tick population's phenology in four one-of-a-kind landscapes. To better understand the biology of the achievable tick species and improve techniques for intervention, they conducted a 3-yr field survey in this region to describe the seasonal abundance of host-seeking *H. longicornis* and *H. flava* in four vegetation types and to estimate the minimum infection rate (MIR) of the SFTS virus in the hard ticks. The seasonal abundance of *H. longicornis* and *H. flava* (Acari: Ixodidae) was monitored from 2015 through 2017 in Gyeonggi-do, South Korea. Furthermore, *H. longicornis* comprised of up to 90% of the ticks collected. Generally, peaks of nymph, adult, and larva numbers had been discovered from April to June, from June to July, and from August to September, respectively. Half of the ticks had been pooled and tested for the SFTS virus's presence with terrible outcomes. The study concluded that molecular analysis suggested that the SFTS virus infection rate in the hard ticks was probably less than 5% in their surveyed location (46).

CHAPTER THREE
METHODOLOGY

3.1. Sampling site and collection:

For bacterial and protozoal detection, 109 samples of ticks collected from three sites Wonju (Gangwondo Province; 37.389545, 127.801770), Gunsan (Jeollabukdo Province; 36.006237, 126.807751), and Yongsan (Gyeongsangnamdo Province; 35.286111, 129,027625) of South Korea during August 2014 to October 2015 (Fig 1, Table 1,3). These sites are in Korea (east, west, south, and north) ,and there are differences in population density and climate. The extracted DNA has been stored in a freezer (-80ºC), Yonsei University College of Medicine Institute of Tropical Medicine, Department of Environmental Medical Biology Lab.For viral pathogen detection, a total of 50 samples of ticks were collected from Samcheok (Gangwondo Province; 37.19241, 129.14014) (Table 2) on April 2020 using the flagging method at ground level. Proper labeling and morphological identification were made using a dissecting microscope.

3.1.1. Flagging, vegetation:

Two flags made of unbleached cotton muslin stapled to timber bases had been used. The small flag was once a 30 • 116 cm (12 in •forty- six in) piece of muslin, stapled at the middle to one quit of a 122-cm (48-in) timber dowel so that the two ends of the muslin (each fifty-eight cm long) hung like flags from the dowel. The dowel used to be used to stir up the leaf litter and low vegetation, and the muslin flag used to be pulled through. The massive flag was once a seventy-six X ll2-cm piece of muslin, stapled alongside the lengthy facet to a wood base, and dragged at the back of the investigator by way of a rope deal with connected to the timber base (47) ,the flagging was employed to trap all stages of the ticks.

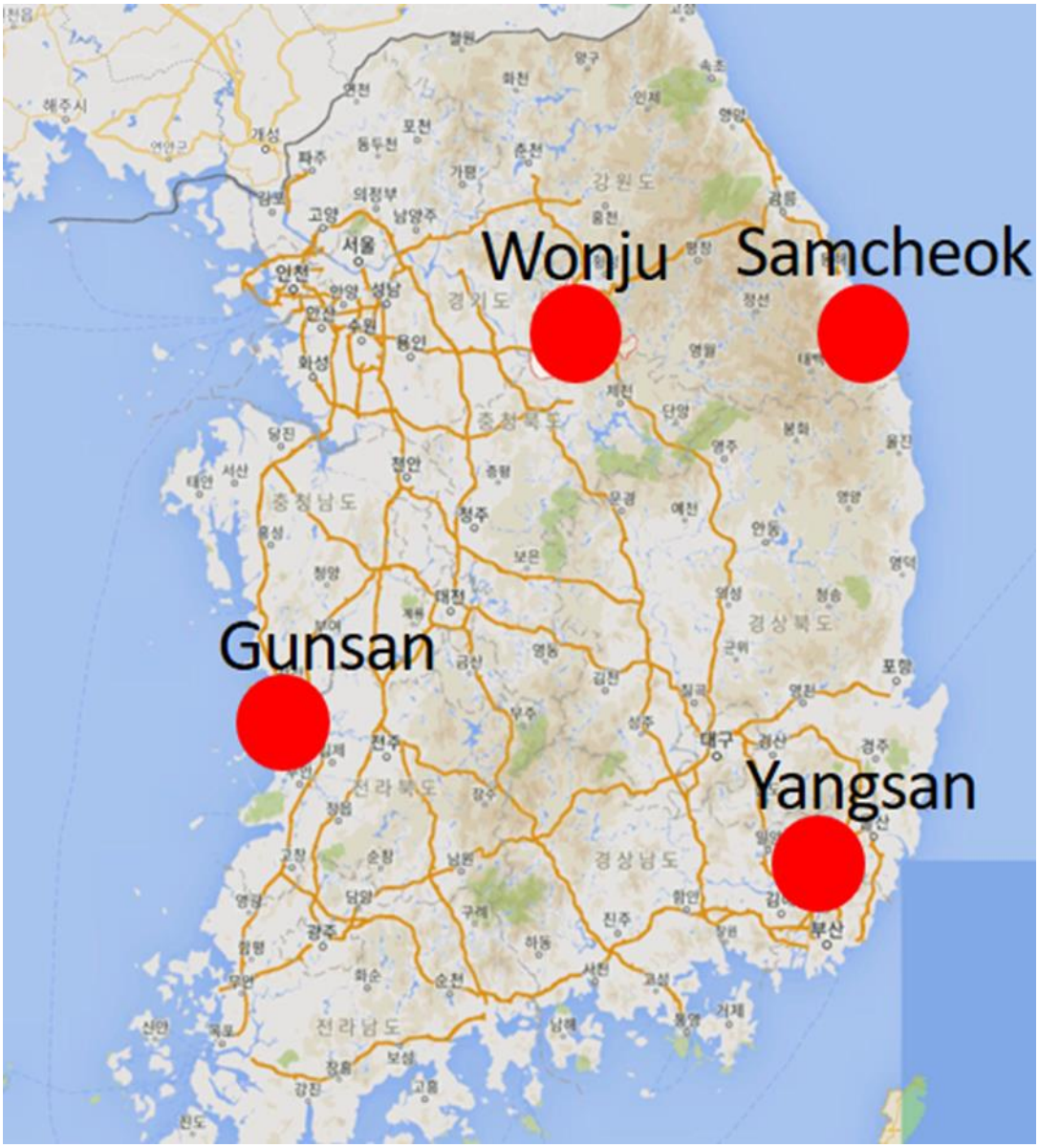


Figure 1. South Korea map, collection of ticks from four places (Wonju, Gunsan, Yangsan, and Samcheok)

Table 1-Summary of *Haemaphysalis* ticks collected for Bacteria and Protozoa detection from vegetation in three localities in South Korea

Site and Location	Climate	No. population	Tick Species		Collection date
			H. longicornis	*H. flava*	
Gunsan (Jeollabukdo Province), the port city, west part 200 km southwest of Seoul on the Midwest coast of the Korean Peninsula.	a cooler version of a humid subtropical climate	278,505	*22*	31	2014-August
Wonju (Gangwondo Province) is the mountainous regions, north middle part, the city is located approximately 140 km east of Seoul.	a hot summer humid continental climate	332,849	*50*	0	2015-July
Yangsan (Gyeongsangnamdo Province) has huge river (fresh water) east south part.	humid in summer and dry in winter	342,371	*0*	6	2015-October
Total no. of ticks109			72 (66.05 %)	37 (33.94 %)	

Table 2. Summary of *Haemaphysalis* ticks collected for SFTS virus detection from vegetation in one locality in Korea

Site and Location	Climate	No. population	Tick Species		Collection date
			H. longicornis	*H. flava*	
Samcheok (Gangwon-do Province) in the east, beach region	a humid subtropical climate	67,575	37	13	2020-April

Table 3. Number of TBDs in 2016-2019. Data was collected from Korea Centers for Disease Control and Prevention

Provinces and site	No. of Lyme disease cases (2016-2019)	No. of Q fever cases (2016-2019)	No. of SFTS cases (2016-2019)
Gangwon (Wonju, Samcheok)	1	2	133
Jeolbuk (Gunsan)	4	30	44
Gyeongsangnam (Yangsan)	5	40	78

3.2. DNA extraction for bacterial and protozoal detection:

DNA of collected ticks were isolated using a NucleoSpin DNA Insect kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions and stored at -20˚C until use.

3.3. RNA extraction and cDNA construction for SFTS:

Total RNA was isolated from 50 tick samples using RiboExTM (Trizol) (GeneAll, Seoul, Korea), according to the manufacturer's instruction. First-strand cDNA was synthesized by reverse transcription-PCR using AMPIGENE® cDNA Synthesis Kit (EnzoLife Sciences, Inc. Farmingdale, NY, USA).

3.4. Primers used for pathogen detection and identification:

PCR had been applied for rapid screening of ticks to detect focused pathogens following by using precise identification of the pathogens the usage of species-specific primers, the use of traditional and nested PCR assays. The DNA and RNA were amplified using specific primers (Table 4)

Table 4. Primers for detection of Bacteria, Protozoa and SFTS virus

Pathogens	Primer	Target gene	Sequences (5-3)	Bp	Ref
Anaplasmataceae	EHR1 F	16SrRNA	5-GAACGAACGCTGGCGGCAAGC-3	610	(48)
	New EHR2Br		5-CACGCTTTCGCACCTCAGTGTC-3		
	EHR3F		5-TGCRTAGGAATCTRCCTAGTAG-3	610	
	new EHR2bR		5-CACGCTTTCGCACCTCAGTGTC-3		
Rickettsiaceae	Rr17k.Ip F	17kDa	5-TTTACAAAATTCTAAAAACCAT-3	539	
	Rr17k.539nR		5-TCAATTCACAACTTGCCATT-3		
	Rr17k.90p F		5-GCTCTTGCAACTTCTATGTT-3	450	
	Rr17k.539nR		5-TCAATTCACAACTTGCCATT-3		
Rickettsia spp	F	16S rRNA	5-TAAGGAGGTAATCCAGCC-3	1482	(49)
	R		5-CCTGGCTCAGAACGAA-3		
	F	gltA	5-GGCTAATGAAGCGGTAATAAATATGCTT-3	341	
	R		5-TTTGCGACGGTATACCCATAGC-3		
	F	OmpA	5-CACYACCTCAACCGCAGC-3	444-438	
	R		5-AAAGTTATATTTCCTAAACCYGTATAAKTATCRGC-3		
Coxiella spp	Q5 F	htpB	5-GCGGGTGATGGTACCACAACA-3	501	(48)
	Q3		5-GGCAATCACCAATAAGGGCCG-3		
	Q6 F		5-TTGCTGGAATGAACCCCA-3	325	
	Q4		5-TCAAGCTCCGCACTCATG-3		
Borrelia spp	new LDfbF	16SrRNA	5-GTAAACGATGCACACTTGGTG-3	524	
	new LDrb R		5-TCCGRCTTATCACCGGCAGTCT-3		

	Outer1 F	fla B	5-AARGAATTGGCAGTTCAATC-3	497	
	Outer2 R		5- GCATTTTCWATTTTAGCAAGTGA TG-3		
	Inner1 F	fla B	5-ACATATTCAGATGCAGACAGAGG TTCTA-3	389	
	Inner2 R		5-GAAGGTGCTGTAGCAGGTGCTGGC TGT-3		
Piroplasmidae	BJ1F	18SrR NA	5-GTCTTGTAATTGGAATGATGG-3	476-520	
	BN2R		5-TAGTTTATGGTTAGGACTACG-3		
Babesia spp	Bab GF2	18SrR NA	5-GYYTTGTAATTGGAATGATGG-3	359	(50)
	BabGR2		5-CCAAAGACTTTGATTTCTCTC-3		
Bartonella spp	QHVE1	ITS	5-TTCAGATGTGATGTGATCCCAA GC-3	735	(51)
	QHVE3		5-AACATGTCTGAATATATCTTC-3		
	QHVE12	ITS	5-GCAGCTAATCTTCCGCAATGG-3	484-569	
	QHVE14		5-CAACCATGCAGCACCTATAT-3		
SFTS virus	BNYS1-F,		5’-TCTTCTCCATCAAGAACAGC-3	491	(52)
	BNYS-R,		5′-TTCGACAAAATTAGACCTCC-3		

3.5. PCR amplification and detection:

The amplification was done for each pathogen, using specific PCR and primers (Table 4). A total of 8 primers was used for all targeted pathogens. PCR conditions were written in the appendix. The PCR products were electrophoresed on 1% agarose gel with gel stain and the result was visualized under UV light for the band's presence with the reference ladder. Finally, the gel product images were documented. The detected pathogen`s products were sequenced, and the sequence results were BLAST on the NCBI database for pathogen identification with the highest percentage similarities were recorded as targeted pathogens.

3.6. Species identification:

The positive samples were analyzed for species identification. Out of the 21 samples for *Rickettsia* spp. 14 were detected positive with primers ompA and gltA to identify the strain types. Each PCR amplicon was sequenced, and for each 17 previously identified species were compared with 14 rickettsia pathogens sequences. The sequence was edited by BioEdit 7.2 version and European molecular biology laboratory (EMBL) online sequence alignment platforms and the neighbor-joining method with the MEGA 4.0 program.

CHAPTER FOUR

RESULTS

4.1. Bacterial and protozoal detection pathogens:

4.1.1. *Haemaphysalis* spp. and total pathogen detection rate:

Among 109 tick samples, the number of *H. longicornis* tick was 72, and *H. flava* was 37. In these examined ticks, 32 (29.35%) ticks were found to have pathogens. Bacterial pathogen detection rate 23 (21.10%) rather than two folds from Protozoal pathogen detection rate 9 (8.25%). *H. longicornis* ticks were associated with a higher total pathogen detection rate, 28 (38.88%) rather than 7 folds from *H. flava* ticks 4 (10.81%) (Table 5).

Table 5. Tick species, total result of Bacterial and Protozoal detection rate

Samples for Bacteria and Protozoa				
Tick Species	No. ticks	No. Bacterial detection (%)	No. Protozoal detection (%)	Total (%)
H. longicornis	72	19(26.38%)	9 (12.50%)	28(38.88%)
H. flava	37	4(10.81%)	0(0%)	4 (10.81%)
Total	109	23(21.10%)	9(8.25%)	32 (29.35%)

H. longicornis ticks were associated with a higher pathogen detection rate rather than 7folds from *H. flava*

4.1.2. *Haemaphysalis* spp. and bacterial pathogen detection rate:

However, the identification rate of *Rickettsia* spp was the most common bacterial pathogens rate 21(19.26%), followed by *C. burnetii* about 2 (1.83%). Among the pathogens identified in *H. longicornis, Rickettsia.* spp was the most common ticks borne disease18 (25.00%) more than threefold from *H. flava* 3 (8.10%) (Fig 2). Total tow *C. burnetii* was detected, one from *H. longicornis* and the other from *H. flava* (Fig 3). While no detection for Anaplasmataceae, *Borrelia* spp., and *Bartonella* spp. for both species (Table 6).

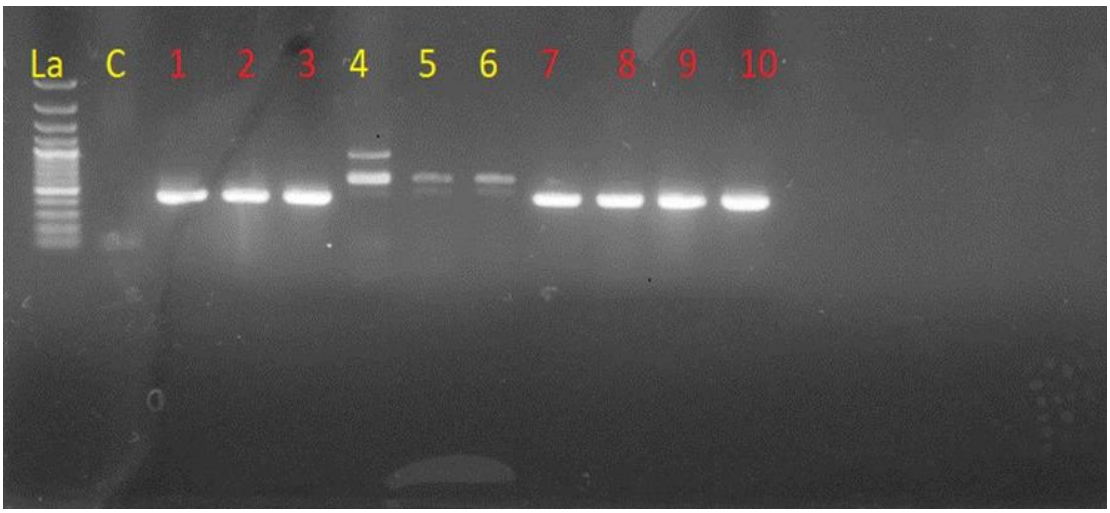


Figure 2. PCR product (DNA fragment band) in 1% agarose gel with base pair (bp 450) that electrophoresed for molecular identification of *Rickettsia.* spp

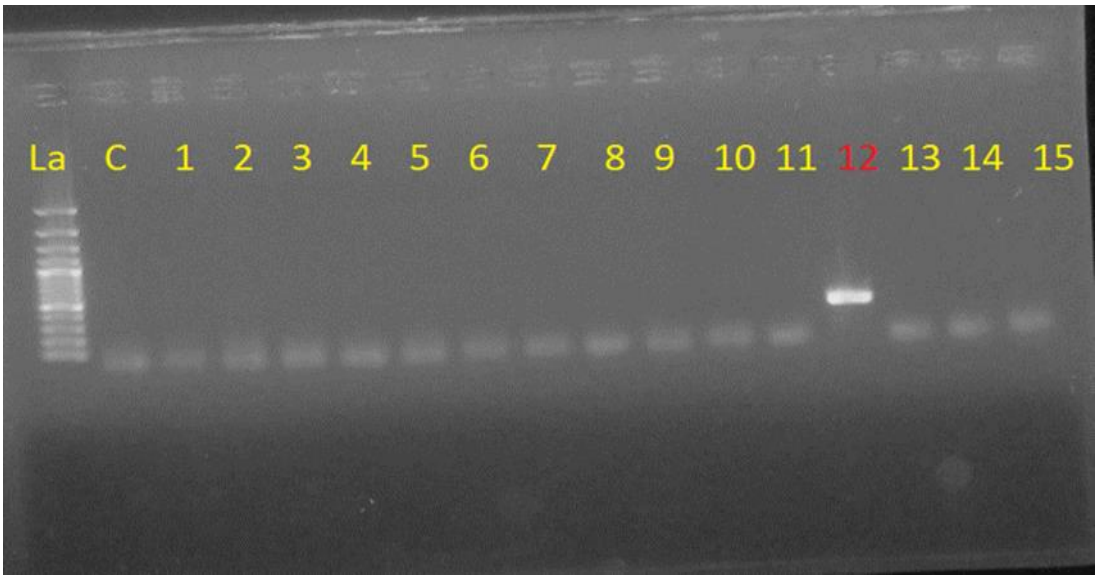


Figure 3. PCR product in 1% agarose gel with base pair(bp) of 325 for molecular identification of *Coxiella burnetii*

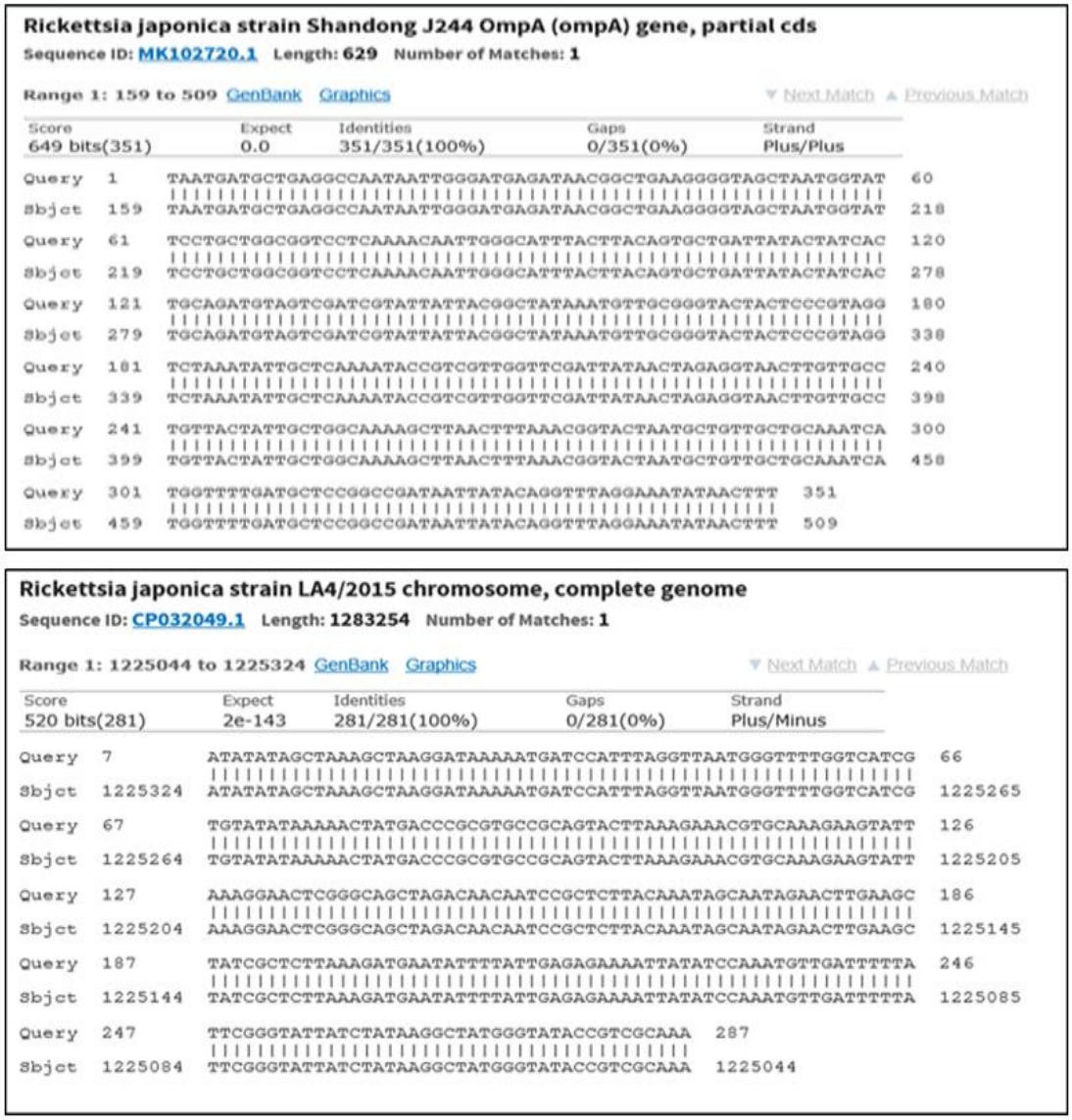
Table 6. Tick species and bacterial total result detection

Sample for Bacteria		No. of infected ticks					
Tick Species	No. Ticks	Anaplasmataceae (%)	*Rickettsia* spp (%)	*Coxiella burnetii* (%)	*Borrelia* spp. (%)	*Bartonella* spp. (%)	Total Result (%)
H. longicornis	72	0 (0%)	18 (25,00%)	1 (1.38%)	0 (0%)	0 (0%)	19 (26.38%)
H. flava	37	0 (0%)	3 (8.10%)	1 (2.70%)	0 (0%)	0 (0%)	4 (10.81%)
Total	109	0 (0%)	21 (19.26%)	2 (1.83%)	0 (0%)	0 (0%)	23 (21.10%)

Rickettsia japonica was the most common ticks borne disease bacteria, followed by *C. burnetii*. The detection rate for *Rickettsia japonica* in *H. longicornis* more than three-fold from *H. flava* but for *C. burnetii* less than one folds in *H. longicornis* from *H. flava*.

4.1.3.Species identification for selected samples:

PCR was performed using ompA and gltA primers to identify the *Rickettsia* strain types. Only 14 samples were amplified and sequenced among 21 *Rickettsia* positive samples. All sequenced samples have been compared with Gene Bank data. Finally, all 14 samples were identified as *Rickettsia japonica* strain with homology 100% identity among the groups (Fig.4).



Rickettsia japonica strain Shandong J244 OmpA (ompA) gene, partial cds

Sequence ID: MK102720.1 Length: 629 Number of Matches: 1

Range 1: 159 to 509 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
649 bits(351)	0.0	351/351(100%)	0/351(0%)	Plus/Plus

```
Query  1    TAATGATGCTGAGGCCAATAATTGGGATGAGATAACGGCTGAAGGGGTAGCTAATGGTAT  60
Sbjct  159  TAATGATGCTGAGGCCAATAATTGGGATGAGATAACGGCTGAAGGGGTAGCTAATGGTAT  218

Query  61   TCCTGCTGGCGGTCCTCAAAACAATTGGGCATTTACTTACAGTGCTGATTATACTATCAC  120
Sbjct  219  TCCTGCTGGCGGTCCTCAAAACAATTGGGCATTTACTTACAGTGCTGATTATACTATCAC  278

Query  121  TGCAGATGTAGTCGATCGTATTATTACGGCTATAAATGTTGCGGGTACTACTCCCGTAGG  180
Sbjct  279  TGCAGATGTAGTCGATCGTATTATTACGGCTATAAATGTTGCGGGTACTACTCCCGTAGG  338

Query  181  TCTAAATATTGCTCAAAATACCGTCGTTGGTTCGATTATAACTAGAGGTAACTTGTTGCC  240
Sbjct  339  TCTAAATATTGCTCAAAATACCGTCGTTGGTTCGATTATAACTAGAGGTAACTTGTTGCC  398

Query  241  TGTTACTATTGCTGGCAAAAGCTTAACTTTAAACGGTACTAATGCTGTTGCTGCAAATCA  300
Sbjct  399  TGTTACTATTGCTGGCAAAAGCTTAACTTTAAACGGTACTAATGCTGTTGCTGCAAATCA  458

Query  301  TGGTTTTGATGCTCCGGCCGATAATTATACAGGTTTAGGAAATATAACTTT  351
Sbjct  459  TGGTTTTGATGCTCCGGCCGATAATTATACAGGTTTAGGAAATATAACTTT  509
```

Rickettsia japonica strain LA4/2015 chromosome, complete genome

Sequence ID: CP032049.1 Length: 1283254 Number of Matches: 1

Range 1: 1225044 to 1225324 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
520 bits(281)	2e-143	281/281(100%)	0/281(0%)	Plus/Minus

```
Query  7        ATATATAGCTAAAGCTAAGGATAAAAATGATCCATTTAGGTTAATGGGTTTTGGTCATCG  66
Sbjct  1225324  ATATATAGCTAAAGCTAAGGATAAAAATGATCCATTTAGGTTAATGGGTTTTGGTCATCG  1225265

Query  67       TGTATATAAAAACTATGACCCGCGTGCCGCAGTACTTAAAGAAACGTGCAAAGAAGTATT  126
Sbjct  1225264  TGTATATAAAAACTATGACCCGCGTGCCGCAGTACTTAAAGAAACGTGCAAAGAAGTATT  1225205

Query  127      AAAGGAACTCGGGCAGCTAGACAACAATCCGCTCTTACAAATAGCAATAGAACTTGAAGC  186
Sbjct  1225204  AAAGGAACTCGGGCAGCTAGACAACAATCCGCTCTTACAAATAGCAATAGAACTTGAAGC  1225145

Query  187      TATCGCTCTTAAAGATGAATATTTTATTGAGAGAAAATTATATCCAAATGTTGATTTTTA  246
Sbjct  1225144  TATCGCTCTTAAAGATGAATATTTTATTGAGAGAAAATTATATCCAAATGTTGATTTTTA  1225085

Query  247      TTCGGGTATTATCTATAAGGCTATGGGTATACCGTCGCAAA  287
Sbjct  1225084  TTCGGGTATTATCTATAAGGCTATGGGTATACCGTCGCAAA  1225044
```

***F*igure 4 .Identification of Rickettsia japonica strain using ompA and gltA gene**

4.1.4. *Haemaphysalis* spp. and protozoal pathogen detection rate:

Nine ticks were positive in PCR analysis using Piroplasmidea primers (BJ1F and BN2R) (Fig 5). Eight ticks among these were also positive in PCR analysis using *Babesia* spp. primers (BabGF2 and BabGR2). All 17 DNA sequences of PCR products were identified as *Thiellaria luwansheni*. Therefore, 9 *T. luwansheni* were detected in this study, and the detection rate for *T. luwansheni* was 8.25%. All *T. luwansheni* were found in *H. longicornis* only (Table 7).

Table 7. Tick species and total protozoal result detection

Sample for Protozoa		No. of infected ticks	
Tick Species	No. Tick	Piroplasmidae BJ1&BN2%	*Babesia* spp BabGF2&BabGR2%
H. longicornis	72	9(12.50%)	8(11.11%)
H. flava	37	0(0%)	0(0%)
Total	109	9(8.25%)	8(7.33%)

Nine *Theilleria. luwansheni* were detected in this study and the prevalence of *T. luwansheni* was 8.25%. All in *T. luwansheni* were found in *H. longicornis* only.

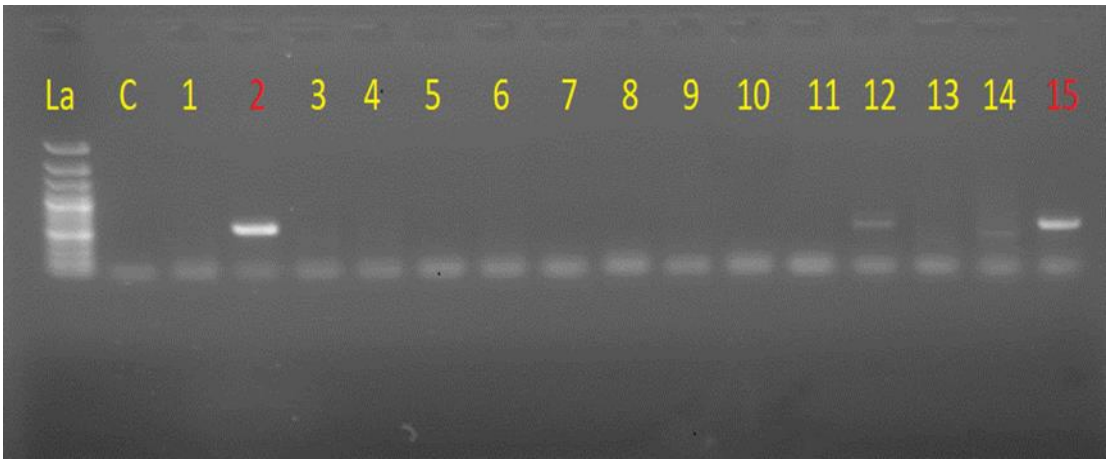


Figure 5. PCR product (DNA fragment band) in 1% agarose gel with base pair(bp) of 476-520 for molecular identification of *T. luwansheni* by BG1&BJ1

4.1.5. Locations and total pathogen detection rate:

The total pathogen detection rate in ticks from Wonju was 26 (52.00%) with hot summer humid continental climate which higher than the rate of Yangsan, and Gunsan; pathogen detection rate in ticks from Yangsan was 2 (33.33%) and Gunsan was 4 (7.54%). This is due to the high bacterial pathogen detection rate of 17(34.00%) in Wonju. The protozoal pathogen was detected only in 9 ticks collected from the Wonju region (Table 8).

Table 8. Total pathogen detection rate in ticks collected from different locations

Location	No. of ticks	No. of bacterial detection (%)	No. of protozoal detection (%)	Total (%)
Gansun	53	4 (7.54%)	0(0%)	4(7.54%)
Wonju	50	17(34.00%)	9(18.00%)	26(52.00%)
Yangsan	6	2(33.33%)	0(0%)	2(33.33%)
Total	109	23(21.10%)	9(8.25 %)	32(29.35%)

4.1.6. Locations and Bacterial pathogen detection rate:

For *Rickettsia japonica*, the detection rate in ticks from Yangsan had two samples (33.33%), Wonju had 16 samples (32,00%), and Gunsan had two samples (5.66%). Ticks from Yangsan had detection rate was higher than Wonju with one-fold and rather than five-fold from Gunsan. Total two *C. burnetii* were detected in ticks: one from in Wonju and the other from Gunsan. No detection for Anaplasmataceae, *Borrelia* spp, and *Bartonella* spp for all regions (Table 9).

Table 9. Location, numbers of ticks and prevalence of bacteria

Sample for Bacteria		No of infected ticks					
Location	No. Ticks	Anaplasmataceae (%)	*Rickettsia* spp. .(%)	*Coxiella Burnetii* (%)	*Borrelia* spp. (%)	*Bartonella* spp. (%)	*Total* (%)
Gansun	53	0 (0%)	3 (5.66%)	1 (1.88%)	0 (0%)	0 (0%)	4 (7.54%)
Wonju	50	0 (0%)	16 (32,00%)	1 (2,00%)	0 (0%)	0 (0%)	17 (34,00%)
Yangsan	6	0 (0%)	2 (33.33%)	0 (0%)	0 (0%)	0 (0%)	2 (33.33%)
Total	109	0 (0%)	21 (19.26%)	2 (1.83%)	0 (0%)	0 (0%)	23 (21.10%)

4.1.7. Locations and Protozoal pathogen detection rate:

All the nine *T. luwansheni* were detected in this study in the Wonju region only, and the detection rate was 8.25% (Table 10).

Table 10. Location and total no. of protozoal infected ticks

Sample for Protozoa		No of infected ticks
Location	No. tick	*T. luwansheni*
Gunsan	53	0 (0%)
Wonju	50	9 (18,00%)
Yangsan	6	0 (0%)
Total	109	9 (8.25%)

4.1.8. Mixed infections:

From 109 tick samples we detect one mixed infection with *R. japonica* and *T. lunwenshuni* in *H. longicornis* spp (Table 11) in the Wonju region (Table 12).

Table 11. Tick species and Mixed bacterial infection

Sample for Bacteria &Protozoa		No. of mixed infected ticks with *Rickettsia japonica*+ *Thiellaria lunwenshuni*
Tick Species	No. tick	
H. longicornis	72	1 (1.38%)
H. flava	37	0 (0%)
Total	109	1 (0.91%)

Table 12. Location and mixed infections

Pathogens Mixed infections	No. of infected ticks from three locations			
	Gansun (N=53) (%)	Wonju (N=50) (%)	Yangsan (N=6) (%)	Total (N=109) (%)
R. japonica+ *T. lunwenshuni*	0 (0%)	1 (2,00%)	0 (0%)	1 (0.91%)

4.2. Virus detection result:

From 50 tick samples collected from Samcheok (37 *H. longicornis* and 13 *H. flava*). The SFTS virus was not detected.

CHAPTER FIVE

5.1 DISCUSSION:

Among the 109 ticks examined, we found that *H. longicornis* (66.05%) was higher than *H. flava* (33,94%) (Table 1). This result was also similar to the previous study on *H. longicornis*, the predominant species in the Korean peninsula (53)(54). In addition to this, the additional findings showed that *H. longicornis* used to be effortlessly discovered in the grasses and herbaceous vegetation of large herbivore habitats (21), which was the same with sample collection procedures. The primary hosts of *H. longicornis* in this finding were vegetation, and grassland were predominant, whereas another study showed that the dominant tick species *H. flava* accumulated directly from wild boars was (45.4%) and (57.9%) from their habitats. *H. flava* was primarily preferred a parasitize from small-to-medium-sized mammals and migratory birds where primarily located in conifer and combined forest habitats (55).

PCR had detected the pathogens detection in 109 ticks was 32 (29.35%) ticks. This finding determined that *H. longicornis* ticks were associated with a higher pathogen detection rate comprised 28 (38.88 %) greater than three folds from *H. flava* ticks 4 (0.11%). As a summary, the detected pathogens include bacterial and protozoal with detection rate 19 (26.38%) and 9 (12.5%) respectively in *H. longicornis* tick species (Table 5). Amongst the bacterial pathogens identified in 109 the samples, *Rickettsia* spp. was the most common ticks borne pathogen 21 (19.26%), followed by *C. burnetii* about 2 (1.83%) (Table 6), the species identification for *Rickettsia* spp. was performed using specific primer for gltA and ompA. All amplified sequences were identified as *R. japonica*.

H. longicornis had 18 (25.00%) for *R. japonica* coverage from the selected tick species and it is also more than three-fold from *H. flava* 3 (8.10%). The first documented finding for identifying *Rickettsia* related spotted fever, 7 were positive in PCR assay of gltA gene among 100 ticks *H. longicornis*, and their homologies were (99.7 to 100%) with that of *R. japonica*

(56). Another similar study stated that the minimum detection rate among 100 ticks *H. longicornis* was 0.93% for the 17 kDa antigen gene and 0.82% for the ompA nPCR assays for *R. Japonica* species with homology of 99.97% (57). The previous study on serosurvey on spotted fever group rickettsiosis among 3,401 patients serum samples, the prevalence was 19.88% ,which was the causes of other fever (58). Incheon, South Korea, on July 9, 2004, the first documentation of Japanese spotted fever has been reported and identified as *R. japonica* from human serum samples using specific primers (ompA) and similar species identity(100%) with this study (59).Therefore, based on the previous finding and this study, generally, SFG rickettsia disease is widely distributed in our study site and other south Korea provinces. To understand the pathogen distribution and reservoirs were different due to tick's bite happened in any means, which indicate that the disease-causing pathogens (*R. japonica*) may be brought public health concerns. Since tick-borne diseases is emerging and zoonotic, special emphasis on different perspectives may minimize the disease burden.

However, for *C. burnetii*, the detection rate was (2.70%) for *H. flava* more than one-fold from *H. longicornis* (Table 5). *C. burnetii* is a bacterial pathogen which causes Q fever disease and infects humans and animals. The first Q fever detected in Korea was in 1993. The numbers of recorded cases became increasing, particularly in Chungbuk Province since 2014 (33), but still, the studies about Q fever in Korea ticks need further efforts, Nonetheless, in a study of 105 *Haemaphysalis* ticks (89 were identified as *H. longicornis*, 8 as *H. flava*, and 8 as just *Haemaphysalis* sp.) were collected from Cheongju City (Chungbuk Province) in 2004, 2 were PCR positive for *Coxiella* (60). Similar to this, in a study conducted by KCDC, only 23% of the reported 65 Q fever patients had high-risk occupations (e.g., slaughtermen and ranchers) from 2006 to 2011 (61). The distributions in different sites, and the seasonal variation for might cause for disease occurrences, this tick-borne disease needs to get special emphasis on prevention and control mechanisms for risk groups.

For *Theilleria luwansheni* identification, we found nine ticks were positive by PCR analysis using specific primers (BJ1F and BN2R). Eight ticks among these were also positive in PCR analysis using *Babesia* spp. primers (BabGF2 and BabGR2). However, the primers used for detection were different; but the result was similar due to nuclear ribosomal rRNA genes are regularly used as targets for species identification. In addition, the conserved nature and repetitive association inside the genome that furnish sample quantities of template DNA for PCR(62). Therefore, all DNA sequences of PCR products were identified as *Thiellaria luwansheni* with a detection rate of 8.25%. All of them were found in *H. longicornis* only (Table 7). In addition to this, the study conducted from 2016 to 2019 on the tick species *H. longicornis*, *Ixodes nipponensis*, and *H. flava*, were obtained from native Korean goat, *Coxiella*-like endosymbiont (CLE, 5.0%), *Candidates Rickettsia longicornii* (45.0%), *Anaplasma bovis* (2.5%), and *T. luwansheni* (5.0%) were detected. *Ehrlichia, Bartonella* spp., and SFTS virus were not detected (63).

In China, a study published in 2014, 227 blood samples were collected from 73 sheep, two dogs, and 152 hedgehogs. *Babesia* spp. was only detected in the two dogs. *Theileria* spp. was detected both in the sheep and the hedgehogs, and the total positive rate of *Theileria* spp. in the sheep and the hedgehogs was 57.53% and 13.82%, respectively. Sequencing and phylogenetic analysis revealed that the *Theileria* spp. detected in the sheep and the hedgehogs were very close to *T. luwansheni* cloned from a small ruminant and *Theileria* spp. isolated from a febrile hospitalized patient in China (64). Therefore, well organized and further representative study on ticks mediated infection such as *Rickettsiaceae (Rickettsia spp), Piroplasmidea,* and *Coxiella* pathogen would be important. In contrast, *Borrelia, Bartonella* spp, Anaplasmataceae had been not detected in ticks in this study (Table 6,7). However, the risk of diseases triggered by Anaplasmataceae, *Bartonella*, and *Borrelia* exist in South Korea, based on the previous studies that detect these pathogens. During a screen for zoonotic tick-borne pathogens in North Korea, 2016, in 292 samples of 77 positive *H. longicornis* samples, they found that *A.*

phagocytophilum (12, 4.1%), *Borrelia* spp. (3, 1.0%), *B. henselae* (10, 3.4%) detected (10). In another study for ticks collected from Korean Water Deer during 2016 in South Korea, they found that 5/363 (1.4%) fed ticks were positive for *A. phagocytophilum*, and 11/363 (3.0%) of the ticks were positive for *Bartonella* spp (18).

The pathogen detection rate in ticks accumulated from Wonju location 52.00% (26/50) had been higher than that in ticks from Yangsan areas 33.33% (2/6) and Gunsan 7.54%(4/53). The pathogen detection rate in ticks from Wonju was one and a half fold from Yangsan and near 7 folds from Gunsan area. According to four-year surveillance on hard tick study indicate that , the climate condition has impact distribution and relation to the pathogen invasion for the environments and the climate of Korea has gradually changed into a subtropical region, and this might make Korean Peninsula environmentally suitable for the proliferation of vector ticks in the near future(65),and due to the excessive pathogen detection rate in *H. longicornis* ticks, which used to be the predominant tick species associated with Wonju region (Table1, 7), bacterial and protozoal pathogen detection rate was 17 (34.00%), 9 (18.00%), respectively. Regarding the pathogen species distribution, *R .japonica* identification in Wonju 16/50 (32.00%) was higher than Yangsan 2/6 (33.33%) and Gunsan 3/52(5.66%) (Table 8). One *C. burnetii* was detected in Wonju and Gunsan with identification rate (2.00%,1.88%), respectively, (Table 9). Nine *T. lunwenshuni* was identified in the Wonju (18.00%) (Table 10). In a previous study surveyed on Jeju, Korea, *T. lunwenshuni* was detected in the blood of 23 roe deer (100%) and *H. longicornis* collected from them (34.8%)(66).

In this study, to screen the SFTS virus belonging to tick species, 37 *H. longicornis* and 13 *H. flava* were used , and the SFTS virus was not detected. However, the major vectors of the SFTS virus in South Korea are *H. longicornis*, *H. flava, Rhipicephalus microplus, Amblyomma testudinarium,* and *Ixodes nipponensis* (11). The SFTS mainly occurs in South Korea in May–September, when tick activity is active, with infected patients reported until November. Gangwon (eastern, mountain area) and Jeju (a southeast island) display a relatively high

incidence. Most patients (80%) are >50 years, and the proportion of agriculture and forestry workers is 70% of patients. The reported SFTS cases have been increasing annually, especially in 2017, reaching 172 cases. The mortality rate was >35% during 2012–2013 but decreased to 11.52% in 2016 (33).

The finding conducted on tick parasitizing native Korean goats (*Capra hircus coreanae*) in South Korea, stated that this virus was also negative (63). In the assay for SFTS virus in Seoul during June-October 2013, they were examined, 732 *H. longicornis*, 62 *H. flava*, and 2 *Ixodes nipponensis* specimens were collected. They found that none of the tick's pools were found to be positive for the SFTS virus gene (67). In another previous study using 21,158 tick samples belonging to 3 genera and 6 species accumulated at 6 provinces and four metropolitan areas in South Korea from March to October 2014 have been assayed for selected tick-borne pathogens. They found the MLE (estimated number of viral RNA positive ticks per 1000 ticks) for SFTSV, by tick species and developmental stage, ranged from 0.45 to 14.70 for selected localities and habitats surveyed. Overall, the highest MLE for SFTSV was observed for *I. nipponen*- sis (4.0; 1 pool/249 individual ticks), followed by *H. flava* (2.42; 8 pools/3317 ticks), and *H. longicornis* (0.63; 11 pools/17,570 ticks). None of the pools of *A. testudinarium*, *I. turdus*, or *H. phasiana* were positive for SFTSV(11).

Another study in the same year for nine provinces in South Korea collected 13,053 tick samples from April till September, and *H. longicornis* (90.8%, 11,856/13,053) used to be once the most considerable among them. The minimal infection rate (MIR) of SFTSV in *H. longicornis* was once 0.46% (55 pools) (8). During the investigated the prevalence of (SFTSV) in shelter dogs and cats in South Korea in 2016, the blood samples were collected from 426 dogs and 215 cats reported as 0.2% and 0.5% positive for SFTSV, respectively (68).

In 2017, a study of one hundred and twenty-six serum samples of feral cats was collected from seven districts (Guro-gu, Geumcheon-gu, Seongdong-gu, Mapogu, Dongdaemoon-gu, Yongsan-gu, Gangnam-gu) reported 22(17%) positives out of 126 samples for SFTSV(69).

Currently, manipulating ticks is hard partly due to the truth of their wide host range, overlapping exercise periods of stadia. Moreover, due to the reality, the higher phase of their annual cycle is spent on pasture. This ability that acaricides through myself no longer satisfactorily limits tick populations or furnishes complete protection to stock, so built-in management combining pasture administration with proper husbandry and chemical prophylaxis is advocated.

Predictions about tick range enlargement also count on the balance of the most necessary biomes, each comprising the similar life-form. However, this is uncertain, in general, when you consider those human things have substantially increased the extent of land areas used for crop manufacturing and grazing of home herbivores. Human-led disturbance of the herbal environment is likely to continue, leading to a growing range expansion of grasslands and croplands at the of expanse of forests and shrublands. Numerous modern-day investigations have counseled that local weather change, specifically unexpectedly increasing global temperatures, has contributed to the range enlargement of many arthropod vectors, along with ticks. Robust evidence is presented in these advisor examples that vary growth is occurring. However, infinite factors host availability (especially for preferred hosts) and host specificity, habitat suitability, relative humidity tolerance, the extent and period of freezing temperatures, and human influence (habitat modification) may limit the possible cost and extent of range growth for one of a kind tick vector species. These elements need as a lousy lot pastime in appreciation tick varies enlargement due to climate change.

In addition to suitable local weather and habitats, tick vary increase is host- dependent. Even small numbers of ticks may also be adequate to set up a new populace so long as hosts, and suitable habitats and temperatures are present days. Moreover, predictions about tick range growth as steadfast in the predominant biomes, each comprising identical life-form. However, this is uncertain, especially because social matters have significantly expanded the extent of land areas used for crop manufacturing and grazing of home herbivores. Human-led

disturbance of the natural environment is likely to continue, leading to growing fluctuate growth of grasslands and croplands at the forests and shrubland's expanse.

5.2 LIMITATION:

This study sample (ticks) has been stored almost from 2014-2015 till this year 2020 in preservatives. Due to this problem, the abundance of bacterial and protozoal pathogens and the excellent of the pattern would have a likelihood to be decreased. Therefore, we collected fresh samples in this year 2020 for viral tick-borne pathogens to include due to long time storage problem. Another limitation is that the mentioned sample size is also small enough, which would have a concern to draw the usual conclusion but scientifically, we format to generate facts and advice for future studies.

CHAPTER SIX

CONCLUSION

- *H. longicornis* had a higher pathogen detection rate than *H. flava*.
- *Rickettsia japonica*, *Coxiella burnetii*, *Theilleria lunwenshuni* were the most commonly detected among the selected pathogens.
- *T. lunwenshuni* were detected higher in ticks collected in Wonju among the target protozoa.
- *Rickettsia japonica* was the most detected from *H. longicornis* among collected tick species in Wonju, Gunsan and Yangsan and the other targeted pathogens(Anaplasmataceae, *Borrelia* spp, *Bartonella* spp, and, SFTS v) .
- The information in this study may provide important information to inform the nearby community, human and animal`s health sectors in order to activate surveillance and monitoring against tick-borne pathogen about the possible emergence should be cautiously monitored. Further research and routine surveillance are required would be important for mitigation the disease prevalence and controlling the transmission in all stages.

APPENDIX:

Table 1. PCR program for primers to detecting Anaplasmataceae

Anaplasmataceae PCR Condition									
1 st					2nd				
	1x		Time	Cycle		1x		Time	Cycle
EHR1 F	1 ul	94 ^{0}C	3 min		EHR3 F	1ul	94^{0}C	3 min	
EHR2 R	1 ul	94 ^{0}C	30 sec		EHR2 R	1ul	94^{0}C	1 min	
d NTP mix	2.5 ul	63^{0}C	30 sec	40 cycles	D TNP mix	2.5 ul	55^{0}C	1 min	35 cycles
10 x taq buffer	2.5 ul	72^{0}C	30 sec		10 x taq buffer	2,5 ul	72^{0}C	1min	
Taq	0.1 ul	72^{0}C	30sec		Taq	0,1 ul	72^{0}C	10min	
DW	16.9ul	4^{0}C	∞		DW	16.9ul	4^{0}C	∞	
Template	0.5				Template	o.5			
Total	25				Total	25			

Table 2. PCR program for primers to detecting Rickettsiaceae

Rickettsiaceae PCR Condition									
1 st					2nd				
	1x		Time	Cycle		1x		Time	Cycle
Rr 17k.Ip F	1 ul	94^{0}C	3min		Rr17k.90p F	1ul	94^{0}C	3min	
Rr 17k. 539n R	1 ul	94^{0}C	30sec		Rr17k.539n R	1ul	94^{0}C	30sec	
d NTP mix	2.5 ul	55^{0}C	30 sec	35 cycles	D TNP mix	2.5 ul	57^{0}C	1min	35 cycles
10 x taq buffer	2.5 ul	72^{0}C	1 min		10 x taq buffer	2,5 ul	72^{0}C	1min	
Taq	0.1 ul	72^{0}C	7 min		Taq	0,1 ul	72^{0}C	7min	
DW	16.9ul	4^{0}C	∞		DW	16.9ul	4^{0}C	∞	
Template	0.5				Template	o.5			
Total	25				Total	25			

Table 3. PCR program to detected *Rickettsia* spp.

Rickettsia spp. PCR Condition

	1x		Time	Cycle
omp A F	1 ul	95 ^{0}C	5 min	
omp A R	1ul	95 ^{0}C	30 sec	40 cycles
d NTP mix	2.5 ul	52 ^{0}C	1 min	
10 x taq buffer	2.5 ul	72 ^{0}C	30 sec	
Taq	0.1 ul	4^{0}C	∞	
DW	16.9 ul			
Template	0.5ul			
Total	25			

Rickettsia spp PCR Condition

	1x		Time	Cycle
glt A F	1 ul	95 ^{0}C	5 min	
glt A R	1ul	95 ^{0}C	30 sec	40 cycles
d NTP mix	2.5 ul	52 ^{0}C	1 min	
10 x taq buffer	2.5 ul	72 ^{0}C	30 sec	
Taq	0.1 ul	4^{0}C	∞	
DW	16.9 ul			
Template	0.5ul			
Total	25			

Rickettsia PCR Condition

	1x		Time	Cycle
16S rRNA F	1 ul	95 ^{0}C	3 min	
16SrRNA R	1ul	95 ^{0}C	30 sec	40 cycles
d NTP mix	2.5 ul	52 ^{0}C	1 min	
10 x taq buffer	2.5 ul	72 ^{0}C	2min	
Taq	0.1 ul	72^{0}C	5min	
DW	16.9 ul	4^{0}C	∞	
Template	0.5ul			
Total	25			

Table 4. PCR program for detection of *Coxiella spp.*

Coxiella spp PCR Condition									
1 st					2nd				
	1x		Time	Cycle		1x		Time	Cycle
Q5 F	1 ul	94^{0}C	3min		Q6 F	1ul	94^{0}C	3min	
Q3 R	1 ul	94^{0}C	0.30sec		Q4 R	1ul	94^{0}C	0.30sec	
d NTP mix	2.5 ul	56^{0}C	0.30sec	35 cycles	D TNP mix	2.5 ul	52^{0}C	0.30sec	35 cycles
10 x taq buffer	2.5 ul	72^{0}C	0.30 sec		10 x taq buffer	2,5 ul	72^{0}C	0.30 sec	
Taq	0.1 ul	72^{0}C	5min		Taq	0,1 ul	72^{0}C	5min	
DW	16.9ul	4^{0}C	∞		DW	16.9ul	4^{0}C	∞	
Template	0.5				Template	o.5			
Total	25				Total	25			

Table 5. PCR for detection of *Borrelia* spp.

Borrelia spp PCR Condition									
1 st					2nd				
	1x	C	Time	Cycle		1x		Time	Cycle
Outer 1 F	1 ul	94^{0}C	3min		Inner 1 F	1ul	94^{0}C	30min	
Outer 2 R	1 ul	94^{0}C	0.30sec		Inner 2R	1ul	94^{0}C	0.30sec	
d NTP mix	2.5 ul	56^{0}C	0.30sec	35 cycles	D TNP mix	2.5 ul	52^{0}C	0.30sec	35 cycles
10 x taq buffer	2.5 ul	72^{0}C	0.30sec		10 x taq buffer	2,5 ul	72^{0}C	0.30sec	
Taq	0.1 ul	72^{0}C	5 min		Taq	0,1 ul	72^{0}C	5 min	
DW	16.9ul	4 ^{0}C	∞		DW	16.9ul	4 ^{0}C	∞	
Template	0.5				Template	o.5			
Total	25				Total	25			

Table 6. PCR program for detection of *Borrelia* spp.

Borrelia spp PCR Condition				
	1x		Time	Cycle
New LDf F	1 ul	94 ^{0}C	3 min	
New LDr R	1ul	94 ^{0}C	30 sec	40 cycles
d NTP mix	2.5 ul	61 ^{0}C	30 sec	
10 x taq buffer	2.5 ul	72 ^{0}C	30 sec	
Taq	0.1 ul	72 ^{0}C	10 min	
DW	16.9 ul	4^{0}C	∞	
Template	0.5ul			
Total	25			

Table7.PCR program for detection of Piroplasmidea

Piroplasmidea PCR Condition				
	1x	C	Time	Cycle
BJ1 F	1ul	94^{0}C	5min	
BN2 R	1ul	94^{0}C	1min	
d NTP mix	2.5ul	55^{0}C	1min	40 cycles
10x taq buffer	2.5ul	72^{0}C	1min	
Taq	0.1ul	72^{0}C	5min	
DW	16.9 ul	4^{0}C	∞	
Template	0.5ul			
Total	25			

Table 8. PCR program for detection of *Babesia* spp

Babesia spp PCR Condition				
	1x	C	Time	Cycle
Bab GF2	1ul	94^{0}C	3min	
BabGR2	1ul	94^{0}C	30 Sec	
d NTP mix	2.5ul	55^{0}C	30 sec	40 cycles
10x taq buffer	2.5ul	72^{0}C	0.30 sec	
Taq	0.1ul	72^{0}C	5min	
DW	16.9 ul	4^{0}C	∞	
Template	0.5ul			
Total	25			

Table 10. PCR program for detection of *Bartonella* spp

Bartonella. spp PCR Condition									
	1 st					2nd			
	1x		Time	Cycle		1x		Time	Cycle
QHVE1 F	1 ul	94^{0}C	3 min		QHVE12 F	1ul	94^{0}C	3 min	
QHVE3 R	1 ul	94^{0}C	30 sec		QHVE 14 R	1ul	94^{0}C	30 sec	
d NTP mix	2.5 ul	55^{0}C	30 sec	40	D TNP mix	2.5 ul	55^{0}C	30sec	40
10 x taq buffer	2.5 ul	72^{0}C	30 sec		10 x taq buffer	2,5 ul	72^{0}C	30 sec	
Taq	0.1 ul	72^{0}C	5 min		Taq	0,1 ul	72^{0}C	5 min	
DW	16.9ul	4^{0}C	∞		DW	16.9ul	4^{0}C	∞	
Template	0.5				Template	o.5			

Table 11. qPCR program for detection of SFTS

RNA virus cDNA				
q PCR				
	1x		Time	Cycle
SFTS R	1ul	94^{0}C	3min	
SFTS F	1ul	94^{0}C	45 sec	
D TNP mix	2.5 ul	56^{0}C	35sec	35 cycles
10 x taq buffer	2,5 ul	72^{0}C	1min	
Taq	0,1 ul	72^{0}C	15min	
DW	16.9ul	4^{0}C	∞	
Template	o.5			
Total	25			

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