PREVALENCE OF TICK-BORNE INFECTIONS IN MILITARY WORKING DOGS IN THE REPUBLIC OF KOREA

by

DENNIS R. BELL

(Under the Direction of Susan Sanchez)

ABSTRACT

Military Working Dogs (MWDs) are not routinely tested for tick-borne infections aside from serology for tick-borne infections at acquisition and just prior to departure to a duty location. However, MWDs are treated on a monthly basis with a topical spot-on tick-prevention product. Currently there are no prevalence studies concerning tick-borne infections in dogs from the Republic of Korea (ROK), although studies in rodents and ticks have demonstrated an abundance of infectious organisms. Our retrospective study utilized banked sera from 1997, 2002, and 2007 for ELISA and IFA testing as well as banked whole blood from 2007 for PCR. Our study revealed that, although many MWDs were seropositive for tick-borne infections, none was positive via PCR amplification. Our results could indicate exposure without contraction of disease, previous infection with residual antibodies, low sensitivity of our PCR, or cross-reactivity of our serologic testing with other antibodies.

INDEX WORDS: *Ehrlichia, Anaplasma, Rickettsia, Babesia, Borrelia*

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VMD, University of Pennsylvania, 1993

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2010

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August 2010

DEDICATION

I would like to dedicate this work to my beautiful wife, Robin, and my three wonderful children, Craig, Douglas, and Evan. This work culminates my initial six years in the US Army, during which time my family has had to tolerate many hardships with me along the way. I did not have the opportunity to spend much time with them during my first 2 years of service due to Army schooling at Fort Sam Houston, TX, a deployment to Iraq, and multiple short trips. My third year was spent in the Republic of Korea without my family. Finally, after a difficult initial three years, I thought that I would have more time to spend with my family during my residency and Master's program. But this was far from the case. The past 3 years have been spent with long hours in the clinics, many hours at night studying, doing homework or research, and not giving my family the attention they deserve. Without their sacrifices this work would not have been possible. Both my wife and children deserve much of the credit for my success at the University of Georgia. I love all of you, and appreciate all of your sacrifices.

ACKNOWLEDGEMENTS

There are so many people that have been involved with my research that I cannot possibly mention everyone. If I have left someone out, it was certainly not intentional. First and foremost, I would like to thank all of the members of my committee: Dr. Susan Sanchez, Dr. Roy Berghaus, and Dr. Cynthia Ward; as well as Dr. Margie Lee, the Director of Graduate Programs and the instigator of the Veterinary and Biomedical Sciences Master's program. Their guidance from the beginning of this project to the completion has been exceptional. I especially want to single out Dr. Sanchez. She was instrumental at getting my project started, providing ideas about research design, and guiding this novice in laboratory testing procedures and proper techniques. Our sessions during journal clubs were sometimes difficult, but she always made these periods very educational and rewarding. I can honestly say that I do not think I would be at this point in my research without her. Dr. Berghaus was always there for me during the last three years. He provided me with excellent ideas for my writings and greatly assisted me regarding the statistics for this project. Dr. Ward was there during this past three years to keep me from dividing my time too unevenly between my research and my residency. At times she had to redirect my focus to keep me headed in the proper direction. I do not think that I would have been able to complete a residency and a Master's degree without the program that Dr. Lee set up. She started me off in the right direction by recommending everyone previously mentioned as people that would be invaluable on my committee. Her suggestions could not have been better. I thank all of you for your help, guidance, patience, and persistence during the last 3 years.

The next group I would like to express my gratitude to is the staff of the Athens Veterinary Diagnostic Laboratory. Everyone was wonderful and always ready to lend a hand. I had many questions for them during my time there, and they were always ready to answer them and to help out whenever possible. I especially want to thank Ingrid Fernandez for her work with my PCR testing. When my clinic duties did not allow me to be in the lab, Ingrid would step up and get the testing done. I would still be running PCRs if were not for her. I also want to thank Paula Bartlett, Amy McKinney, Ashley Phillips, and Sarah Bates for assisting me.

The next group that needs to be mentioned is all of my laboratory partners. Chrissy Still is one person that really guided me along the way. At times, she would step away from her own work to guide me in the right direction. Chrissy was our leader in the lab, and as such was always there when we needed help, no matter in what area. Everyone should be so lucky as to have a lab partner as selfless and dedicated as Chrissy. Other people that have assisted me during my laboratory time include Charles Hong, Hajung Roh, Shreena Patel, and Stephanie Beavers.

Many individuals in the US Army helped make this project happen. These include LTC Roger Parker, Mr. Edwin Cooper, and Mr. Michael Gray from the Food Analysis and Diagnostic Laboratory at Fort Sam Houston, TX, MAJ Douglas Owens from Lackland Air Force Base in San Antonio, TX, COL David Rolfe, VETCOM Commander, Fort Sam Houston, TX, and COL Mack Fudge, Commander, 106th Medical Detachment, Republic of Korea. This project took a significant amount of coordination to just get approval, and without these individuals, none of this would have been possible.

Two individuals provided me with positive control samples for use in my diagnostic testing. Dr. Edward Breitschwerdt from North Carolina State University provided positive

control samples for all of the ELISA testing that was done. Dr. Michael Yabsley provided the positive control samples that were utilized in all of the PCR testing that was done, including positive controls for *Anaplasma platys* and *Ehrlichia ewingii*, which cannot be cultured.

A thank you also goes out to IDEXX for donating all the ELISA test kits used in this research. Fuller Laboratories also needs to be acknowledged for providing discounted IFA tests for completion of this research.

Lastly, I would like to thank God for giving me the ability and perseverance to see this project through to completion. Without my faith to guide me, I would have lost hope many times, but God was always there to nudge me forward.

At this point I would like to provide some additional background about what inspired this project. Being a veterinarian on active duty in the US Army, our top priority is the proper care and treatment of military working dogs (MWDs). From the time that a new veterinarian participates in the veterinary track at Officer Basic Leadership Course, to the mandatory participation in the Clinical Proficiency Course, to the proposed development of the new First Year Graduate Medical Education (which will basically be an Army internship for new officers), it is quite obvious that the Army wants to ensure these amazing animals receive the best care possible from well-trained veterinarians. In addition to all this required training, the Veterinary Corps also has a manual entitled The Handbook of Veterinary Care and Management of the Military Working Dog, which some of us in the Veterinary Corps affectionately refer to as the MWD Bible. This manual goes into great detail pertaining to MWDs including proper record keeping, preventative care, nutrition, anesthesia, exercise, emergency care, surgery, dentistry, and behavior, just to list some of the contents. There are also sections regarding training of the dog handlers on emergency first-aid, prevention of heat injuries, proper administration and

application of medications as well as proper feeding. The Veterinary Corps also has board certified and residency-trained officers that are available for consultations and referrals. In 2007, a new state-of-the-art referral center was completed at Lackland Air Force Base (AFB) in San Antonio, TX. This facility, staffed by multiple veterinary specialists, has several functions that include referrals from other military installations in the US and abroad, caring for all dogs that are stationed at Lackland AFB for initial training, and sustainment of a breeding program. It is quite obvious, given the required training of new Army veterinarians, to the residency training of specialists, to the new specialty hospital, that the Army is dedicated to the advancement of MWD care.

Since joining the Army, I have had the opportunity to witness first-hand the types of services that MWDs provide at bases in the US, the Republic of Korea (ROK), and in Iraq in support of Operation Iraqi Freedom. While being stationed in the ROK, I had the opportunity to attend and participate in a joint US-ROK veterinary conference. It was during this conference that the seed for this research was planted. Research was presented by Sang-Ho Seo regarding *Ehrlichia* and *Borrelia* infections in German shepherd dogs in Korea. This research, which I later learned was Sang-Ho Seo's Master's Thesis (Sang-Ho Seo, Seoul National University, 2006), showed a seroprevalence for *Ehrlichia* antibodies on ELISA of 7.56% (22 or 291) and prevalence of *E. chaffeensis* based on PCR of 3.09% (9 of 291). No DNA was amplified for *E. canis* or *E. ewingii*. After this presentation, many thoughts were going through my mind. What is the actual prevalence of tick-borne infections in the ROK? What tick-borne organisms are present in the ROK? Were the dogs in this study treated with monthly flea and tick preventative preparations, and if so, which one? What was the prevalence of tick-borne infections in the US MWD population in the ROK? Was our tick-prevention program adequate?

Upon leaving the ROK in July 2007, I was fortunate to have been selected by the Army and the University of Georgia to participate in a small animal internal medicine residency which included a Master's program in Veterinary and Biomedical Sciences. This opportunity has allowed me, over the past 3 years, to attempt to answer as many of these questions as possible.

TABLE OF CONTENTS

Page

ACKNOWLEDGEMENTS v

LIST OF TABLES xi

LIST OF FIGURES xii

CHAPTER

1 INTRODUCTION AND LITERATURE REVIEW 1

Tick-Borne Organisms 1

Ticks as Vectors 4

Diagnosis of Tick-Borne Infections 7

Treatment and Prevention of Tick-Borne Infections 13

2 SEROPREVALENCE OF TICK-BORNE INFECTIONS IN MILITARY WORKING DOGS IN THE REPUBLIC OF KOREA 20

Abstract 21

Introduction 22

Materials and Methods 24

Results 27

Discussion 29

3 CONCLUSIONS AND FUTURE DIRECTION 37

REFERENCES 39

LIST OF TABLES

Page

Table 1: Oligonucleotide Primers for PCR assays.....33

Table 2: IFA and ELISA results for *Anaplasma phagocytophilum*.....34

Table 3: IFA and ELISA results for *Ehrlichia canis*.....35

LIST OF FIGURES

Page

Figure 1: Location of US Military Installations in the Republic of Korea 36

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Tick-Borne Organisms

Many infections that dogs acquire are known to be transmitted by the bites of infected ticks. This transmission occurs during the consumption of a blood meal by an arthropod. Prior to 2001, tick-borne organisms in the order *Rickettsiales* were grouped based on characteristics such as morphology, epidemiology, or clinical signs. But in 2001, the basis for grouping these organisms was changed to reflect their relatedness to each other according to DNA sequencing of the 16S rRNA and groESL genes.[1] This work provided the classification for these organisms that is used today. This study emended the classification of the family *Anaplasmataceae* to include species from the genera *Ehrlichia*, *Anaplasma*, *Neorickettsia*, and *Cowdria*, and the family *Rickettsiaceae* to include only the genera *Rickettsia* and *Orientia*.[1]

Ehrlichia species

In the genus *Ehrlichia*, several species are known to infect the dog including *Ehrlichia canis*, *E. chaffeensis*, and *E. ewingii*. *Ehrlichia canis* was first discovered in 1935[2,3] and later became a significant concern for the United States military during the Vietnam War, where hundreds of dogs were becoming severely ill or dying as a result of *E. canis* infections.[2] *E. canis* is a small, gram negative coccoid to ellipsoidal bacteria that is known to infect canine mononuclear cells. This organism is usually found contained in intracytoplasmic vacuoles in the form of morulae, which are clusters of organisms. This organism has been found worldwide. *Ehrlichia chaffeensis* is the causative agent for human monocytic ehrlichiosis. It was first

identified in US Army reservists that presented for acute illness to a medical clinic at Fort Chaffee, Arkansas in 1990.[4] Like *E. canis*, *E. chaffeensis* is a small, gram negative coccoid to ellipsoidal bacteria that also has been shown to infect canine mononuclear cells. However, unlike *E. canis*, this organism is not typically associated with severe disease in dogs, which could implicate dogs as a natural host for this organism.[5] The distribution of *E. chaffeensis* is similar to that for *E. canis*.[6,7] *Ehrlichia ewingii*, like *E. canis* and *E. chaffeensis*, is a small, gram negative coccoid to ellipsoidal bacteria. However, *E. ewingii* differs from *E. canis* and *E. chaffeensis* because it typically infects granulocytic cells such as neutrophils and eosinophils as opposed to mononuclear cells. The first case report regarding this organism was in 1971 from a dog in Arkansas.[8] Later, in 1994, PCR was used to identify this organism from infected dogs.[9] Similar to *E. canis*, dogs infected with *E. ewingii* can develop significant illness, although *E. ewingii* infection is rarely fatal. This organism is typically thought to be found in the southeastern and southern United States, but there is PCR evidence of *E. ewingii* in ticks and small mammals in the Republic of Korea (ROK).[10,11]

Anaplasma species

The genus *Anaplasma* has two species that are known to infect dogs; *Anaplasma phagocytophilum* and *A. platys*. Like *E. ewingii*, *Anaplasma phagocytophilum* is a small, gram negative coccoid to ellipsoidal bacteria that mainly infects granulocytic cells, with neutrophils predominating over eosinophils. However, unlike *E. ewingii*, *A. phagocytophilum* is distributed in the United States mainly in the Northeast, upper Midwest, and in northern California.[12] This organism has also been identified in dogs in Europe and South America.[13,14] *Anaplasma platys* is also a small, gram negative coccoid to ellipsoidal bacteria, which differs from *A. phagocytophilum* with respect to the cells it infects; platelets as opposed to granulocytes. *A.*

platys was first reported in dogs in the United States in 1978,[15] and since that time the global distribution of this organism has been shown to be similar to that of *E. canis*,[16-18] to include finding DNA evidence of *A. platys* in rodents and small mammals in the ROK.[10,19]

Rickettsia species

Other organisms in the order *Rickettsiales* that are important canine tick-borne pathogens are the Spotted Fever Group (SFG) *Rickettsiae*, of which *Rickettsia rickettsii* is of importance in the United States[20,21] and Brazil[22,23] while *R. japonica* is of importance in Japan.[24] Both *R. rickettsii* and *R. japonica* DNA have been identified in the ROK.[25,26] The illness caused by the SFG *Rickettsiae* dates all the way back to the late 19th Century and was first described by Howard T. Ricketts in 1909.[21] These organisms, similar to those described previously, are small, gram negative coccoid to ellipsoidal bacteria, but unlike organisms from the family *Anaplasmataceae*, which infect blood cells, the SFG *Rickettsiae* usually infect vascular endothelial cells.

Borrelia burgdorferi

Besides *Rickettsiales*, bacteria from another order, *Spirochaetales*, namely the genus *Borrelia*, are known to be transmitted to dogs via arthropods. The condition caused by this organism, known as Lyme disease, was first described in humans in 1975 in Lyme, Connecticut,[27] and was subsequently associated with similar clinical signs in a dog in 1984.[28] *Borrelia* are small, spiral shaped, gram negative organisms that are best visualized by dark-field microscopy. Lyme disease has been reported mostly in Europe and the US,[12] with the Northeastern, upper Midwestern, and Western areas being the main areas for occurrence in the United States.[29] Recently a suspected case of Lyme borreliosis was noted in a dog in the ROK.[30]

Babesia species

In addition to bacterial organisms, protozoal organisms from the order *Haemosporidia*, family *Babesiidae,* genus *Babesia* are also known to be transmitted by the bite of infected ticks. The two most common forms of *Babesia* species found in dogs are *B. canis* and *B. gibsoni*, with both species being parasites of red blood cells, but with *B. canis* seen as much larger piriform structures in the cytoplasm of red blood cells compared to *B. gibsoni*. *Babesia* organisms are found worldwide[31] including reports of *Babesia gibsoni* identified in dogs in the ROK.[32,33]

Ticks as Vectors

There are a large number of tick species throughout the world. Ticks belong to the order *Ixodida*, with two primary tick families represented: *Argasidae* (soft ticks) and *Ixodidae* (hard ticks), with the *Ixodidae* being the most important for transmission of tick-borne infections. The majority of the *Ixodidae* are three-host ticks, meaning each stage feeds on a different host. One exception is the brown dog tick, *Rhipicephalus sanguineus*, which can complete its entire life cycle on dogs. This tick is also unique because it can complete its entire life cycle totally indoors.[34] In the United States, other common ticks besides *Rhipicephalus sanguineus* include *Dermacentor variabilis* (American dog tick), *Dermacentor andersoni* (Rocky Mountain wood tick), *Dermacentor occidentalis* (Western dog tick), *Amblyomma americanum* (Lone Star tick), *Amblyomma maculatum* (Gulf Coast tick), *Ixodes scapularus* (black-legged tick), and *Ixodes pacificus* (Western black-legged tick).[35,36] Most of these ticks, however, are not common to eastern Asia. In Japan, *Haemaphysalis longicornis* is the most common tick, but other tick species are found in lesser numbers and include other *Haemaphysalis* spp., *Rhipicephalus sanguineus*, and several *Ixodes* spp.,[37] while in the ROK, *H. longicornis* is by far the most common species identified with fewer numbers of other *Haemaphysalis* spp. and *Ixodes*

spp.,[11,19,26] and in China, *H. longicornis* and several *Rhipicephalus* spp. are of concern for transmission of tick-borne pathogens.[38]

Rhipicephalus sanguineus

As stated previously, *R. sanguineus* is able to complete its entire life cycle indoors, which makes this tick a concern in kennels and homes.[36] Although the natural host for *R. sanguineus* is the dog, it has been known to feed on other hosts including humans, cats, rodents, and birds.[39] The life cycle of this tick, from egg to adult, can be completed in as little as two months with favorable conditions.[40] The mode of transmission of organisms in this tick is transstadial, meaning the organism is passed from infected larva to nymph and from infected nymph to adult. There is no transovarial transmission, passing organisms from adult females to the eggs, in this species. *R. sanguineus* is known to be the vector for several organisms including *Anaplasma platys*, *Babesia canis*, *B. gibsoni*, and *Ehrlichia canis*.[41] Recently, it was discovered to be responsible for the transmission of *Rickettsia rickettsii* in humans and dogs in northeastern Arizona.[20,42]

Dermacentor species

Dermacentor spp. are typical *Ixodidae*, requiring three different hosts for completion of their life cycle. Depending on the species and climate, the life cycle can be completed anywhere from 3 months to 2 years. Unlike other *Ixodidae*, the larvae of *Dermacentor* spp. only infest rodents and do not feed on dogs, but nymphs and adults have been known to feed on dogs, cats, cattle, deer, and humans.[35] Transstadial transmission along with transovarial transmission occurs in *Dermacentor* spp.[21] This species of tick has been implicated in the transmission of many organisms including *Babesia canis, Ehrlichia chaffeensis*, and *Rickettsia rickettsii*.[43]

Amblyomma species

Amblyomma spp., similar to *Dermacentor* spp., are typical three-host ticks. The best recognized tick in this genus is the Lone Star tick (*Amblyomma americanum*) which has a characteristic white spot on its back. These ticks are usually active and feeding in the spring and fall, and the life cycle normally takes two years to complete, but can be completed in one year if conditions are favorable. The preferred host for *Amblyomma* ticks is the white-tailed deer, with all stages potentially using this host for feeding.[36] When white-tailed deer are not available, the larvae and nymphs will feed on ground birds like wild turkey and quail, and have also been found feeding on rabbits, squirrels, raccoons, and foxes. The adults will feed on larger mammals like cattle, horses, and sheep. All stages may feed on dogs, cats, and humans.[41] *Amblyomma* ticks can transmit *Ehrlichia chaffeensis* and are the only confirmed ticks that can transmit *E. ewingii*.[41,44]

Ixodes species

Ixodes spp. are similar to *Amblyomma* spp. because of a preference for the white-tailed deer as a host. *Ixodes scapularis* is found mainly in the eastern and central United States while *I. pacificus* is found principally in the western portion.[36] Unlike other *Ixodidae*, completion of the life cycle takes at least 2 years. *I. scapularis* larvae will feed on small mammals, birds, and lizards, and nymphs will typically feed on mice, squirrels, raccoons, opossums, cats, and humans.[41] Of considerable importance in the transmission of *Borrelia burgdorferi* is the white-footed mouse, which is thought to be the reservoir for this organism.[29] Adults of this species feed primarily on white-tailed deer, but will select other hosts such as dogs or humans if no deer are available.[36] *I. pacificus* larvae and nymphs will feed mainly on lizards, but may select alternate hosts such as small rodents, birds, or humans if a lizard is not available. *I. pacificus*

adults are normally found on deer or elk but will utilize humans, dogs, cats, or other larger mammals as hosts if deer are not available.[41] Only transstadial transmission of organisms occurs in Ixodes spp.[29] Both *I. scapularis* and *I. pacificus* are known to transmit both *Borrelia burgdorferi* and *Anaplasma phagocytophilum*.[41]

Haemaphysalis species

Haemaphysalis ticks are similar to the previously mentioned *Ixodidae* ticks that are found in the United States. These ticks commonly feed on medium to large mammals and birds during all of their life stages.[45] Common hosts from the medium to large mammal group include cattle and deer.[46,47] There is also evidence of these ticks infesting dogs and cats[37,47] as well as small mammals and mustelids,[10,11] and also humans.[48] *Haemaphysalis* ticks have been known to transmit *Rickettsia japonica* and *Babesia gibsoni*[37,46] however, DNA has been identified from ticks collected in the ROK for *Anaplasma phagocytophilum, A. platys, Ehrlichia canis*, *E. chaffeensis*, and *E. ewingii*.[11,19]

Diagnosis of Tick-Borne Infections

As discussed in previous sections, many tick-borne organisms are found to infect dogs. These infections can cause a myriad of clinical signs which can range from apparently healthy dogs to ones with severe illness.[5,21,44,49,50] When considering diagnostic testing for tick-borne infections, one must take into account several factors including living in an endemic area, known exposure to a tick, and appropriate clinical and biochemical abnormalities.[51] If signs and symptoms are consistent for a tick-borne infection, then further diagnostic testing with either an enzyme-linked immunosorbent assay (ELISA), indirect Immunofluorescence antibody (IFA) test, or polymerase chain reaction (PCR) test would be indicated. Western immunoblotting has been used previously for differentiating between several species of organisms, but with PCR

becoming more readily available, it has replaced Western blots.[43] Culturing some tick-borne organisms is possible, but this is generally difficult and cell cultures are often required, limiting the usefulness of this procedure.[43] Limiting testing to when there is a likelihood of disease will increase the positive-predictive value of the tests.[52] Diagnosis of tick-borne infections can be difficult because dogs often present with varying clinical signs, and co-infection with multiple organisms is possible.[34]

Canine Monocytic Ehrlichiosis

Canine monocytic ehrlichiosis is caused by *Ehrlichia canis* or *E. chaffeensis*, but, since *E. chaffeensis* infected dogs rarely develop clinical signs except for thrombocytopenia, this discussion will focus mainly on *E. canis*. Infection with *E. canis* is typically thought of as occurring in 3 phases; acute, sub-clinical, and chronic. The acute phase is characterized by fever, anorexia, lethargy, depression, ecchymosis, petechia, enlarged lymph nodes, and weight loss.[51] This phase typically last from two to four weeks. Clinical laboratory abnormalities commonly seen in the acute phase include anemia and elevated liver enzymes.[7] Around 4% of dogs will have morulae present on a blood smear.[53] Unless appropriate treatment is rendered, dogs will enter the sub-clinical phase in which they appear to be healthy. This phase can last months to years. Once these dogs progress to the chronic phase of infection, the prognosis is poor.[7] Dogs in the chronic phase will have severe illness with clinical signs similar to the acute phase, however, laboratory results will be significantly worse and can include pancytopenia, hyperglobulinemia (usually polyclonal but occasionally will be monoclonal), and elevated renal values.[54] Dogs in the chronic phase often suffer from bone marrow hypoplasia, which causes them to die from bacterial septicemia, severe bleeding, or both.[55] It has been noted that German shepherd dogs are more prone to developing severe forms of disease compared to other breeds,

which was found to be related to depressed cell-mediated immunity.[56] Diagnosis of canine monocytic ehrlichiosis can be difficult. Serology is often the initial diagnostic testing modality, with IFA being considered the "gold standard."[57] IgM antibodies appear from 4-7 days post-infection with class switching to IgG antibodies occurring around 15 days post-infection. Dogs in the sub-clinical and chronic phases will continue to have high IgG titers.[56] ELISA tests are available for *E. canis* antibody identification. Multiple ELISAs have been developed including ones that use whole cultured organisms as a source of antigen, one that uses the *E. canis* recombinant major antigenic protein 2 as the antigen source, and one that targets the *E. canis* p30 and p30-1, which are proteins specific to *E. canis*.[58] Serology results for *E. canis* can be difficult to interpret, given that there is significant cross-reactivity between other ehrlichial organisms, with *E. chaffeensis* having the most pronounced cross-reactivity.[56] Therefore, to accurately diagnose active *E. canis* infection, PCR amplification of specific DNA sequences for *E. canis* may be necessary. Unfortunately, if a small amount of DNA is present, negative PCR results may not completely rule out *E. canis* infection.[52]

Canine Granulocytic Ehrlichiosis

Canine granulocytic ehrlichiosis and anaplasmosis are caused by *E. ewingii* and *A. phagocytophilum* respectively. The most common clinical signs of these conditions include fever, lethargy, anorexia, and lameness, while less frequently gastrointestinal signs including vomiting and diarrhea have been noted.[44,50,59,60] By far the most common laboratory abnormality is thrombocytopenia, but other common findings include anemia, hypoalbuminemia, and elevated liver enzymes.[44,60] The diagnosis of *E. ewingii* can be difficult in most cases because the organism has not been cultured successfully and because of high false-negative results for direct visualization of morulae in infected cells.[61] There is no specific serologic assay for *E.*

ewingii,[62] but there can be cross-reactivity with *E. canis* assays.[44,59,63] Therefore, PCR amplification of *E. ewingii* needs to be performed for accurate diagnosis.[61] *A. phagocytophilum*, unlike *E. ewingii*, can be cultured from blood, but this is not routinely used for diagnosis.[50] Routine diagnostics for *A. phagocytophilum* consist of ELISA, IFA or PCR analysis. ELISA and IFA have been shown to cross-react with *A. platys* antibodies, and the finding of morulae in granulocytes is not definitive for *A. phagocytophilum*, therefore, as with *E. ewingii*, PCR is necessary for definitive diagnosis.[50] PCR for *A. phagocytophilum* is routinely performed for msp2 or 16S rRNA genes, but other genes have been used for this organism as well.[10,49,50,64]

Canine Cyclic Thrombocytopenia

Canine cyclic thrombocytopenia is caused by *Anaplasma platys*. This condition is often sub-clinical, but clinical signs such as fever, depression, and lethargy have been observed and may spontaneously resolve.[35] *A. platys* results in a cyclic thrombocytopenia that may be severe enough to cause clinical signs of bleeding such as petechia and ecchymosis.[16] Diagnosis of *A. platys* can be done by visualization of the morulae in platelets, but decreased numbers of platelets and the cyclic nature of this condition make false-negative results common.[18,65] The IFA for serum antibodies is the test of choice for *A. platys* diagnosis, but cross-reactivity with *A. phagocytophilum* antibodies is possible.[66] Therefore, given the previously mentioned difficulties with diagnosis, sensitive and specific protocols for PCR amplification of *A. platys* DNA have been developed for definitive diagnosis of this condition.[17,18,66]

Canine Rocky Mountain Spotted Fever

Rocky Mountain spotted fever is caused by the organism *Rickettsia rickettsii*. There are other *Rickettsia* spp. that cause similar conditions to those of Rocky Mountain spotted fever. These include *R. japonica*, which causes Japanese spotted fever, and *R. conorii*, the causative

agent for Mediterranean spotted fever. Since all Spotted Fever Group (SFG) Rickettsiae cause similar disease, this section will focus on *R. rickettsii*. Clinical signs consistently seen with *R. rickettsii* infection include fever, petechia and ecchymosis, along with edema of the extremities. Other less common clinical signs anorexia, polyarthritis, vestibular deficits,[21] as well as neurologic signs such as tetraparesis, ataxia, and seizures.[67] Typical clinical laboratory abnormalities include thrombocytopenia, elevated white blood count, anemia, hypoproteinemia (mainly hypoalbuminemia), and elevated alkaline phosphatase.[67,68] Diagnosis of *R. rickettsii* is done via serology with IFA and/or ELISA. IgG antibodies develop within one to three weeks of infection, therefore, if it is early in the course of infection, false-negative results may be obtained.[68] With that being known, testing for IgM antibodies via IFA, with a titer of greater than or equal to 64, is considered diagnostic.[21] Identification of organisms by immunofluorescence from a biopsy specimen or by amplification of *Rickettsia* spp. DNA by PCR are also considered diagnostic.[21,67]

Canine Lyme Disease

Lyme disease is caused by *Borrelia burgdorferi*. The most common clinical signs in dogs are fever, lameness, and anorexia.[29] No specific laboratory abnormalities are noted in dogs with Lyme disease, but proteinuria is commonly seen in dogs that have positive titers for *B. burgdorferi*.[27] Lyme nephropathy is an unusual presentation for dogs with Lyme disease, and Labrador retrievers and Golden retrievers are over-represented.[69,70] It is suspected that this is an immune-mediated nephropathy, but it is unclear why some dogs develop this condition and others do not. Dogs with Lyme nephropathy present with acute renal failure, and many of these dogs die within days to weeks.[27] Whole-cell-based ELISA and Western blots are available to diagnose Lyme disease, but tests utilizing a protein from an invariable region of an outer

membrane lipoprotein (C6) have been shown to be as good or better than the whole-cell-based tests. A qualitative ELISA utilizing the C6 sequence is available for in-house testing, and a quantitative C6 ELISA is available in a laboratory setting. This test can not only be used for diagnosis, but it can also be utilized to monitor treatment success by documenting a substantial drop in antibody levels.[71] PCR is available for diagnosis of Lyme disease, but since the organism is not commonly found in blood, urine, or joint fluid, false-negative results may be common. Therefore, serologic tests are usually performed.[27]

Canine Babesiosis

Canine babesiosis may be caused by many *Babesia* spp., with the most common being *Babesia canis* and *B. gibsoni*. Three subspecies of *B. canis* are known: *B. canis canis*, *B. canis rossi*, and *B. canis vogeli*.[72] *B. canis vogeli* has been shown to be present in a large number of racing greyhounds particularly in the southeastern United States, and American Pit Bull Terriers seem to be over-represented with respect to *B. gibsoni* infections.[73] There is evidence suggesting that *B. gibsoni* can be transmitted by dog bites, and there is also evidence that both *B. gibsoni* and *B. canis* organisms can be transmitted through contaminated blood transfusions.[73-75] Of the *B. canis* organisms, *B. canis rossi* causes the most severe disease, while infection with *B. canis canis* is often subclinical. Infection with *B. canis vogeli* can cause a wide range of clinical signs.[76] Clinical signs from *B. canis* infections can vary, but typical signs, when present, include lethargy, depression, anorexia, fever, pale mucus membranes, and jaundice.[51,72] Clinical signs seen with *B. gibsoni* infection include fever, lethargy, and depression; however, dogs that survive the acute phase often become carriers.[77] Typical abnormalities seen on laboratory tests include hemolytic anemia, thrombocytopenia, and elevated liver enzymes.[51] Diagnosis of canine babesiosis is often made by visualization of the organisms on a blood smear.[72] Serologic testing

for *Babesia* spp. is available, as well as PCR testing. Unfortunately there is no proven "gold standard" test for diagnosing canine babesiosis.[73] Serologic results may be difficult to interpret because antibodies may not be present early in the course of infection, antibodies may have cross-reactivity, or the animals may live in an endemic area. PCR has helped, but DNA may be lower than the detectable limit on any given test.[72]

One final point to mention when considering tick-borne infections is the screening of potential blood donors. The incidence of transmission of tick-borne organisms via blood transfusion is low,[74] but *B. gibsoni* has been successfully transmitted experimentally to a dog from infected blood and has been associated with a blood transfusion in a German shepherd.[75] Therefore, screening of potential blood donors should consist of at least screening for *Babesia* spp., and screening for *Ehrlichia* and *Anaplasma* spp. should strongly be considered.[74]

Treatment and Prevention of Tick-Borne Infections

Ehrlichiosis and Anaplasmosis

Once a diagnosis of a tick-borne infection has been made, it is imperative to institute appropriate therapy quickly. For ehrlichiosis and anaplasmosis, tetracycline antibiotics are usually the preferred treatment.[66,78-80] According to the 2002 American College of Veterinary Internal Medicine Consensus Statement regarding ehrlichial disease, the recommendation is to prescribe doxycycline at 10 mg/kg per day orally for 28 days.[79] Other tetracycline antibiotics may be effective as well including tetracycline, oxytetracycline, and minocycline. However, doxycycline is associated with fewer side effects and has the convenience of once or twice daily dosing as opposed to other tetracyclines that require dosing three times daily.[81] There is some controversy regarding the successful clearance of *Ehrlichia canis* infection with doxycycline treatment. Several studies have either isolated organisms or recovered DNA evidence of

infection after appropriate treatment with doxycycline.[80,82,83] In a more recent study, however, it was shown that doxycycline treatment was effective at clearing organisms from experimentally infected dogs based on PCR of blood and tissues including liver, spleen, and bone marrow.[66] Other drugs that have been used successfully include chloramphenicol and imidocarb dipropionate.[78,79] Unfortunately, a recent study showed that treatment with two intramuscular doses of imidocarb dipropionate given at 6.6 mg/kg two weeks apart failed to clear the organism from dogs experimentally infected with *E. canis*.[81] Treatment success for ehrlichiosis and anaplasmosis should include resolving clinical signs and normalization of the complete blood count.[78] Serology, noting a drop in titers, may be used to monitor treatment success; however some successfully treated dogs may continue to have high titers, therefore PCR would be a more reliable indicator that the organism has been cleared.[78,79,83]

Canine Rocky Mountain Spotted Fever

Therapy for Rocky Mountain spotted fever is similar to that for ehrlichiosis and anaplasmosis. Treatment with tetracycline antibiotics as well as chloramphenicol for one week results in a successful outcome for most dogs.[21,67] Unlike for ehrlichiosis and anaplasmosis, enrofloxacin has been shown to be an effective alternative to tetracycline antibiotics to treat Rocky Mountain spotted fever.[67,79] Prompt therapy with appropriate antibiotics prior to tissue necrosis or coagulopathies will decrease the mortality associated with Rocky Mountain spotted fever.[21]

Canine Lyme Disease

As with the previously mentioned conditions, the treatment of choice for Lyme disease is doxycycline at 10 mg/kg per day for at least one month.[27,29] Other antibiotics that have been used successfully to treat Lyme disease include amoxicillin, azithromycin, and ceftriaxone, with

the latter recommended for dogs with neurologic signs.[27,29,71] Treatment should be monitored for a resolution of clinical signs including resolution of lameness and proteinuria.[27] A recent study using a quantitative C6 ELISA showed that antibodies for the C6 peptide dropped at six months in treated animals compared to baseline, suggesting that this test may be useful for determining success of treatment.[71]

Canine Babesiosis

Treatment of babesiosis depends on the species of the organism infecting the dog. *Babesia canis* infections seem to be cleared easily while complete elimination of *Babesia gibsoni* is not easily or commonly achieved. The treatment of choice for *B. canis* infection is imidocarb dipropionate, which is usually effective in eliminating the organism.[72,75] However, for *B. gibsoni* infections, imidocarb will alleviate clinical signs of infection, but it will not eliminate the organism.[75,84,85] Multiple drugs have been used to treat *B. gibsoni* infections including atovaquone, azithromycin, doxycycline, metronidazole, and clindamycin, with little success in clearing the organism.[72,75,86-89] It has been suggested that the combination treatment with atovaquone and azithromycin may have a good chance of curing the infection,[85] but another study found that this combination therapy did not eliminate the parasite.[86] A triple drug combination therapy has had some efficacy in treating *B. gibsoni* infection, however, this combination took a long time until clinical improvement was seen.[90] Therefore, regardless of the treatment protocol used, owners should be cautioned that a chronic carrier state may exist.

Organophosphate compounds

Rather than attempting to treat a tick-borne infection after it occurs, it is advisable to consider the implementation of tick-prevention strategies to reduce the likelihood of a tick-borne infection. Many options are available and include the use of dips, collars, spot-on preparations,

and vaccinations. Prior to the advent of spot-on preparations, organophosphate dips were the treatment of choice.[35] Organophosphate compounds are neutral esters of phosphoric acid which act by inhibiting the action of acetylcholinesterase at cholinergic synapses and neuromuscular junctions.[91] These compounds are active against ticks, but they can be extremely toxic to animals.[91] Hence, alternative compounds have been developed to prevent and control tick infestations while minimizing the deleterious side effects that occur.

Amitraz

One such compound for tick prevention is amitraz. This comes in amitraz impregnated collars or amitraz containing spot-on preparations. Amitraz affects the control of the nervous system of ticks by inhibiting mixed function oxidases, preventing tick attachment and feeding.[36,92] This chemical has also been shown to be effective in repelling ticks.[93] Additionally, amitraz has proven to reduce hatchability of eggs and to decrease the number of surviving and feeding larvae.[92,93] In experimental and natural conditions, amitraz impregnated collars were shown to be effective at preventing feeding, repelling, and killing the brown dog tick, *Rhipicephalus sanguineus*.[93,94] Besides a collar preparation, amitraz is also available in a spot-on preparation combined with metaflumizone. This product has been shown to be highly effective in controlling existing tick infestations as well as preventing reinfestation.[95]

Pyrethrins and Synthetic Pyrethroids

Pyrethrins and synthetic pyrethroids, namely deltamethrin and permethrin, are also used for their acaricidal properties. These compounds work by modulating sodium channels in the nerves, resulting in repetitive nerve discharges and ultimately death of the tick.[36] Synthetic pyrethroids not only quickly kill ticks but also have tick repellant activity as well.[36,96-99] In experimental studies, deltamethrin collars were proven to be effective for greater than five

months at controlling infestations with *Ixodes ricinus* and *Rhipicephalus sanguineus* ticks.[100] There have been numerous studies looking at the efficacy of a spot-on preparation of imidacloprid plus permethrin with regards to control of ticks.[96,97,99,101-105] These studies found this combination to be effective for control of tick infestations for multiple species of ticks for a three to four week period post-application.

Fipronil

Fipronil is another compound that has been found to be an effective acaricide. It comes in either a spray or spot-on formulation. Fipronil acts on gamma-aminobutyric acid and glutamate-gated chloride ion channels in the tick nervous system.[36] This compound has been shown to be effective in the control of tick infestations for up to four weeks.[96,97,99,101-105] However, unlike the imidacloprid plus permethrin combination, fipronil did not demonstrate any tick-repellant activity.[96-99]

Selamectin

Another compound useful in tick control is an avermectin called selamectin. This chemical causes neuromuscular paralysis by altering glutamate-gated channels, increasing chloride permeability.[106] Selamectin has been shown to give excellent control of *Rhipicephalus sanguineus* and *Dermacentor variabilis* for up to 30 days, with efficacy being enhanced by the addition of a second dose fourteen days after the initial dose. Unfortunately, is did take up to five days for tick counts on the dogs to drop significantly.[107] Like fipronil, selamectin has not been shown to have any repellant activity.[106]

Vaccinations for Tick-Borne Infections

A different approach to the prevention of tick-borne infections besides chemicals is the use of vaccinations. Currently, there are only two tick-borne organisms that have commercial

vaccines available for use in dogs; *Borrelia burgdorferi* and *Babesia canis*.[27,29,35,43,72,108-110] There was a human vaccine developed by the US Army for Rocky Mountain spotted fever, but it had a high rate of reactions among vaccinates, thus it is no longer available.[2] Work has been done attempting to develop a vaccine against ticks. Although this work has been promising, no anti-tick vaccine is currently available.[40,111] It should be reinforced that vaccines should be used only as one part of tick-borne disease prevention and that routine application of topical acaricides and routine grooming should still be included.[35]

Canine Lyme Disease Vaccines

There are multiple vaccine types available for Lyme disease prevention in dogs; bacterins (monovalent and bivalent) and recombinant outer surface protein (Osp) A vaccines (adjuvanted and nonadjuvanted).[27,29,35,43] Osp A is expressed by *B. burgdorferi* only in the midgut of the tick, therefore antibodies against Osp A must be ingested by the tick during feeding. These antibodies are thought to work by complement-mediated lysis of *B. burgdorferi* in the tick.[29,108] These vaccines have shown some efficacy, however vaccine failure is possible because *B. burgdorferi* will undergo a shift from Osp A to Osp C as the tick begins feeding.[108] Previous bacterins have failed to demonstrate anti-Osp C bands on Western blots.[27] Recently a seven amino acid portion of the carboxy terminus of Osp C has been identified that appears to be conserved over many *Borrelia* spp. This has resulted in the development of a bivalent bacterin vaccine against *B. burgdorferi* that induces both anti-Osp A and anti-Osp C antibodies. It is hoped that this new vaccine will improve the efficacy of vaccination for canine Lyme disease.[108]

Canine Babesiosis Vaccine

A vaccine against *Babesia canis* infection has been developed using soluble parasite antigens from cultures of *B. canis* and *B. rossi*.[72,109,110] Unfortunately this vaccine does not

prevent infection, however, it does reduce the parasite load and the severity of anemia and splenomegaly associated with infection.[43,72,109] Also, this vaccine does not protect against other *Babesia* spp. and is not available in the United States.[43,110]

At the time of this writing, there were no published prevalence studies from dogs in the Republic of Korea (ROK). The primary goal of this retrospective study was to determine the seroprevalence of tick-borne infections in the military working dog (MWD) population in the ROK. In addition, we hoped to determine whether there was a breed, sex, or age predisposition for these various tick-borne infections, and if location of the MWDs on the peninsula was correlated with the seroprevalence. A final objective was to compare the seroprevalence of tick-borne infections in recent years, when fipronil has been the primary flea and tick preventative used in MWDs, to the seroprevalence in a previous year when monthly organophosphate dips were used.

CHAPTER 2

SEROPREVALENCE OF TICK-BORNE INFECTIONS IN MILITARY WORKING DOGS IN THE REPUBLIC OF KOREA[1]

[1] Bell, D.R., Berghaus, R.D., Patel, S., Beavers, S., Fernandez, I., Sanchez, S. To be submitted to *American Journal of Veterinary Research*.

Abstract

Objective – To determine the seroprevalence of tick-borne infections in the military working dog (MWD) population in the Republic of Korea (ROK).

Sample Population – 182 serum samples were available from MWDs during 3 different years (1996, 2002, and 2007). In addition, 63 whole blood samples from 2007 were available for PCR.

Procedure – Serum samples were evaluated by a commercially available ELISA and IFA for *Ehrlichia canis* and *Anaplasma phagocytophilum*, and by ELISA only for *Borrelia burgdorferi*. PCR amplification of DNA was performed to screen for multiple tick-borne organisms using previously published primers and probes.

Results –A total of 56 (30.8%) MWDs were positive by at least one serologic test. Seroprevalences for *Anaplasma* and *Ehrlichia* were 4.4% and 0.6% based on the ELISA, and 24.7% and 22.5% based on the IFA, respectively. ELISA testing for Borrelia yielded 2 (1.1%) positive results. In parallel testing using both the ELISA and IFA tests, the percentage of dogs with one or more positive results was 34.1%, 25.9%, and 28.4% for 1996, 2002, and 2007, respectively. There was no statistical difference in seroprevalence based on location, year, breed, or sex of the MWD. There was poor agreement between IFA and ELISA test results. No MWD samples had a positive PCR result.

Conclusions and Clinical Relevance – MWDs stationed in Korea had serologic evidence of exposure to several tick-borne pathogens, but PCR testing did not identify any active infections.

Introduction

Ticks and tick-borne infections are a problem of global concern.[2,112-118] The US Army first recognized the importance of tick-borne infections for military working dogs (MWDs) during the Vietnam War when many MWDs were becoming ill or dying from an unknown condition which was later determined to be caused by *Ehrlichia canis*.[2] Since that time, there have been many advances in diagnosis, prevention and treatment of tick-borne infections. Even with these advances, tick-borne infections are still persistent world-wide.

Military Working Dogs (MWDs) are important for all branches of the United States military. These highly trained animals provide such services as explosive, mine, and drug detection; security and patrol; search and rescue; and guard duty. The tasks these dogs perform are critical to the fight in the global war on terrorism, demonstrated by the MWD presence in both Operation Iraqi Freedom and Operation Enduring Freedom.[a] Therefore, the health and well-being of these indispensable animals is of the upmost importance, which prompted the development of strict guidelines for their care and management. Proper procedures for preventative care and record-keeping are documented in The Handbook of Veterinary Care and Management of the Military Working Dog. To help control tick-borne infections, MWDs are required to have a monthly topical treatment with a commercially available flea and tick-prevention product. [b] Many studies looking at the efficacy of fipronil [c] along with other more commonly used preventatives, i.e. imidacloprid and permethrin[d] and amitraz collars[e], have shown that these products are not 100% effective.[94,98,99,102,103] These products are marketed to offer residual protection against tick infestation for up to 30 days.

There are several published reports from the Republic of Korea (ROK) of ticks, small mammals, and rodents harboring organisms that are considered tick-borne

pathogens.[6,10,11,19,26,119] These studies have demonstrated the presence of *Ehrlichia canis*, *E. chaffeensis*, *E. ewingii*, *Anaplasma phagocytophilum*, *A. platys*, *Borrelia burgdorferi*, *Rickettsia rickettsii*, *R. japonica*, as well as several others. By far, the most common tick identified in these studies is the *Haemaphysalis longicornis* tick, but other *Haemaphysalis* species and several *Ixodes* species of ticks are present. There have been several case reports of tick-borne infections in wild animals, dogs, and humans in the ROK.[25,30,33,120-126] Given the fact that tick-preventative measures may not be 100% effective, the possibility of dogs serving as a potential reservoir for tick-borne infections for humans and the risk of illness in the MWD population are valid concerns. These facts raised questions concerning the prevalence of tick-borne infections in the MWD population in the ROK. The MWDs are sent to the ROK immediately after initial training at Lackland Air Force Base in San Antonio, Texas, and these animals rarely if ever leave the Korean peninsula. All MWDs are screened for tick-borne infections by IFA prior to departure from Lackland Air Force Base, ensuring that any tick-borne infection detected in the animals stationed in the ROK would have been acquired after arrival on the peninsula.

To date, there have been no published prevalence studies regarding tick-borne infections in dogs in the ROK. Our primary objective of this retrospective study was to determine the seroprevalence of tick-borne infections in the MWD population in the ROK. We further wanted to examine whether there was a breed, sex or age predisposition for these various tick-borne infections, and if location of the MWDs on the peninsula was correlated with the seroprevalence. We also sought to compare the seroprevalence of tick-borne infections in recent years, when fipronil has been the primary flea and tick preventative used in MWDs, to the seroprevalence in a previous year when monthly organophosphate dips were used.

Materials and Methods

Sample selection

The analysis included serum samples from three separate years; 1996, the year prior to the use of fipronil; and from 2002 and 2007, when fipronil was used. All MWDs are required to have blood drawn on an annual basis for banking purposes. These samples are submitted to the Food and Animal Diagnostic Laboratory (FADL), Fort Sam Houston, TX, for storage. In June 2008, all samples from MWDs stationed in the ROK for the above mentioned years were identified. Samples were excluded if there was insufficient serum available for analysis. When multiple submissions were available for an individual dog in the same year, only the first submitted sample was utilized for analysis. In the instance that an individual dog was present in multiple years, the only sample included for analysis was from the first year that serum was available for that animal. All samples from later years were excluded. Serum samples were drawn from the MWD at the duty location and shipped to FADL, where the samples were stored at -70^{o}C. Samples from all dogs for the study were identified and shipped by overnight delivery to the Athens Veterinary Diagnostic Laboratory where the samples were thawed, an aliquot of the serum was separated, and the remainder of the serum was returned to FADL for storage. The selected aliquots of serum were refrozen and stored at -70^{o}C until needed.

The only year during our study when EDTA anti-coagulated whole blood was banked from the MWDs in the ROK was 2007. As with the serum, samples were collected at the duty location of the MWD, shipped to FADL, and stored at -70^{o}C. The samples were thawed, aliquots of the EDTA anti-coagulated whole blood were collected, and the remainder of the sample was shipped back to FADL. If an animal had multiple banked EDTA anti-coagulated whole blood samples, only the first collected sample was utilized.

Serum and whole blood samples were collected from MWDs at eight US military locations throughout the ROK. Military installations that submitted samples during the study periods included Camp Casey, located in a rural, mountainous area in the northwest; Yongsan Garrison, located in the city of Seoul; Osan Air Base, located in a suburban area south of the city of Osan; Camp Humphreys, located in a suburban area near the city of Pyeongtaek; Kunsan Air Base, located on the southwestern coastline near the city of Kunsan; Camp Henry, located in the city of Daegu; Camp Carroll, located in a semi-rural area west of Daegu; and Camp Hialeah, located on the southeastern coast in the city of Pusan. (Figure 1) The location, age, breed, sex, and year of sample collection were recorded for each animal, if available.

Enzyme-Linked Immunosorbent Assay

ELISA tests, using a commercially available ELISA, were performed according to manufacturer instructions.[f] This ELISA-based test identifies IgM and IgG antibodies to *Ehrlichia canis*, *Anaplasma phagocytophilum*, and *Borrelia burgdorferi*, as well as antigen of *Dirofilaria immitis*. Test results were read at 8 minutes. Animals were recorded as positive for a particular antibody if a color change was present at the appropriate sample spot. In addition a sample was considered positive for *Dirofilaria immitis* antigen if the appropriate sample spot showed a color change. No color change was considered as negative. Tests having equivocal results were repeated. Tests were also repeated if the positive control spot showed no color change.

Indirect Immunofluorescence Assay

A commercially available microimmunoflouresence (MIF) assay[g] was used for detection of *E. canis* and *A. phagocytophilum* antibodies. All testing materials and sera were allowed to equilibrate to room temperature prior to testing. Serum samples were diluted using phosphate

buffered saline (PBS) to a dilution of 1:80. Only samples demonstrating fluorescence at this dilution were considered positive. Tests were performed according to manufacturer's instructions. Slides were read using a fluorescence microscope at 400x magnification. Sample wells were compared to the appearance of the positive and negative controls that were provided with the tests. End-point titers were not performed.

Polymerase Chain Reaction Assay

DNA was extracted from 200ul of EDTA anti-coagulated whole blood using a commercially available product according to manufacturer's instructions.[h] A housekeeping gene for canine glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was used as an extraction positive control.[127] All protocols used for PCR amplification were described elsewhere (Table 1). For detection of all *Anaplasma* and *Ehrlichia* species, real-time primers and a probe were utilized to amplify a portion of the 16S rRNA gene.[10] If any sample was positive on the real-time PCR for *Ehrlichia* or *Anaplasma* species, a conventional nested PCR protocol amplifying a portion of the 16S rRNA gene was utilized to identify the species of the organism. Organisms detectable using this nested PCR protocol include *Ehrlichia canis, E. chaffeensis, E. ewingii*, and *Anaplasma platys*.[128] Real-time primers and probes were used to amplify a portion of the *msp2* gene of *A. phagocytophilum* and a portion of the 23S rRNA gene of *Borrelia burgdorferi*.[64] For detection of *Rickettsia rickettsii*, real-time PCR was performed to amplify a portion of the *gltA* gene.[129] Conventional PCR was performed to amplify a fragment of the 18s rRNA gene common to *Babesia* and *Theileria* spp.[128] Master mix for PCR and the PCR set up were performed in separate biosafety cabinets to prevent potential contamination. Specific pathogen free water was used as a negative control for each PCR reaction. Positive controls were included with each PCR reaction to ensure the procedure was performed appropriately.

Statistical Analysis

Animals were grouped by age, sex, breed, duty location, and year of sampling. For statistical analysis, age was evaluated as a categorical variable with groups consisting of dogs from 1-4 years, 5-8 years, 9-12 years, and over 12 years of age. MWDs were placed into 1 of 3 groups according to breed; Belgian Malinois, German shepherd, and others. Duty locations were grouped according to geographic location. The northwestern region consisted of Camp Casey and Yongsan Garrison, the mid-western region consisted of Osan Air Base and Camp Humphreys, the southwest region contained only Kunsan Air Base, and the southeastern region consisted of Camp Henry, Camp Carroll, and Camp Hialeah. An exact test of homogeneity was used to evaluate the associations between prevalence and potential risk factors. McNemar's test was used to compare the proportions of positive results for ELISA and IFA tests performed on the same samples, and the Kappa statistic was used to estimate agreement. Seroprevalence was calculated by taking the number of dogs testing positive in each year divided by the number of dogs tested that year. All statistical analyses were performed using commercially available software.[i] A p-value < 0.05 was considered significant for all tests performed.

Results

Population Characteristics

A total of 182 dogs were included in the study. Sixty-two dogs were from the northwestern region [Yongsan Garrison (55) and Camp Casey (7)], 71 were from the mid-western region [Camp Humphreys (11) and Osan Air Base (60)], 30 were from the southwestern region [Kunsan Air Base (30)], and 19 were from the southeastern region [Camp Henry (8), Camp Hialeah (10), and Camp Carroll (1)]. There were 45 dogs in the 1-4 year age group, 74 in the 5-8 year age group, and 56 in the 9-12 year age group. Age could not be determined for 7

dogs. The breed distribution included 92 Belgian Malinois, 78 German shepherd dogs, and 12 others, which consisted of Dutch shepherds (8), Belgian shepherds (2), and Labrador retrievers (2). The sex distribution included 31 female dogs and 119 males. Sex was not reported for 32 dogs. There were 88 dogs enrolled from 1996, 27 from 2002, and 67 from 2007.

Serologic Testing

The results of serologic testing for *A. phagocytophilum* are summarized in Table 2. The ELISA test yielded significantly fewer positive results than did the IFA ($P < 0.001$), and the agreement between the two tests was poor (kappa = 0.12). When using the results of both tests in parallel, age was the only characteristic that was significantly associated with *A. phagocytophilum* prevalence, with dogs in the 1-4 year age group having the highest percentage of positive results.

The results of serologic testing for *E. canis* are summarized in Table 3. As was the case for *A. phagocytophilum*, the ELISA test yielded significantly fewer positive *E. canis* results than did the IFA ($P < 0.001$), and the agreement between the two tests was similarly poor (kappa = 0.04). None of the evaluated characteristics was significantly associated with *E. canis* prevalence.

Serologic testing for *B. burgdorferi* was performed using only the ELISA. Two (1.1%) dogs had a positive ELISA result for *B. burgdorferi*, with both being Belgian Malinois dogs sampled in 1996. One was a 10 year-old spayed female from Camp Hialeah, and the other was a 9 year-old dog of undetermined sex from Kunsan Air Base. All dogs were negative for *D. immitis* based on ELISA antigen testing.

No dogs had a positive ELISA result for more than one organism, while 33 (18.1%) dogs had positive IFA results for both *A. phagocytophilum* and *E. canis*. When considering the results

of all tests in parallel, the percentage of dogs having a positive result did not differ significantly between 1996 (34.1%), 2002 (25.9%), and 2007 (28.4%; P = 0.655). Likewise, the percentage of dogs with a positive result during the year when organophosphate dips were used as the primary preventative (1996) did not differ significantly from the percentage of positives during the combined years when fipronil was used for tick prevention (2002 and 2007) (34.1% versus 27.7%; P = 0.422).

Polymerase Chain Reaction Assay

There were 63 EDTA-anticoagulated whole blood samples available for PCR testing from 2007. All samples were negative for *Ehrlichia* spp. or *Anaplasma* spp. All positive controls displayed appropriate amplification. Furthermore, nested PCRs specific for *A. platys, E. canis, E. chaffeensis,* and *E. ewingii* were negative. In addition, PCR amplifications for *A. phagocytophilum, B. burgdorferi, R. rickettsii,* and *Babesia* and *Theileria* spp. were negative. Internal controls for all samples displayed appropriate amplification.

Discussion

This study utilized banked serum from 3 different years (1996, 2002, and 2007) from MWDs in the ROK for serologic testing for multiple tick-borne infections as well as banked whole blood from 2007 for PCR analysis for the presence of DNA from multiple tick-borne organisms. Given the large number of dogs with a positive IFA result in 2007, it was surprising to find that there were no PCR positive dogs. It is possible that the specificity of the IFA test was low, or that MWDs were exposed to ticks and tick-borne pathogens, but did not have an active infection at the time of sampling. The presence of DNA inhibitors, such as hemoglobin or immunoglobulins, may have prevented adequate amplification of DNA from any tick-borne organisms. DNA could have been present in quantities that were below the detection limit of the

assay, however, all internal controls performed as expected. Storage of the whole blood samples may also have adversely affected DNA quality, but frozen EDTA anticoagulated whole blood has previously been used successfully.[130,131]

There was a large discrepancy between the results of the IFA and ELISA testing. Based on the ELISA testing, 8 (4.4%) dogs were seropositive for *A. phagocytophilum* and 1 (0.6%) had a positive *E. canis* result. This is in contrast to the IFA testing, which identified 45 (24.7%) dogs that were seropositive for *A. phagocytophilum* and 41 (22.5%) that had a positive *E. canis* result. These results are similar to a previous study that showed poor correlation of IFA and ELISA results when a single IFA *E. canis* titer of 1:80 to 1:160 was used.[132] Another study compared IFA and ELISA results, finding poor agreement between the two testing modalities.[133] It has been suggested that using an IFA titer of 1:320 as opposed to 1:80 may yield better agreement between the ELISA and IFA results.[58] However, multiple serum dilutions were not evaluated in the current study because sample quantities were limited.

There are several reports from the ROK that document tick-borne infections in dogs. One recent publication documented PCR evidence of *B. gibsoni* in dogs.[33] Still another report has documented *E. chaffeensis* infection in two dogs.[121] Finally, a case of Lyme borreliosis was recently documented using a quantitative C6 assay.[30] The dog with Lyme borreliosis in that report was found to be PCR negative, but had appropriate clinical signs, responded to appropriate therapy, and the quantitative C6 assay documented a reducing titer, suggesting that this dog had active borreliosis. These reports document that tick-borne infections are a valid concern for dogs in the ROK. None of these reports document whether or not these animals were receiving adequate tick prevention. The most common tick in the ROK is *H. longicornis*. There has only been 1 report documenting efficacy of fipronil against this particular tick.[98] This study

documented 94.4% efficacy of fipronil in killing adult female *H. longicornis* ticks after 4 days. Given this data, it is plausible that the MWDs in this study could have become exposed to various tick-borne pathogens and become seropositive even while receiving appropriate tick preventative treatment according to manufacturer instructions.

Of even greater concern than dogs developing tick-borne infections is the likelihood that humans may develop these same infections. There have been several case reports from the ROK of humans developing antibodies to *E. chaffeensis, A. phagocytophilum* and spotted fever group rickettsioses.[25,122-125] Given that dogs and humans interact so closely, it is reasonable to infer that dogs could act as either a reservoir for these infections, or that the dogs can possibly bring in ticks from outside that could potentially result in transmission of these tick-borne organisms to humans. Therefore, it is imperative that proper screening of dogs for ticks as well as appropriate preventative medications be utilized to prevent human cases of tick-borne infections.

Although there was not a statistically significant difference between the seroprevalences observed in different years, the point estimates were numerically lower during the later years when fipronil was used as a tick preventative compared to 1996 when organophosphate dips were used. Many factors other than tick prevention methods may influence seroprevalence, however, such as the effect of climatological changes on the tick and rodent populations. Changes in humidity as well as average annual rainfall have been shown to influence the rates that ticks become infected with *A. phagocytophilum* and *E. chaffeensis*.[134]

There are several limitations of the current study. The first is that there was no concurrent control population to allow for the comparison of dogs on different types of tick prevention programs, or to allow the comparison of military dogs to local civilian dogs that were not using tick prevention. Secondly, EDTA anticoagulated whole blood was only available for

dogs sampled in 2007. Having whole blood available from dogs that were sampled in all years would have increased the sample size and may have allowed the detection of DNA from tick-borne pathogens. The primers used in this study were not developed specifically for tick-borne organisms in the ROK, which could have lead to false negative PCR results. However, many of the primers used here were also used successfully in other studies for tick-borne organisms in the ROK.[10,11,19] Therefore, this was likely not an important issue.

In conclusion, this study found serologic evidence of tick-borne infections in the MWD population in the ROK. However, there was no DNA evidence of active tick-borne infections. Further studies, to include prospective studies, examining the prevalence in the MWD population globally could further advance our understanding of tick-borne infections as well as enhance the overall health and well-being of our MWDs.

Footnotes

a. The Handbook of Veterinary Care and Management of the Military Working Dog, 05 March 2004, DOD MWD Veterinary Services, Lackland Air Force Base, TX, pgs. 2, 8-9.

b. The Handbook of Veterinary Care and Management of the Military Working Dog, 05 March 2004, DOD MWD Veterinary Services, Lackland Air Force Base, TX, pg. 26.

c. Frontline Plus®, Merial Limited, Duluth, GA. All rights reserved.

d. Advantix®, Bayer HealthCare, Animal Health Division, Shawnee, KS.

e. Preventic Collars®, Virbac Animal Health, Inc., Fort Worth, TX.

f. IDEXX SANP® 4Dx®, IDEXX Laboratories, Inc., Westbrook, ME.

g. Fuller Laboratories, Fullerton, CA.

h. UltraClean DNA BloodSpin Kit, MO BIO Laboratories, Inc., Carlsbad, CA.

i. Stata version 10, StataCorp LP, College Station, TX.

Table 1. Oligonucleotide primers for polymerase chain reaction assays

Target organism	Target Gene	Primer	Primer sequence (5'-3')	Product (bp)	Annealing temp. (^{o}C)	Reference
Ehrlichia spp. and *Anaplasma* spp.	16S rRNA	ESP-F ESP-R ESP-P	agtccacgctgtaaacgatgag ttcctttgagttttagtcttgcgac 6-FAM-acgcgttaagcactccgcctgg-TAMARA	114	55	Chae et al. 2003
Outer primers *Ehrlichia* spp. and *Anaplasma* spp.	16S rRNA	ECC ECB	agaacgaacgctggcggcaagcc cgtattaccgcggctgctggca	450	65	Yabsley et al. 2008
Nested Primers 1. *E. canis*	16S rRNA	ECA HE3	caattatttatagcctctggctatagg tataggtaccgtcattatcttccctat	365	55	
2. *E. chaffeensis*	16S rRNA	HE1 HE3	caattgcttataacctttggttataaat tataggtaccgtcattatcttccctat	389	55	
3. *E. ewingii*	16S rRNA	EE72 HE3	caattcctaaatagtctctgactatt tataggtaccgtcattatcttccctat	350	55	
4. *A. platys*	16S rRNA	PLATYS GA1UR	gatttttgtcgtagcttgcta gagtttgccgggacttcttct	405	55	
Anaplasma phagocytophilum	*msp2*	Apmsp2f Apmsp2r Apmsp2p-HEX	atggaaggtagtgttggttatggtatt ttggtcttgaagcgctcgta tggtgccagggttgagcttgagattg	77	60	Courtney et al. 2004
Borrelia burgdorferi	23S rRNA	Bb23Sf Bb23Sr Bb23Sp-FAM	cgagtcttaaaagggcgatttagt gcttcagcctggccataaatag agatgtggtagacccgaagccgagtg	75	60	
Rickettsia spp.	*gltA*	CS-F CS-R CS-P	tcgcaaatgttcacggtacttt tcgtgcatttctttccattgtg 6-FAM-tgcaatagcaagaaccgtaggctggatg-BHQ-1	632	50	Stenos et al. 2005
Babesia spp. and *Theileria* spp.	18S rRNA	3.1(1^{o}) 5.1(1^{o}) RLB-F (2^{o}) RLB-R (2^{o})	ctccttcctttaagtgataag cctggttgatcctgccagtagt gaggtagtgacaagaaataacaata tcttcgatcccctaactttc	450	55	Yabsley et al. 2008
Canine Housekeeping gene	G3pdh	G3pdh-F G3pdh-R G3pdh-P	tcaacggatttggccgtattgg tgaaggggtcattgatggcg HEX-cagggctgcttttaactctgg-BHQ-1	90	60	Peters et al. 2003

Table 2. Serum IFA and ELISA testing results for the detection of antibodies to *Anaplasma phagocytophilum* in 182 military working dogs stationed in South Korea by region, age, breed, sex, and year of sampling.

Factor	*n*	No. Positive (%)			†*P*-value
		IFA	ELISA	IFA or ELISA	
Region					
Northwestern	62	22 (35.5)	3 (4.8)	24 (38.7)	0.063
Mid-Western	71	13 (18.3)	3 (4.2)	14 (19.7)	
Southwestern	30	7 (23.3)	1 (3.3)	7 (23.3)	
Southeastern	19	3 (15.8)	1 (5.3)	3 (15.8)	
Age (yrs)					
1-4	45	18 (40.0)	3 (6.7)	18 (40.0)	0.016
5-8	74	10 (13.5)	4 (5.4)	12 (16.2)	
9-12	56	16 (28.6)	0 (0.0)	16 (28.6)	
‡NR	7	1 (14.3)	1 (14.3)	2 (28.6)	
Breed					
Belgian Malinois	92	24 (26.1)	2 (2.2)	26 (28.3)	0.748
German Shepherd	78	19 (24.4)	6 (7.7)	20 (25.6)	
Other	12	2 (16.7)	0 (0.0)	2 (16.7)	
Sex					
Female	31	6 (19.4)	0 (0.0)	6 (19.4)	0.367
Male	119	33 (27.7)	6 (5.0)	34 (28.6)	
‡NR	32	6 (18.8)	2 (6.3)	8 (25.0)	
Year Sampled					
1996	88	26 (29.6)	4 (4.6)	28 (31.8)	0.422
2002 & 2007	94	19 (20.2)	4 (4.3)	20 (21.3)	
Total	182	45 (24.7)	8 (4.4)	48 (26.4)	

†Exact test of homogeneity for the proportion of dogs positive by IFA or ELISA
‡Not recorded – dogs in these categories were excluded from group comparisons

Table 3. Serum IFA and ELISA testing results for the detection of antibodies to *Ehrlichia canis* in 182 military working dogs stationed in South Korea by region, age, breed, sex, and year of sampling.

Factor	*n*	No. Positive (%)			†*P*-value
		IFA	ELISA	IFA or ELISA	
Region					
Northwestern	62	19 (30.7)	0 (0.0)	19 (30.7)	0.275
Mid-Western	71	12 (16.9)	0 (0.0)	12 (16.9)	
Southwestern	30	7 (23.3)	0 (0.0)	7 (23.3)	
Southeastern	19	3 (15.8)	1 (5.3)	3 (15.8)	
Age (yrs)					
1-4	45	14 (31.1)	1 (2.2)	14 (31.1)	0.293
5-8	74	14 (18.9)	0 (0.0)	14 (18.9)	
9-12	56	12 (21.4)	0 (0.0)	12 (21.4)	
‡NR	7	1 (14.3)	0 (0.0)	1 (14.3)	
Breed					
Belgian Malinois	92	19 (20.7)	0 (0.0)	19 (20.7)	0.352
German Shepherd	78	21 (26.9)	0 (0.0)	21 (26.9)	
Other	12	1 (8.3)	1 (8.3)	1 (8.3)	
Sex					
Female	31	5 (16.1)	1 (3.2)	5 (16.1)	0.347
Male	119	30 (25.2)	0 (0.0)	30 (25.2)	
‡NR	32	6 (18.8)	0 (0.0)	6 (18.8)	
Year Sampled					
1996	88	23 (26.1)	0 (0.0)	23 (26.1)	0.290
2002 & 2007	94	18 (19.2)	1 (1.1)	18 (19.2)	
Total	182	41 (22.5)	1 (0.6)	41 (22.5)	

†Exact test of homogeneity for the proportion of dogs positive by IFA or ELISA
‡Not recorded – dogs in these categories were excluded from group comparisons

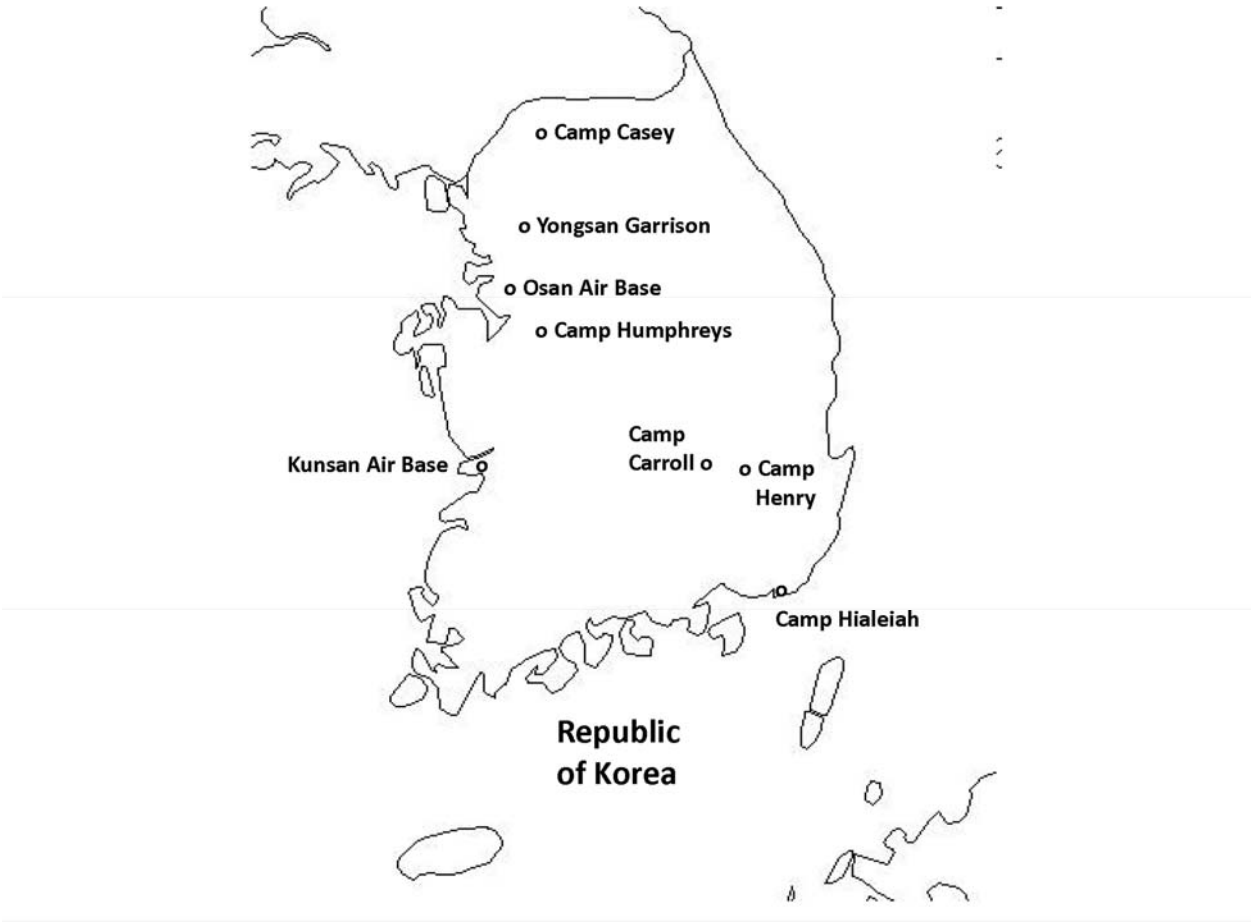

Figure 1. Location of 8 bases in the Republic of Korea with military working dogs that contributed serum samples for a seroprevalence study of tick borne illnesses.

CHAPTER 3

CONSLUSIONS AND FUTURE DIRECTION

This study utilized banked sera from MWDs from the ROK from the years 1996, 2002, and 2007, for ELISA and IFA testing for tick-borne infections. ELISA was performed for *E. canis*, *A. phagocytophilum*, and *B. burgdorferi*. IFA testing was performed for both *E. canis* and *A. phagocytophilum*. Results of serologic testing revealed a surprising number of positive dogs by IFA, while a much smaller number tested positive by ELISA. Results of positive tests were compared utilizing a Fisher exact test for homogeneity. Comparisons were performed based on region of the country where MWDs were stationed, age, breed, sex of the MWD, and year the sample was collected. Other than age group for *A. phagocytophilum* ($p = 0.016$), none of the other comparisons for *A. phagocytophilum* or *E. canis* were statistically significant ($p < 0.05$).

A second portion of this study was carried out utilizing banked EDTA anticoagulated whole blood for PCR analysis to identify DNA of multiple tick-borne pathogens including *E. canis*, *E. chaffeensis*, *E. ewingii*, *A. phagocytophilum*, *A. platys*, *R. rickettsii*, *B. burgdorferi*, and *Babesia* spp. Other than positive controls, all PCR tests were negative for DNA for any of the aforementioned tick-borne organisms. This finding was unexpected given the number of MWDs positive by IFA. These discordant results could indicate exposure to tick-borne organisms without contraction of disease, previous infection with residual antibodies, low sensitivity of the PCRs, or cross-reactivity of the serologic testing with antibodies against some other pathogen. Given that there was no control group of dogs available that were not treated with tick-preventatives, coupled with the fact that other confounding variables would need to be

addressed, no conclusion can be made regarding the efficacy of the tick-prevention protocol utilized. That being said, the data would suggest that the use of fipronil for prevention of tick-borne infections is useful for preventing active infection with tick-borne organisms. However, it may not prevent exposure to these organisms.

Based on the data collected in this study, it would be interesting to conduct similar studies examining a much larger number of MWDs from military installations world-wide. Unfortunately, any such study would have to be a retrospective study because prospective studies using MWDs are prohibited at this time. If larger scale studies provide similar results based on serologic testing and PCR results indicate the presence of DNA from tick-borne organisms, the US Army may want to consider re-evaluating the current standard for tick-prevention.

The ultimate goal of this study or any study examining the health or well-being of MWDs is to ensure that these canine Soldiers receive the best care possible. Given the sacrifices these animals make for the protection of our armed service personnel, they deserve nothing less.

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