Exploring Tick Borne Pathogens Circulating Mongolia Through Collection of Ticks

by

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Thesis submitted in partial fulfillment of
the requirements for the degree of
Master of Science in the Duke Global Health Institute
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2017

ABSTRACT

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Abstract

Introduction: Mongolia is a country known for its rich nomadic and pastoral culture, with populations of people who work in environments that are densely populated with ticks and TBP animal reservoirs. TBPs typically undergo transstadial transmission, but transovarial transmission may also occur. Transovarial transmission events have been demonstrated in laboratory settings, but few studies have evaluated transovarial transmission of TBPs in field settings within the host-vector ecosystem. Tick borne pathogens of most concern in Mongolia are *Rickettsia spp*., *Anaplasma spp*., *Borrelia spp*., *Babesia spp*., *Ehrlichia spp*., and tick-borne encephalitis. In this study, specific aims were: 1) To determine the prevalence of tick borne pathogens, particularly *Rickettsia spp.*, *Anaplasma spp*., and *Ehrlichia spp*. among various tick species at different developmental life stages; and 2) To understand the role of animal reservoirs and vertical transmission of TBPs among feeding ticks at different life stages using larval and nymph ticks collected in the wild from small mammal reservoirs, as well as eggs laid by engorged wild-caught adult female ticks and reared in the laboratory.

Methods: In this cross-sectional design, there were two components of this study to evaluate ticks at separate life stages. For the first component, engorged adult ticks were collected from livestock located in three soums (districts) within three aimags

(provinces) from May 6th to 22nd, 2016. Tick eggs were collected from engorged ticks from May 9th to June 1st, 2016. For the second component of this study, ticks in their larvae and nymph life stages were collected off of captured rodents across seven soums in three aimags situated in the Northern region of Mongolia from June 20th to July 23rd, 2016. Ticks were tested by PCR to detect the presence of *Rickettsia spp.*, *Anaplasma spp., and Ehrlichia spp.* and sequenced to determine species of pathogen detected.

Results: There were 546 (88%) larval and 72 (12%) nymphal *Dermacentor spp.* ticks collected. There were 588 (95%) of 618 ticks allocated into 42 larvae and 18 nymph pools (60 pools total). All tick pools were PCR-positive for *Rickettsia spp.* and no tick pools were PCR-positive for *Anaplasma/Ehrlichia spp*. minimum infection rate (MIR) for *R. raoultii* ranged from 6.7% to 28.6%. Of the 60 tick pools, 50 (522 ticks total) were matched with rodent rickettsial infection history status. There were 31 (62%) tick pools or 362 (69%) of individual ticks found on rodents with no history of rickettsial infection. The majority of ticks discovered to have no association with rodents with rickettsial infection history were larvae (352/362 individual ticks). There were 38 adult fed female ticks collected. All adult fed ticks were PCR-positive for *Rickettsia spp.* and 2 (5%) were PCR-positive for *Anaplasma/Ehrlichia spp.* There were 33 ticks that laid eggs. PCR testing of eggs showed a 91% (30/33) positivity for *Rickettsia spp*. and one pool of eggs was PCR-

positive for *Anaplasma/Ehrlichia spp*. All sequenced *Rickettsia spp.* products were identified to be *R. raoultii* and all sequenced *Anaplasma/Ehrlichia spp.* were *An. ovis.*

Conclusions**:** This study identified transovarial transmission of *Rickettsia spp.* and *Anaplasma spp*. among *D. nuttalli* ticks. This study also found a low association between rodents with history of *Rickettsia spp*. infection and infection status of biting ticks. Additional study is needed to further assess the proportion of transovarial transmission found in nature. Specifically, testing of individual tick eggs and larvae should be conducted. A better understanding of the ecology of TBPs in nature can provide public health and human and veterinary medicine with a greater awareness of the burden of TBPs in Mongolia.

Dedication

I dedicate this thesis to my loving wife Brittney, who has stood by me through this adventure. Without her support, this thesis would not have been possible. I also dedicate this to my mother, who has always encouraged me to explore the many cultures of the world.

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1. Introduction

While significant effort has been directed to better understand the risk of tick-borne pathogens (TBPs), there still remains much to learn. This is particularly so in the developing regions of the world, where resources to study TBPs are often limited. Interestingly, recent reports indicate that the global burden of TBPs has increased over the last three decades, in part due to changes in land use and climate change.[1-5] Seasonal durations for ticks and other ectoparasites that carry disease have become much longer. For example, there has been reported increased winter activity and distribution of ticks in Sweden due to milder winter months and increased humidity during summer months.[6] Additionally, there has been a change in host range and expansion of tick distribution due to warming temperatures. Throughout the mountains of the Czech Republic there has been a marked increase of tick habitat elevation due to the spread of roe deer to previously uninhabitable environments.[7] There have also been changes in human development and behavior, which have resulted in an increase in contact with ticks and their animal host habitats, proliferating TBP exposures and subsequent infections.[2, 3, 8, 9]

Mongolia is a country known for its rich nomadic and pastoral culture, with populations of people who work very closely with their livestock in environments that are often densely populated with ticks and animal reservoirs that can harbor TBPs.[10]

Additionally, ecotourism is a rapidly growing industry in Mongolia, where international visitors could be at risk of being exposed to TBPs.[10, 11] This public health challenge is further complicated by a limited knowledge and understanding of tick and TBP ecology, both globally and within Mongolia.[8, 11, 12]

Disease ecology is the study of interactions between host, pathogen, and the environment, including vectors.[13] In the context of multiple-host tick species, adult ticks typically feed on larger mammals while larvae and nymph generally feed on small mammals.[14-16] Ticks of every life stage have been found feeding on humans.[17] Infections of TBPs in humans usually occur in environments with a higher index of vegetation.[10, 17] The mobility of ticks is restricted to questing and travelling via feeding on animals and humans.[17] TBPs typically undergo transstadial transmission before being vectored by a competent tick host. However, depending on the tick species and the type of TBP, transovarial transmission may also occur.[14] The event of transovarial transmission, also known as vertical transmission, occurs when a pathogen spreads to the eggs of an infected female tick.[18] While transovarial transmission events have been demonstrated in laboratory settings for several TBPs[17, 19-21], few studies have evaluated transovarial transmission of TBPs in field settings. Research related to transovarial transmission has been particularly limited within the Asian and Eurasian regions of the world.

Several TBP surveillance and case studies have been conducted throughout Russia, China and Mongolia, which have tested human samples[22-24], livestock[16, 25-28], wildlife[29-31], and ticks.[19, 32-39] However, most of these studies have only looked at ticks in their adult life stage, either fed or unfed. Few studies have examined the larvae and nymph stages of ticks in the Eurasian environment. Larvae and nymph life stages of ticks are of special interest in regards to exposure risk, as their small size can lead to less readily detectable feeding on human hosts. It is known that nymphs traditionally pose the greatest risk to humans if they have fed on an infected animal in a previous life stage. However, larvae may pose similar risks if transovarial transmission of TBPs were to occur.[40-42]

Tick-borne pathogens of most concern in Asian and the Eurasian regions of the world are *Rickettsia spp*. pathogens, which are known to cause febrile illness.[33, 35] Such diseases include North Asian tick typhus or Siberian tick Typhus (STT), caused by infection of *R. sibirica*,[17, 34, 43] Tick-borne lymphadenopathy (TIBOLA) or Dermacentor-borne necrosis erythema and lymphadenopathy (DEBONEL), which has been associated with the etiological agent *R. raoultii*[20, 44-46], and most recently *Candidatus R. tarasevichiae* (CRT), which was originally thought to be nonpathogenic and thus resulting in asymptomatic infections.[47, 48] Other TBPs of notable concern, which have been identified throughout Russia, China and Mongolia, include *Anaplasma spp*.[25, 27, 29, 36, 38, 49], *Borrelia*

spp.[39, 49, 50], *Babesia spp*.[26, 51-53], *Ehrlichia spp*.[30, 36], and tick-borne encephalitis virus[49, 54-56]. Many of these pathogens, similar to other TBPs, have been associated with small mammal and rodent reservoirs.[14, 30] However, little research has been conducted to compare the role of potential reservoir transmission to vertical transmission of TBPs within the host-vector ecosystem. Collectively, the objective of this study was to further elucidate the ecology and life cycle of TBPs between reservoir and host, examine the propensity of certain TBPs to undergo transstadial and transovarial transmission, and evaluate the infection prevalence of TBP infections from early life stage ticks throughout the Northern Mongolia region. The specific aims of this study were: 1) To determine the prevalence of tick borne pathogens, particularly *Rickettsia spp*., *Anaplasma spp*., and *Ehrlichia spp*., among various tick species at different developmental life stages; and 2) To understand the role of animal reservoirs and vertical transmission of TBPs among feeding ticks at different life stages using larval and nymphal ticks collected in the wild from small mammal reservoirs, as well as eggs laid by engorged wild-caught adult female ticks and reared in the laboratory.

2. Methods

2.1 Study Design and Setting

In this cross-sectional study, design, there were two components of this study to evaluate ticks at separate life stages (Figure 1). For the first component, engorged adult ticks were collected from livestock located in three soums within three aimags (Figure 2) from May 6th to 22nd, 2016. Tick eggs were collected from laboratory reared engorged ticks at the Laboratory of Arachno-Entomology and Protozoolgy, Institute of Veterinary Medicine in Ulaanbaatar from May 9th to June 1st, 2016.

For the second component of this study, ticks in their larvae and nymph life stages were collected off of captured rodents across seven soums (districts) in three aimags (provinces) situated in the Northern region of Mongolia from June 20th to July 23rd, 2016. Using a handheld global positioning system (GPS) unit (Juno Trimble Positions System, Sunnyvale, CA) provided by the Nicholas School of the Environment, Duke University, latitudinal and longitudinal coordinates were collected at each collection site (Figure 3). Additional information recorded for this study at each collection site consisted of ecosystem type, species of animal collected, and reports of any irregularities during rodent capture or tick collection. See Table 1 for summary of tick collection locations.

All-terrain vehicles were used to travel to and from each collection site for about week at a time. During fieldwork, team members assisted with rodent capture and tick collection. This study was parallel to a team member's analysis of potential rodent reservoirs.

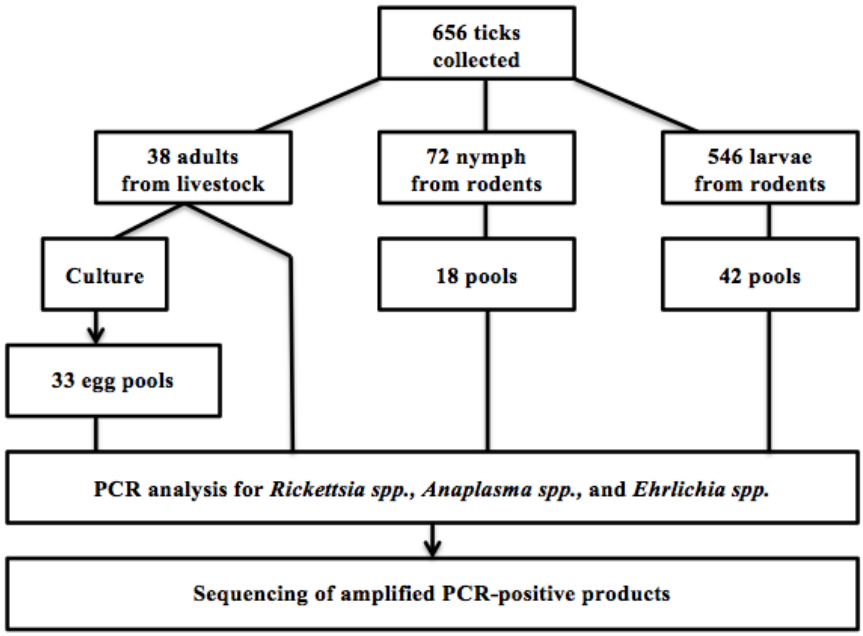

Figure 1: Summary of study design.

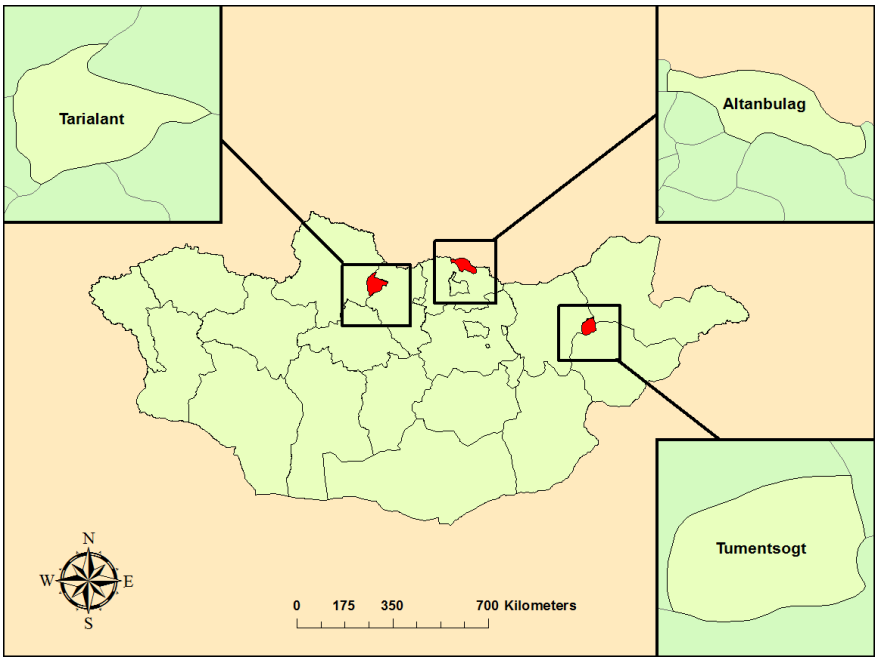

Figure 2: Map of soums where engorged ticks were collected, Mongolia

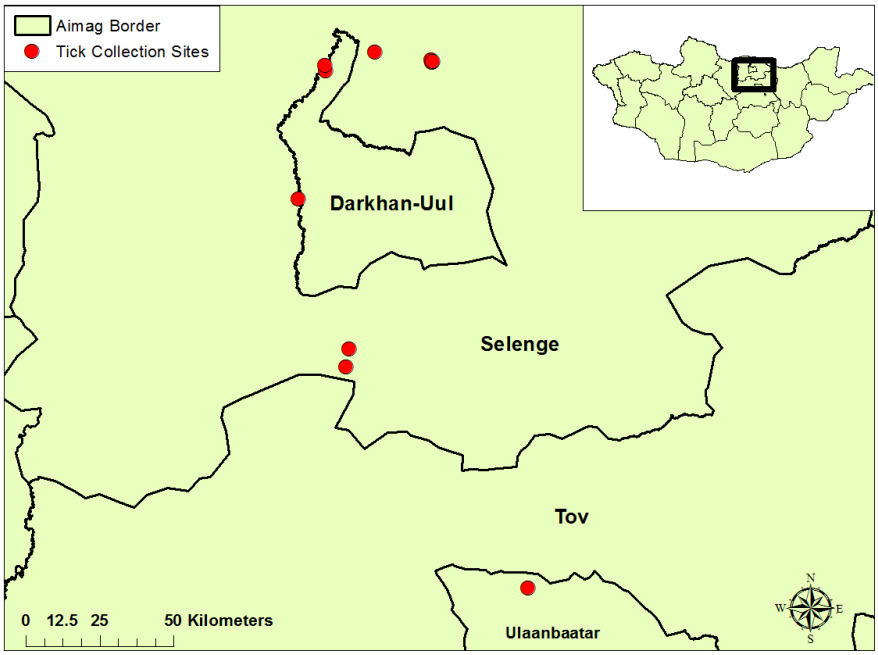

Figure 3: Map of larvae/nymph tick collection sites in Mongolia.

Table 1: Summary of tick collection locations.

Aimag	Soum	Tick Life stage Collected
Tov	Batsumber	Larva/Nymph
Darkhan-Uul	Orkhon	Larva/Nymph
	Darkhan	Larvae
Selenge	Bayangol	Larva
	Eruu	Larva/Nymph
	Javkhlant	Larva/Nymph
	Altanbulag	Adult
Khuvsgul	Tarialan	Adult
Sukhbaatar	Tumentsogt	Adult

2.2 Engorged Adult Tick Collection and Rearing

Female adult fed ticks were collected from livestock and kept alive at room temperature until researchers returned to the laboratory facilities at the Institute of Veterinary Medicine in Ulaanbaatar. Ticks were kept at room temperature in storage containers. Moist cotton was placed near ventilation of storage containers to replicate environmental humidity conditions. Once female ticks laid eggs (between 2-7 days of cultivation), both adult tick and eggs were stored separately at -28°C until morphological identification of adult ticks was completed. Ticks and eggs were subsequently stored at -80°C until DNA extraction procedures.

2.3 Adult Tick and Egg Pool Processing

Tick eggs were pooled based on the adult female tick they were laid by and were weighed to measure the amount laid. Eggs and adult ticks were briefly rinsed with 70%

ETOH in sterile 1 mL vials to remove contamination and then air dried on a sterile dish.[58, 59] Before DNA extraction, ticks were ground using a sterile pre-chilled mortar and pestle with 500 μL of sterile PBS and 50 mg sterile sand for friction.[57] Contents were then centrifuged in a 1.5 mL vial at 9,500 g for 5 minutes. Supernatant was then pipetted from the sand deposit and inserted into a new vial for storage. Tick supernatant was subsequently stored at -20°C until DNA extraction procedures.

2.4 Rodent Trapping and Tick Collection

Trapping and handling procedures were approved by the Duke University Institutional Animal Care and Use Committee (#A086-16-04) and directed by veterinary staff from the Institute of Veterinary Medicine in Mongolia to ensure safety of the animals. Live Tomahawk or Sherman traps were used at each location and placed near holes where there were signs of current rodent occupation. All captured rodents were sedated with ketamine (50 mg/Kg).

Once the anesthetic had taken hold, the sedated rodent was inspected for ticks. When identified, a tick was removed from the rodent using sterile forceps, immediately placed in 1 mL vials of 70% ethanol (ETOH), and stored at room temperature within the vehicle at each collection site until returned to laboratory facilities. Each vial was labeled with an ID that could be matched to the ID of the small mammal in which the tick was

removed and the location of collection. Protective rodent handling gloves and Tyvex suits were worn at all times during the collection of ticks from rodents.

2.5 Tick Pool Storage and Processing

Upon returning to the Institute of Veterinary Medicine, tick specimens, still in 70% ETOH, were placed in 4°C storage until they could be taxonomically identified to genus by an entomologist at the institute. After identification was completed, specimens were air dried to remove ETOH and were pooled based on their life stages (larvae up to 15; nymph up to 5), animal species collected, collection location and genus of tick. Once ticks were pooled (60 pools total) they were stored at -20°C into new sterile 1 mL vials before genomic DNA extractions. See Table 2 for tick pool characteristics.

Prior to the genomic DNA extractions, frozen ticks were ground using a sterile pre-chilled mortar and pestle with 500 μL of sterile PBS and 50 mg sterile sand for friction.[57] Contents were then centrifuged in a 1.5 mL vial at 9,500 g for 5 minutes. Supernatant was then pipetted from the sand deposit and inserted into a new vial for storage. Tick supernatant immediately underwent DNA extraction procedures.

Table 2: Summary of tick pool characteristics.

Aimag	Soum	Species Collected	Number of Pools	Pool Size Range
Selenge	Bayangol	Ground Squirrel	2	1-15
		Mongolian Gerbil	1	6
	Eruu	Ground Squirrel	2	1-13
		Striped Dwarf Hamster	12	1-15
	Javkhlant	Ground Squirrel	8	1-15
		Siberian Chipmunk	2	10-11
Darkhan-Uul	Orkhon	Ground Squirrel	6	1-15
		Striped Dwarf Hamster	1	15
	Darkhan	Mongolian Gerbil	1	15
Tov	Batsumber	Ground Squirrel	10	3-15
		Mongolian Gerbil	1	5
		Pika	8	5-11
		Korean Field Mouse	1	1
		Striped Dwarf Hamster	4	5-15

2.6 DNA Extraction and Polymerase Chain Reaction Analysis

Genomic DNA was extracted from tick supernatants using a TIANamp Genomic DNA Kit (Tiangen Biotech (Beijing) Co., LTD, Beijing, China), per the manufacturer's instructions. Briefly, 20 μL of Proteinase K was added to 200 μL of tick supernatant and mixed thoroughly by vortex. Next, 200 μL of Buffer GB was added to the sample, mixed by vortex, and then incubated at 70°C for 10 minutes. ETOH (100%) was added to the sample and vortexed for 15 seconds. DNA was separated from the mixture through a

repeated combination of spin columns and addition of kit buffers under the following conditions: Centrifuge with spin column at 12,000 rpm for 30 seconds, addition of Buffer GD (with 100% ETOH) to spin column centrifuged at 12,000 rpm for 30 seconds, and addition of Buffer PW (with 100% ETOH) to spin column centrifuged at 12,000 rpm for 30 seconds, twice repeated. Last, Buffer TE was added to the dried spin column and left to incubate at room temperature for 5 minutes followed by a final centrifuge cycle for 2 minutes at 12,000 rpm. Once DNA extractions were complete, a subset of samples were selected and measured with a Nano Drop spectrophotometer (Thermo Scientific, Wilmington, Delaware) to verify successful extraction of genomic DNA. Extracts remained stored at -80°C until shipment of samples from the Institute of Veterinary Medicine in Ulaanbaatar, Mongolia to Duke's One Health Research Laboratory, Duke University, North Carolina, USA for molecular analysis.

2.6.1 *Rickettsia spp.*

For the molecular detection of *Rickettsia spp.*, nested PCR reactions with primers designed to target the citrate synthase gene (*gltA*) were used (Table 3). All reactions were performed using 50 μL of the reaction mixture containing 35.8 μL ultra-pure water, 1X PCR Buffer, 1.0 mM $MgCl_2$, 0.2 mM dNTP each, 0.2 uM of each primer, 2 U Platinum Taq DNA polymerase (cat# 10966034, Invitrogen, Carlsbad, CA) and 10 μL of DNA for primary reactions or 10 μL of the primary PCR product for nested reactions. The PCR

conditions were as follows: initial denaturation for 2 minutes at 95°C followed by 35 cycles of denaturation for 30 seconds at 95°C, annealing for 30 seconds at 50°C for primary reaction and 54°C for nested reaction, and extension for 1 minute at 72°C. Final extension was carried out for 5 minutes at 72°C, followed by an indefinite hold at 10°C.[60] DNA from *R. parkeri* and *R. rickettsii* (kindly provided by Dr. Barbara Qurollo at North Carolina State University) were used as positive controls.

In an effort to speciate rickettsia-positives, nested PCR reactions with primers designed to target the outer-membrane protein gene (*ompA*) were also used (Table 3). All reactions were performed using 50 μL of the reaction mixture containing 35.8 μL ultra-pure water, 1X PCR Buffer, 1.0 mM $MgCl_2$, 0.2 mM dNTP each, 0.2 uM of each primer, 2 U Platinum Taq DNA polymerase (cat# 10966034, Invitrogen, Carlsbad, CA) and 10 μL of DNA for primary reactions or 10 μL of the primary PCR product for nested reactions. The PCR conditions for primary and nested reaction were as follows: initial denaturation for 2 minutes at 95°C followed by 35 cycles of denaturation for 30 seconds at 95°C, annealing for 30 seconds at 50°C for both primary and nested reactions, and extension for 30 seconds at 72°C. Final extension was carried out for 5 minutes at 72°C followed by an indefinite hold at 10°C.[47] Again, DNA from *R. parkeri* and *R. rickettsii* were used as positive controls.

2.6.2 *Anaplasma/Ehrlichia spp.*

For the molecular detection of *Anaplasma spp.* and *Ehrlichia spp.*, primers designed to target the 16S rRNA gene were used (Table 3). All reactions were performed using 25 μL of the reaction mixture containing 15.4 μL ultra-pure water, 1X PCR Buffer, 1.0 mM $MgCl_2$, 0.2 mM dNTP each, 0.2 uM of each primer, 1 U Platinum Taq DNA polymerase (cat# 10966034, Invitrogen, Carlsbad, CA) and 5 μL of DNA for primary reactions or 5 μL of the primary PCR product for nested reactions. The PCR conditions were as follows: initial denaturation for 1 minute at 94°C followed by 35 cycles of denaturation for 30 seconds at 94°C, annealing for 30 seconds at 57°C for primary reaction and 60°C for nested reaction, and extension for 1 minute for primary reaction and 30 seconds for nested reaction at 72°C. Final extension was for 1 minute at 72°C followed by an indefinite hold at 10°C.[30] The DNA from *An. phagocytophilum* (kindly provided by Dr. Barbara Qurollo at North Carolina State University) was used as a positive control.

Table 3: Summary of primers used in molecular assays.

Pathogen	Gene	Primers	Sequence (5' - 3')	Size	Ref.
Rickettsia spp.	*gltA*	CS2d	ATG ACC AAT GAA AAT AAT AAT	381bp	(60)
		CSEndr	CTT ATA CTC TCT ATG TAC A		
		RpCS877p	GGG GAC CTG CTC ACG GCG G		
		RpCS1258n	ATT GCA AAA AGT ACA GTG AAC A		
	ompA	Rr190.70p	ATG GCG AAT ATT TCT CCA AAA	346bp	(47)
		Rr190.602n	AGT GCA GCA TTC GCT CCC CCT		
		190.70-38s1	AAA ACC GCT TTA TTC ACC		
		190.602-384r1	GGC AAC AAG TTA CCT CCT		
Anaplasma/ Ehrlichia spp.	16S rRNA	Ehr1	AAC GAA CGC TGG CGG CAA GC	524bp	(30)
		Ehr2	AGT AYC GRA CCA GAT AGC CGC		
		Ehr3	TGC ATA GGA ATC TAC CTA GTA G		
		Ehr4	CTA GGA ATT CCG CTA TCC TCT		

2.7 Electrophoresis and Sequencing

Gel electrophoresis was used to evaluate PCR reactions. Products were loaded on 1% (w/v) agarose gels stained with ethidium bromide for detection of amplified product. Upon completion of electrophoresis at 120 V, gels were evaluated using the Gel Doc EZ System (Bio-Rad, Hercules, California) ultra-violet illumination.

Amplified products of PCR -positives were selected as a sample subset and submitted for Sanger sequencing (Eton Biosciences, Inc., NC, USA). Sequencing results were then compared against the NCBI nucleotide database using the Standard Nucleotide BLAST application (http://www.ncbi.nlm.nih.gov/BLAST/) for species identification.

2.8 Data analysis

2.8.1 Geospatial Analysis

GPS data points of tick collection sites were downloaded into ArcGIS 10.4 (ESRI, Redlands, CA). Locations were grouped by soum into six sites. For each soum site, 10 km buffers were created to evaluate maximum normal density vegetation index (NDVI), minimum land surface temperature (LST), and mean elevation data as described by Hay et al [61]. Maps were downloaded for geospatial analysis from Mongolian Environmental Health Geodatabase (http://www.eic.mn/). Using zonal statistics in ArcGIS, geospatial statistics for maximum NDVI, minimum LST, and mean elevation were calculated and selected for comparison between sites.

2.8.2 Minimum Infection Rate

Minimum infection rate (MIR) was calculated using PooledinfRate software (http://www.cdc.gov/westnile/resource pages/mosqSurvSoft.html) based on the number of ticks per pool and pool infection status for *Rickettsia spp.* and/or *Anaplasma/Ehrlichia spp*.[12] This analysis was used to estimate the proportion of infected ticks in each soum, aimag, and across each species rodent captured. Briefly, for this study MIR was calculated by using the following formula: [(number of positive pools)/(total ticks tested) x 100]. This analysis assumes that each positive tick pool only has one infected tick. One sample analysis was used with group variables to calculate MIR by soum, aimag and species of rodent collected.

2.8.3 Transovarial Analysis

Engorged adult tick infection status was compared to corresponding egg infection status for PCR-positive *Rickettsia spp.* and *Anaplasma/Ehrlichia spp.,* as well as sequence data. Tick egg samples positive for a target pathogen that was the same as was identified in the associated adult tick from which they were laid, was considered transmission by vertical route. Statistical analyses, including two-way frequencies with measures of association, were conducted using STATA 14.1 (StataCorp, College Station, TX).

2.8.4 Matched Rodent *Rickettsia spp*. Status

Ticks were paired with rodent data collected from a parallel study evaluating potential rodent reservoirs. Data consisted of active infection of *Rickettsia spp.* or *Anaplasma/Ehrlichia spp*. and seroprevalence for *Rickettsia spp.* or *Anaplasma/Ehrlichia spp*. Tick pools were evaluated by corresponding rodent's history of rickettsial infection. Rodent history was defined as having either active infection or seroprevalence of target pathogen. Statistical analyses, including two-way frequencies with measures of association, were conducted using STATA 14.1 (StataCorp, College Station, TX).

3. Results

Over the course of this study, a total of 656 ticks were collected from 15 different locations across nine soums in five aimags. Geospatial analysis was calculated for larvae and nymph tick collection sites (Table 4). There was no GPS latitudinal and longitudinal data taken during collection of adult ticks. All ticks were morphologically identified as *Dermacentor spp.* Due to the size of larvae and nymph ticks collected and the variety of *Dermacentor spp.* found in Mongolia, early life stage ticks were only identified to genus. All adult-fed ticks were morphologically identified to be *D. nuttalli*.

Of the early life stage ticks collected, 546 (88%) were larvae, 72 (12%) were nymph. Larvae and nymphs were collected from rodents consisting of 287 (46.44%) ticks from ground squirrels (*Spermophilus spp.*), 52 (8.41%) from Daurian pika (*Ochotona dauurica*), 1 (0.16%) from a field mouse (*Apodemus spp.*), 26 (4.21%) from Mongolian gerbils (*Meriones unguiculatus*), 21 (3.40%) from Siberian chipmunks (*Tamius sibiricus*), and 231 (37.38%) from striped dwarf hamsters (*Cricetulus barabensis*). There were no adult ticks collected from rodents. There were 588 (95%) of 618 ticks that were allocated into 42 larvae and 18 nymph pools (60 pools total).

All adult ticks were collected from livestock. A total of 38 adult fed female ticks were collected. Tick distribution consisted of 30 (86.84%) adult ticks collected from sheep and eight (21.05%) from cattle. Of the 38 adult ticks collected, there were 33 ticks that laid eggs when reared in the laboratory setting. All specimens underwent PCR analysis.

Table 4: Summary of tick collection location characteristics.

Soum (Aimag)	Characteristics		
	Elevation	NDVI	Ecosystem
Bayangol (Selenge)	890.58	1554.69	Grassland
Orkhon (Darkhan-Uul)	749.501	1569.27	Mixed Forest/Grassland
Eruu (Selenge)	772.613	1566.49	Mixed Forest/Grassland
Javkhlant (Selenge)	797.302	1572.05	Mixed Forest/Grassland
Darkhan (Darkhan-Uul)	744.714	1532.78	Grassland
Batsumber (Tov)	1363.44	1642.08	Mixed Forest/Grassland

NDVI: Normalized Density Vegetation Index

3.1 Rickettsia spp. PCR Analysis

All tick pools were PCR-positive for *Rickettsia spp.* Calculations of MIR for *R. raoultii* ranged from 6.7% to 28.6% across the 6 tick pool collection sites (Table 5). The highest MIR of all soums larvae/nymph collected was in the Selenge aimag at Bayangol. However, in calculating the MIR for *R. raoultii* by aimag, Tov had the highest rate with 14.4% (95% CI: 9.1-19.7), followed by Darkhan-Uul with 8.6% (95% CI: 3.5-13.7), and Selenge with 8.5% (95% CI: 5.4-11.7). Of all animals ticks were collected from, the species with the greatest associated MIR was Pika with 15.4% (95% CI: 5.6-25.1) and the lowest associated MIR was with Mongolian gerbils at 7.4% (95% CI: 4.0-10.7). Korean field mouse species were excluded from animal species MIR analysis because only one tick was collected.

All engorged adult ticks tested positive by PCR for *Rickettsia spp.* Subsequent PCR testing of paired eggs resulted in 91% (30/33) PCR positivity among tick egg pools for *Rickettsia spp*. All three tick egg samples that were PCR-negative for *Rickettsia spp.* were laid by ticks collected from sheep from Tarialan soum located in Khuvsgul aimag.

Table 5: Summary of minimum infection rates by soum (aimag).

Soum (Aimag)	Minimum Infection Rate (95% CI)
Bayangol (Selenge)	28.6 (0.0-62.0)
Orkhon (Darkhan-Uul)	8.9 (3.4-14.5)
Eruu (Selenge)	7.8 (3.9-11.7)
Javkhlant (Selenge)	8.5 (3.5-13.5)
Darkhan (Darkhan-Uul)	6.7 (0.0-19.3)
Batsumber (Tov)	14.4 (9.1-19.7)

3.2 Anaplasma/Ehrlichia spp. Analysis

There were no tick pools that tested PCR-positive for *Anaplasma/Ehrlichia spp*. See Table 6 for summary of tick pool PCR status. Of the 38 engorged ticks collected, 2 (5%) ticks were PCR-positive for *Anaplasma/Ehrlichia spp.* Both engorged ticks were collected from sheep located in Tarialan soum located in the Khuvsgul aimag. Additionally, one pool of eggs laid by an *Anaplasma/Ehrlichia spp*.-positive engorged adult female tick, was also found to be PCR-positive for *Anaplasma/Ehrlichia spp*. This same *Anaplasma/Ehrlichia spp.* PCR-positive egg sample was one of the three egg samples that tested PCR-negative for *Rickettsia spp.*

Table 6: Summary of molecular results for larvae/nymph pools.

Soum (Aimag)	Pathogens Detected (%)	
	Rickettsia spp.	*Anaplasma/Ehrlichia spp.*
Bayangol (Selenge)	3/3 (100)	0
Orkhon (Darkhan-Uul)	8/8 (100)	0
Eruu (Selenge)	14/14 (100)	0
Yavkhlant (Selenge)	10/10 (100)	0
Darkhan (Darkhan-Uul)	2/2 (100)	0
Batsumber (Tov)	23/23 (100)	0

3.3 Sequencing

A select subset of PCR amplified products were sequenced based on *Rickettsia spp. gltA* and *ompA* genes and evaluated using the NCBI BLAST application. All sequenced products were identified to be *R. raoultii*. The sequenced products of a subset of engorged ticks with PCR-positive matched egg samples were shown to be *R. raoultii*. *Anaplasma/Ehrlichia spp.* PCR-positive engorged ticks and corresponding tick egg samples were sequenced and evaluated using the NCBI BLAST application. All three samples were positive for *An. ovis.*

3.4 Matched Rodent Rickettsia spp. Status

Tick pools were matched with rodent rickettsial infection data collected from a parallel study observing potential rodent reservoirs. Of the 60 tick pools collected from rodents, there were only 50 pools (522 ticks total) that were matched with rodent rickettsial infection history. There were 31 (62%) tick pools or 362 (69%) of individual ticks that were found on rodents with no history of rickettsial infection. Only 19 (38%)

tick pools or 160 (31%) of individual ticks were associated with a known status of rickettsial infection history. Additionally, only 4 nymph life stage pools or 10 individual nymph ticks accounted for the total 31 tick pools associated with rodents of no rickettsial infection history. This suggests that the majority of ticks discovered to have no association with rodents with rickettsial infection history were of the larva life stage (352/362 individual ticks).

4. Discussion

Few studies have evaluated transovarial transmission of TBPs in Mongolia.[51, 62] Considering these few studies, data regarding transovarial transmission of *Rickettsia spp.* pathogens[63] are particularly sparse and it remains unclear what role transovarial transmission of *Rickettsia spp*. and *Anaplasma/Ehrlichia spp*. may play in the maintenance of TBPs in Mongolia. Additionally, few studies have assessed TBPs in tick larvae collected from animals.

4.1 Detection of Rickettsia spp.

It is often difficult to compare field surveys on TBPs, because of varying sampling methods and sample sizes. However, the detection of *R. raoultii* found in this study show similarity with other surveillance studies conducted in Mongolia with 100% of *Dermacentor spp.* tick pools testing positive for *R. raoultii.*[19, 32] The molecular positivity of all of the adult ticks was 100% for *R. raoultii*, suggesting that the prevalence of *R. raoultii* in the areas in which the ticks were collected is quite high.

Testing larvae collected from animal hosts can prove advantageous as an indirect method of determining if pathogens infect ticks by transovarial or transstadial transmission. Interestingly, a substantial proportion of ticks that tested positive for *Rickettsia spp.*, were collected from rodents that were molecularly and serologically negative for rickettsial infection. Potential explanations for this observed event may include co-feeding. Studies have shown that *Rickettsia spp*. can be transmitted from an

infected tick to an uninfected tick without infecting the host.[14, 64-66] This event occurs when ticks are clustered together while feeding on a small mammal host and one infected tick inoculates the other ticks that are in close proximity.

The alternative explanation is by transovarial transmission of *Rickettsia spp.* pathogen into larvae found on small mammals. It is possible that larvae infected with *Rickettsia spp*. had attached to the non-infected rodent hosts for their first blood meal. Rodents may not have shown to be PCR-positive for *Rickettsia spp*., because ticks were not attached to the rodent host long enough to cause infection[67] or infected tick density on the host was too low for transmission of pathogen.[68]

4.2 Transovarial transmission of Rickettsia spp.

Transovarial transmission of *Rickettsia spp*. has been both demonstrated in controlled laboratory settings and observed in nature. Laboratory demonstration of transovarial transmission has often included controlled concentration of the pathogen, artificial climactic conditions (temperature, humidity, etc.) and use of experimental hosts such as rabbits[63], mice or guinea pigs or capillary feeding. [69] Among these laboratory-based transovarial studies, transmission of *Rickettsia spp*. from mother to progeny has shown to be up to 100% across various tick species.[18, 63] Additionally, many laboratory-based transovarial studies have demonstrated the efficiency of vertical transmission of *Rickettsia spp*. over multiple generations of tick.[21, 63] However, among studies observing naturally infected ticks transmitting *Rickettsia spp*. to progeny, prevalence has ranged

from 30% to 100%.[18] Though, laboratory-based studies are able to determine the capability of transovarial transmission, observational studies of transovarial events can provide a real-world picture illustrating burden of transovarial transmission of TBPs in a given region.

There may be reason to believe that naturally occurring *Rickettsia spp.* is sustained through both transovarial and transstadial transmission in *Dermacentor spp.* ticks in Mongolia based on previous epidemiological research of *Rickettsia spp*. in Inner Mongolia, China, which shares similar ecosystems.[70] Previous studies have demonstrated transovarial transmission of *R. raoultii* in *D. nuttalli*, *D. silvarum, D. marginatus,* and *D. reticulatus* ticks. Transovarial transmission ranged from 43% to 100% prevalence depending on generation of tick infection, species of tick, and strain of *R. raoultii.* In comparison to the previous laboratory-based research reporting 43% to 99.5% of transovarial transmission of *R. raoultii* in *D. nuttalli* ticks[21], this study reports comparable transovarial transmission prevalence between adult and pooled eggs in *D. nuttalli* ticks at 91%. Though there has been no conclusive data reporting vertebrate hosts as a reservoir for *R. raoultii*, it has been suggested that *D. marginatus* and *D. reticulatus* serve as both vector and reservoir.[45] In Mongolia, the reservoir for *R. raoultii* has not been identified. Our study's high prevalence of transovarial occurrence in conjunction with the substantial number of infected larvae found on rodents with no history of rickettsial infection suggests that *D. nuttalli* ticks serves both as a vector and as

a reservoir for *R. raoultii* in the northern region of Mongolia. If this is the case, it may be beneficial to direct future research and prevention efforts for *R. raoultii* through control of *D. nuttalli* ticks.

4.3 Transovarial transmission of Anaplasma spp.

Though this study did not identify *Anaplasma spp.* or *Ehrlichia spp.* pathogens in larvae or nymph, there was detection of *A. ovis* in two *D. nuttalli* fed adult ticks and one tick egg pool. There have been reports of *A. phagocytophilum* transmitted transovarially with efficiencies ranging from 10% to 40%[71], however transovarial transmission of most *Anaplasma spp.* and *Ehrlichia spp*. are believed to not occur or occur at low frequencies.[72, 73] There has been research evaluating the role of transovarial transmission of *Anaplasma spp*. and *Ehrlichia spp*. with little success.[74] As far as we know, this may be the first documented report identifying transovarial transmission of *A. ovis* in *D. nuttalli* ticks.[75] There has been documentation of this particular *Anaplasma spp.* in *Dermacentor spp.* ticks[76] with history of infection in goats[16, 25], sheep[16, 27], cattle[16] and reindeer[29] throughout Mongolia and China. *A. ovis* has not been confirmed to cause human infection, however there have been reports of suspect human anaplasmosis associated with an *A. ovis* variant in the USA.[75] Though this particular *Anaplasma spp*. is not known to cause human disease, the economic burden is great for individuals who rely on raising livestock for their income.[77] Further research is needed to evaluate the efficiency and role of potential transovarial transmission of *A. ovis.*

4.4 Strengths and Weaknesses

This study used two *Rickettsia spp.* PCR assays, which both increase the sensitivity and specificity of laboratory methods. *Rickettsia spp.* PCR analysis has been shown to cross-react with *Anaplasma spp.* and *Ehrlichia spp*. However, this study also utilized a general screening assay for *Anaplasma/Ehrlichia spp*. and confirmation by sequencing, which allowed for greater confidence in the *Rickettsia spp*. PCR assay.

Like many tick pool studies, it is difficult to determine the exact prevalence of disease using this approach. Though MIR was calculated, it was not possible to calculate the maximum likelihood estimation (MLE). Due to the nature of the MLE calculation, the proportion of infected ticks with maximum likelihood of being *Rickettsia spp*. infected within tick pools cannot be calculated if there is 100% positivity of sample pools.[78] Though MLE is not a required calculation to determine infection rate among sample pools, it is one less calculation to determine the burden of *R. raoultii*. Additionally, due to the pooling of tick eggs, this study was unable to determine a more precise proportion of transovarial transmission from an infected female tick to at least one progeny. Though data suggest that transovarial transmission for *R. raoultii* did occur, we were unable to determine how many progeny were infected.

Though paired rodent data strengthens the investigation of the early life stage of ticks, there is also the potential for under-reporting rickettsial infections among rodent

populations. Blood extraction from rodents may not have yielded enough sample for DNA extraction and PCR detection of *Rickettsia spp*. pathogen.

Lastly, as in any cross-sectional design, this study does not assess prevalence over time or seasons. Therefore, this study was unable to investigate difference of infection rates over time and may have missed seasonal feeding periods of other tick species at study collection sites.

4.5 Future Research

The indication that *D. nuttalli* ticks can serve as reservoirs for *R. raoultii* may warrant additional evaluation of transovarial transmission of *R. raoultii*. Studies should focus on assessing tick eggs, either in smaller egg pools or individually, to determine the proportion of vertical transmission for *R. raoultii* in the natural foci of Mongolia. Additionally, the testing of larvae from animal hosts and the environment should be further examined, preferably testing individual ticks instead of tick pools. Also, this report has identified a potentially novel transovarial transmission of *A. ovis*. Further investigation would be needed to determine the efficiency and prevalence of vertical transmission of this pathogen.

5. Conclusion

In conclusion, this study identified transovarial transmission of *Rickettsia spp.* and *Anaplasma spp*. among *D. nuttalli* ticks. This study also found low prevalence of rodents with history of *Rickettsia spp*. infection and infection status of biting ticks. Additional study is needed to further assess the proportion of transovarial transmission found in nature. Specifically, testing of individual tick eggs and larvae should be conducted. A better understanding of the ecology of TBPs in nature can provide public health and human and veterinary medicine with a greater awareness of the burden of TBPs in Mongolia. Studies similar to this can inform control methods of vector borne disease, specifically TBPs.

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