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Unearthing an Emerging Neglected Bacterial Disease: Evidence of Spotted Fever Group Rickettsioses in El Salvador

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UNEARTHING AN EMERGING NEGLECTED BACTERIAL DISEASE: EVIDENCE OF SPOTTED FEVER GROUP RICKETTSIOSES IN EL SALVADOR

by

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ABSTRACT

The main objectives of this dissertation were to investigate the seroprevalence of Spotted Fever Group Rickettsioses (SFGR) antibodies in a specific vulnerable population at high risk for vector-borne disease in El Salvador, understand the relevant risk factors for SFGR past and acute infection in this same population through logistic regression modeling, and clarify the presence of SFGR bacteria in locally collected ticks.

SFGR are a group of bacterial diseases caused by species in the Rickettsia genus of the phylogenetic spotted fever group. These are tick-borne diseases (TBD) that have been described in almost every region of the world. SFGR are categorized as neglected bacterial pathogens due to a lack of tools for definitive diagnoses, their underreported nature, and their tendency to impact marginalized populations and those in poverty more seriously.

Our previous 2018 study examined the prevalence of Chagas disease and gastrointestinal (GI) parasites among a cohort of children in the Department of Sonsonate, El Salvador. Banked human sera samples from this study were used for understanding seroprevalence of antibodies to SFGR bacteria through two antibody assays, the focus of Chapter 4. Our results found an overall immunoglobulin G (IgG) antibody prevalence (representing past infection) of 2.5% and an immunoglobulin M (IgM) antibody prevalence (representing acute infection) of 10.7%. Previous Chagas disease positive status was associated with

SFGR antibody prevalence, and a hotspot of antibody positive and equivocal results was identified. Chapter 5 provided a more in-depth picture of SFGR risk through examination the association of environmental risk factors and antibody positive status. We found enrollment in the wet season, increased age, male sex, increased maternal education, and previous household infection increased odds of SFGR antibodies. Finally, Chapter 6 investigated the presence of SFGR bacteria in collected ticks from El Salvador. Traditional and quantitative polymerase chain reaction (PCR) yielded multiple pathogenic bacteria including SFGR pathogens in up to 48.8% of ticks. Multiple ticks were simultaneously infected with more than one pathogenic bacterial species.

These findings provide crucial information regarding the presence of pathogenic SFGR bacteria circulating in the environment in El Salvador. This is the first report of these bacteria in the country. Additionally, identified risk factors of antibody presence in this vulnerable population of children adds to the small body of research regarding human risk to SFGR in Central America. Future research should aim to measure antibody presence in populations in adults as well as children to increase generalizability, encompass a larger geographical area of El Salvador, and study the relationships between infected animals, ticks, and humans for a more holistic epidemiologic frame of reference.

Keywords: spotted fever group rickettsioses, risk factors, antibodies, tick-borne disease, El Salvador

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CHAPTER ONE

INTRODUCTION AND BACKGROUND

1.1. INTRODUCTION TO SPOTTED FEVER GROUP RICKETTSIOSES

Rickettsia bacteria are separated into 4 separate phylogenetic groups: the spotted fever group (SFGR), typhus group (TG), transitional group (TRG), and the ancestral group (AG) (Figure 1.1)[1-3]. The placement into these groups is not only phylogenetic in nature, but also dependent on phenotypic characteristics measured with ecological, epidemiological, and clinical data[1]. All of these bacteria are Gram-negative, obligate intracellular bacteria and vectored to hosts by different arthropods including hard ticks (Acari: Ixodidae), mites (Acari: Dermanyssidae), fleas (Siphonaptera), and body lice (Phthiraptera: Pediculidae)[4]. A majority, approximately 60%, of these bacteria are recognized human pathogens, especially those in the SFGR and TG groups[4-6]. Some *Rickettsia* bacteria with unknown human pathogenicity are considered potentially pathogenic with repeated exposure into the human body[5,7]. SFGR and TG *Rickettsia* spp. are among the oldest known vector-borne pathogens in the world[5,6]. Although only approximately 30 species are described fully and validated, there are potentially hundreds of bacteria in this genus that have yet to be fully characterized[1].

Rickettsia bacteria have a global distribution and are most common in tropical and semi-tropical areas. Table 1.1. lists the recognized pathogenic

Rickettsia species' global distribution along with their respective known vector species, animal hosts beyond humans (if applicable), and associated human disease. Rickettsial diseases are emerging and increasing in incidence worldwide[8]. Seventeen of the twenty-one (81%) described pathogenic *Rickettsia* species are vectored by hard ticks in the family Ixodidae (Order: Acari), indicating the vast evolutionary history between hard ticks and these bacteria. Vectors of pathogenic *Rickettsia* spp. have been described in 7 tick genera in the Ixodidae family. Multiple vertebrate animal hosts—including humans—act as reservoir or amplification hosts for these *Rickettsia* spp. bacteria.

Note that there are more *Rickettsia* spp. in the SFGR group than any other group, this group has a large number of pathogenic species, and more species are newly discovered from this group than any other[1,4]. Western Hemisphere notable and deadly pathogens included in the SFGR group include *R. rickettsii* (causative agent for Rocky Mountain Spotted Fever [RMSF]) and *R. africae* (causative agent for African tick-bite fever [ATBF]). In the past two decades, advances in diagnostics and surveillance have revealed numerous *Rickettsia* that fall within this group that (1) are now recognized as human pathogens and (2) are novel species[5,9-12]. New species and pathogens continue to be isolated and detected from ticks globally.

When *Rickettsia* spp. are introduced into a human host through the bite of an arthropod vector, they are transmitted into the skin via the bite wound. Once inoculated into the skin, the bacteria are phagocytized by dendritic cells and subsequently transported to local lymph nodes through the lymphatic system[11,13].

Replication follows immediately in the lymph nodes, and the bacteria can then enter the bloodstream. Eventually, the bacteria disseminate and infect the endothelium, causing damage and increased permeability in the vascular system[8]. The bacteria can continue to spread throughout the body through the bloodstream, infecting nearly all tissues and organs—but especially monocyte and macrophage cells[8,13]. This damage manifests in symptoms such as rash—which is seen in most SFGR infections—and sometimes meningoencephalitis, acute kidney injury, multiorgan failure, and even death in the most severe species and cases[13]. Clinical symptoms generally begin 4-10 days following the tick bite[14]. In general, pathogenic species present with undifferentiated febrile illness (UFI) with prominent symptoms including fever, headache, and myalgia; sometimes nausea, vomiting, and abdominal pain are also seen in more severe cases[11]. Most infections present these nonspecific symptoms and even mimic viral illness[3]. Inoculation eschars at the site of the tick bite can be seen in a majority of infections, except those of *Rickettsia rickettsii*[3]. Sometimes, different rickettsioses can manifest with their own additional specific features based on species, including the severity and inoculation rate[14]. Immunity against reinfection by the same SFGR agent after natural infection is not well understood or studied, but the literature suggests prior infection can lead to immunity for approximately 1 year[3,8].

Multiple antibiotics are active against rickettsial diseases including tetracyclines, macrolides, and rifamycins, however doxycycline (a tetracycline) is the antibiotic of choice for all rickettsial infections[3,8]. Treatment is stressed for the

first week of infection, if possible, as complications are more likely to occur when disease remains untreated into the second week[3].

In the United States (US), SFGR is the second leading cause of tick-borne disease (TBD) cases reported annually—second to Lyme disease cases; SFGR incidence in the US has steadily increased every year[15,16]. RMSF is the deadliest and arguably the most concerning SFGR species in the Western Hemisphere. Reported annual incidence RMSF has seen a dramatic increase in the past two decades[17]. Case fatality rates for untreated RMSF cases for can reach up to 20-55% in Latin America, compared to 5-10% in the US[7,18-21]. One region in Brazil reported up to 80% case fatality when diagnosis and antibiotic treatment was significantly delayed[22]. Median time to death for untreated cases is reportedly 8 days[3]. Comparatively, case fatality rates for those that receive treatment in the US rarely reach higher than 0.3%, underscoring the importance for accurate and prompt treatment[3,11]. The most prominent symptom of RMSF is a macular or maculopapular rash appearing centrally on the trunk, usually migrating to the limbs. Approximately 10% of cases will not develop this key characteristic rash, however[3]. As the rash progresses on the body, it becomes petechial, indicative of the progression of severe disease—a risk-factor for misdiagnosis and poor outcome[3]. Lymphadenopathy is also commonly seen in patients with RMSF, and in the most severe cases pulmonary involvement may manifest as dyspnea or respiratory failure, neurologic involvement including delirium, stupor, or coma, and even gangrenous appendages or digits[11,23]. An eschar at the site of the tick bite is not associated with RMSF. Long-term sequelae can also occur in patients

with severe illness, even if treated with antibiotics. These long-term symptoms include hearing loss, peripheral neuropathy, development and language delays, motor dysfunction, and bladder or bowel incontinence[3].

Another species of concern in both the Western and Eastern Hemispheres is *R. africae*, which has traversed the globe, and it is now found in most regions of the world (Table 1). This species is of concern for traveling tourists and deployed military members when visiting endemic regions[24-26]. Additionally, approximately two decades ago, this species was detected in the Western Hemisphere for the first time, and it has since become established in local tick species and has spread throughout the Caribbean into Central America[27-31]. This species has also emerged in local ticks collected from humans in Oceania, found in New Caledonia[32]. In addition to the UFI symptoms, ATBF infection is associated with papulovesicular and papulopustular rashes and multiple eschars—as multiple tick bites are common[11,14,33].

In Europe, the most important SFGR species in regard to morbidity and mortality are *R. conorii* (subspecies *conorii*), the causative agent for Mediterranean Spotted Fever (MSF) and *R. conorii* (subspecies *israelensis*), causative agent for Israeli Spotted Fever (ISF). MSF has reported relatively high seroprevalence in Europe, reported between 4% in Italy and Spain to 23% in some regions of the Serbian mountains[5]. Recently, fatal cases have significantly increased in the Mediterranean region (also including North Africa), with the case fatality rate reaching 13-54% in patients with severe complications[5,34]. MSF case fatality rate is typically 2.5%[35]. In most MSF cases, patients immediately exhibit

fever, flu-like symptoms, a rash on the hands and soles of feet, and extreme exhaustion along with an eschar at the bite site[14]. Characteristics that increase likelihood of severe disease include advanced age, suppressed immune system, alcoholism, and delayed treatment[14]. ISF presents with similar cases/symptoms in the same region—however gastrointestinal problems such as nausea and vomiting are more frequently reported. Case fatality rates can be as high as 29%, reported in Portugal[5]. Both *R. conorii conorii* and *R. conorii israelensis* are the top SFGR species of concern in North Africa as well.

In sub-Saharan Africa, *R. africae* is the most important SFGR pathogen of concern. In its endemic region, *R. africae* causes high incidence of disease, where seroprevalence rates have been documented between 12-52%[5,36]. Also important is *R. conorii conorii,* which is capable of 'mimicking' a fatal hemorrhagic viral fever in some cases in this region[37].

The Asian species of highest importance is *R. sibirica sibirica,* causative agent for Siberian tick typhus (STT). Spanning the entirety of the Asian continent, incidence of STT has continuously increased every year, reaching rates as high as 60 cases per thousand people in certain regions[5,6,38]. Although it is fairly common throughout the region, STT is not known to cause significant disease, rarely causing severe complications. Typical symptoms include UFI, inoculation eschar, lymphadenopathy, and maculopapular rash[14].

In Australia and the Pacific, *R. australis*, causative agent for Queensland tick typhus (QTT) is of most concern. This species can cause disease similar to murine typhus (fever, headache, malaise, shivering) along with a maculopapular

rash and inoculation eschar; most cases are mild, however fatal cases have been reported in Australia in patients with underlying co-morbidities[14,39-41]. Although historically uncommonly reported in Australia, it is thought that *R. australis* infection is much more common than previously thought, and a lack of epidemiologic surveillance has resulted in the relative little available information on this species[42].

As described in Table 1.1., seven of the 12 different tick genera serve as vectors for pathogenic SFGR bacteria. All of these different rickettsioses typically follow a pattern of disease emergence based on the seasonality and life history of their associated Ixodid tick vectors. It is highly anticipated that globalization and climate change will continue to increase the transmission and expand the range of these rickettsioses[14]. Increased temperatures can cause tick habitat expansion, can influence the aggressiveness of ticks and their tendency to bite humans, and thus can impact the 'typical' seasonality of disease for a specific region[14,43,44]. Additionally, urbanization and globalization both lead to the increased movement of humans into sylvatic environments and in new habitats, also leading to an increased risk for coming into contact with new vectors and new pathogens, like tick-borne diseases[14,43].

The life history of Ixodid ticks varies significantly across the different genera. However, similar to all hard ticks there are the 4 different life stages: egg, larva, nymph, and adult, and all life stages (except for the egg) require a blood meal before reaching the next life stage, to survive and to stimulate oogenesis or spermatogenesis. A limited number of species have un-feeding adult males

which do not take a blood meal[19]. Ixodid ticks are thus obligate ectoparasites. These ticks can follow one of three basic lifecycles which are associated with their preferred host animal(s): one-host ticks, two-host ticks, and three-host ticks. This is indicative of the number of hosts each species feeds on their entire lifespan. Typically, the tick vectors listed in Table 1.1. associated with SFGR pathogens are two- or three-host ticks. These lifecycles indicate that these ticks will attach to, feed on, and develop on at least two different hosts; epidemiologically, humans play the part of one of these hosts when pathogen transmission occurs.

The ticks associated with these SFGR pathogens not only serve as vectors, but many also serve as reservoir hosts by transmitting *Rickettsia* pathogens from one life stage to the next (i.e. when an infected immature tick molts to the subsequent immature stage or the adult stage), called transstadial transmission, and from an infected adult female to her progeny, called transovarial transmission[11,14,19]. Transovarial transmission can be significantly impacted if an infected female is host to more than one *Rickettsia* spp.; more research is needed to understand the prevalence of multiple infections in ticks and the potential epidemiological impacts of this modulation[45]. These additional ways of spreading infective *Rickettsia* spp. ensure survival of the pathogens in nature and continued evolutionary success[14].

In general, vulnerable populations to tick-borne SFGR include children, those living in poverty, and people with the least access to modern healthcare[46]. Children are generally at higher risk for tick bite exposures due to outside or

environmental behaviors, including play time and increased exposure to tick vectors[47-49]. In addition, due to their underdeveloped immune system, children can also be at higher risk for developing symptoms to infectious diseases, including those vectored by ticks. Children infected with SFGR pathogens develop more severe illness compared to adults including rapidly developed neurologic disease, leading to higher possibilities of death[3,50-52]. Many individuals in developing countries are also living in poverty. Shared risk factors include close proximity to rodent or stray dog populations—which serve as reservoirs and hosts to ticks and agricultural or outdoor work with close proximity to infested animals and vectors[3,14,46,53-57]. In rural, vulnerable communities dependent on farming or outdoor work, SFGR or other *Rickettsia* infection is likely to compound the impacts of poverty as sick individuals are unable to work, reducing productivity, burdening communities and increasing poverty. Due to the non-descript nature of UFI symptoms, it can be very difficult to diagnose patients including children and those living in poverty, leading to a reduced possibility that these patients will receive the needed timely treatment, if treatment can be administered[11,58].

It is challenging to define the worldwide or even regional burden of SFGR because of the commonly misdiagnosed symptomology. As such, these infections have been classified as neglected bacterial pathogens in many of the tropical and subtropical endemic regions, in both rural and urban populations[8,58,59]. Neglected bacterial diseases—or just neglected infectious diseases in general—cannot be compared if they are not accurately diagnosed

nor reported[58]. These infections are widely unrecognized and underreported epidemiologically[46]. Because these infections are so dangerous yet easy to treat, the consequences of undiagnosed disease or misdiagnosis are great[46]. In addition to preventable morbidity and mortality, neglect of treatment and diagnostic capability leads to a lack of widespread recognition of the problem. Increased human travel, urbanization, and climate change will all impact the spread of SFGR-causing bacteria, and more species will likely be discovered in new areas as spread is inevitable. Vulnerable populations in endemic regions of the world need a better understanding of risk and epidemiological characteristics of each SFGR bacteria in order to prevent transmission and reduce morbidity and mortality.

Central America is a region in need of further understanding of the impact of SFGR on morbidity, mortality, and productivity[60]. Despite large numbers of vulnerable individuals, high rates of poverty, and high prevalence of vector-borne diseases causing UFI symptoms, the true geographical range, risk, and burden of SFGR remains largely unknown. A recent review article described 15 tick-borne pathogens affecting humans in the Central American and Caribbean regions[60]. Of these pathogens, 7 (47%) are bacteria from the SFGR group. Additional *Rickettsia* bacteria from the Ancestral group, *R. bellii* and *R. canadensis*, with unknown human pathogenicity, have been found in both ticks and animals in the region as well[7,60-62]. Figure 1.2. shows the current known geographic distribution of *Rickettsia* spp. from the Ancestral and SFGR Groups in Central America. Both the transitional and typhus groups of *Rickettsia* have

pathogens found in this region, but none of these are vectored by hard ticks. The literature describes few human serology studies from Central American countries, and the causative SFGR *Rickettsia* species were not always identified[7,63-68]. Additionally, the few studies investigating bacteria in animals and ticks are not always successful in narrowing down past genus level. In this region of the world, specific diagnostic methods are lacking, combined with relatively little pressure to identify species when addressing clinical disease. These infections remain commonly underdiagnosed and underreported, especially when most infectious disease research and diagnostic approaches in Central America focus on dengue, malaria, or Chagas disease[7,54,64,69]. Public health resources are constrained in this region of the world, and when facing a group of diseases in which diagnostics are not (1) readily available, (2) easy to use, or (3) feasible to dedicate resources to—the umbrella of UFI symptomology seems like a unbreachable barrier[70,71]. It is estimated that rickettsial disease diagnosis is missed in 60% of patients who are truly infected with these pathogens[72].

1.2. Aims and Hypotheses

The overall goals of this investigation are to (1) understand the seroprevalence of SFGR in a specific vulnerable population, (2) estimate the prevalence of infection in collected ticks, and (3) understand environmental risk factors. This will lay the foundation of understanding the burden and risk of spotted fever group rickettsioses in El Salvador. This project will result in a better understanding of where and with whom epidemiological investigations of rickettsial diseases should be focused for increased SFGR awareness and

acquisition of treatment for those who need it. Data includes previously collected qualitative survey information and banked human samples collected from a previous study in El Salvador involving a cohort of high-risk children for vector-borne diseases and primarily collected local ticks. The **Specific Aims** of this project are:

Aim 1. Conduct a cross-sectional study to define the prevalence of SFGR in Salvadoran non-febrile and acute undifferentiated febrile illness (UFI) children from banked sera samples using commercially available antibody assays.

Hypothesis 1: Children presenting with UFI will have approximately 5% burden of SFGR infection.

Aim 2. Quantify the association between environmental risk factors (i.e. children with a fever, living with animals in the home, agriculture as main source of income) related to SFGR in Salvadoran children through a case-control study.

Hypothesis 2: Children presenting with a fever, living with animals, and those within a household with agriculture as the main source of income will be more likely to be infected with SFGR bacteria compared to those without a fever, not living with animals, or with a non-agriculture focused main source of income.

Aim 3. Determine species and prevalence of bacterial pathogens, including SFGR bacteria, in Salvadoran locally collected ticks.

Hypothesis 3: Locally collected ticks will have a relatively high burden of SFGR bacteria (approximately 15% or more), and infection prevalence will be associated with tick vector sex, species, and other bacterial pathogen prevalence.

1.3. IMPORTANCE OF RESEARCH

This research is significant for the country of El Salvador as there is little existing research on ticks and tick-borne disease in this country, a large portion of the country is vulnerable to vector-borne diseases including SFGR, and almost 25% of the population is living below the national poverty line[73]. To date, SFGR prevalence in humans and the local tick populations is largely unknown in El Salvador. The implicated tick vectors of SFGR for human disease in Central America include *Rhipicephalus sanguineus, Amblyomma maculatum, A. cajennense, A. mixtum, A. dissimile, A. sculptum, A. pacae, A. ovale, A. geayi, A. longirostre, Dermacentor nitens, D. latus, D. dissimilis, Haemaphysalis juxtakochi,* and *H. leporispalustris*[7]. Every country in Central America has reported the presence of at least one of these tick species, and six of these have been reported in El Salvador[61,74]. However, only two surveys of ticks have been published in El Salvador, a bachelor's thesis in 2014 and a 2021 survey, neither providing long-term baseline tick population information or species movement over time. Despite the lack of consistent surveillance information, these recent surveys do provide guidance for potential hotspots of SFGR distribution in the country[61,74]. These surveys reported the presence of three SFGR bacteria: *R. canadensis colombianensi* strain, *R. bellii*, and *R. amblyommatis*—all species of unknown human pathogenicity. It is crucial to understand native versus invasive vector species and which species are competent vectors for pathogens capable of causing human disease. Invasive tick species can be brought across borders on migratory birds, mammals, and even humans[75-77]. In contrast to these

arthropod surveys, only two human serology reports have been published from the country: a WHO 1993 survey where 32.5% of participants were positive for SFGR antibodies, and a later 1996 survey reporting 40.0% positive participants for SFGR antibodies[69,78]. Clearly, SFGR pathogens are circulating in humans and animals alike in the Central American region, including in El Salvador, but without baseline and consistent surveillance, risk is largely unknown.

The relative few serology reports from El Salvador and neighboring countries in Central America are sometimes singular in nature and do not accurately identify risk areas for SFGR, leaving a gap in the understanding of *Rickettsia* spp. geographic risk distribution, true incidence, and molecular profile of SFGR pathogens in Central America. If invasive tick species are brought over the border by migratory birds, these could also harbor and bring novel strains of (*Rickettsia*) pathogens into an area. The introduction of new pathogens could easily equate to pathogen spillover into native mammalian hosts, increasing both pathogen richness and prevalence, leading to potentially increased risk for vulnerable populations in the local area[79]. With little to no baseline information on *Rickettsia* spp., invasive or native, El Salvador lies in a particularly vast gap of knowledge. Molecular profiling of pathogens would be key in understanding SFGR relationships across countries to target specific locations for enhanced prevention, interventions, and even monitoring for novel pathogens. Utilizing molecular tools like whole genome sequencing is significant and necessary for enhancing epidemiological surveillance by discriminating between bacterial isolates which cause disease and those that do not in addition to identifying

relatedness to other isolates[80,81]. This molecular epidemiology is essential in other vector-borne disease surveillance and response, such as arboviruses Zika and Chikungunya, and is used extensively in monitoring outbreaks of high-impact viruses like Ebola and during the current global pandemic of SARS-CoV-2[80-86].

Antibiotic resistance of specific strains of *Rickettsia* pathogens is critically important for various species which can cause significant morbidity and mortality, like *Rickettsia rickettsii*[8,87-90]. An understanding of distinct species recovered in El Salvador will also help further scientific investigations and clinical treatment of SFGR in the region as resistance is monitored. Using the population structure of *Rickettsia* spp. in infected individuals from the study will ultimately lead to better understanding of the overall dynamics of transmission and future outbreaks[81]. Any documentation of pathogenic bacterial species in the environment will only add valuable information to veterinarians, public health professionals, and entomologists as SFGR movement and infection potential is monitored globally. Additionally, the identification of specific strains of pathogenic SFGR bacteria in Central America will help bolster vector-borne disease preparedness in the US due to the large influx of potentially infected individuals immigrating and traveling from Central America annually[91,92]. The US hosts multiple tick species which are competent vectors to most of the *Rickettsia* pathogens reported in Central America, and development of clear diagnostic clinical manifestations will also aid in surveillance efforts leading to more efficient treatment in both Central America and the US.

Table 1.1. Global distribution of pathogenic *Rickettsia spp.* with associated characteristics. Adapted from multiple sources[5,12,14,19,93-96].

***Rickettsia* spp.**	**Disease**	**Primary Vector Species (Taxonomic Order or Family)**	**Animal Host(s) Beyond Humans†**	**Global Distribution**
R. africae	African tick-bite fever	*Hyalomma, Amblyomma,* and *Rhipicephalus* spp. (Ixodidae)	mammals	North and Central America, North Africa, Sub-Saharan Africa, Asia, Australia and the Pacific
*R. conorii**	Mediterranean spotted fever, Israeli spotted fever	*Rhipicephalus, Dermacentor,* and *Haemaphysalis* spp. (Ixodidae)	domestic mammals, hedgehogs, dogs	Europe, North Africa, Sub-Saharan Africa, Asia
R. heilongjiangensis	Far-Eastern spotted fever	*Haemaphysalis, Hyalomma, Ixodes, Rhipicephalus,* and *Dermacentor* spp. (Ixodidae)	wild rodents	Asia
*R. honei**	Flinders island spotted fever	*Bothriocroton hydrosauri*, *H. novaeguineae, I.* spp. (Ixodidae)	black rat	Asia, Australia and the Pacific
R. japonica	Japanese spotted fever	*Haemaphysalis* spp., *I. ovatus,* and *D. taiwanensis* (Ixodidae)	wild rodents, small carnivores	Asia
R. rickettsii	Rocky Mountain spotted fever	*Dermacentor* spp., *Amblyomma* spp., *R. sanguineus,* and *H. leporispalustrus* (Ixodidae)	small mammals, carnivores, dogs, rabbits, others	North and Central America, South America, Asia
*R. sibirica**	lymphangitis-associated rickettsiosis (LAR)	*Hyalomma, Rhipicephalus, Dermacentor, Ixodes,* and *Haemaphysalis* spp. (Ixodidae)	migratory birds, cattle, small mammals	Europe, North Africa, Sub-Saharan Africa, Asia
R. slovaca	SENLAT (scalp eschar and neck lymphadonepathy after a tick bite)	*Dermacentor* spp., *Hy. aegyptium,* and *R. sanguineus* (Ixodidae)	domestic ruminants	Europe, North Africa, Asia
R. parkeri	*Rickettsia parkeri* rickettsiosis	*A. maculatum* group, *D. variabilis* (Ixodidae)	cotton rats and others, cotton mice, dogs	North and Central America, South America

R. philipii (*Rickettsia* 364D)	Pacific coast fever	*D. occidentalis* (Ixodidae)	unknown, likely rodents	North and Central America
R. aeschlimannii	spotted fever	*Hyalomma, Amblyomma, Haemaphysalis, Dermacentor, Ixodes,* and *Rhipicephalus* spp. (Ixodidae)	migratory birds, cattle	Europe, North Africa, Sub-Saharan Africa, Asia
R. massiliae	spotted fever	*Rhipicephalus* spp., *Haemaphysalis* spp., *A. sylvaticum, D. variabilis, H. paraleachi,* and *I. ricinus* (Ixodidae)	dogs, foxes, other carnivores	North and Central America, South America, Europe, North Africa, Sub-Saharan Africa, Asia
R. monacensis	spotted fever	*A. dissimile, D. variabilis, R. sanguineus,* and *Ixodes* spp. (Ixodidae)	lizards, wild rodents	Europe, North Africa, Asia
R. raoultii	SENLAT	*Dermacentor, Ixodes, Amblyomma, Rhipicephalus, Hyalomma,* and *Haemaphysalis* spp. (Ixodidae)	domestic ruminants	Europe, North Africa, Asia
R. helvetica	unnamed rickettsiosis	*Ixodes ricinus* and *I. persulcatus, D. reticulatus* (Ixodidae)	lizards, deer, wild boar, wild rodents	Europe, North Africa, Asia
R. tamurae	unnamed rickettsiosis	*Amblyomma testudinarium* (Ixodidae)	wild and domestic pigs, livestock	Asia
R. australis	Queensland tick typhus	*I. holocyclus, I. tasmani, I. cornatus* (Ixodidae)	wild rodents, deer	Australia and the Pacific
R. akari	rickettsialpox	*Liponyssoides sanguineus* (Dermanyssidae)	house mouse	(cosmopolitan) North and Central America, Europe, Sub-Saharan Africa, Asia
R. felis	cat flea rickettsiosis	*Ctenocephalides felis* (Siphonaptera)	cats	North and Central America, South America,

				Europe, North Africa, Sub-Saharan Africa, Asia, Australia and the Pacific
R. prowazekii	sporadic epidemic typhus	*Orchopeas howardi* (Siphonaptera)	flying squirrels	North and Central America, South America
R. prowazekii	epidemic typhus	*Pediculus humanus corporis* (Pediculidae)	N/A	(focal) North and Central America, South America, Europe, North Africa, Sub-Saharan Africa, Asia, Australia and the Pacific
R. typhi	murine typhus	*Xenopsylla* spp., *Ctenocephalides* spp. (Siphonaptera)	cats	North and Central America, South America, Europe, North Africa, Sub-Saharan Africa, Asia, Australia and the Pacific

*****These species have multiple subspecies with different names given for disease or distinct geographical distributions; most common disease name is given and entire geographical distribution from all subspecies is listed

†Many of these animal hosts are suggested, and have not been proven

Green shading = vectored by mites; yellow shading = vectored by lice; blue shading = vectored by fleas; no shading = vectored by hard ticks

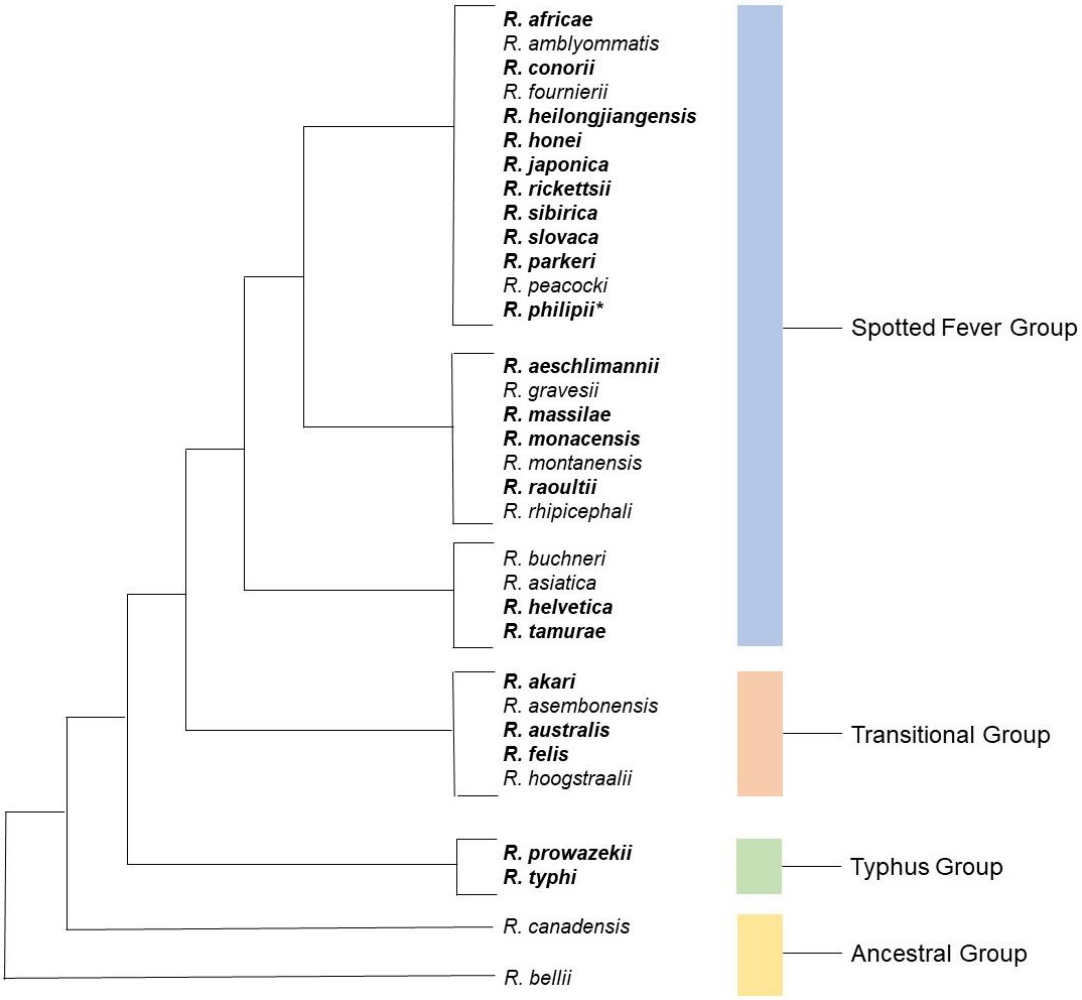

Figure 1.1. Phylogenetic tree of *Rickettsia* species based on previous literature. Bold indicates pathogenic species. Asterisk represents not fully-validated species.

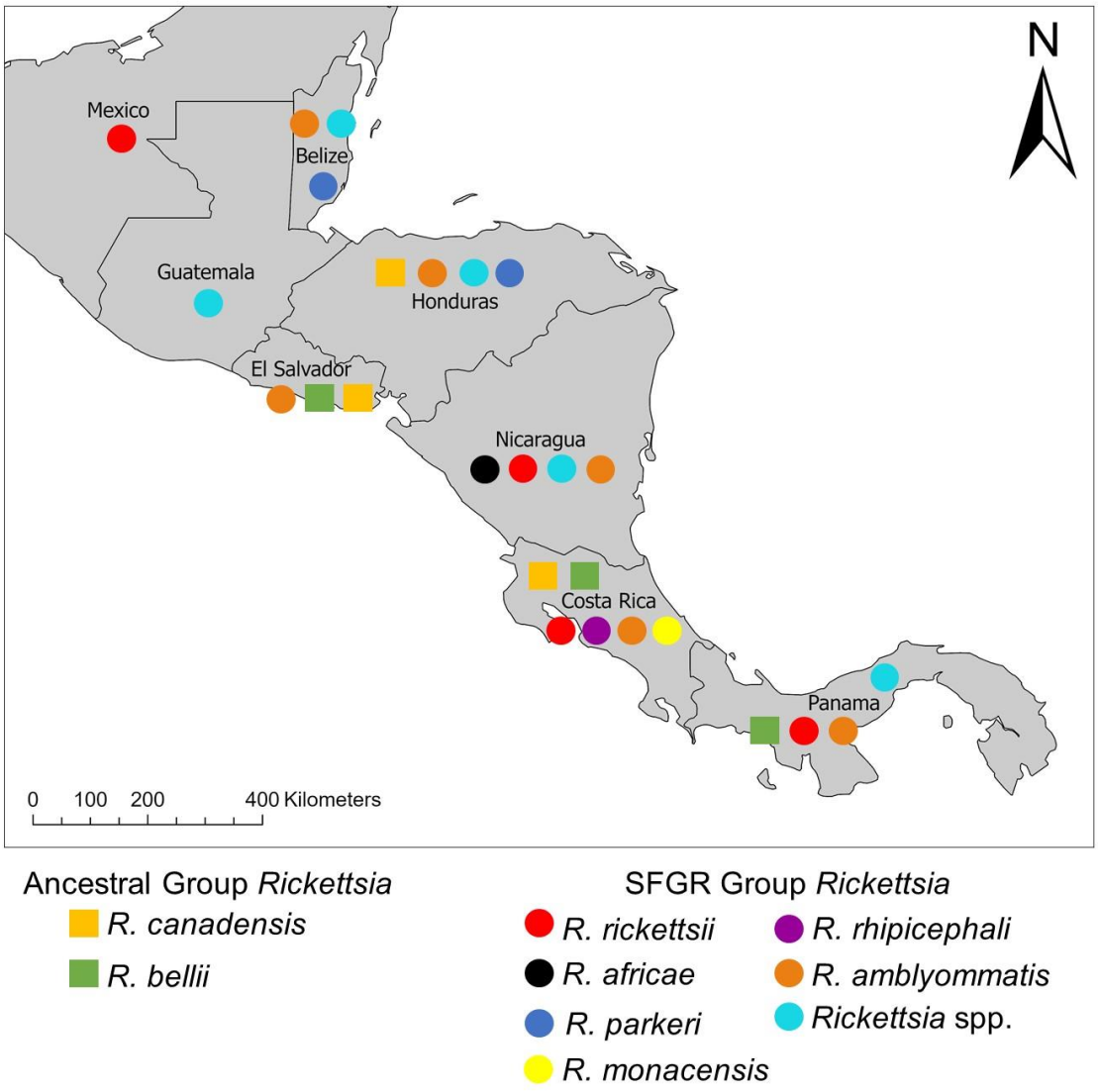

Figure 1.2. Current known geographic distribution of *Rickettsia* spp. from SFGR and Ancestral Groups in the Central America region, and Mexico. Adapted from Parola et al. (2005), Parola et al. (2013), and Charles et al. (2021).

CHAPTER TWO

LITERATURE REVIEW

The goal of this literature review was to: systematically describe what is currently known about 1) SFGR prevalence in humans, ticks, and animals and 2) risk factors related to SFGR human disease in El Salvador and neighboring Central American countries Guatemala, Honduras, and Nicaragua. The additional 3 countries in Central America (Costa Rica, Panama, and Belize) were excluded from this review due to their overall difference regarding general human development, poverty, and health. El Salvador, Guatemala, Honduras, and Nicaragua all have similar vulnerability to multidimensional poverty and United Nations Human Development Index values compared to Costa Rica, Panama, and Belize (Table 2.1.).

2.1. SEARCH TERMS

A comprehensive systematic literature review was conducted using the following databases: PubMed, Web of Science, SciELO, and Latin American and Caribbean Health Sciences Literature. Additional articles were recovered from references of selected articles when applicable and searches within bachelor's and master's thesis databases from public universities in the selected countries. There were no limits set for publication date, although more recent articles (e.g. published within the past 10 years) were favored. The following search terms were used to locate studies evaluating the presence of SFGR *Rickettsia* bacteria

in humans, animals, and ticks, serology results of humans or animals to antibodies to SFGR *Rickettsia* in the selected countries: El Salvador AND Rickettsia OR tick surveillance, Guatemala AND Rickettsia OR tick surveillance, Honduras AND Rickettsia OR tick surveillance, Nicaragua AND Rickettsia OR tick surveillance, “Central America” AND Rickettsia OR tick surveillance.

A total of 103 publications were initially accumulated from all databases. Following title and abstract review, 17 duplicates and 67 non-relevant publications were removed for multiple reasons including: no mention of SFGR bacteria or SFGR disease and information regarding non-selected countries from Central America. The remaining 19 publications were reviewed, and 3 additional publications were added from references. Following the complete read-through, 6 publications were removed as 4 were review articles with no original content that was already reported, 1 used the same sample population as another article, and 1 did not test for SFGR pathogens in the manuscript. The 16 final publications were used for the following literature review.

2.2. History of and Risk Factors Related to SFGR in El Salvador, Guatemala, and Nicaragua

El Salvador

The first investigation into SFGR in El Salvador was published in 1993 when the World Health Organization (WHO) conducted a global serological investigation into human rickettsial diseases in the early 1990s. Forty human serum samples from patients with fever from unknown origin were submitted to this study from El Salvador, and 13 (32.5%) were positive through an indirect fluorescence antibody (IFA) assay for SFGR antibodies[69]. Although identification

of the SFGR species was not conducted, the WHO concluded that this study demonstrated the extensive spread of rickettsial infection and the need to continue surveillance[69]. A second global serological investigation was conducted around the same time looking at rickettsial antibodies in humans and animals using both an enzyme-linked immunosorbent assay (ELISA) and IFA. Sixteen of 40 (40.0%) of the human samples were positive by ELISA to SFGR antibodies and 13 (32.5%) were positive by IFA to *R. conorii* antibodies from El Salvador[78]. The positive samples for *R. conorii*, the causative agent for both Mediterranean and Israeli spotted fevers (not known in the Central American region) was noted as most likely an indication of cross-reactivity for SFGR antibodies. The authors conclude that the relatively high prevalence of antibodies in El Salvador compared with the relatively few reports of clinical disease lead to the probability that cases are not recognized or not reported in this country[78].

The next investigations into SFGR in this country are not reported until nearly 20 years later: when a singular investigation into ticks parasitizing turtles was published in 2012. This investigation was the first published report to document a species of tick and rickettsial bacteria in the country. Four *Amblyomma sabanerae* ticks found on a turtle in the western portion of the country were all positive for *R. bellii* and negative for any SFGR bacteria[97]. Although *R. bellii* is not in the SFGR group, this is a significant report as the first documentation of *Rickettsia* in the country. No environmental risk factors for infection in humans were noted.

In both 2014 and 2021, two wide-scale tick and tick-borne disease surveillance investigations were published increasing the number of known Salvadoran tick species from 1 to 12 and increasing the number of *Rickettsia* species from 1 to 3. Both studies collected ticks from peridomestic and livestock animals for identification and analysis, then tested select ticks for presence of *Rickettsia* through PCR followed by sequencing. The 2014 study did not report exact percentages of ticks positive for *Rickettsia*, however of the 250 ticks tested, 3 tick species were positive for the SFGR Group species *R. amblyommatis* (*A. auricularium, A. cajennense,* and *A. parvum*), two positive for the ancestral group species *R. canadensis colombianensi* (*A. dissimile* and *A. scutatum*), and two positive for *R. bellii* (*A. dissimile* and *A. ovale*)[74]. The 2021 study expanded tick collections across the entire country, collecting 1,181 ticks and tested a large majority of them for pathogens[61]. *R. amblyommatis* was found in 10/13 (77%) *A. mixtum* ticks, 8/16 (50%) *A. parvum* ticks, 1/9 (11%) unknown *Amblyomma* spp. nymphal ticks, and in 1/13 (8%) *D. nitens* ticks. *R. canadensis colombianensi* was found in 3/31 (10%) *A. dissimile* ticks and in 2/18 (11%) *A. scutatum* ticks. *R. bellii* was found in 1/31 (3.2%) *A. dissimile* ticks and in 1/6 (17%) *A. ovale* ticks. Of note for the 2021 study was that multiple ticks were collected from humans—both *Rhipicephalus sanguineus* and *R. microplus*—but these were negative for any SFGR pathogens.

GUATEMALA

Only one publication reports the presence of SFGR bacteria in Guatemala, published in 2013. This study documents an outbreak investigation

conducted in 2007 during which 17 persons from a farming community in the southeastern Department of Jutiapa reported an acute febrile illness, of which 2 died[57]. Sixteen of the 17 patients were tested through IFA, Western Blotting, and PCR to detect acute rickettsial infection. Seven of the 16 tested (40%) were positive for both IgG and IgM SFGR antibodies, and 1 (6.25%) individual was positive by PCR for SFGR DNA. The study concluded that 10/16 tested individuals were confirmed or probable cases of SFGR. In addition, 97 ticks were collected from the area and tested for SFGR DNA through PCR. One of the 12 (8.3%) of *A. cajennense* and 0/85 *R. microplus* ticks were positive for SFGR DNA. Although the authors did not determine the species of SFGR bacteria, they do comment that the most likely culprit was *R. rickettsii* or *R. africae*[57]. Additionally, risk factors noted for all patients included contact with animals like dogs and rats, and a majority of the cases were farm workers and had seen ticks on animals routinely.

HONDURAS

A 1971 study documents the first known presence of SFGR in Honduras after a large Central American human serological survey. Samples from El Salvador, Honduras, Guatemala, and Nicaragua were submitted, however only samples from Honduras and Nicaragua were positive for SFGR antibodies. Samples were tested through complement-fixation (CF) and microscopic agglutination tests (MA); of the 348 samples submitted from Honduras, 3 (0.9%) and 1 (0.3%) were positive for SFGR antibodies through CF and MA,

respectively. The location of patients who submitted samples was not given, and no risk factors were identified from the data.

The next documentation related to SFGR in Honduras was reported forty years later, when a single tick-bite investigation was initiated regarding a US traveler who developed SFGR disease after reporting a tick bite from Honduras during his stay[53]. The man reported typical SFGR symptoms including erythema, flu-like symptoms, fever, headache, and a possible eschar at the tick bite site. He was positive through serology for antibodies to SFGR bacteria, and the authors suggested *R. rickettsii* as the causative agent. Risk factors included reported frequent contact with horses and dogs, as well as spending a lot of time outdoors in Honduras.

Two additional SFGR serological studies were published in 2010 and 2021 regarding risk to US military personnel stationed in Honduras (locations not described). The first study investigated the presence of antibodies to *R. rickettsii* in feral cats that frequently came into contact with military personnel on base[98]. Only 12 cats were tested through IFA, and 2 (16%) were positive for *R. rickettsii* antibodies. Because these cats were being handled by troops, the authors noted that close contact with feral animals increased risk for exposure to this tick-borne SFGR, although no ticks were found[98]. The second study investigated 1,000 US military personnel deployed in Honduras for greater than 6 months between 2000-2016. Included participants were evaluated pre- and post-deployment to Honduras for antibodies to SFGR through both MA and IFA[99]. Results revealed 39/1000 (3.9%) personnel seroconverted while on deployment to Honduras, and

9 of these 39 individuals were also positive for antibodies to 1 or 2 additional pathogens[99]. Minority race was associated with increased rate of SFGR seroconversion, and all military personnel spent copious amounts of time outdoors.

Lastly, a tick surveillance investigation was conducted in the northwestern portion of Honduras in 2014 where ticks were collected from animals and humans and tested for SFGR bacteria presence through PCR[100]. SFGR Group *R. amblyommatis* was found in 4/9 (44.4%) *A. mixtum* ticks (found biting humans), 1/9 (11.1%) *A. longirostre* ticks, and 12/67 *Amblyomma* spp. larval ticks. Ancestral Group *R. canadensis colombianensi* was found in 3/12 (25%) *A. dissimile* and *Amblyomma* spp. larval ticks. The authors made note that many of the people handling animals found ticks on themselves, and animal contact was a notable risk factor for exposure[100]. This study was the first to document *R. amblyommatis* and *R. canadensis colombianensi* in Honduras.

NICARAGUA

The same 1971 Central American serological study mentioned for Honduras reported only 1/312 (0.3%) of patients tested positive for SFGR antibodies through CF only in Nicaragua[66]. No other reports were published from Nicaragua regarding SFGR bacteria or disease until the late 2010s.

One such study, a human serological investigation, was conducted from 2008-2009 through enrolling febrile patients with undiagnosed febrile illness from hospitals. Patient sera was tested for IgG antibodies to *R. rickettsii* through ELISA, followed by IFA for confirmation and PCR to confirm acute infection:

results indicated 51/748 (6.8%) of patients had antibodies to *R. rickettsii* from ELISA only and 6 acute infected patients with *R. rickettsii* were identified through PCR[54]. Risk factors associated with SFGR antibodies or SFGR infection included rural residence, less education, river water exposure, exposure to farm animals and domestic pets, and exposure to ectoparasites[54].

Three additional studies carried out between 2013-2016 investigated the health of dogs in relation to humans and risk of rickettsial infection in Nicaragua. The first, conducted near the north-central border of Nicaragua and Honduras, focused on only ticks from peridomestic and domestic dogs. Of these community dogs, 146 ticks were collected (comprising 3 species) and tested by real time PCR (RT-PCR) for presence of SFGR DNA. Eighteen of 127 (14.2%) of *A. ovale* ticks were positive for *R. africae*; 5/12 (41.7%) of *A. sculptum* were positive for *R. amblyommatis*, and 1/7 (14.3%) of *A. triste* ticks were positive for unknown rickettsial DNA[31]. This was the first report of SFGR group *R. africae* in Nicaragua. The second study, also conducted in 2013, tested ticks collected from dogs at various animal shelters in the western portion of the country. The authors tested 316 pools of ticks (1,023 individual ticks) through quantitative PCR (qPCR) for presence of *Rickettsia* spp. DNA, then tested all positive pools for targeted *Rickettsia* spp.. In total, 22/316 (7%) of the pools were positive for *Rickettsia* DNA, and 1/199 (0.5%) *R. sanguineus* and 1/3 (33.3%) *A. ovale* ticks were positive for *R. amblyommatis*[55]. The same investigators from the initial 2013 study expanded their investigation to testing the community dogs for evidence of SFGR infection in 2016. The investigators tested 77 dogs in total from 3

communities using IFA reporting prevalence of antibodies to *R. rickettsii* ranging from 70–92.6%[56]. In all three of these studies, the authors made mention of the proximity of these dogs to humans, increasing risk for ectoparasite and pathogen exposure as risk factors for human disease[31,55,56].

Lastly, a review article conducted in 2019 cites an additional report of SFGR group *A. amblyommatis* in collected *A. ovale* tick from companion animals in Nicaragua, however no number of ticks collected or percentage of infection was given[101].

Overall, the information on SFGR rickettsial pathogens and clinical disease in these selected countries is very limited. In total, this search produced: 5 studies for El Salvador, 1 for Guatemala, 5 studies for Honduras, and 6 studies for Nicaragua documenting evidence of SFGR pathogen presence or clinical disease. Common risk factors mentioned in many all studies included contact with animals and spending time outdoors—which all fit the profile of acquiring tick-borne pathogens like SFGR.

Table 2.1. Human Development Indicators for Central America. Accessed directly from the United Nations Human Development Programme[102].

Country	Human Development Index (HDI) Rank	HDI Score	Life Expectancy at Birth (avg.)	% Child Malnutrition (under age 5)	% In or Vulnerable to Multidimensional Poverty	% Below national Poverty Line
Panama	57	0.815	78.5	19.0	N/A	1.2
Costa Rica	62	0.810	80.3	5.6	2.4	1.0
Belize	110	0.716	74.6	15.0	9.0	N/A
El Salvador	124	0.673	73.3	13.6	11.6	22.8
Guatemala	127	0.663	74.3	46.7	32.3	59.3
Nicaragua	128	0.660	74.5	17.3	19.0	24.9
Honduras	132	0.634	75.3	22.6	29.1	48.3

CHAPTER THREE

METHODS

3.1. OVERVIEW OF STUDY

Both primary and secondary data analyses were conducted to estimate the prevalence of SFGR in children in the Department of Sonsonate and in ticks, along with estimating risk factors for pediatric infection in this same Department. Primary data for analysis included a large (n~250) sample of ticks collected from three locations in El Salvador and a biorepository of Salvadoran febrile and afebrile pediatric patient serum and whole blood, collected from a previous vector-borne disease study from 2018[103]. Secondary data for analysis consisted of survey data obtained when the pediatric patients were enrolled and biological samples were collected. Molecular testing and antibody screening results were combined with qualitative data from the survey in order to form an epidemiological frame of reference for risk of tick-borne SFGR in El Salvador in a vulnerable population and select locally collected ticks.

Our overall goals were to: conduct a cross-sectional study to estimate the SFGR burden in vulnerable Salvadoran children through serology screening, identify environmental risk factors for tick-borne SFGR for this specific group of individuals, and conduct molecular pathogen testing in locally collected ticks to corroborate human serology data with entomological pathogen data to lay the foundation for understanding the spread and burden of bacterial pathogens in El

Salvador. This served to increase awareness of tick-borne pathogens in the country, identified risk factors which can be used in future epidemiological investigations to target populations at risk, and will—ultimately—lead to preventing future disease transmission. Foundational knowledge of tick-borne pathogens in El Salvador along with understanding where resources can be allocated can aid public health officials make decisions regarding the health of vulnerable populations in this country.

PREVIOUS STUDY

Our previous work in El Salvador identified a relatively high prevalence of Chagas disease and gastrointestinal (GI) parasitic infections—other neglected tropical diseases of poverty—in a pediatric cohort of approximately 1,000 children from the study area in the Department of Sonsonate (Figure 3.1.)[103]. We worked closely with the University of El Salvador and the Ministry of Health for this project, and we continue to have a working relationship with these organizations through continued research on Chagas disease and other vector-borne diseases. This study found that children in underserved and rural areas of the country were at higher risk for vector-borne disease and provided baseline knowledge of the prevalence of parasitic diseases infecting children in the cohort. In a subset of the studied pediatric participants, 60% of children were infected with at least one gastrointestinal parasite, and 15% were concomitantly infected with multiple parasites. The highlight of this study demonstrated the relatively high proportion of participants with polyparasitism. This dissertation adds

necessary information regarding neglected tropical diseases in vulnerable populations in this region of Central America.

3.2. Specific Aim 1

To define the prevalence and geographical spread of SFGR bacteria in Salvadoran pediatric patients from a cross-sectional study utilizing banked serum samples from previous study. The completion of this aim will further expand the literature on human exposure to SFGR bacteria, notably *R. rickettsii*, which has not been documented in El Salvador to date. A targeted cohort of at-risk pediatric participants will provide a strong baseline for understanding the risk for exposure to SFGR bacteria. Additionally, recognizing a moderate to high prevalence of antibodies to SFGR infection will bolster awareness of tick-borne diseases in vulnerable populations.

Sample. The banked biological samples are from the previous pediatric study mentioned above. This previous study was approved by the El Salvadoran Ministry of Health, University of South Carolina, and Baylor College of Medicine Institutional Review Boards. Parental or legal guardian signed consent and verbal child consent was secured through study nurses prior to participation or sample collection. All approached participants and guardians were provided a non-monetary (food) appreciation gift for their time and consideration. The associated consent forms explicitly stated that banked biological samples would be used for future infectious disease studies and those results would not be shared with participants given the delayed surveillance nature and lack of clinical relevance. Children were recruited by local study nurses in collaboration with

trusted community promotoras through two different scenarios: (1) children lived in a home with documented recent or current infestation with triatomine insects (vectors of the causative agent for Chagas disease), or (2) presenting with an objectively measured fever (≥100.4°F or ≥38°C) in clinic with acute febrile disease with unknown etiology. Inclusion criteria included anyone aged 9 to 18 years and living within the Department of Sonsonate, El Salvador at the time of enrollment. Children were not excluded based on gender or pregnancy status. Participants without parent or legal guardian or who were not available for follow-up phone calls were excluded. Approximately 1,100 pediatric patients were enrolled and provided biological samples throughout the year 2018.

Enrolled participants provided two whole blood samples for diagnostics, which were collected by a trained study nurse or phlebotomist. Whole blood was collected in Serum Separator Tubes (SSTs) and EDTA Blood Tubes. Upon collection in EDTA tubes, DNA/RNA Shield was added to the whole blood. Plasma was separated from the whole blood in EDTA tubes following centrifugation, aliquoted into labeled cryogenic tubes with the participant's ID, date collected, and project name, and stored in a -80°C freezer in the Center for Health and Research and Development (CENSALUD) at the University of El Salvador, San Salvador. Serum was separated from whole blood samples through centrifugation in the SSTs, aliquoted into labeled cryogenic storage tubes and stored in the same freezer. Samples were shipped to the Laboratory of Vector-Borne and Zoonotic Diseases at the University of South Carolina, Columbia, SC following completion of sample collection. Each participant's legal

guardian or parent was asked to complete a household risk factor questionnaire related to vector-borne disease. This questionnaire was adapted from a published Central American Chagas household risk factor survey and developed in collaboration with local experts from the University of El Salvador, the University of San Carlos (Guatemala City, Guatemala), and the Sonsonate health department[104].

Methods. Antibody responses to SFGR infection was assessed through use of both IgM and IgG ELISA assays (Spotted Fever Rickettsia IgM EIA kit & Spotted Fever Rickettsia EIA IgG Antibody Test Kit, Fuller Laboratories, Fullerton, CA). The IgM antibody kit measures an early immune response (acute or current infection) to SFGR antigens, while the IgG kit measures any immune response (cumulative lifetime exposure) to SFGR antigens. The IgM antibody kit utilizes antigens purified from *R. rickettsii* specifically, however these antigens will react with 4 other pathogenic SFGR species: *R. montanensis, R. parkeri, R. conorii,* and *R. africae*. The IgG antibody kit utilizes a group-specific lipopolysaccharide antigen extracted from SFGR species, which will react to the following: *R. rickettsii*, *R. parkeri, R. amblyommatis, R. conorii, R. siberica, R. australis,* and *R. akari*.

ELISA testing was as follows: patient sera will be diluted with Sample Diluent, incubated for 1 hour at ambient temperature (22-25°C), followed by a wash with Wash Buffer. Enzyme-labeled goat anti-human IgM or IgG enzyme conjugate was added for the appropriate ELISA, followed by a second incubation period of 30 minutes in the same conditions as before. Lastly, an enzyme

substrate was added to each reaction well to allow for any antigen-antibody reactions in a darkened environment. We used the same incubator conditions as recommended by the manufacturer, but each plate was placed in a box covered with aluminum foil to ensure no light entered the incubator environment. Reactions were stopped following a 10 minute-reaction time, and the absorbance of each sample was read through a 96-well microplate reader. A Cutoff Calibrator was used for discrimination between reactive and non-reactive sera. By dividing the absorbance values of patient sera by the average absorbance values of the Cutoff Calibrator, an index value was derived, and the Cutoff Calibrator was set at an index of 1.0.

For the IgM antibody kit: Indices from 0.8-1.2 were considered equivocal. Indices >1.2 were considered positive, and indices <0.8 were considered negative. For the IgG antibody kit: Indices from 0.9-1.1 were considered equivocal. Indices >1.1 were considered positive, and indices <0.9 were considered negative.

Antibody serostatus was used as the outcome variable for the hot-spot analysis. This outcome measure was controlled by the number of recruited individuals from each municipality to create a proportion of antibody positives.

Analysis. Prevalence of SFGR acute infection (results of IgM antibody kit) in at-risk pediatric participants was determined as well as past SFGR exposure (from IgG antibody kit) of pediatric participants. This information was compared to human serological studies from the selected neighboring countries (Guatemala, Honduras, and Nicaragua). Although there is substantial uncertainty

regarding SFGR pathogen prevalence in El Salvador considering the previous two serological surveys from this country included only n=80 participants in total over 25 years ago, this study provides crucial information to help fill this gap. Because these two kits detect antibodies to multiple pathogenic SFGR bacteria, and only 3 rickettsial pathogens have been identified in neighboring countries (*R. parkeri, R. rickettsii,* and *R. africae*), we were able to make an informed estimate to narrow down the potential species responsible for infecting pediatric participants. We expected to see a moderate to high prevalence in these children, approximately 5-10% reactivity to SFGR antigens, based on serological studies from neighboring countries. Additionally, further comparisons through chi-square tests and Fisher's exact tests were made with previous neglected tropical disease prevalence in this same pediatric cohort to understand the correlation between Chagas disease, GI parasitic infection, and SFGR exposure.

The Getis-Ord Gi* statistic was used to conduct a hot-spot analysis of SFGR infection (IgG and IgM antibody seroprevalence) from the previous study's dataset. This analysis revealed statistically significant SFGR hot- and cold-spots throughout the department of Sonsonate. This spatial analysis was conducted with ArcGIS Pro (ESRI, West Redlands, CA). We anticipated to find significant hotspots of SFGR infection in similar municipalities where Chagas disease was found in the previous study.

3.3. Specific Aim 2

To conduct a case-control study to quantify the association between previously recorded environmental risk factors related to tick-borne SFGR

infection and exposure in a cohort of Salvadoran febrile and afebrile children utilizing qualitative survey information collected from the previous study[103]. This aim allowed us to investigate multiple potential environmental risk factors that have not been explored before regarding increased odds of tick-borne disease in this context. Given the paucity of human infection data in El Salvador compared to tick surveys, this aim provided needed information to fill in the gap of SFGR exposure and infection in a vulnerable population of pediatric patients who are more at-risk for tick-borne diseases. Quantifying this relationship leads to a better understanding of the healthcare needs of similar populations in El Salvador and surrounding countries where tick-borne disease is typically neglected compared to other neglected tropical diseases in the region.

Sample. SFGR infection and exposure results from pediatric samples assayed in Specific Aim 1 were utilized as the outcome in this case-control study: pediatric participants with either IgM (acute) or IgG (past, non-acute exposure) antibodies to SFGR *Rickettsia* species were considered cases, and pediatric participants with no evidence of IgM or IgG antibodies to SFGR *Rickettsia* species were considered controls. Various exposures were examined through the survey performed at enrollment for each participant (approximately 1,100). The survey consisted of approximately 60 questions to evaluate participant demographics and risk factors related to vector-borne disease including: socioeconomic status and characteristics, home building materials, proximity to livestock or pet animals, and knowledge of vectors of other diseases, such as Chagas disease.

Methods. Patient demographic data included sex, age, number of persons living in the home, number of children living in the home, and febrile status. Additional variables that were extracted as potential exposures/risk factors included: season of enrollment (categorical: wet and dry season), household has agriculture as the main source of income (binary: yes/no), mother's and father's last year of education (continuous), type of bedroom floors (categorical: bare earth and cement), type of wall material in the bedroom (categorical: blocks, adobe, other), type of cooking fuel (categorical: wood, gas, both), type of water in the home (categorical: potable and non-potable), access to electricity (binary: yes/no), household has dogs (binary: yes/no), household has cats (binary: yes/no), household has birds (binary: yes/no), use of outside latrine (binary: yes/no), knowledge of triatomines (vector of the causative agent for Chagas disease: called "chinches" in the survey) (binary: yes/no), exposure to triatomine bites (binary: yes/no), fumigation treatment history of the house (binary: yes/no), and whether or not a family member has been to the hospital in the past year for general reasons (binary: yes/no) or for an infection (binary: yes/no). Results from this previous study regarding infectious diseases were also utilized as potential exposures: positive for GI parasites (binary: yes/no), positive for Chagas disease parasite (binary: yes/no).

Analysis. Multivariate logistic regression models were used to determine the associations—quantified as odds ratios (OR)—between the outcome of interest (antibodies to SFGR *Rickettsia* pathogens) and the above listed potential exposures among the pediatric cohort. We expected to see increased odds for

SFGR antibodies in pediatric patients who presented with a fever at enrollment, had dogs and cats, lived in a household with agriculture as the main source of income, had previous exposure to triatomines, and had a household history of seeking clinical at the hospital for an infection. To our knowledge, this was the first case-control study evaluating tick-borne SFGR odds in the selected countries El Salvador, Guatemala, Honduras, or Nicaragua.

3.4. Specific Aim 3

To determine the prevalence and molecular identity of SFGR bacteria in Salvadoran locally collected ticks. Only two tick surveillance studies have been conducted in El Salvador to date[61,74]. Both studies documented the presence of SFGR *R. amblyommatis*, a species with unknown human pathogenicity. However, given the presence of *R. parkeri* and *R. africae*, species with known human pathogenicity in neighboring countries Nicaragua, Belize, and Honduras, and potentially *R. rickettsii* in Guatemala, Honduras, and Nicaragua, it is likely that pathogenic species are circulating in local ticks in this country. By examining ticks through species-specific PCR and sequencing, this aim adds to the very small body of research on SFGR in El Salvador.

Sample. Approximately 250 ticks were collected from three different sites in El Salvador in May 2021 by the author and additional study team members. These sites were (1) the University of El Salvador's Department of Agriculture's (UES DAG) cattle farm, (2) a private cattle farm, and (3) a veterinary office. All three sites were in separate geographical areas of the country near the state capital, San Salvador. Ticks were collected from animals from each location and

immediately placed in 75% ethanol in labeled tubes according to each host animal.

Methods. Collected tick samples were transported back to the Laboratory of Vector-Borne and Zoonotic Diseases, and morphologically identified to species using dichotomous keys[105-110]. All ticks were taken to the University of Tennessee's Medical and Veterinary Entomology Laboratory for processing and pathogen screening. Each tick was dissected in half, and one half was used for DNA extraction using QIAamp 96 DNA QIAcube HT Kits (Qiagen, Hilden, Germany); the other half was saved as a voucher specimen when available. Detection of SFGR DNA was performed by traditional PCR amplification of the fragment of the 190kDA protein gene (*ompA*) and the citrate synthase gene (*gltA*). A third gene, outer membrane B (*ompB*), specific to *R. rickettsii* was targeted for quantitative PCR (qPCR). These protocols for SFGR PCR testing have been proven effective in reliably detecting species in environmentally collected ticks globally[111-113]. All PCR products were identified via gel electrophoresis. DNA sequencing was conducted on randomly selected SFGR positives to differentiate species.

Analysis. A multivariate logistic regression model was used to estimate the odds of SFGR infection prevalence in collected ticks by species, sex, and collection site. A second multivariate logistic regression model was used to estimate the odds of *R. rickettsii* infection prevalence collected in ticks by species, sex, and collection site. Additionally, chi-squared tests with Fisher's exact tests were used to determine any associations between tick species,

animal host species, and infection status. Sequencing results were used to phylogenetically link previously published SFGR sequences from Central America. Results were compared with previously published tick pathogen results from El Salvador and neighboring countries in Central America, with a focus on Guatemala, Honduras, and Nicaragua. We expected to find a relatively high burden of SFGR pathogens (approximately 15% or more) in locally collected ticks—based on previously published rickettsial prevalence in neighboring countries.

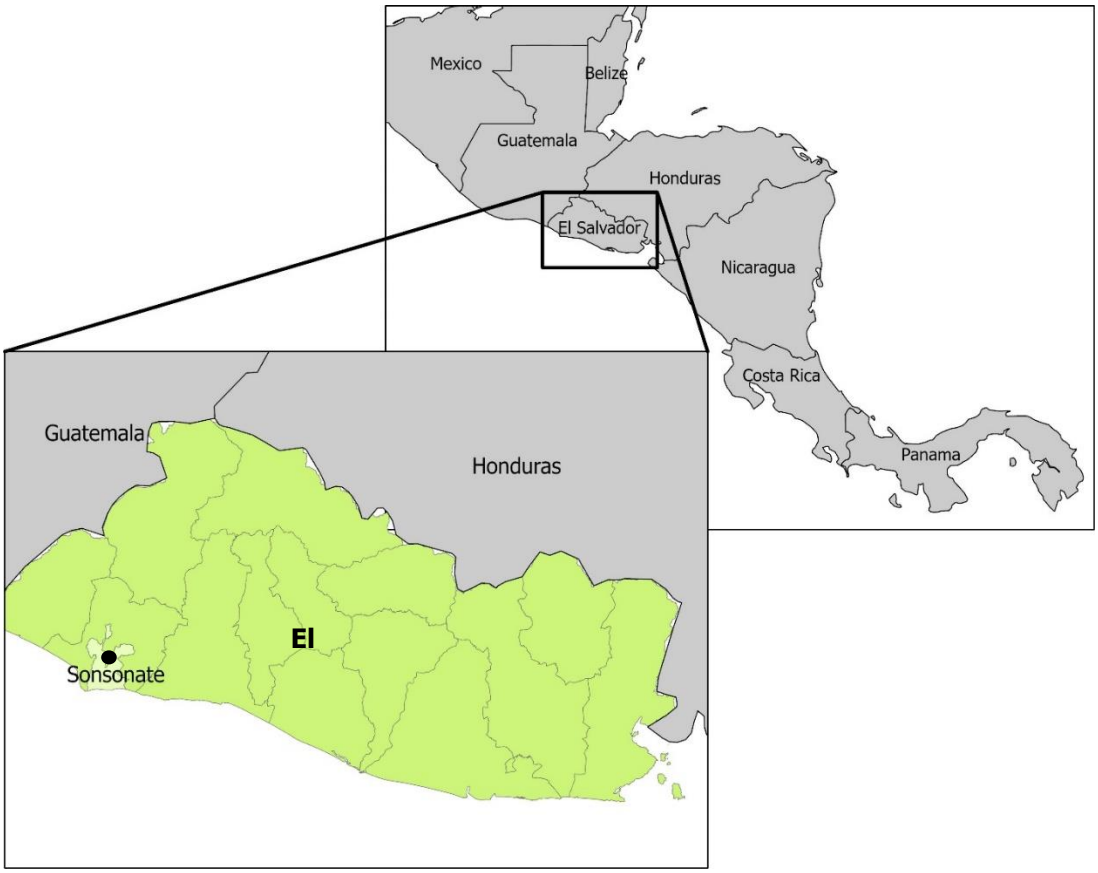

Figure 3.1. Map and inset of the previous study area, Sonsonate, in El Salvador.

CHAPTER FOUR

EVIDENCE OF AN EMERGING NEGLECTED TROPICAL DISEASE IN EL SALVADOR: ANTIBODY PREVALENCE OF RICKETTSIA SPP. IN AFEBRILE AND FEBRILE CHILDREN

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[1]Dye-Braumuller KC, Rodriguez Aquino MS, Zellars K, Waltz H, Meyer M, Gual-Gonzalez L, Self SCW, Kanyangarara M, Nolan MS. To be submitted to J*ournal of Tropical Medicine and Infectious Disease*

4.1 ABSTRACT

Spotted Fever Group Rickettsioses (SFGR) are caused by a group of tick-borne pathogens that are increasing in incidence globally. These diseases are typically underreported and undiagnosed in Central America, and thus, have been classified as neglected bacterial pathogens. Countries with high poverty, low human development index score, and limited health infrastructure—like El Salvador—lack necessary surveillance for SFGR and other tick-borne pathogens. This paucity of baseline information on SFGR infection prevalence leaves vulnerable populations at risk of misdiagnosis. Knowledge of tick-borne disease burdens in El Salvador is severely limited. To expand the knowledge base on prevalence and potential risk of tick-borne SFGR in El Salvador, our team conducted two different enzyme-linked immunosorbent assays (ELISA) on banked human sera samples from a cohort of approximately 1,000 pediatric participants from a previous risk study of a population at high risk for vector-borne disease. Commercially available SFGR IgG and IgM kits were utilized following manufacturer recommended protocols. Eleven percent of all tested banked pediatric sera were positive for at least one ELISA assay, and 14.0% were equivocal for at least one ELISA assay. This broke down as 2.5% and 1.0% of pediatric samples were positive and equivocal for IgG antibodies to SFGR, respectively, indicating a past SFGR infection. Additionally, 10.7% and 15.2% of participants were positive and equivocal for IgM antibodies to SFGR, respectively, indicating an acute SFGR infection at time of enrollment. Antibody seroprevalence was associated with febrile disease at time of enrollment. The

large percentage of acute SFGR infections indicates that it continues to remain an underreported and undiagnosed issue in El Salvador and the Central American region. Public health officials in this region need to consider SFGR as a diagnosis for vulnerable populations to prevent future morbidity and mortality.

4.2 INTRODUCTION

Spotted Fever Group Rickettsioses (SFGR) are a group of diseases caused by multiple species of *Rickettsia* bacteria transmitted by hard ticks (Acari: Ixodidae). Clinical symptoms typically present with rash, fever, headache, myalgia, and in more severe cases meningoencephalitis, multiorgan failure, or death[11,13,14]. The most common symptomology falls under the large umbrella of undifferentiated febrile illness (UFI). These bacteria have a global distribution but are most common in subtropical or tropical areas. SFGR incidence is increasing globally due to multiple factors including urbanization, climate change, and weak tick-borne disease surveillance[70,114,115]. SFGR as a pathogen family comprises species causing disease along the clinical spectrum with *Rickettsia amblyommatis* believed to cause mild infection versus *Rickettsia rickettsii* causing severe morbidity, and a variety of clinically-relevant species in between[116]. Although easily treatable with antibiotics, if cases are not diagnosed and remain untreated, the likelihood of severe disease increases[70]. In higher income countries, case fatality rates rarely rise above 10%; however, in low- and middle-income countries, especially in Latin America, case fatality rates have reached between 20-55%[7,18,20,21]. Unfortunately, the common UFI symptoms displayed are shared among multiple neglected tropical pathogens in areas

including Central America, and specific diagnostic methods for tick-borne disease are either unreliable or too costly for countries with constrained public health resources[70,71]. This is the case for El Salvador, where most neglected tropical disease public health resources are devoted to mosquito-borne disease treatment and surveillance[7]. Without a baseline understanding of tick-borne disease burden, infected individuals are more likely to remain undiagnosed and the true burden of disease will be underreported[70].

Serologic determination of rickettsial infections has been utilized since its inception in the late 1910s when the Weil-Felix (WF) test was developed[117]. Multiple techniques have been developed since; antibodies to Rickettsia species can be detected using complement fixation[118], latex agglutination[119] enzyme-linked immunosorbent assay (ELISA)[120], and immunofluorescence assay (IFA)[121]. Both the ELISA and IFA assays have proven to be adequate antibody screening tools for low- and middle-income countries. The IFA is considered slightly more advantageous due to its higher sensitivity and specificity; however, the ELISA is simpler and readings are made in a more objective manner compared to the IFA[78].

Two previous serological reports from the early 1990s of *Rickettsia* antibodies in El Salvador utilized both ELISA and IFA tests, and prevalence of antibodies to SFGR ranged from 32.5 – 40% of the tested population (n=40 in each report)[69,78]. However, neither of these reports evaluated reactive antibody type, therefore infection stage or temporal pathogen exposure could not be determined. While not always exclusive in infection-timing determination, this

lack of detail in surveillance can place public health officials at a disadvantage when there is little to no time sequence of infection. This, and the lack of tick-borne disease surveillance in this country combined, has created a large gap in the literature and in the public health surveillance of neglected tropical diseases of this Central American region. To understand the burden of tick-borne rickettsial pathogens—and potential time sequence of infection—in a vulnerable population in El Salvador, our team conducted IgM and IgG ELISA antibody serological assays on banked pediatric participant samples from a previous vector-borne disease cohort investigation. Given the vulnerability of this pediatric population, we were also interested in the relationships between all infectious disease outcomes from the previous study and the current study: Chagas disease, gastrointestinal (GI) parasite infection, and SFGR infection.

4.3 METHODS

STUDY RECRUITMENT

Participants recruited for the original study were from the Department of Sonsonate, El Salvador, located in the western region of the country. This region is made up of 16 municipalities, and participants were approached for enrollment from the entire department. Detailed information on the study site and recruitment of pediatric participants for this research are described in the previous study[103]. Briefly, this previous study prospectively enrolled approximately 1,200 children aged 9 months to 18 years from January to December 2018 for vector-borne disease surveillance to investigate concomitant intestinal parasitic infection and malnutrition. Acute undifferentiated febrile

children were recruited from state pediatric clinics and asymptomatic children were recruited from homes known to the state vector control agency to have triatomine infestation. All sera samples have been bio-banked at -80°C since initial collection.

ETHICS STATEMENT

Institutional Review Board or human ethics committee approvals were secured from the El Salvadoran Ministry of Health, University of South Carolina, and Baylor College of Medicine. Parental or legal guardian signed consent and verbal child consent was secured through study nurses prior to participation or sample collection. Recruitment, consent, and data collection were all conducted in Spanish, by fluent, native Spanish-speaking study personnel who were fully trained in the protection of human research subjects and in interviewing techniques to cater to local cultural appropriateness. All approached participants and guardians were provided a non-monetary (food) appreciation gift for their time and consideration. Our prior study was not an interventional and/or clinical trial.

ANTIBODY SCREENING

Commercially available ELISA kits for both IgG and IgM antibodies to SFGR pathogens were utilized (Fuller Laboratories, Fullerton, CA, USA). All patient samples were screened at a 1:100 dilution and the manufacturer's recommended methods and protocols were followed. In short, ELISA testing proceeded as follows: patient sera were diluted with sample diluent, incubated for 1 hour at ambient temperature (22-25°C), followed by a wash with wash buffer.

Enzyme-labeled goat anti-human IgM or IgG enzyme conjugate was added for the appropriate ELISA, followed by a second incubation period of 30 minutes in the same conditions as before. Lastly, an enzyme substrate was added to each reaction well to allow for any antigen-antibody reactions in a darkened environment. We used the same incubator conditions, but the ELISA plate was placed in a box covered with aluminum foil to prevent any light from entering. Reactions were stopped following 10 minute-reaction time, and the absorbance of each sample was read through a 96-well microplate reader at 450 nm (Accuris Instruments, London, UK).

The IgM antibody kit measures an early immune response (acute or current infection) to SFGR antigens, while the IgG kit measures any immune response (cumulative lifetime exposure) to SFGR antigens. The IgM antibody kit utilizes antigens purified from *R. rickettsii* specifically, however these antigens will react with other closely related SFGR species including *R. parkeri* and *R. africae*. The IgG antibody kit utilizes a group-specific lipopolysaccharide antigen extracted from SFGR species, which will react to multiple SFGR species including: *R. rickettsii, R. parkeri, R. amblyommatis, R. conorii, R. siberica, R. australis*, and *R. akari*. A Cutoff Calibrator was used for discrimination between reactive and non-reactive sera. By dividing the absorbance values of patient sera by the average absorbance values of the Cutoff Calibrator, an index value was derived, and the Cutoff Calibrator was set at an index of 1.0. According to the IgG kit, samples with index values >1.1 were considered positive, index values 0.9 – 1.1 were considered equivocal, and index values <0.9 were considered

negative for IgG antibodies. According to the IgM kit, samples with index values >1.2 were considered positive, index values 0.8 – 1.2 were considered equivocal, and index values <0.8 were considered negative.

DATA ANALYSIS

SFGR antibody prevalence was determined for both acute or current infection (IgM positives) and historical infection (IgG positives). A hotspot analysis using Getis Local Gi* statistic was performed in ArcGIS Pro (ESRI Corporation, Redlands, CA). The analysis evaluated spatial autocorrelation of SFGR infection for both acute and past infection in the participant population throughout the multiple municipalities of the study area. The hotspot analysis was conducted utilizing the proportion of positive or equivalent participants out of the number of tested participants in each municipality to account for the unequal recruitment of participants by municipality. Chi-square and Fisher's exact tests were performed to assess independence regarding all infectious disease outcomes from this cohort, specifically: between SFGR infection with previously determined Chagas disease infection, gastrointestinal parasite infection, and febrile children. The total number of Chagas-positive individuals (n=25) from the entire pediatric cohort was low, and there were relatively few individuals tested for GI parasites, and thus Fisher's exact tests were necessary to interpret these comparisons. Statistics were executed in SAS statistical software (version 14.1; StatCorp LP, College Station, TX, USA).

4.4 RESULTS

A total of n=1,175 eligible participants were approached for enrollment, and a final n=1,074 consented participants with complete interviews and provided serum samples were included in the original study[103]. Of the available 1,074 participants' banked serum samples, a total of n=1,049 (97.7%) were tested for either IgG or IgM antibodies or both. Due to the availability of reagents, overall cost, or amount of serum, not every participant's serum samples could be tested with both antibody assays. All 1,049 samples were tested for IgG antibodies, and 900 samples were tested for IgM antibodies. A total of 899 participants' samples were tested for both antibody assays.

From the banked sera tested for IgG antibodies, 2.48% (n=26) and 0.95% (n=10) tested positive and equivocal, respectively, indicating a past convalescent infection to SFGR species. From the sera tested for IgM antibodies, 10.67% (n=96) and 15.22% (n=137) tested positive and equivocal, respectively, indicating a current or acute infection with SFGR bacteria. Figure 4.1 includes additional information on the breakdown of sera samples which were positive and/or equivocal for multiple assays or those positive or equivocal for at least one assay. Approximately 11% of all tested samples were positive for at least one ELISA assay, and 25% of samples tested either positive or equivocal on at least one ELISA assay. A total of 5 (0.56%) participants' sera samples were positive for one ELISA and equivocal for another. Only one participant tested positive on both IgG and IgM assays (0.11%). Of those tested for both antibodies, 14.91% (n=134) tested negative for IgG antibodies and equivocal for

IgM antibodies. Additionally, 0.89% (n=8) individuals tested negative for IgM antibodies and equivocal for IgG antibodies.

The geographic distribution of ELISA assay results in the Sonsonate Department is displayed in Figure 4.2. The proportion of IgG and IgM positives or equivocals are displayed in Figure 4.2 (a) by municipality. Although positive and equivocal participants are seen throughout the department, the highest concentration of seropositive and equivocal individuals appears to be in both Nahuizalco and Sonzacate municipalities, in the north central region. The neighboring municipalities Santa Catarina Masahuat directly to the west, Santo Domingo de Guzman to the southwest, Juayua to the north, and Izalco to the east have the second highest proportions of positives and equivocals. The distribution of both IgM and IgG serology results are displayed separately in Figure 4.2 (b) and (c), which follow a similar pattern. The municipalities located on the southwest (Acajutla) and southeast corners also stand out regarding both IgG and IgM antibody serology results (Santa Isabel Ishuatán).

The hotspot analysis results are presented in Figure 4.3. Considering the proportion of positive or equivocal participants, one hotspot with 95% confidence was identified in the northwest region of the Sonsonate Department, in the Juayua municipality, with an adjacent hotspot with 90% confidence in both Nahuizalco and Sonzacate municipalities.

The results of the chi-square tests for independence and Fisher's exact tests are presented in Table 4.1. No significant associations at the 0.05 level were noted. There was an association between IgG positive and Chagas disease

positive individuals where there were more individuals testing positive for both than expected at the 0.10 level (p-value = 0.0785). There was an association between Chagas disease positive status and those positive for at least one ELISA (p-value = 0.0744), between those positive for at least one ELISA and febrile status (p-value = 0.082), and between those testing positive or equivocal for at least one ELISA and febrile status (p-value = 0.0869) at the 0.10 significance level. Higher numbers of participants positive for both Chagas disease and for at least one antibody assay were observed than expected. Fewer numbers of individuals tested positive for at least one ELISA assay or tested positive or equivocal for at least one assay and were febrile were observed than expected. No significant associations were seen between individuals positive for IgM antibodies and any other infection or febrile status. Further investigation of the difference between febrile status and antibody seroprevalence yielded no differences between the mean temperature recorded for each participant who tested negative, positive, or equivalent for IgG or IgM. Twenty-six percent (n=25) of those positive for IgM antibodies presented with a fever at enrollment.

4.6 Discussion

This is the first report in over 25 years investigating the serological evidence of SFGR infection in El Salvador, adding necessary and updated information to the literature regarding human infection with rickettsial disease in Central America. Our results indicate a smaller potential burden of past or current infections with SFGR pathogens than previously reported. In 1987, the World Health Organization (WHO) developed an IFA assay for detection of antibodies

to multiple rickettsial pathogens and distributed this with protocols to laboratories in 37 countries, and one laboratory in El Salvador participated in this effort. From n=40 human sera samples tested, 13 (32.5%) tested positive for SFGR antibodies, but the type of antibodies was not described[69]. A second study also surveyed n=40 human sera samples from El Salvador and found 16 (40.0%) positive with presence of SFGR antibodies without describing the specific antibodies surveilled[78]. A description of these previous studies' population demographics (pediatric vs. adult, febrile vs. afebrile, etc.) is not provided, so our results are not directly comparable. However, our study does provide a baseline understanding of past and acute SFGR infection for children in Sonsonate less than 18 years old. Because IgM antibodies typically peak following 3-4 weeks post infection—and are detectable for 3-4 months—we believe IgM positive participants were infected with SFGR bacteria within a 3-4 month window of enrollment[122-125]. IgG antibodies peak 10-12 weeks post infection and are detectable for up to 1 year, so all IgG positive participants could have been infected in a much larger window[122-125]. Our results indicated a small proportion of pediatric participants with a past (1 calendar year) infection through IgG antibody detection (2.48% positive and 0.95% equivocal), but a surprisingly high number of acute or current infections through IgM antibody detection (10.67% positive and 15.22% equivocal). The age of participants ranged from 9 months to 18 years, thus in our study population, a relatively high proportion of children were acutely sick with SFGR infection.

Our results also demonstrate that approximately a quarter (25.07%) of participants were either positive or equivocal on either ELISA assay, indicating seroconversion transition may be continuing in this population. For those individuals whose serum was tested for both assays, nearly 15% were IgG negative, but IgM equivocal, suggesting these additional 134 individuals were very recently infected. Another 1.78% were IgM negative but IgG equivocal, suggesting a waning antibody response to a past infection. One individual from the entire cohort was positive for both IgM and IgG antibodies, suggesting this individual was likely in the middle of seroconversion. Overall, this is the first report to highlight the proportion of acute infections and potentially seroconverting acute infections in children in El Salvador, a known vulnerable group for SFGR infections and severe disease[46,50-52]. Although our study only includes information from one department, this is crucial information for public health in this country providing a semblance of baseline information for additional areas in the country.

The geographic distribution of SFGR antibody positive and equivocals generally reflected the distribution of parasitic infections earlier reported from this department[103]. The municipalities in the northcentral municipalities of Sonsonate had the highest proportion of antibody positive and equivocal individuals, with the northernmost municipality, Juyua, as the single hotspot at 95% confidence and Nahuizalco and Sonsacate as hotspots of 90% confidence. With this information, public health professionals can potentially begin to narrow down areas in the

country where tick-borne disease investigations can be initiated, or risk factor analyses can be attempted.

Although we found no statistical evidence of associations between parasitic infection and SFGR antibody presence, the cold spots of SFGR positives or equivocals indicated by the hotspot analysis are areas where our previous study found very few Chagas disease cases and the lowest amounts of GI parasite infection[103]. It is important to note that only 25 children were identified as Chagas disease positive from this previous study, and only 168 individuals were screened for GI parasites, thus limiting the number of participants whose SFGR results could be compared.

Additionally, there was only an association between serological positivity of either antibody and febrile status at the 0.10 significance level, and no statistically significant difference mean recorded temperature among the serological groups. We had anticipated that those with potential evidence of an acute infection (IgM positive or equivocal) would be likely to present with a fever, but this was not observed in the data at the 0.05 significance level[20]. IgM antibodies were detected outside the clinical window of febrile disease, closer to the end of acute disease. Nonetheless, the lack of fever correlation to IgM-indicated acute infection in this pediatric cohort increases the misdiagnosis likelihood of an already underdiagnosed disease[46]. It is also possible that individuals presenting with a fever and no seropositivity to rickettsial antibodies were infected with other pathogens.

Human antibody presence to SFGR rickettsial pathogens have been documented in neighboring countries Guatemala and Honduras, along with other Central American countries Belize and Nicaragua. Our results fit within the range of neighboring countries' reports of SFGR antibodies: one study investigating an outbreak in 2007 reported 40% of tested individuals with antibodies to SFGR[57], and two others reporting serological surveys from Honduras reported individuals with antibodies to SFGR from 0.3%-0.9%[66] to 3.9%[99]. In Belize, one study reported human antibody prevalence at 33.5%[126], and studies in Nicaragua have reported SFGR antibody presence in 0.3%[66] to 6.8%[54] of individuals tested. Use of ELISA versus IFA assays was evenly distributed throughout these reports. These previous studies further support our suggestion that human infection with tick-borne rickettsial pathogens is probably more common than previously thought, and constant throughout the tropical Central American region.

Limitations to this study include the fact that SFGR serological assays are diagnostic when paired with acute and convalescent samples, and the cross-sectional nature of this study design inhibited formal diagnosis. Additionally, both commercially available IgG and IgM ELISAs used in this study do not have the ability to differentiate between rickettsial pathogens and will indicate reactions for antibodies to multiple species of *Rickettsia*. It is thus impossible to determine what species of SFGR individuals may have been infected with. The original study did not include additional pathogenic indicators of SFGR species, such as eschar, rash, etc., that could have yielded additional clinical correlate information. The large number of febrile participants also makes comparisons to the rest of

the country less generalizable. Selection bias is a possibility due to the recruiting mechanism of participants: where those who were febrile at clinics were actively recruited over individuals who were not febrile. Also, the small number of participants tested for parasitic infections from the original study reduced the reliability of comparisons we could make from this study. Lastly, because this cohort was limited to one department in El Salvador, these results may not be directly comparable to the rest of the country.

The results of this study are important for neglected tropical disease in the Central American region. We demonstrate a relatively high proportion of children with evidence of acute infection or developing seroconversion with SFGR pathogens in El Salvador. Based on these results, we can suggest that there is a large proportion of vulnerable individuals that are at risk for SFGR infection in this country that are potentially remaining undiagnosed. The few serological investigations for El Salvador emphasized the same claim: that SFGR infection in this region is most likely remaining largely unknown and infected individuals are underreported and undiagnosed. Rickettsial antibodies in the body decline over a year[127], and thus the window for identifying infected individuals will wane over time, leaving these individuals with a smaller chance of receiving treatment. Certain rickettsial species, if undiagnosed, can result in severe cases of disease, sometimes leading to death[7,11,13,18]. This risk is elevated in children, marginalized populations, women, and those in poverty[46]. Diagnostic methods for SFGR infection are not ideal, and specifically in this region, rickettsial disease presents with typical undifferentiated febrile illness, making these infections notoriously

difficult to diagnose[11,70,71]. Additional difficulties arise from the fact that most neglected tropical disease surveillance and treatment in this region is targeted towards other arthropod-borne pathogens including dengue, malaria, Chagas disease, and leishmaniasis[7,54,64]. Further studies should investigate the burden of rickettsial pathogens in humans, animals, and ticks through molecular diagnostic methods. With this information, we hope that public health officials and infectious disease physicians consider rickettsial infections as a cause of disease in El Salvador to increase the awareness of these tick-borne pathogens and the number of individuals receiving treatment.

Table 4.1. Chi-Square Test of Independence on SFGR ELISA Serology and Parasite Infections.

	χ^2 Test Statistic	Degrees of Freedom	χ^2 P-value	Fisher' Exact Test Probability
Positive for at least one ELISA (IgG or IgM)				
Chagas disease positive[1]	3.48	1	--	0.0744
Any GI parasite positive[1]	0.0008	1	--	1.0000
Febrile	3.02	1	0.082	0.0959
Positive for one ELISA or Equivocal in another ELISA (At least one positive or equivocal)				
Chagas disease positive	0.11	1	0.7405	0.8003
Any GI parasite positive	0.38	1	0.55	0.6141
Febrile	2.93	1	0.0869	0.0909
IgG Positive				
Chagas disease positive[1]	5.15	1	--	0.0785
Any GI parasite positive[1]	0.74	1	--	0.4265
Febrile	0.71	1	0.398	0.5316
IgM Positive				
Chagas disease positive[1]	1.53	1	--	0.1981
Any GI parasite positive[1]	0.21	1	--	1.00
Febrile	2.28	1	0.131	0.1572

[1]At least one cell had expected counts less than 5, thus Fisher's exact test probability is provided.

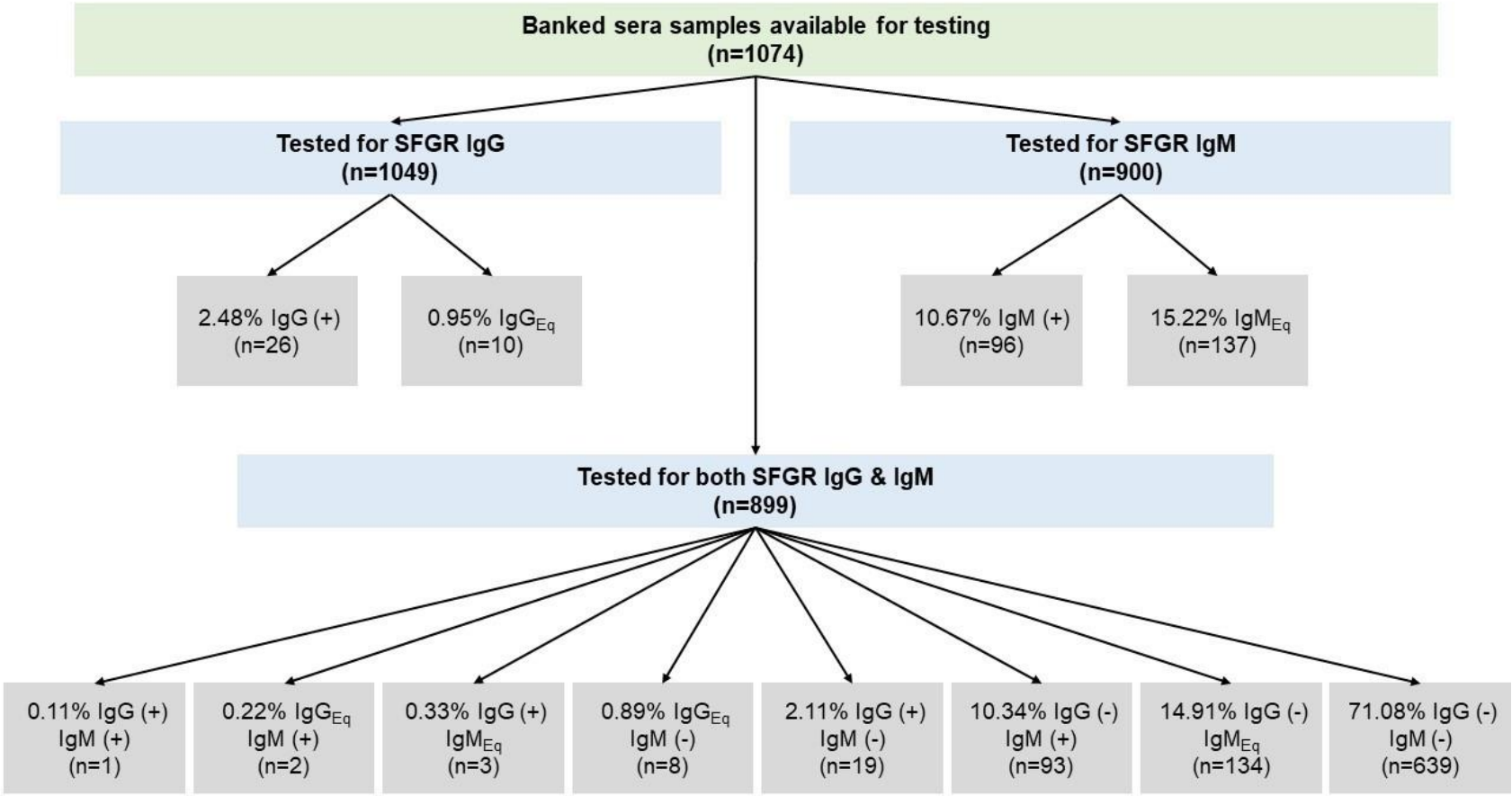

Figure 4.1. Breakdown: Prevalence of IgG and IgM serology results.

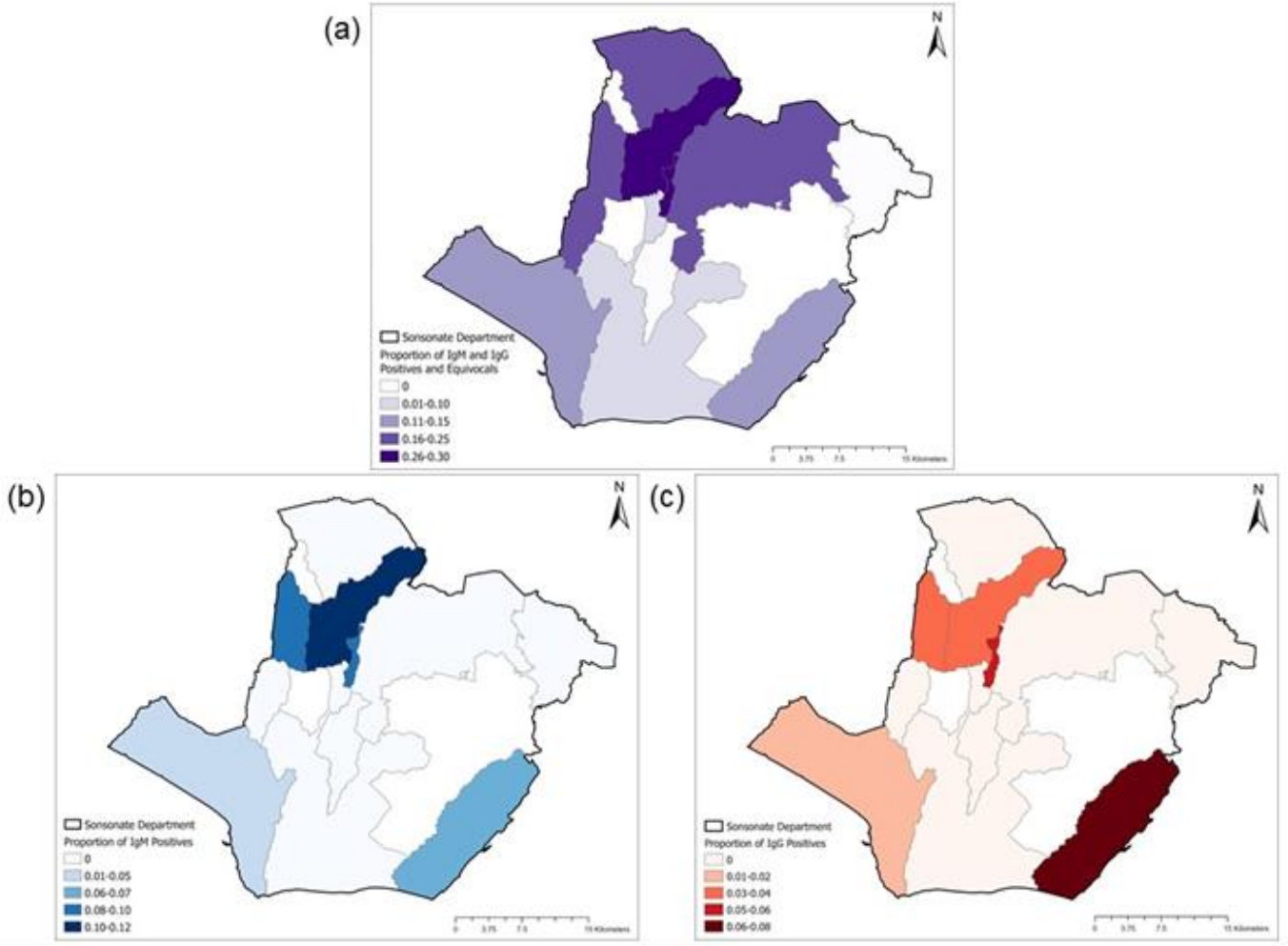

Figure 4.2. Geographic distribution of serology proportion results from ELISA assays: (a) SFGR IgG and IgM positives and equivocals; (b) IgM positives only; (c) IgG positives only.

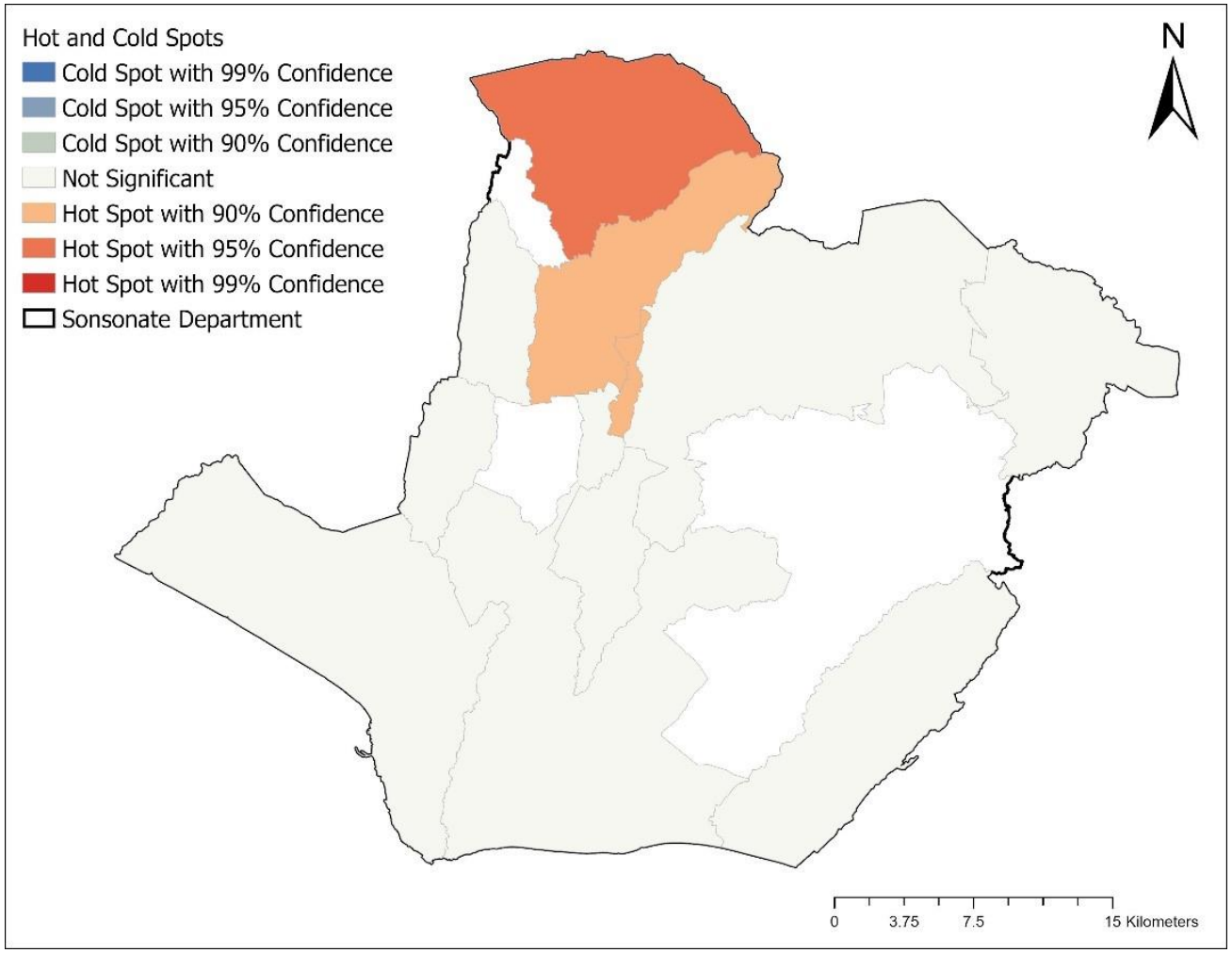

Figure 4.3. Hotspot cluster analysis of IgG and IgM serology results from ELISA assays.

CHAPTER FIVE

NEGLECTED BACTERIAL PATHOGENS IN CENTRAL AMERICA: RISK FACTORS ASSOCIATED WITH SPOTTED FEVER GROUP RICKETTSIOSES ANTIBODIES IN A PEDIATRIC COHORT FROM EL SALVADOR

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5.1 Abstract

Spotted Fever Group Rickettsioses are a group of bacterial zoonoses that are considered neglected diseases due to their typical undifferentiated febrile illness symptomology, underreported nature, and association with those in poverty. Unfortunately, SFGR diagnostic methods are severely lacking. The methods that are available are either too complicated or too expensive for low- to middle-income countries to routinely integrate, especially when public health resources are constrained and focused on other diseases such as malaria and dengue. There is little to no understanding of who is at risk for SFGR in El Salvador, where no epidemiological investigations have been conducted to determine who is most at risk for SFGR or other tick-borne diseases. To lay the foundation for tick-borne disease epidemiology in this country, our team conducted multiple statistical analyses on SFGR IgG and IgM seroprevalence data from banked pediatric samples from a previous vector-borne disease high-risk study. Older, male, children enrolled during the wet season, with a household history of infectious disease and higher maternal education level had higher odds of SFGR antibodies. Additionally, children from households with birds and previous knowledge of other vector-borne diseases had significantly reduced odds of SFGR antibodies. This study highlights that much is unknown regarding the complexity of the tick, animal host, and human host ecology surrounding movement of SFGR pathogens in El Salvador. Further studies investigating this system are warranted to complete the picture of tick-borne disease in El Salvador.

5.2 INTRODUCTION

Neglected tropical diseases (NTDs) are infectious diseases which impact those in poverty, marginalized populations, populations in geographical isolation, and populations which have insufficient and unstable financial and political resources to control them, oftentimes leading to devastating impacts on society and development[58,128]. Many bacterial zoonoses fit this definition but are commonly not classified as NTDs due to issues with definitive etiological diagnosis and reporting as their symptomology typically falls under undifferentiated febrile illness (UFI), and differential diagnosis will not be made[58]. One group of neglected bacterial diseases commonly misdiagnosed and underreported are spotted fever group rickettsioses (SFGR)[11,46,59]. Although easily treatable, these tick-borne bacterial infections can cause significant disease if untreated, especially for the most virulent species, *Rickettsia rickettsii*, the causative agent for Rocky Mountain spotted fever (RMSF)[58,116].

In tropical and semitropical regions of the world, including Central America, SFGR is commonly placed on the proverbial ‘backburner’ in comparison to NTDs or other vector-borne diseases such as malaria, dengue, Chagas disease, and soil-transmitted helminths[7,58]. Although there are multiple molecular and serological methods available for diagnosing SFGR infection, typically the use of a singular test or the use of a singular sample is not recommended. Additionally, many of the kits or diagnostics require expensive equipment, expensive reagents, or technical expertise, which are not always affordable for low- and middle-income countries. Due to these requirements of

use and resources, countries with limited resources may not be able to dedicate public health resources to diagnosis of SFGR or other bacterial that fall under the UFI umbrella of symptomology[70,71]. Use of IgG and IgM ELISA antibody tests for SFGR are considered one of the easiest options available, while also maintaining relatively high sensitivity and specificity, and the combination of these with convalescent sera is thought to be one of the best methods for surveillance of SFGR[122]. For countries with no epidemiological information on risk factors to SFGR, like El Salvador, this gap leaves vulnerable populations at continued risk for remaining undiagnosed and untreated.

Only two serological surveys have been published for the country of El Salvador in the past 30 years[69,78], and neither of these investigated epidemiological characteristics of positive individuals. In contrast, 4 reports from neighboring Honduras have identified frequent contact with animals (specifically dogs) and large amounts of time spent outdoors as significant risk factors associated with SFGR antibody seroprevalence[53,98-100]. In Nicaragua, exposure to farm animals and dogs, rural residence, and lower education were all found to significantly increase the odds of SFGR infection in humans[54-56]. Lastly, a fatal outbreak of SFGR in Guatemala also suggested those in close contact with dogs, rats, and farm animals were at highest risk for infection[57]. Without this information in El Salvador, public health officials are left at an impasse when determining where to dedicate limited resources to break the cycle of infection.

To close this information gap and inform public health officials regarding those at risk for SFGR infection in El Salvador, our team conducted SFGR IgG

and IgM ELISA antibody serological assays on banked pediatric samples from a previous study, determined seroprevalence of SFGR antibodies, and conducted an epidemiological investigation into risk factors of this vulnerable population.

5.3 METHODS

DATA

The dataset utilized in this study was described previously, in Chapter 4. Primary data analysis was conducted to determine outcome measurements of SFGR antibody results through commercially available ELISA kits for both IgG and IgM antibodies (Fuller Laboratories, Fullerton, CA, USA). These outcome measurements were then added to the previously collected household survey data from the original study on Chagas disease, gastrointestinal parasite infection, and nutrition[103].

DATA ANALYSIS

The participant household survey from the original study included multiple variables that were analyzed as potential risk factors for SFGR antibodies including time of enrollment, demographic characteristics, poverty indicators, knowledge of vector-borne disease, association with animals, and health information. Descriptive statistics were ascertained on all participants, stratified by the outcome: antibody positive and antibody negative individuals. Three separate multivariate logistic regression models with stepwise selection were run to estimate associations between potential risk factors and antibody status. Initial inclusion into each model was set to $p<0.35$, and final inclusion was set to $p<0.10$. Three models were run to determine risk factor differences between

individuals positive for the two separate SFGR antibodies. Model 1 regressed the outcome of positive/negative on either SFGR ELISA against various risk factors, Model 2 utilized the outcome of positive/negative on IgG ELISA only, and Model 3 utilized the outcome of positive/negative on IgM ELISA only. Multicollinearity was assessed for all variables included in the stepwise selection models through variance inflation factors (VIF); no multicollinearity was found among any variables. All statistical models were run using SAS software (version 14.1; SAS Institute, Inc., Cary, NC, USA).

5.4 Results

A graph of the enrolled pediatric participants' SFGR antibody positive status over the 2018 calendar year is presented in Figure 5.1. The majority of IgG and IgM positive individuals were enrolled during the wet season, from Epidemiological Weeks 18-44. There was a steady increase in participants testing positive for IgM antibodies, with a peak at week 30 during this wet season. The IgG positive individuals did not have as noticeable of a peak comparatively. Positive participants to both antibodies seemed to drop-off around week 36, however positive individuals continued to be enrolled until week 45. In comparison, Figure 5.2 displays the proportion of positive enrolled pediatric participants over the 2018 calendar year by Epidemiological Week. This noticeable increase in positives over the wet season is conserved when considering the proportion positive of pediatric participants. The highest proportion of IgM positives occurred at week 22, nearly reaching 40% of all

enrolled participants that week. The highest proportion of IgG positives occurred at week 41, with 20% of enrolled participants.

Table 5.1 contains demographic and risk factor information related to all enrolled participants from the Chagas disease enrollment survey from the original study[103]. Results are broken down into three SFGR antibody status groups: positive/negative on at least one ELISA, positive/negative on IgG ELISA only, and positive/negative on IgM ELISA only. The majority of antibody positive individuals were female, not febrile, enrolled during the wet season, and were slightly older than those who were antibody negative. IgG positive individuals were the only group that was not mostly female. Household occupancy and composition was also very similar among groups, with slightly more individuals living in antibody negative participants' homes and IgG antibody positive individuals with the lowest average last year of maternal education comparatively. Poverty indicators appeared to be evenly split between antibody positive and negative individuals, except a larger proportion of positive individuals had adobe walls in their bedrooms, potable water, and no electricity, while a smaller proportion of positive individuals consistently reported having fowl (chickens, ducks, and/or turkeys). Additionally, a smaller proportion of antibody positives consistently reported knowing what a "chinche" is, someone in the household being bitten by a chinche, and having the house treated/fumigated in the past year, compared to antibody negative individuals. Lastly, more antibody positive participants reported someone in their household needing medical

attention for an infectious disease in the past year than antibody negative participants.

Multiple risk factors were significantly associated with Rickettsia antibody positive status in the pediatric cohort, displayed in Table 5.2 from the multivariate logistic models utilizing stepwise selection. One variable was significantly associated with antibody positive status in all three models: participant age. As age increases by one year, participants' odds of testing positive for either SFGR antibody (Model 1) significantly increased (p-value <.0001; OR: 1.155, 95% CI: 1.081 to 1.232). A similar increase in odds was seen in the model for IgG ELISA positive only (p-value = 0.0159; OR: 1.179, 95% CI: 1.031 to 1.348) and IgM positive only participants as well (p-value<.0001; OR: 1.152, 95% CI: 1.075 to 1.235).

The odds of testing positive for Rickettsia antibodies among males was between 1.8 and 5.3 times higher than females in the first (p-value = 0.0456; OR: 1.813, 95% CI: 1.012, 3.251) and second models (p-value = 0.0203; OR: 5.31, 95% CI: 1.404, 20.246). For every one year increase in mother's last year of education, Model 1 and Model 3 indicated participants' odds of testing positive for SFGR antibodies increased by 6.8% (Model 1 p-value = 0.0738; OR: 1.068, 95% CI: 0.994 to 1.148) and 12% (Model 3 p-value = 0.0115; OR: 1.112, 95% CI: 1.024 to 1.208). Though, Model 1's OR is not significant, this trend is of note. A household with reported member(s) seeking clinical care for an infection origin in the past year had 2.174 (p-value = 0.0094; 95% CI: 1.21 to 3.906) times the odds of antibody positive status for either ELISA, and the same pattern was seen for

the odds of IgM antibody positive participants only (p-value = 0.0516; OR: 1.898, 95% CI: 0.996 to 3.619)—although this was slightly higher than a 0.05 significance level. Both Models 1 and 3 also indicated that participants who were febrile at enrollment had a decreased odds of either antibody positivity (Model 1 p-value = 0.0035; OR: 0.357, 95% CI: 0.176 to 0.713) and IgM antibody positivity only (Model 3 p-value = 0.0132; OR: 0.377, 95% CI: 0.174 to 0.815). The odds of antibody positive status on either ELISA assay for those who indicated they used an outside latrine for a bathroom were 0.249 times the odds of those who did not use this type of bathroom (p-value = 0.0011; OR: 0.249, 95% CI: 0.108 to 0.575). This same pattern was seen in IgG positive participants (p-value = 0.0027; OR: 0.104, 95% CI: 0.026 to 0.415).

For Model 1 only, those who indicated they did not have electricity had an increased odds (p-value = 0.0048; OR: 2.413, 95% CI: 1.309 to 4.45) of SFGR antibody positive status compared to those who did have electricity. Households with birds had a decreased odds (p-value = 0.0156; OR: 0.486, 95% CI: 0.27 to 0.872) of testing positive for either antibody, and those reporting someone in the household bitten by chinches in the past year had a decreased odds (p-value = 0.0491; OR: 0.393, 95% CI: 0.155 to 0.997) of testing positive for either antibody.

For Model 2 only, households with a cement bedroom floor had a decreased odds of SFGR IgG antibodies (p-value = 0.0276, OR: 0.26, 95% CI: 0.076 to 0.862) compared to those with a bare earth bedroom floor. Additionally, those reporting that they knew what a chinche was had a decreased odds for IgG

antibodies (p-value = 0.0027, OR: 0.13, 95% CI: 0.034 to 0.492) compared to those who did not know what a chinche was.

For Model 3 only, participants enrolled during the wet season had 2.527 times the odds of testing positive for SFGR antibodies compared to those enrolled during the dry season (p-value = 0.0099; OR: 2.527, 95% CI: 1.25, 5.108).

5.5 Discussion

This is the first epidemiological investigation of potential risk factors for SFGR infection in El Salvador. We found significant associations between multiple demographic, poverty-related, and human behavior characteristics and evidence of SFGR infection from this pediatric cohort. Additionally, there were some risk and protective factors that do not follow the traditional story of neglected infectious diseases, and this warrants further investigation into the complex natural history and ecology of SFGR pathogens and human and animal hosts. A conceptual model of risk factors determined from these analyses is displayed in Figure 5.3.

Overall, an increase of the proportion and number of acute infected (IgM positive) participants was seen as the 2018 year progressed and pediatric participants were enrolled, and the number and proportion of positive participants declined in the middle of the wet season. Seasonality played a statistically significant role in accumulation of IgM positive individuals, as this variable was a risk factor for IgM antibody positive status. In contrast, seasonality did not play a significant role for IgG positive individuals, as expected since these individuals

were infected a much earlier time compared to those positive for IgM. Following infection with SFGR *Rickettsia* spp., IgM antibodies typically peak around 3-4 weeks and are detectable until 3-4 months post infection, and IgG antibodies peak around 10-12 weeks and are detectable until 12 months post infection[122-125]. Kinetics of antibody responses will vary by person, and by the species of SFGR bacteria infecting the individual[124,125]. The wet season in El Salvador is hotter and more humid, providing a preferable habitat for tick vectors' movement and reproduction. With more ticks in the environment questing for blood meals, there is an increased chance for children to come into contact with them, thus increasing the chances for infection with SFGR bacteria. This pattern is most likely seasonal, as a previously fatal SFGR outbreak occurred in neighboring Guatemala—in a department on the El Salvador border—at the beginning of the wet season in 2007[57].

All three of the multivariate logistic models identified at least five risk or protective factors associated with SFGR antibody presence in this pediatric cohort. Increased age was the only variable consistently seen to increase participants' odds for SFGR antibody presence for all three models by approximately 15-18%, suggesting widespread transmission. A similar age-dependent increase has been documented in two separate studies in Peru, where older participants (older children and adults) had consistently greater odds of antibodies to SFGR[129,130]. Both Models 1 and 2 also indicated that males had increased odds for SFGR antibodies, which has also been reported in previous studies in South America[130-132]. As children age and grow, their propensity to

explore outdoors increases, increasing chances of encountering ticks in the environment—especially from infested dogs[133], and males in general typically participate in activities associated with higher tick exposure due to gender roles and playtime[130,131,134-136].

Family use of an outdoor latrine was a significant protective factor against either SFGR antibody or IgG antibodies. This rural poverty indicator is a surprising finding, as poverty indicators are commonly found associated with increased odds of neglected tropical diseases. In previous vector-borne disease studies, outdoor latrine use has been associated with increased odds of visceral leishmaniasis[137], but also decreased odds of lymphatic filariasis[138]. One study in Mexico found an outside latrine was associated with decreased risk for SFGR-infected animals around households but did not offer an explanation as to why this could be the case[139]. It is possible outdoor latrines are avoided by feral animals hosting ticks, and this could reduce potential infected tick exposure around these households. Obviously, additional factors related to the ecology of SFGR transmission between ticks and host animals are related to outdoor latrine use and need to be explored.

Another variable with an unanticipated impact on odds of SFGR antibodies was increasing maternal educational level had a positive impact on odds of at least one ELISA positive and IgM antibodies (Models 1 and 3). Few studies have included education level in epidemiological investigations regarding SFGR serology, and results either suggest no association between education level and positive serology[140], or an increase in odds for SFGR antibodies with

lower education level[54,141,142]. However, these reports reference the respondents' own level of education, and not maternal education level. It is possible that mothers with a higher education level have jobs outside of the home, meaning they might leave their children at home more often than those at home. This could lead to children potentially encountering ticks from infested animals or from the environment more as they are unsupervised more often than others. More recent serological investigations in Latin America focus on parental occupation rather than education level[129,130]. One study found occupations outside of the home (agriculture or non-agriculture based) had elevated odds of SFGR serostatus compared to those who were students or at home[129].

For both Models 1 and 3, participants with a fever (temperature >38°C) at time of enrollment, had a significant reduction in odds of SFGR serology compared to those without a fever. This finding was unanticipated for those with IgM antibodies—or evidence of an acute infection. Two possible explanations could account for this observation. First, most acutely infected participants were outside of the window of fever, as this typically develops within 1-2 weeks of infection and lasts for approximately 1 week[143], and IgM antibodies peek around 3-4 weeks—thus this symptom can be missed if serology evaluation is not timed just right. Second, a larger proportion of those acutely infected never developed a fever than anticipated. A growing body of evidence has suggested that many SFGR infections' symptomologies will not always include fever, and this has been seen in multiple cases[144-146]. Given the large differences in symptoms from the various SFGR species, it is important to note that multiple studies have

indicated that relying on most common symptoms of SFGR infections or strict case definitions will miss many infections where symptomology is not 'typical'[125,145]. Lastly, it is important to mention the recruitment of this population (from the original study) indicated that participants were either presenting at a clinic or known to live in a home infested with triatomines. A selection bias could have occurred here where this selection mechanism could be related to febrile status, and this was not indicated through our statistical models.

Odds for SFGR serostatus increased significantly when respondents indicated someone in the household had sought clinical care for an infection origin in the past year. This could indicate that residents of the same household are at increased risk for multiple vector-borne diseases due to similar characteristics such as poverty indicators, as seen in the previous Chagas disease study[103]. Additionally, illness from an infectious origin could be relatively common in these households, leading to a sort of complacency regarding preventing infectious diseases of any cause.

Our study also indicates that household having birds such as chickens, ducks, or turkeys had significantly lower odds of either SFGR antibody. Similarly, pet bird (macaws and parakeets) ownership was found to be a significant protective factor for SFGR antibodies in one Peru study[129]. The presence of birds may serve as a zooprophylactic protective factor to reduce the contact between infected ticks and humans[129], but it is also postulated that the non-pet species mentioned specifically in El Salvador could directly protect household members through predation on ticks[147,148].

Households without electricity had significantly increased odds of either SFGR antibody positive status. This poverty indicator is probably directly related to the amount of time children are spending outdoors: increased outdoor playtime is anticipated when there is no electricity in the household, increasing chances of encountering infested animals or infected ticks in the environment. Previous studies have seen increased likelihood of SFGR infection within households without electricity[149] or an increase of neglected tropical vector-borne disease risk with increased poverty indicators including lack of electricity[150].

Participants reporting someone had been bitten by a chinche insect in the past year and those reporting knowing what a chinche is had significantly reduced odds for SFGR antibody status in Model 1 and Model 2, respectively.

It is plausible that individuals in households with previous experience with other vectors (triatomines) will be more observant or will notice other vectors, i.e. ticks, inside their home or on their loved ones. This would lead to a reduction in either the number of ticks brought in one's household, or the amount of time ticks could spend on children or family members inside, as more vigilant parents are more aware of arthropod vectors and will remove them.

Another poverty indicator, type of floor in the bedrooms, was a significant predictor for IgG antibodies in participants. Cement floors, as opposed to bare earth floors, significantly reduced the odds of SFGR antibody serology. This indicator is common in more rural areas, which is a typical risk factor for SFGR infection[151].

The most surprising results from these models are what each of these models did not indicate as statistically important: whether or not the household had dogs and whether the primary household income source was agriculture-based. Exposure to dogs is one of the most commonly reported risk factors for SFGR infection for the Central American region[53-57] Although, dog ownership was not found to be significantly associated with increased odds for SFGR antibodies in some studies in South America[129]. Dogs, and canines in general, are thought to be important bridges from the sylvatic environment to the domestic, great hosts for multiple species of ticks, and they can develop high enough bacteremia to sustain transmission of SFGR bacteria for 4-8 days on average post infection[152]. In addition to exposure to dogs, previous reports have indicated copious amounts of time spent outdoors[53,99], agricultural work[54,57] and exposure to other animals like rodents[57,98,100] are all significant risk factors for SFGR infection. The original study of this pediatric did find dogs were a significant risk factor for Chagas disease infection[103], but it seems as though households reporting having dogs were spread relatively evenly among the SFGR antibody positive and negative groups. Additional information regarding animals in and around the home would be beneficial to further understand this ecological dynamic.

Limitations to this study include the fact that the risk factor survey for the original study was developed to investigate Chagas disease risk factors and was not specifically made for investigating SFGR risk factors or symptomology. Due to this, we were unable to investigate other factors that could fill in gaps to the

SFGR story of this pediatric population. To better understand potential etiologic agents, specific SFGR symptomology could have been determined to narrow down species as this is an important aspect of acute SFGR infection[153]. Additionally, questions regarding exposure to ticks on respondents and their children would be beneficial to understand participants' knowledge of ticks and tick-borne disease in this region and help in creating a clearer picture of a potential exposure timeline. Only one question from this survey inquired about dogs, and given that dogs are typically considered a major risk factor for infection with SFGR, additional questions about participant relationships with feral, domestic, and community dogs could reveal significant information for this cohort[154]. In addition, the reliability of ELISA antibody assays varies depending on the species of SFGR infecting cases: delay or prevention of antibody development has even been seen in patients infected with less pathogenic species compared to those infected with species like *R. rickettsii*[122,146,155]. Lastly, the generalizability of this population is low for the rest of the country, as the recruiting mechanism targeted those presenting to clinics or those living in infested homes.

Despite evidence of SFGR infection prevalence in El Salvador from the previous two studies in the early to mid-1990's, no epidemiological investigation into risk factors of SFGR had been conducted until this study[69,78]. We found multiple characteristics that increased participants' odds of SFGR antibody positivity status, some of which do not fit the typical mold of tick-borne disease ecology and need to be explored even further. The evidence of relevant human

behavior and characteristics related to poverty and tick ecology indicate that there are avenues where epidemiological intervention can succeed in preventing future SFGR infections in those most at risk in El Salvador. Although singular antibody assay results are not 100% reliable, we present evidence that SFGR infection is probably endemic in El Salvador, and significantly associated with other vector-borne disease concerns. Further molecular and serological studies of vectors, humans, and host animals are necessary to understand the epidemiological and ecological relevance of these findings. In addition, diagnostics are limited for SFGR infection in general, and there is a need for less expensive and easy-to-use diagnostic tests—especially in regions where there is little to no resources formally dedicated to tick-borne disease surveillance[7,122]. Patients presenting with undifferentiated febrile illness or fever of unknown origin with any of these presented risk factors should be screened for rickettsial pathogens in El Salvador to ensure misdiagnosis of SFGR does not continue in this country, and those who need antibiotics are treated.

Table 5.1 Descriptive Statistics of Enrolled Pediatric Participants, by Antibody Status.

	ELISA Positive (IgG or IgM) N=121 (%) or Mean +/- SD	ELISA Negative (IgG or IgM) N=887 (%) or Mean +/- SD	IgG Positive N=26 (%) or Mean +/- SD	IgG Negative N=971 (%) or Mean +/- SD	IgM Positive N=95 (%) or Mean +/- SD	IgM Negative N=662 (%) or Mean +/- SD
Participant characteristics						
Female	63 (52.1)	409 (46.1)	9 (34.6)	529 (54.5)	54 (56.8)	344 (52.0)
Febrile at time of enrollment	32/117 (27.4)	540 (64.2)	7 (26.2)	322/922 (34.9)	25/91 (27.5)	228/642 (35.5)
Enrolled in the wet season	81 (66.9)	503 (56.7)	14/26 (53.8)	561 (57.8)	67 (70.5)	380 (57.4)
Age (years)	10.6 +/- 4.6	8.5 +/- 4.7	12.0 +/- 4.1	8.6 +/- 4.7	10.2 +/- 4.7	8.3 +/- 4.8
Household occupancy and composition						
N living in household	5.2 +/- 2.1	5.5 +/- 3.0	5.0 +/- 2.0	5.5 +/- 3.0	5.3 +/- 2.1	5.5 +/- 3.1
N kids in household	2.5 +/- 1.3	2.5 +/- 1.4	2.4 +/- 1.4	2.5 +/- 1.4	2.6 +/- 1.3	2.5 +/- 1.3
Mother's last year of education	5.1 +/- 4.0	5.4 +/- 4.1	4.2 +/- 4.6	5.4 +/-4.1	5.5 +/- 4.0	5.4 +/- 4.0
Father's last year of education	5.5 +/- 4.2	6.0 +/- 4.4	5.0 +/- 4.5	5.9 +/- 4.4	5.7 +/- 4.1	6.1 +/- 4.4
Number of beds in home	4.7 +/- 1.9	4.8 +/- 2.3	4.7 +/- 1.9	4.8 +/- 2.2	4.7 +/- 1.9	4.8 +/- 2.3
Household poverty indicators						
Agriculture as primary household income source	69/110 (62.7)	513/816 (62.9)	13/22 (59.1)	564/894 (63.1)	57/88 (64.8)	389/607 (64.1)
Type of cooking fuel used						
Leñas (a type of wood)	14/119 (11.8)	126/886 (14.2)	1/25 (4.0)	139/970 (14.3)	13 (13.7)	95/660 (14.4)
Gas	6/119 (5.0)	50/886 (5.6)	1/25 (4.0)	53/970 (5.5)	5 (5.3)	37/660 (5.6)
Both	99/119 (83.2)	710/886 (80.1)	23/25 (92.0)	778/970 (80.2)	77 (81.0)	248/660 (37.6)
Type of floor in bedroom						

Bare earth	96/119 (80.7)	695 (78.4)	18/25 (72.0)	765 (78.8)	79 (83.2)	514/661 (77.8)
Cement	23/119 (19.3)	192 (21.7)	7/25 (28.0)	206 (21.2)	16 (16.8)	147/661 (22.2)
Type of wall material in bedroom						
Blocks	21/119 (17.6)	177/885 (20.0)	2/25 (8.0)	191/969 (19.7)	19 (20.0)	132/559 (20.0)
Adobe	58/119 (48.7)	357/885 (40.3)	12/25 (48.0)	401/969 (41.4)	46 (48.4)	279/559 (42.3)
Other	40/119 (33.6)	351/885 (39.7)	11/25 (44.0)	377/969 (38.9)	30 (31.6)	248/559 (37.6)
Family uses outside latrine for bathroom	108/120 (90.0)	823/886 (92.9)	21/25 (84.0)	901/970 (92.9)	88 (92.6)	618/660 (93.6)
Potable water in the house	112/120 (93.3)	776 (87.5)	24/25 (96.0)	854 (88.0)	89 (93.7)	581/660 (87.9)
House has electricity	58/116 (50.0)	568/876 (64.8)	12/24 (50.0)	606/957 (63.3)	46/92 (50.0)	418/651 (64.2)
House has fowl (chickens, turkeys, and/or ducks)	53/113 (46.9)	471/860 (54.8)	9/25 (36.0)	511/937 (54.5)	44/88 (50.0)	336/636 (52.8)
House has cats	17/113 (15.0)	121/860 (14.1)	2/25 (8.0)	135/937 (14.4)	15/88 (15.8)	88/636 (13.3)
House has dogs	60/113 (53.1)	462/860 (53.7)	11/25 (44.0)	507/937 (54.1)	50/88 (56.8)	335/636 (52.7)
Household Chagas disease vector exposure and parent or legal guardian Chagas disease knowledge						
Knows what a "chinche" is	74/120 (61.7)	610 (68.8)	13/25 (52.0)	663 (68.3)	61 (64.2)	449/661 (67.9)
Knows about Chagas disease	17/120 (14.2)	178 (20.1)	6/25 (24.0)	188 (19.4)	11 (11.6)	126/661 (19.1)
Has seen chinches inside house within the past year	28/120 (23.3)	283 (31.9)	6/25 (24.0)	302 (31.1)	22 (23.2)	218/661 (33.0)
Someone in household has been bitten by a chinche in the past year	18/120 (15.0)	161/884 (18.2)	3/25 (12.0)	176/968 (18.2)	15 (15.8)	132/660 (20.0)
House has been fumigated in the past year	7/120 (5.8)	90/885 (10.2)	2/25 (8.0)	94/969 (9.7)	5 (5.3)	66/660 (10.0)

Health concerns						
Someone in the household sought clinical care in the past year	90/119 (16.0)	599/867 (69.1)	18/25 (72.0)	665/950 (70.0)	73/94 (77.7)	455/646 (70.4)
Someone in the household sought clinical care for an infection origin in the past year	59/118 (50.0)	341/865 (39.4)	12/25 (48.0)	386/948 (40.7)	48/93 (51.6)	257/645 (39.8)

Table 5.2. Multivariate Logistic Regression Models with Stepwise Selection.

Variable	Odds Ratio	95% CI	P-value
Model 1: At Least one ELISA Positive (IgG and IgM)			
Male vs. Female	1.813	(1.012, 3.251)	0.0456
Age	1.155	(1.081, 1.232)	<.0001
Febrile at time of enrollment	0.357	(0.179, 0.713)	0.0035
Mother's last year of education	1.068	(0.994, 1.148)	0.0738
Family uses outside latrine for bathroom	0.249	(0.108, 0.575)	0.0011
No electricity	2.413	(1.309, 4.45)	0.0048
House has birds	0.486	(0.27, 0.872)	0.0156
Someone in household has been bitten by a chinche in the past year	0.393	(0.155, 0.997)	0.0491
Someone in the household sought clinical care for an infection origin in the past year	2.174	(1.21, 3.906)	0.0094
Model 2: IgG ELISA Positive Only			
Male vs. Female	5.331	(1.404, 20.246)	0.0203
Age	1.179	(1.031, 1.348)	0.0159
Cement vs. bare earth floor in bedroom	0.26	(0.079, 0.862)	0.0276
Family uses outside latrine for bathroom	0.104	(0.026, 0.415)	0.0027
Knows what a "chinche" is	0.13	(0.034, 0.492)	0.0027
Model 3: IgM ELISA Positive Only			
Age	1.152	(1.075, 1.235)	<.0001
Febrile at time of enrollment	0.377	(0.174, 0.815)	0.0132
Enrolled during the wet season	2.527	(1.25, 5.108)	0.0099
Mother's last year of education	1.112	(1.024, 1.208)	0.0115
Someone in the household sought clinical care for an infection origin in the past year	1.898	(0.996, 3.619)	0.0516

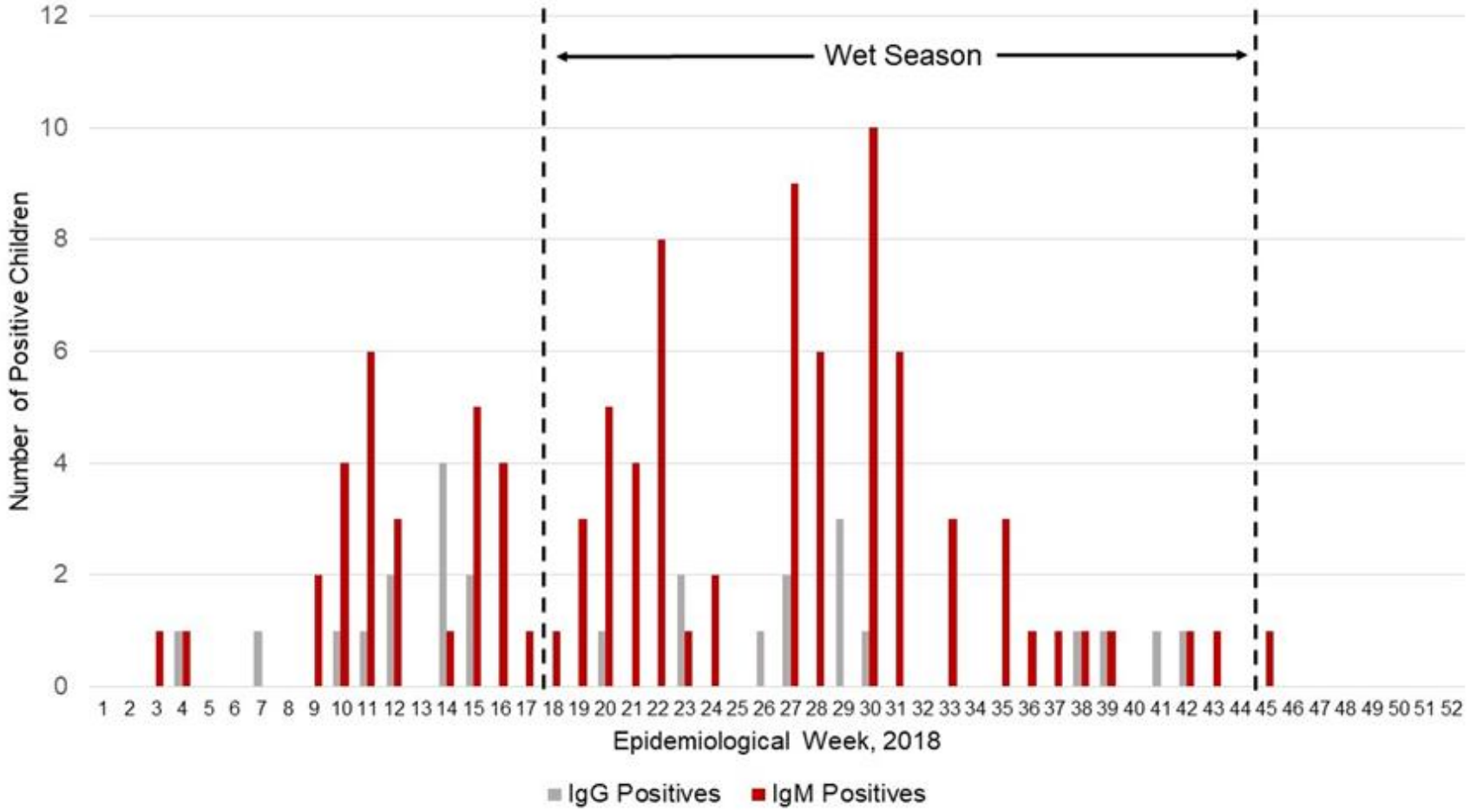

Figure 5.1. Seasonality of SFGR Serology in Enrolled Pediatric Participants

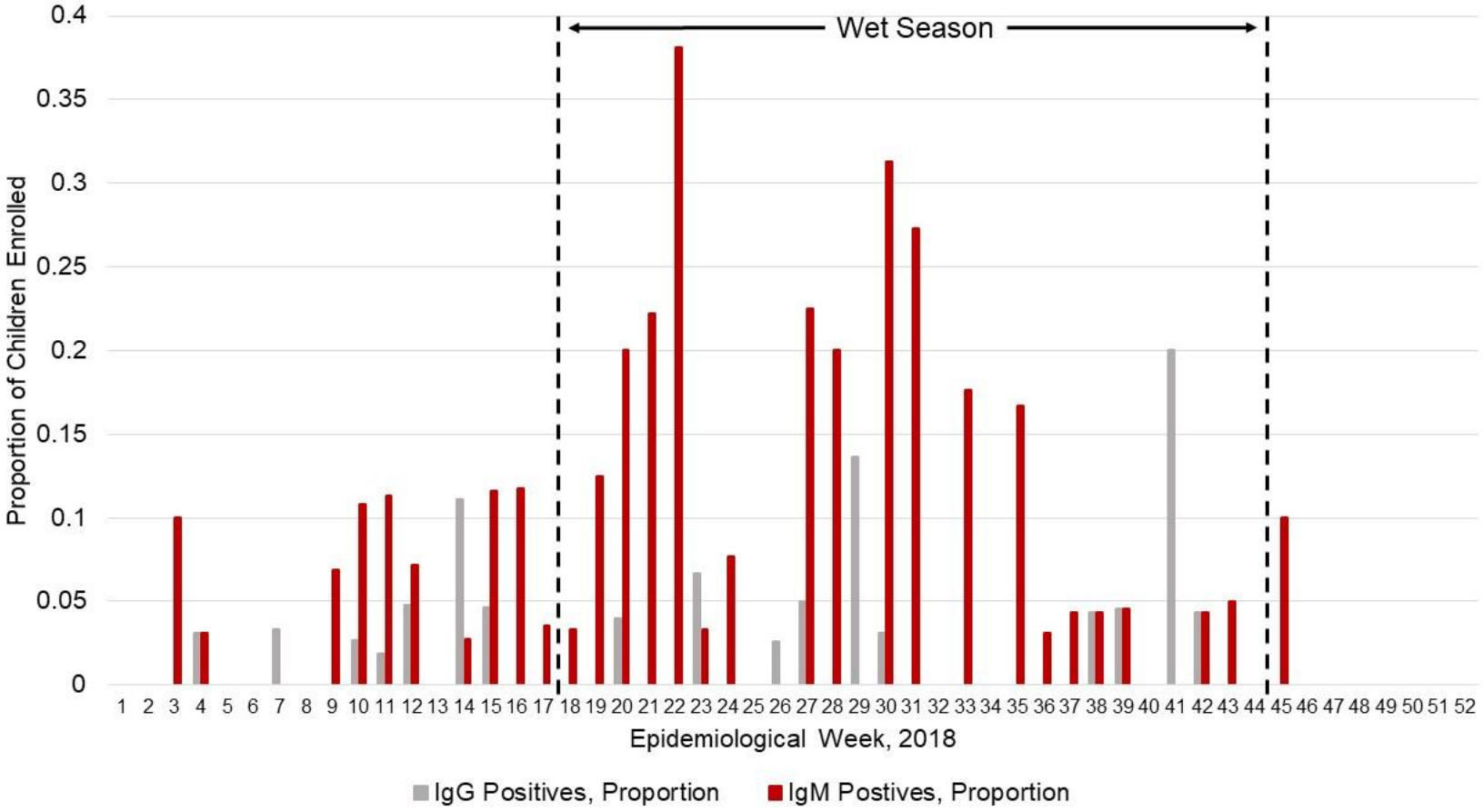

Figure 5.2. Seasonality of SFGR Serology in Enrolled Pediatric Participants, Proportion Positive of all Enrolled

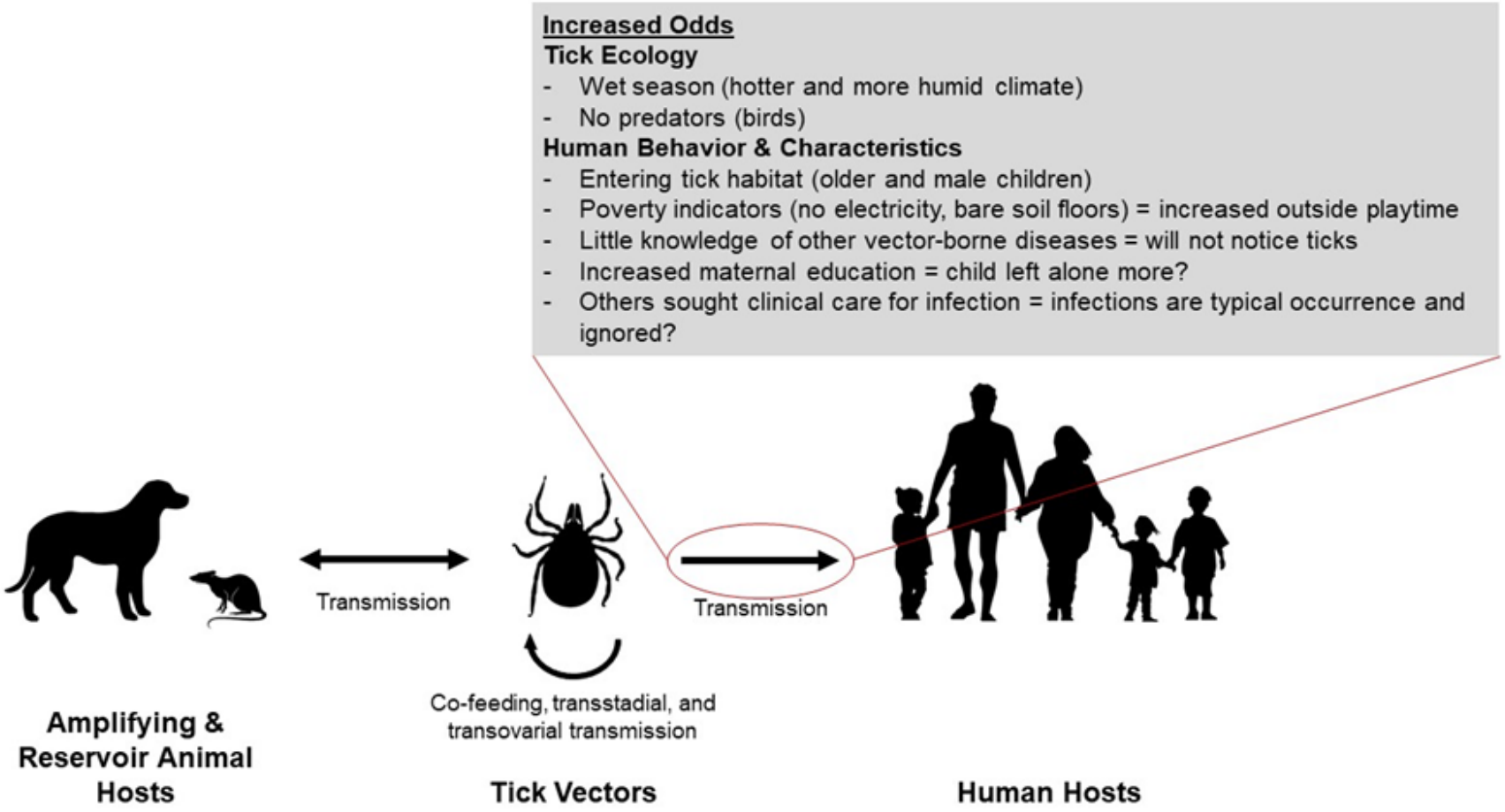

Figure 5.3. Conceptual Model of SFGR Pathogen Movement and Results of Logistic Regression Models

CHAPTER SIX

FIRST REPORT OF *RICKETTSIA RICKETTSII, ANAPLASMA* SPP. AND *EHRLICHIA* SPP. IN EL SALVADOR: GLOBAL IMPLICATIONS FOR UNDERDIAGNOSIS OF NEGLECTED BACTERIAL PATHOGENS IN LOW- AND MIDDLE-INCOME COUNTRIES

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[3]Dye-Braumuller,KC, Lynn MK, Cornejo Rivas PM, Rodriguez Aquino MS, Chandler JG, Trout Fryxell RT, Self SCW, Kanyangarara M, Nolan MS. To be submitted to *Journal of Medical and Veterinary Entomology*

6.1 Abstract

Neglected bacterial zoonoses are a group of Neglected Tropical Diseases (NTDs) that are commonly underdiagnosed and underreported due to their undifferentiated febrile illness symptomology. Spotted fever group rickettsioses (SFGR), a subset of tick-borne bacterial zoonoses, belong in this group. There is a dichotomy in the reporting and recognition of these pathogens in Central America: countries with reduced human development scores—like El Salvador—have little to no research or surveillance dedicated to these pathogens and the diseases they cause. Our team conducted the third-ever tick survey in El Salvador to investigate the presence of SFGR pathogens in local ticks. Traditional and quantitative PCR were used to detect presence of SFGR, *Ehrlichia*, and *Anaplasma* spp. of pathogens in ticks. *Ehrlichia* spp. were detected in up to 4.8% of ticks, *Anaplasma* spp. were detected in up to 5.2% of ticks, and SFGR pathogens were detected in up to 48.8% of ticks. *Rickettsia rickettsii* was specifically found in up to 25.6% of ticks. This is the first report of *R. rickettsii* in El Salvador. Our study emphasizes the need for further surveillance and research including incorporating additional human seroprevalence and testing to understand the public health burden in this country.

6.2 Introduction

Neglected Tropical Diseases (NTDs) cause morbidity and mortality with high endemicity in low-and middle-income countries of Africa, Asia, and the Americas. These infectious diseases disproportionately affect those in poverty, commonly resulting in chronic conditions or disabilities, and adversely affecting

not only health—but childhood education and economic productivity[156]. Nevertheless, NTDs are underdiagnosed and underreported as they impact the poorest and most disadvantaged populations globally. The Central American region has faced considerable challenges to combating NTDs including the drug trade, food, political, and socioeconomic insecurity, and urbanization[157]. Multiple NTDs of concern are vectored by arthropods; the highest concern and priority in Central America placed on Chagas disease, dengue, malaria, and leishmaniasis[64,157].

Although not included in the World Health Organization's list of NTDs, bacterial diseases caused by *Rickettsia* bacteria cause significant acute febrile illness with high rates of potentially severe or fatal complications in disproportionately poor and marginalized communities globally—especially in the Americas[46]. The species with the highest case fatality rate is *R. rickettsii*, the causative agent for Rocky Mountain spotted fever (RMSF). Most rickettsial diseases are widely unrecognized and underreported epidemiologically, despite being easily treatable[46]. Consequently, these diseases have been classified as neglected bacterial pathogens[59]. Populations at risk for vector-borne rickettsial diseases include children, those in poverty, marginalized peoples, and those in rural areas or who work in agricultural jobs[46]. Of special interest are bacteria that fall into the spotted fever group rickettsioses (SFGR). Pathogens in this group cause higher rates of human mortality and severe complications—but are generally underdiagnosed due to a paucity in diagnostics as most research and

prevention efforts are funneled towards other NTDs, especially those that are mosquito-borne, which cause similar acute febrile symptoms[7,46,54,64].

In Central America, there seems to be a distinct dichotomy in the reporting and recognition of these SFGR pathogens: countries with higher poverty and reduced human development and health, like El Salvador, Guatemala, Honduras, and Nicaragua, have little to no research or surveillance dedicated to these pathogens and the diseases they cause. In contrast, the Central American countries with lower amounts of poverty and higher human development (Belize, Costa Rica, and Panama), seem to have a better understanding of the SFGR risk to their people due to the surveillance and/or research efforts dedicated to these pathogens[7]. This difference in reporting and surveillance renders those in the more impoverished countries at higher risk for outbreaks, which further promotes the cycle of neglected bacterial diseases.

This study focused on SFGR in El Salvador: where the prevalence of tick-borne disease is largely unknown. Two serological studies investigating the prevalence of antibodies to SFGR Rickettsia bacteria have been reported from El Salvador, both from over 25 years ago[69,78]. Human SFGR antibody prevalence ranged from 32.5-40.0% with sample sizes of n=40 for each survey[69,78]. Given this relatively high prevalence of antibodies with few reports of clinical disease, it is highly likely that widespread cases are neither recognized nor reported in El Salvador[78]. In contrast, two tick surveys performed in 2013 documented the tick species in the country and identified multiple *Rickettsia* species infecting these ticks, but none of the species found were pathogenic to humans[61,74]. This

serological history of human infection and lack of documented pathogenic *Rickettsia* bacteria circulating in the environment leads to a misalignment in the epidemiological profile of tick-borne disease in the country. The epidemiology of SFGR presence does not match previous entomological investigations. To bridge this knowledge gap and strengthen the baseline knowledge of pathogenic bacteria circulating in local ticks, our team collected ticks from various locations in El Salvador to further investigate the prevalence of pathogenic *Rickettsia* species in local ticks.

6.3 METHODS

TICK COLLECTIONS AND PROCESSING

El Salvadoran climate is characterized by two seasons: the dry season and rainy season, with two additional 30-day transitional periods between them. The dry season typically falls between November and April, and the rainy season is usually from May to October. Our collections were within the 30-day transitional period between dry season and rainy season.

In May 2021, ticks were collected from three locations in El Salvador: a large experimental agricultural farm part of the University of El Salvador (Campo Experimental Facultad Multidisciplinaria de Oriente Universidad El Salvador San Miguel), a private agricultural farm, and a veterinary office (Veterinaria El Semental). Before collection, permission was obtained from the farm managers, owners, and veterinary manager. The university experimental station is located in the municipality of San Miguel, San Miguel Department (Figure 6.1). This farm has facilities for livestock, agriculture, and agroindustry; it houses 178 cattle, 6

pigs, 16 goats, 31 sheep, and 46 rabbits for various agricultural programs. The private farm (27.5 hectares), located in El Progresso, San Miguel, houses 40 cattle, 2 horses, and 3 dogs. The private veterinary office is located in the municipality of San Miguel, San Miguel Department; the office takes in all domestic animals, however only one dog had ticks when we visited the office.

Ticks were directly collected from the cattle corralled by farm hands into stalls to protect the safety of both the investigator and the animal. Farm hands surveyed all cattle, then brought any cows with ticks to the stalls, so an attempt was made to collect all ticks from all infested cattle. Investigators used their hands to detect ticks, along with visual inspection—paying special attention to common attachment sites including the ears, withers, udders, genital region, and the underside of legs[158,159]. The dog from the veterinary clinic was examined by a veterinary technician, and investigators further checked for ticks on common locations such as the head and neck, ears, between digital pads, belly, rump, and tail[160,161]. Collected ticks were placed in labeled vials containing 75% ethanol. Information about each animal was recorded including breed, age, and sex.

All ticks were transported to the Laboratory of Vector-Borne and Zoonotic Diseases at the University of South Carolina in Columbia, SC and morphologically identified to sex, life stage, and species utilizing various dichotomous keys[106,110,162-164]. Following identification, all ticks were taken to the Department of Entomology and Plant Pathology at the University of Tennessee in Knoxville, TN for pathogen testing and sequencing.

DNA EXTRACTION

Nymph and adult ticks were analyzed individually. All ticks were bisected longitudinally with a sterilized scalpel blade; total DNA was extracted from one half of each tick using the QIAamp 96 DNA kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol using the QIAcube HT (Qiagen, Hilden, Germany). The second half of each tick was stored in 80% ethanol as a voucher. Extracted DNA was stored at -20°C until further processing.

PCR FOR DETECTION OF RICKETTSIA *SPECIES*

Two genes were utilized to detect the presence of *Rickettsia* species in all tick samples: through amplification of the outer membrane protein A (ompA) gene and outer membrane protein B (*ompB*) gene of multiple SFGR species. For the *ompA* gene amplification: reactions in 40 µl total volume using 20 µl of DreamTaq Hot Start Green PCR Master Mix (2X) (Thermo Fisher Scientific, Waltham, MA), 16 µl nuclease-free water, 2 µl template DNA, and 0.25 µM previously published forward and reverse primers (Table 6.1)[111,112]. PCR reactions were performed in a Veriti 96 Well Thermalcycler (Applied Biosystems, Foster City, CA) with conditions set to: 95ºC for 12 min, followed by 35 cycles of 95ºC for 30 s, 60ºC for 30 s, 72ºC for 60 s, and a final extension at 72ºC for 3 min. A positive control (previously *ompA*-positive tick DNA) and two negative controls (first: no template control, and second: previously *ompA*-negative tick DNA) were used in all reactions.

All tick samples were subjected to a second screening specifically for *R. rickettsii* through qPCR, targeting previously published outer membrane protein B

(*ompB*) gene (Table 6.1)[165,166]. For each sample, 15 µl reactions using TaqMan Environmental Master Mix 2.0 (7.5 µl), 200 nM probe, 400 nM each primer, 3 µl template DNA and nuclease free water were performed with a QuantStudio 6 Flex Real Time PCR system (Applied Biosystems, Foster City, CA). PCR conditions were set as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 59°C for 1 min. A positive control of *R. rickettsii* genomic DNA and three negative controls (first: nuclease-free water, second: no template control with all PCR components without sample DNA; and third: *R. parkeri*-positive tick DNA) were used in all reactions. Samples were considered positive if the cycle threshold (Ct) value was less than 40[167].

NESTED PCR FOR DETECTION OF ANAPLASMA *AND* EHRLICHIA *SPECIES*

For detection of both *Anaplasma* and *Ehrlichia* species targeting the previously published *groEL* gene, primary reactions of 20 µl total volume consisting of 10 µl DreamTaq Hot Start Clear PCR Master Mix (2X) (Thermo Fisher Scientific, Waltham, MA), 0.5 µM of each forward and reverse primers (Table 6.1), 6 µl nuclease-free water, and 2 µl template DNA were conducted[168]. The following conditions were used for these PCR reactions: 95°C for 15 min followed by 40 cycles, 58°C for 30 s, 72°C for 30 s and a final extension at 72°C for 3 min. Nested PCR reactions of 30 µl total volume consisting of 15 µl DreamTaq Hot Start Green PCR Master Mix (2X), 0.25 µM of each forward and reverse nested primers (Table 6.1), 11 µl of nuclease-free water, and 2 µl of amplified product from the primary reaction were conducted. Conditions for the nested reaction were as follows: 95°C for 15 min, 35 cycles of 95°C for 30 s,

58°C for 30 s, 72°C for 30 s and a final extension at 72°C for 3 min. Both primary and nested reactions were conducted in the Veriti 96 Well Thermalcycler. Nested amplicons were visualized via gel electrophoresis (1.5% agarose gel: 1xTAE buffer stained with ethidium bromide for 1.5h at 100 V).

CONFIRMATION SEQUENCING

Due to cost constrains, a subset of 65% randomly selected positive amplicons for SFGR *Rickettsia ompA* and 50% randomly selected positive amplicons for *R. rickettsii ompB* and all positive amplicons for *Ehrlichia* or *Anaplasma* were submitted for sequencing. To remove excess dNTPs and residual single-stranded primers or DNA, products were cleaned using 1.5 µl ExoSAP-IT (Thermo Fisher Scientific, Waltham, MA) per 5 µl of PCR product. Final cleaned products were sent to Eurofins Genomics (Louisville, KY) for bi-directional Sanger sequencing. Resulting sequences were aligned in Sequencher 5.1 (Gene Codes Corporation, Ann Arbor, MI) and compared to GenBank deposits via NCBI BLAST using default conditions (other database, non-model organism optimized for highly similar sequences)[169].

STATISTICAL ANALYSES

To detect any univariate associations between tick species, animal host, location of collection, and infection status, chi-square tests and Fisher's exact tests were conducted. Additionally, two multivariate logistic regression models were fit to evaluate between these factors and SFGR and *R. rickettsii* infection status of ticks. All statistical analyses were conducted in SAS statistical software (version 14.1; SAS Institute, Cary, NC, USA).

6.4 RESULTS

In total, 253 ticks were collected from eleven host animals in the three locations in San Miguel, El Salvador. The species breakdown by location is shown in Figure 6.2 (a). Two species were collected: *Rhipicephalus microplus* (Canestrini), the southern cattle tick (N=211), and *Rhipicephalus sanguineus* (Latreille), the brown dog tick (N=21). An additional 21 unknown species were collected which were either partial tick bodies or their integument was too destroyed to make a species determination. All of these unknown ticks were collected from cattle, so it is highly likely that these were R*. microplus*. The private farm yielded the largest number of ticks (N=189), followed by the University of El Salvador's Farm (Campo Experimental de Universidad de El Salvador) (N=51), and lastly, the private veterinarian office (Veterinaria El Semental) (N=13). *R. microplus* was collected from both farms but was not collected from the veterinarian office. However, *R. sanguineus* was collected from dogs on the private farm and at the veterinary office, but not from the University of El Salvador's farm.

Figure 6.2 (b) displays the percent of ticks positive for pathogens collected from each location in San Miguel. Ticks from all three locations were found positive for all targeted pathogens: *Ehrlichia*, *Anaplasma*, and *Rickettsia*. Approximately 8% of ticks collected from Veterinaria Semental were found positive for *Ehrlichia* or *Anaplasma* spp. (n=1) and *Rickettsia rickettsii* (n=1); sequencing identified the first as *Ehrlichia canis*. GenBank accession numbers, percent alignment, and corresponding references for pathogens sequenced are

included in Table 6.2 for randomly selected ticks. Sequencing of the SFGR *ompA* gene did not produce a recognized sequence in GenBank, and the sequencing of R. rickettsii *ompB* positives were not successful. Exact accession numbers are unavailable for these *ompB* positive ticks. The University of El Salvador's farm yielded the highest percentage of positive ticks for any pathogen. Approximately 26% of the 51 ticks collected from this location were positive for *Ehrlichia* or *Anaplasma* pathogens: 12 positive for *Anaplasma margina* and 1 positive for uncultured *Ehrlichia*. Approximately 75% (n=38) of these ticks were positive for SFGR *Rickettsia* and 39% (n=20) were found positive for *R. rickettsii*. Of the 38 SFGR-positive ticks, 13 (34.2%) were attributed to *R. rickettsii*. From the private farm, approximately 3% of the 189 ticks (n=6) were positive for *Ehrlichia* or *Anaplasma* (2 ticks positive for *Anaplasma platys*; 3 unknown *Ehrlichia* spp.; 1 unknown). Another 38% (n=71) of ticks were found positive with SFGR *Rickettsia* bacteria, and 20% (n=37) were positive for *R. rickettsii*. Of these 71 SFGR-positive ticks, 32 (45.1%) are attributed to *R. rickettsii*. Percentages do not add to 100% for each location as multiple ticks collected from each location were found with multiple pathogens, as detailed below.

Table 6.3 describes pathogen prevalence by species. Ticks simultaneously positive for multiple pathogens are included in the calculated percentages of this table; Table 6.4 details information regarding only simultaneously positive ticks. Two of the 21 collected *R. sanguineus* were positive for pathogens: 1 positive for *Ehrlichia canis* (4.8%) and the other positive for *R. rickettsii* (4.8%). Of the collected cattle ticks, a total of 175 were positive for

any pathogens: 5 (2.4%) found positive for uncultured or unknown *Ehrlichia* spp., 2 (0.9%) positive for *Anaplasma platys*, 11 (5.2%) positive for *Anaplasma margina*, 103 (48.8%) positive for SFGR *Rickettsia* spp., and 54 (25.6%) positive for *R. rickettsii* specifically. Of the unknown ticks, 1 (4.8%) was positive for *Anaplasma margina*, 6 (28.6%) tested positive for SFGR *Rickettsia* spp., and 3 (14.3%) were positive for *R. rickettsii*.

Of the simultaneously positive ticks (n=55), almost all were identified as *R. microplus*, and all ticks were positive for SFGR *Rickettsia* plus at least one other pathogen (Table 6.4). Of these ticks, if they were positive for *R. rickettsii*, they were also always positive for SFGR *Rickettsia*. Two ticks were simultaneously infected with uncultured *Ehrlichia* spp. and SFGR *Rickettsia*. One tick was simultaneously infected with both *Anaplasma platys* and SFGR *Rickettsia*, and 8 were simultaneously infected with *Anaplasma margina* and SFGR *Rickettsia*; one tick was positive for a simultaneous infection with three pathogens: *Anaplasma margina*, SFGR *Rickettsia*, and *R. rickettsii*. A majority (n=32) of simultaneously infected ticks were collected from the private farm, followed by the University of El Salvador's farm; no multiple-pathogen-infected ticks were collected from the veterinary office. Of the simultaneously positive ticks identified as adults, n=37 were female, compared to only 17 male ticks.

Tick pathogen prevalence by host animal is displayed in Table 6.5. All five of the cows from the University of El Salvador's Farm had ticks that were positive for SFGR, and four had ticks positive for *R. rickettsii*. One cow hosted ticks positive for uncultured *Ehrlichia*, three cows had ticks positive for *A. marginale*

(Palmeira), and two cows had ticks positive for *A. marginale* (Jaboticabal). At the private farm, all three cows had ticks positive for SFGR, and two of these also had ticks which tested positive for *R. rickettsii*. Two cows hosted ticks positive for *Ehrlichia* sp. and uncultured *Ehrlichia*. One cow hosted ticks positive for *A. platys*. Only one dog of the two surveyed from the private farm had ticks positive for SFGR pathogens. The one dog from the veterinary office had ticks positive for SFGR, *R. rickettsii*, and *E. canis*.

Table 6.6 displays the results of the chi-square and Fisher's exact tests. There was a significant association between: SFGR infection status and host animal (p-value = <.0001), SFGR infection status and collection location (p-value = <.0001), and SFGR infection status and tick species (p-value = <.0001), where higher numbers of SFGR-infected ticks were collected from cattle, from the University of El Salvador's farm, and identified as *R. microplus* than expected. Significant associations were found between: *R. rickettsii* infection status and host animal (p-value = 0.0469) and *R. rickettsii* infection status and collection location (p-value = 0.0076), where higher numbers of *R. rickettsii*-infected ticks were collected from cattle and from the University of El Salvador's farm than expected. Lastly, significant associations were found between: *Ehrlichia* or *Anaplasma* infection status and collection location (p-value = <.0001), and *Ehrlichia* or *Anaplasma* infection status and tick sex (p-value = 0.0189), where higher numbers of *Ehrlichia* or *Anaplasma*-infected ticks were collected from the University of El Salvador's farm and were identified as male than expected. Pertinent null statistical associations were: Tick species with *R. rickettsii* infection

status, tick species with *Ehrlichia* or *Anaplasma* infection, tick sex with SFGR or *R. rickettsii* infection status, and host animal with *Ehrlichia* or *Anaplasma* infection status.

Multivariate logistic regression model results are displayed in Table 6.7. Quasi-complete separation of data points was detected when tick location, species, and host animal variables were included, and consequently these needed to be excluded from the models. Final models for SFGR infection and *R. rickettsii* infection included the variables *Ehrlichia* or *Anaplasma* infection and tick sex. The SFGR logistic model found an approximate 3-fold [Odds Ratio (OR) 3.15, 95% Confidence Interval (CI): 1.14, 8.71] increase in odds of SFGR infection with positive *Ehrlichia* or *Anaplasma* ticks and a nearly 8-fold increase of odds in SFGR infection with positive R. rickettsii ticks (OR 7.78, 95% CI: 3.84, 15.78). The *R. rickettsii* model did not find a significant relationship between *Ehrlichia* or *Anaplasma* infection status and odds of *R. rickettsii* infection (OR 0.41, 95% CI: 0.11, 1.56), but there was a 7.7-fold increase in odds for *R. rickettsii* infection with positive SFGR ticks (OR 7.71, 95% CI: 3.81, 15.62). Tick sex was not significantly associated with SFGR or *R. rickettsii* infection in either model.

4.5 Discussion

This is the first report of pathogenic *Rickettsia* bacteria infecting local ticks in El Salvador: specifically, *R. rickettsii*. Approximately 23% of all (58/253) ticks (4.8% R*. sanguineus* and 25.6% *R. microplus*) collected were positive for *R. rickettsii*, which is a relatively high positive tick burden compared to the only other

countries in Central America where this pathogen has been found infecting ticks: in Costa Rica (range of positive ticks: 3.2 – 5.3%) and Panama (range of positive ticks: 0.8 – 8.7%). In Costa Rica, *R. rickettsii* has been documented in the following tick species: *Haemaphysalis leporispalustris* (Packard), the rabbit tick, *Amblyomma mixtum* Koch, and *A. varium* Koch, sloth's giant tick[170-172]. In Panama, R. rickettsii has been reported in: *R. sanguineus*, *Dermacenter nitens* Neumann the tropical horse tick, *A. cajennense* (Fabricius), the Cayenne tick, and *A. mixtum* Koch[173-176]. *R. rickettsii* antibodies have also been reported in humans and dogs from neighboring country Nicaragua, however no ticks have been found infected with this bacterium[54,56].

Additionally, this is the first report of both *Anaplasma* and *Ehrlichia* pathogens infecting ticks in El Salvador. We found approximately 8% (20/253) of all collected ticks were positive for either pathogenic genus: 4.8% of *R. sanguineus* and 2.4% of *R. microplus* were positive for *Ehrlichia* pathogens, and 6.2% of R. microplus were positive for *Anaplasma* species. The two previous tick survey efforts from El Salvador did not test ticks for these zoonotic pathogens[61,74].

Interestingly, all pathogenic SFGR were identified in ticks collected from only the farms, and not from the veterinary office. The large majority of the *Rickettsia* spp. found in ticks originated from Campo Experimental de Universidad de El Salvador, which did not have the highest number of ticks collected in total. *Ehrlichia* and *Anaplasma* bacteria were found in all three locations, however the University of El Salvador's farm still represented the

majority of infected ticks. All of the ticks identified as simultaneously positive for pathogens were collected from both of the farms, but not the veterinary office. Surprisingly, the private farm had the highest number of concomitantly positive ticks (n=32) compared to the University of El Salvador's farm (n=23). This is interesting considering Campo Experimental de Universidad de El Salvador had a larger diversity of potential host animals for ticks to feed on compared to the private farm. This perhaps explains why the University of El Salvador's ticks were found to have a higher infection burden of all pathogenic bacteria detected.

Both tick species identified in this study have been previously recorded and are thought to be native to El Salvador[61,74]. *R. sanguineus* is considered a major vector of public health significance due to its capability of transmitting for *R. rickettsii* to humans across the western hemisphere[177,178]. It is not surprising that we found some of this species infected with this pathogen. However, the tick species comprising the majority of our collections and burden of infection, *R. microplus*, is not typically considered a tick of public health importance, rather one of veterinary importance. *R. microplus* has a world-wide distribution and is a primary vector for bovine babesiosis, bovine ehrlichiosis, anaplasmosis, and heartwater disease in cattle and ruminants[19,179]. Two reports from Brazil have reported southern cattle ticks infected with *R. rickettsii* to date[180,181]; yet our results indicate that this pathogen may be more widespread than previously thought in cattle. In addition, the southern cattle tick is a one-host tick, indicating this tick species spends the entirety of its life on one host (primarily cattle). This brings an interesting context regarding *R. rickettsii*-infected one-host ticks: is risk

to humans reduced because these ticks will not leave to find another host to feed on? There are multiple capable *R. rickettsii* vectors found in the country[61,74], thus other tick species may feed on infected cattle to continue transmission to humans, but this question is important to consider for the larger tick ecology perspective. Additionally, recent reports have documented this species occasionally feeding on humans where farmers and herdsmen were indicated at highest risk for *R. microplus* bites[179,182,183].

Although the number of host animals that produced ticks for this study was small (n=11), a significant finding was that 9/11 (81.8%) of these animals were hosts to ticks with multiple pathogens. Additionally, multiple cows and one dog hosted ticks with pathogens in the SFGR group and *Ehrlichia* and/or *Anaplasma* spp.. One cow from the University of El Salvador's farm was suspected as ill with anaplasmosis, and two *A. marginale* strains were found in ticks on this cow. Additionally, the dog from the veterinary office was suspected as ill with rickettsiosis, and both SFGR and *R. rickettsii* was found in ticks on this dog. This dog hosted ticks positive for both rickettsial pathogens and *E. canis*. *Ehrlichia canis* is the causative agent for canine monocytic ehrlichiosis, with clinical symptoms including fever, weight loss, weakness, anorexia, splenomegaly, thrombocytopenia, and nose bleeds[184-186]. Dogs are commonly infected with both pathogens worldwide, and this simultaneous infection in animals leads to an increase in risk for secondary infections and transfer of infected ticks to humans[184,185]. *Anaplasma marginale* is the most common and pathogenic species of *Anaplasma* which causes bovine anaplasmosis in cattle worldwide,

causing significant morbidity and mortality[187-189]. Prominent symptoms of bovine anaplasmosis include fever, weight loss, haemolytic anemia, abortion, and death[187,190]. Both the tick vectors and cows are considered reservoir hosts and can become persistently infected[189]. Multiple infections of pathogens in the *Anaplasma* and *Ehrlichia* genera are common in cattle, but little is known about the impacts of multiple infections[187,189,191,192]. *Anaplasma platys* is the causative agent for canine cyclic thrombocytopenia, with symptoms very similar to *E. canis* with the addition of petechiae[184,193]. Interestingly, this was detected in a tick on a cow, not from one of the dogs—indicating there is potential for spillover of pathogens between these animals. With a wide variety of pathogens detected in ticks collected from both host animals, it would be beneficial to test the animals and handlers or farmers for exposure to these pathogens as well.

In this study, we found significant associations between multiple variables related to tick collections. Host animal (majority being cattle) was important for both SFGR and *R. rickettsii* infection status, collection location was important for all pathogens, tick species was important for SFGR infection, and tick sex was important for *Ehrlichia* or *Anaplasma* infection status. Interestingly, approximately 57% of the ticks collected were female, and this might have important implications for transmission of pathogens among animals at the farms. A majority of the simultaneously infected concomitantly ticks were female, which could also be explained by the necessity of these ticks to feed for longer periods of time to acquire an appropriate amount of blood for egg production. Longer

blood-feeding times increases the probability of pathogen transfer during co-feeding from infected ticks to uninfected ticks on the same host animal[194,195].

The logistic regression models indicated that sex was not an important predictor for either SFGR or *R. rickettsii* infection status, in contrast to the associations seen in the chi-square results. Interestingly, *Ehrlichia* or *Anaplasma* infection was only indicative of an increase in odds of SFGR infection in ticks, and not for *R. rickettsii* infection. This suggests that *Ehrlichia* or *Anaplasma* spp. infection is a better predictor for SFGR infection than tick sex. After we controlled for the *Ehrlichia* or *Anaplasma* infection status, the association between rickettsial infection and tick sex was not evident anymore. It is also not surprising to see *R. rickettsii* and SFGR were statistically important for predicting one another, further supporting the screening and testing methods used to identify pathogens. Having SFGR infection makes a tick more likely to have *R. rickettsii* infection.

The results of this study are important for tick-borne disease research and surveillance in El Salvador and Central America. Given the peri-domestic and domestic nature of the host animals where positive ticks were collected, new records of a potentially fatal human pathogen, *R. rickettsii*, and the larger burden of positive ticks relative to previous research in the surrounding area, more effort should be placed on surveillance for tick-borne disease in this country. Currently, there is no tick or tick-borne disease surveillance conducted in El Salvador; most of the public health funding for arthropod-borne disease is focused on mosquito-borne pathogens such as dengue and malaria. While these vector-borne

pathogens are very important for human health, ignoring tick-borne disease will continue to promote the cycle of neglected bacterial pathogens in El Salvador. As a country with relative low human development, high numbers of vulnerable populations, and high rates of poverty, neglected rickettsial diseases are probably vastly more common in El Salvador than previously thought. It is often difficult to justify funding allocated to a disease group without proper baseline information, and our study shows how much information is potentially missing in the epidemiological profile of SFGR in El Salvador. Given that our study only encompassed ticks collected from one municipality in this country, it is very likely that ticks throughout El Salvador are circulating SFGR pathogens in similar peri-domestic and agricultural environments.

Future studies should continue to investigate tick species and the pathogens they carry throughout the country. An additional need is human- and animal-based serology or molecular testing studies which would complement the previous tick surveys and our pathogen prevalence investigation. To complete the SFGR ecological investigation, humans in close contact with infected ticks and potentially infected host animals should also be tested for exposure to SFGR pathogens. A full, holistic, understanding of the tick-borne disease burden in El Salvador can lead to future prevention efforts, timely treatment, and ultimately a reduction in morbidity and mortality in vulnerable populations.

Table 6.1 Primer and Probe Sequences for Targeted Genes

Species	Gene Target	Reaction	Primer or Probe Nucleotide sequence (5'-3')	Size (bp)	Reference
R. rickettsii	*ompA*	forward	Rr 190.70p ATG GCG AAT ATT TCT CCA AAA	532	Regnery et al. 1991; Eremeeva & Raoult 1994
		reverse	Rr 190.602n AGT GCA TTC GCT CCC CCT		
SFGR spp.	*gltA*	forward	Rr CS.372 TTT GTA GCT CTT CTC ATC CTA TGG C	617	Kollars & Kengluecha 2001
		reverse	Rr CS.989 CCC AAG TTC CTT TAA TAC TTC TTT GC		
R. rickettsii	*ompB*	probe	*6FAM*-TCC TAA TGC TAT AAC CCT TAC C-*MGBNFQ*	116	Jiang et al. 2005; Egizi et al. 2020
			GAC TCA AAC TTT GGT AAT ACT GAT TTT G		
			Rr 1494 GCA GTG TTA CCG GGA TTG CT		
Ehrlichia or *Anaplasma* spp.	*groEL*	primary forward	Gro607 GAA GAT GCW GTW GGW TGT ACK GC	664	Tabara et al. 2007
		primary reverse	Gro1294 AGM GCT TCW CCT TCW ACR TCY TC		
		nested forward	Gro677 ATT ACT CAG AGT GCT TCT CAR TG	315	
		nested reverse	Gro1121 TGC ATA CCR TCA GTY TTT TCA AC		

Table 6.2. Homology of isolate consensus sequences from GenBank

Species	Gene Target	Percent Identity (Range)	GenBank Accession Numbers	Reference
SFGR spp.†	*ompA*	--	--	--
Ehrlichia canis	*groEL*	100%	MG953295, LC373041	[196]
Ehrlichia species	*groEL*	88.11%	KF977220	
Uncultured *Ehrlichia*	*groEL*	97.57 - 100%	MW054558, MW054557	[196]
Anaplasma marginale-Jaboticabal	*groEL*	99.31 – 100%	CP023731, KM091070	[197]
Anaplasma marginale-Palmeria	*groEL*	99.65 – 100%	CP023730, KM091069	[198]
Anaplasma platys	*groEL*	100%	MN202021, MN202019	[199]

†Sequencing results for SFGR species did not produce a recognized or previously identified *Rickettsia* species from GenBank.

Table 6.3. Pathogen Prevalence by Species.

	Total Collected	***Ehrlichia* or *Anaplasma* spp. (%)***				SFGR spp. (%)*	*R. rickettsii* (%)*
		Uncultured / Unknown *Ehrlichia*	*Ehrlichia canis*	*Anaplasma platys*	*Anaplasma margina*		
R. sanguineus	21	0	1 (4.8)	0	0	0	1 (4.8)
R. microplus	211	5 (2.4)	0	2 (0.9)	11 (5.2)	103 (48.8)	54 (25.6)
Unknown†	21	0	0	0	1 (4.8)	6 (28.6)	3 (14.3)

*Percentages are calculated as: N positive for target pathogen / N total collected of each species. Ticks positive for multiple genera of pathogens are included within calculated percentages, and these percentages are not exclusive.
†Unknown ticks were either too destroyed or partial bodies, and species identification could not be determined.

Table 6.4. Ticks Simultaneously Positive for Multiple Pathogens.

	Number	Sex	Location*	Host	Uncultured / Unknown *Ehrlichia*	*Anaplasma platys*	*Anaplasma margina*	SFGR spp.	*Rickettsia rickettsii*
R. microplus	7	F	B	cattle	-	-	+	+	-
R. microplus	1	F	B	cattle	-	-	+	+	+
R. microplus	2	F	B	cattle	+	-	-	+	-
R. microplus	8	F	B	cattle	-	-	-	+	+
R. microplus	4	M	B	cattle	-	-	-	+	+
R. microplus	1	F	C	cattle	-	+	-	+	-
R. microplus	14	F	C	cattle	-	-	-	+	+
R. microplus	13	M	C	cattle	-	-	-	+	+
R. microplus	1	I	C	cattle	-	-	-	+	+
Unknown	1	U	B	cattle	-	-	+	+	-
Unknown	3	U	C	cattle	-	-	-	+	+

*Location: (A) Veterinaria El Semental; (B) Campo Experimental de Universidad de El Salvador; (C) Private Farm.

Table 6.5. Distribution of Pathogens in Ticks Collected from Host Animals

	University of El Salvador Farm					Private Farm					Veterinary Office
	Cow #1	Cow #2*	Cow #3	Cow #4	Cow #5	Cow #1	Cow #2	Cow #3	Dog #1	Dog #2	Dog #1†
SFGR	+	+	+	+	+	+	+	+	+		+
R. rickettsii	+	+	+	+			+	+			+
E. canis											+
Uncultured ehrlichia			+			+	+	+			
Ehrlichia sp.						+					
A. marginale (Palmeira)		+		+	+						
A. marginale (Jaboticabal)		+		+							
A. platys							+				

*This cow was suspected ill with anaplasmosis.
†This dog was suspected ill with rickettsiosis.

Table 6.6. Multivariate Logistic Regression Models of Predicted SFGR and *R. rickettsii* infection.

Variable		SFGR Infection OR (95% CI)	*R. rickettsii* Infection OR (95% CI)
Tick Sex			
	Female	ref	ref
	Male	0.67 (0.36, 1.25)	1.73 (0.85, 3.51)
	Unknown	0.40 (0.16, 1.01)	0.77 (0.23, 2.57)
Ehrlichia or *Anaplasma* infection			
	Yes	3.15 (1.14, 8.71)**	0.41 (0.11, 1.56)
	No	ref	ref
SFGR infection			
	Yes	--	7.71 (3.81, 15.62)**
	No	--	ref
R. rickettsii infection			
	Yes	7.78 (3.84, 15.78)**	--
	No	ref	--

**Statistically significant (alpha = 0.05)

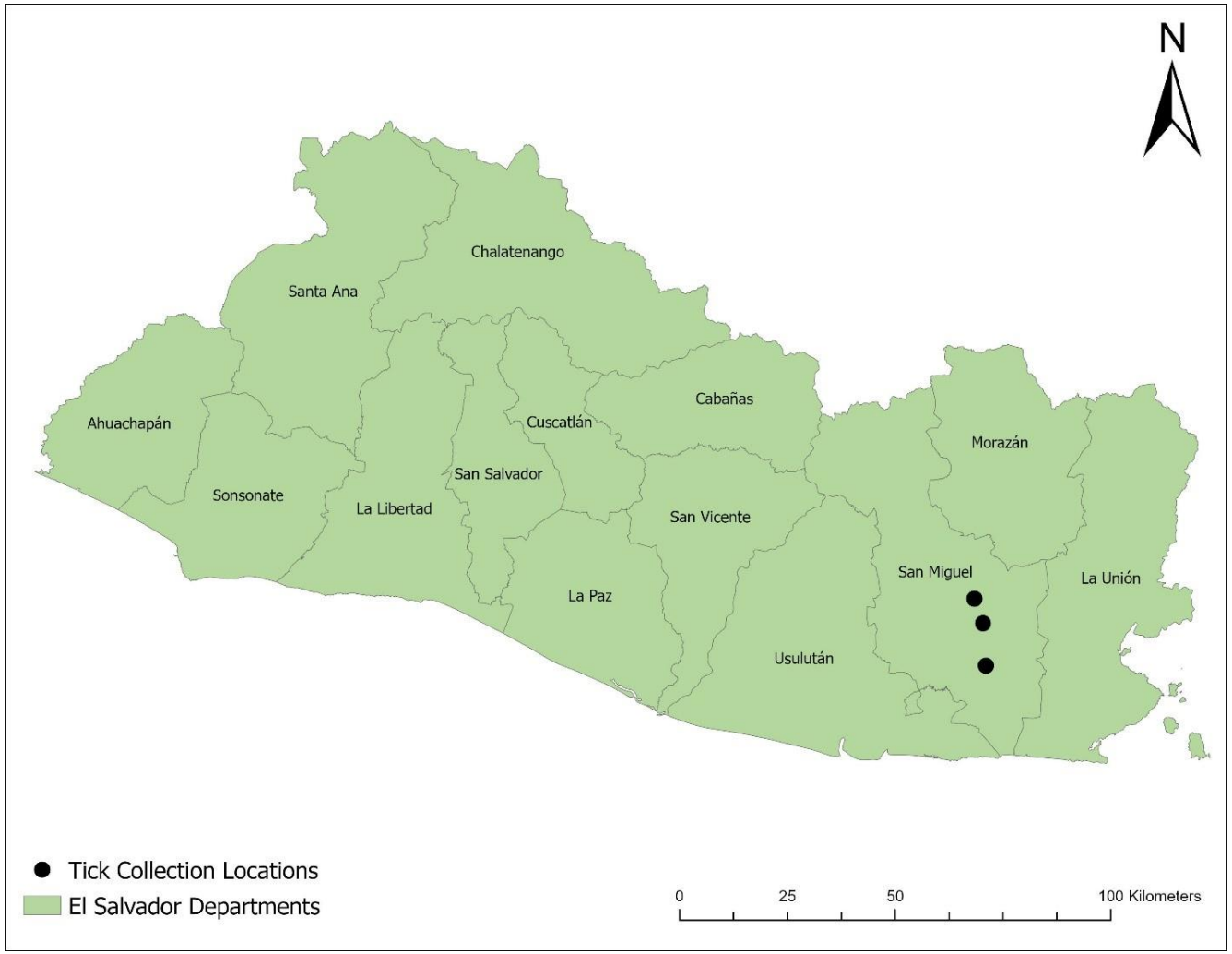

Figure 6.1. Tick Collection Locations in El Salvador. Tick collection locations: (A) Veterinaria El Semental; (B) Campo Experimental de Universidad de El Salvador; (C) Private Farm.

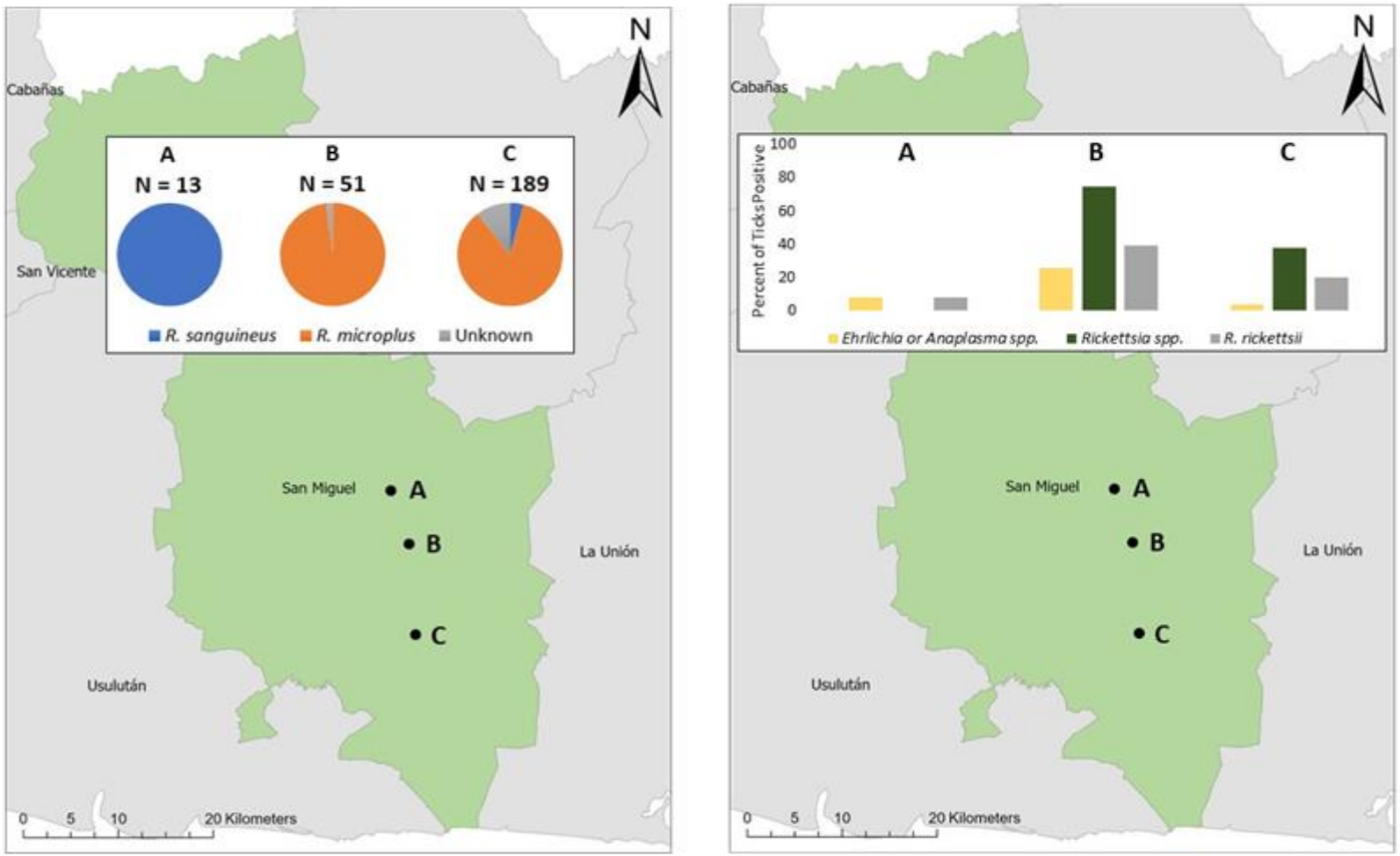

Figure 6.2. Tick species and pathogen prevalence by location in San Miguel, El Salvador. (a) Number of ticks and by species for each collection location. (b) Pathogen prevalence of collected ticks from each location.

CHAPTER SEVEN

SUMMARY

The findings from this dissertation have provided crucial information about spotted fever group rickettsioses (SFGR) infection prevalence in a vulnerable, high-risk population in El Salvador, relevant risk factors regarding odds of past and acute infection, and the presence of circulating SFGR bacteria in tick vectors. We conducted antibody assays on banked biological samples from a population of children from the Sonsonate Department of El Salvador to assess SFGR infection and investigated various demographic, household, poverty, and behavioral characteristics' associations with SFGR infection from this same population from the enrollment questionnaire. This risk factor assessment was conducted through logistic regression analyses. Furthermore, we collected ticks from El Salvador and tested them for pathogenic bacteria, and we assessed infection prevalence with various tick characteristics.

Overall, the critical findings throughout each chapter unearth the story of neglected bacterial pathogens and risk for specific regions and populations in El Salvador. We found an overall prevalence of past infection (IgG antibodies) with SFGR in 2.48% of the pediatric cohort from Sonsonate Department and a prevalence of acute infection (IgM antibodies) with SFGR in 10.67%. The proportion of positive individuals formed a geographic hotspot in the municipalities in the northcentral region of this department, similar to the findings

of Chagas disease infection in the previous study. There were also association trends (at the 0.10 significance level) between those positive for either IgG or IgM antibodies and infection with Chagas disease and febrile status at enrollment. Higher numbers than expected of those positive for both Chagas disease and SFGR antibodies were observed in this cohort. Fewer individuals than expected were enrolled with a fever and had SFGR antibodies were observed. Our study was the first investigation into SFGR antibodies in humans in El Salvador in over 25 years, and the first to investigate this infection in a specific, vector-borne disease vulnerable population of pediatric participants. We also provided an in-depth investigation into the various risk factors related to SFGR antibody prevalence in this specific cohort through multiple logistic regression models. Individuals with increased age consistently had greater odds for either IgG or IgM SFGR antibodies compared to those who were younger. Those who were male, were enrolled during the wet season, had a reported higher maternal education level, did not have electricity, and had a household member seek clinical care for an infection in the past year had significantly greater odds for SFGR antibodies. Those who were febrile at time of enrollment, used an outdoor latrine, had birds, cement bedroom floors, and previous exposure to Chagas disease vectors had lower odds for SFGR antibodies. Many of these risk factors are commonly reported in global SFGR investigations, however a few—including febrile status, maternal education level, and outside latrine use did not fit the typical narrative for neglected bacterial infections. Clearly there are more variables and aspects of the natural history of SFGR in the environment that are important for

transmission in El Salvador that warrant future investigations. Nonetheless, this was the first epidemiological SFGR risk factor study in El Salvador.

Lastly, we detected multiple pathogenic bacteria in ticks collected from two agricultural farms and one veterinary office in one Salvadoran department. Approximately 5% of brown dog ticks (*Rhipicephalus sanguineus*) and 2% of cattle fever ticks (*R. microplus*) were positive for *Ehrlichia* spp., and 6% of cattle fever ticks were positive for *Anaplasma* spp. Both of these genera host species of bacteria that can cause significant disease in animals and humans. Additionally, approximately 5% of brown dog ticks and 26% of cattle fever ticks were positive for *Rickettsia rickettsii*, the SFGR causative agent for Rocky Mountain spotted fever. Significant associations were found between SFGR infection and tick species and sex. *Ehrlichia*/*Anaplsama* spp. infection status was also found as an accurate predictor for SFGR infection—ticks had increased odds of SFGR infection if they were also infected with *Ehrlichia* or *Anaplasma* bacteria. This study is the first to report *R. rickettsii*, *Ehrlichia* spp., and *Anaplasma* spp. in El Salvador, and the fourth report in Central America for *R. rickettsii.*

These results are important for El Salvador and the Central American region as a whole. With evidence of infected pediatric participants and circulating bacteria in local ticks, it is very likely that SFGR infection in humans and ticks is more prevalent than previously thought. Many countries in this region do not have the public health resources to dedicate to tick and tick-borne disease research, especially when many of these diseases, like SFGR, largely result in

clinically ambiguous diseases with undifferentiated febrile illness or those that mimic viral disease. This then makes these diseases more difficult and potentially more costly to accurately diagnose. Neglected tropical disease and vector-borne disease resources are thus allocated to issues such as malaria, dengue, Chagas disease, and soil transmitted helminths. With evidence of circulating SFGR bacteria in the environment, we hope that public health officials and infectious disease physicians become more aware of tick-borne disease in El Salvador and consider rickettsial infections as etiologic agents for future patients. An increase in awareness can lead to advocacy for better and definitive diagnostics which will in turn increase the acquisition of treatment for those who become infected.

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