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Original Article

Diversity and Distribution of Bacterial and Parasitic Tick-Borne Pathogens in Armenia, Transcaucasia

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Abstract

Background: Variations in the distribution and prevalence of pathogens in ticks can have significant consequences for human health. Information on these variables in Transcaucasia is scarce, so the aim of our study was to conduct a large-scale study to detect selected tick-borne infectious agents in Armenia.

Methods: Overall, 209 adult ticks were collected from different hosts including 4 samples from human clothes. We tested ticks using high-throughput microfluidic single-cell real-time PCR to detect 42 genospecies of pathogens. We used GIS to determine biotic and abiotic factors governing the prevalence of pathogens and applied statistical analyses to test the association between prevalence of pathogens depending on hosts, locality and environment.

Results: From 209 samples, 134 were positive to targeted pathogens. *Anaplasma phagocytophilum* Foggie, 1949 was the most prevalent case (44%). The highest overall prevalence was observed in ticks from sheep (74%), followed by cows (67%) and calves (60%). The highest multiple infection rates were also detected in sheep (40%) and calves (40%) followed by cows (28%). One statistically significant association was found among co-infections ($P<0.05$). The prevalence of pathogens varied according to locality. The abundance of *Anaplasma* spp. is significantly correlated with "slope" and "vegetation" factors. Similar patterns were detected for other pathogens.

Conclusion: This was the first large-scale survey of multiple tick-borne pathogens in Armenia and Transcaucasia. The results of this study shed light on spatial variations in pathogen infection rate among adult ticks found on hosts and underline a number of environmental determinants of pathogen distribution among ticks.

Keywords: Tick-borne pathogen; Bacterial pathogen; Special variation; Parasite ecology; Armenia

Introduction

Ticks serve as the primary vectors for infectious diseases impacting both humans and animals across Europe, transmitting a greater number of pathogens compared to other arthropods (1, 2). Among these, Lyme borreliosis, caused by spirochetes from the *Borrelia burgdorferi sensu lato* complex, stands out as the most widespread tickborne disease (3). Various bacterial pathogens transmitted by ticks contribute to emerging diseases, including *Anaplasma phagocytophilum* (causing human and animal anaplasmosis), *Rickettsia helvetica* (responsible for nonspecific fevers in humans), *Bartonella henselae*, *B. quintana*, *Coxiella burnetii*, and *Francisella tularensis* subspecies, among others (5–7). Additionally, ticks can transmit several protozoan parasites, such as *Babesia* spp., *Theileria spp.*, affecting both humans and animals (8).

To mitigate the risk of exposure to infected tick bites, it is crucial to identify environmental conditions associated with a heightened risk of infection (9, 10). Understanding the environmental determinants of tick abundance and the associated pathogenic agents proves valuable in estimating future pathogen distribution and prevalence, particularly under scenarios of environmental change, such as alterations in climate, land use, habitat, and hosts (11). Given the variations in climate, vector ecology, and socioeconomics across continents, regional analyses are essential (12–14).

Located in the Transcaucasian region, Armenia has a wide range of climatic conditions and many biotopes, as well as a set of tick species and host animals (15–17). It is also important to mention that the last tick surveys seeking bacterial or parasitic pathogens conducted in the country date back to 1994 and 1995 (18, 19), so the primary aim of our investigation was to conduct a large-scale study to detect several selected bacterial and parasitic agents among ticks in Armenia.

Materials and Methods

Sampling

We collected a total of 209 adult ticks from different regions of Armenia (Fig. 1). Most of the ticks were collected from different hosts including dogs, cows/calves, sheep, and goats. Four samples were collected from clothes. We kept the samples in 70% ETOH for further DNA extraction. The ticks were morphologically identified to species level.

DNA extraction and PCR

DNA was extracted using the Dneasy Blood and Tissue Kit and the NucleoSpin Tissue kit.

The TaqMan PreAmp MasterMix was used for DNA pre amplification according to the manufacturer's instructions. The final volume of the pre amplification was 5 µL including 1 µL Perfecta PreAmp SuperMix (5X), 1.25 µL pooled primers mix, 1.5 µL H_2O and 1.25 µL DNA with one cycle at 95 °C for 2 min, 14 cycles at 95 °C for 10 sec then one cycle at 60°C for 3 min. At the end of the cycling program, the reactions were diluted 1:10 by adding 45 µl of sterile deionized water to obtain the final volume of 50 µL: Pre amplified DNAs were further processed immediately.

High-throughput real-time PCR system

The BioMark™ real-time PCR system (Fluidigm, USA) was used for high-throughput microfluidic real-time PCR amplification using 48.48 dynamic arrays. These chips dispense 48 real-time PCR mixes and 48 samples into individual wells, after which on-chip microfluidics assemble real-time PCR reactions in individual chambers prior to thermal cycling, resulting in 2304 individual reactions (20, 21).

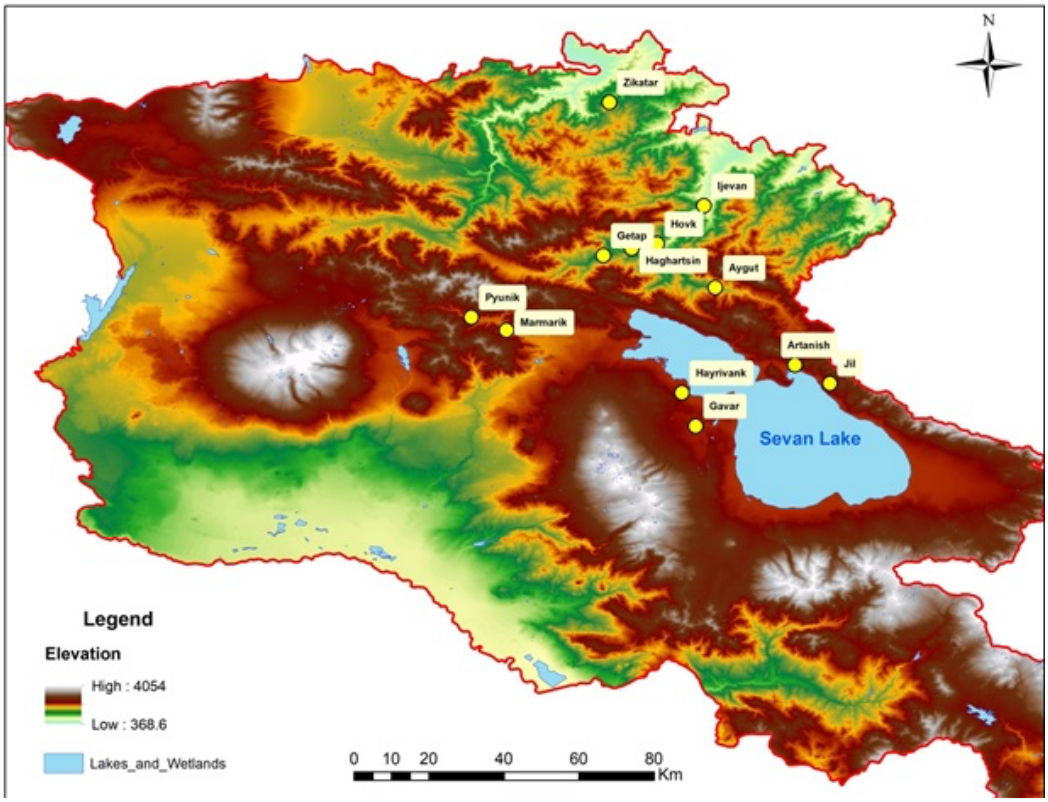

Fig. 1: Sampling localities

Thermal cycling comprised 2 min at 50 °C, 10 min at 95°C, followed by 40 cycles of 2-step amplification of 15 sec at 95 °C, and 1 min at 60°C. Data were acquired on the BioMark™ real-time PCR system and analyzed using the Fluidigm real-time PCR analysis software. Each array included one negative control of H_2O. To determine whether internal factors present in the sample itself could inhibit the PCR, *Escherichia coli* Castellani et. Chalmers, 1919 strain EDL933 DNA was added to each sample as an internal inhibition control.

GIS analyses

To study the role and influence of environmental components on the prevalence of tick-borne pathogens, we selected the following components, recognized as the most appropriate for mountainous areas (13, 14, 22): slope, elevation and aspect, monthly precipitation, temperature, and area vegetation. For the latter we used the normalized difference vegetation index (NDVI). We used ArcGIS 10.1 with the 3D Analyst expansion module to obtain the slope, aspect and elevation data for the country based on a digital elevation model (DEM) (Earth Explorer. https://earthexplorer.usgs.gov/ (23). Vegetation maps were generated from Landsat OLI multispectral satellite images using the NDVI, which ranges from -1.0 to 1.0 respectively indicating cloudiness and humidity to dense green vegetation (24). Temperature and precipitation data were freely available from www.aua.am (25). The GPS coordinates were plotted on maps and a GIS database was created for further analyses (Table 1).

Table 1: Data on localities

Locality	*NDVI*	*Aspect*	*Slope (%)*	*Elev. (m)*	*Temp. (°C)*	*Prec. (mm)*
Artanish	0.04	S	2	1946	5	500
Aygut	0.32	SW	5.8	1401	5	600
Gavar	0.15	SW	0.8	1949	5	500
Getap	0.31	S	13.3	1152	9	600
Haghartsin	0.35	SE	12.4	1018	9	600
Hayrivank	0.26	SE	2.2	1940	5	400
Hovk	0.48	S	11.2	1155	9	600
Ijevan	0.11	N	0.67	675	11	600
Jil	0.39	SW	4.05	2016	3	600
Marmarik	0.22	NE	0.41	1748	5	700
Pyunik	0.4	S	7.84	1824	5	800
Zikatar	0.54	W	13.47	1228	7	700

Statistical analyses

The associations between tick-borne pathogen distribution and environmental conditions were made using a linear regression method. We tested the influence of host species and locality on adult ticks' disease infection rates with Chi-square tests. The statistical analyses were processed using the Realstatistics add-in for MS Excel and Statistica 7 software. We grouped the pathogens into *Borrelia, Anaplasma, Ehrlichia, Candidatus, Rickettsia, Francisella*, *Apicomplexa, Babesia*, and *Theileria* for some of the analyses including influence of locality, hosts on which ticks were obtained, and environmental variables on the prevalence of pathogens.

We used the association screening approach to test potential associations between TBP (tick-borne pathogen) species. For a given number of pathogen species tested (NP), the number of possible combinations (NC) was calculated as $NC = 2^{NP}$. Assuming similar pathogen prevalence as those observed, a simulated dataset was built in the form of an absence/presence matrix with hosts in lines and pathogen combinations in columns. We obtained the NC statistical distributions from 5000 simulations. We estimated a 95% confidence interval to obtain a profile that includes all the combinations at the same time. From this profile, we inferred two quantiles Qinf and Qsup for each combination. A global test was based on the 95% confidence envelope. When H0 was rejected, the local tests were based on the NC confidence intervals.

Results

Detection and prevalence of pathogens

In all, 134 out of 209 adult ticks were positive to the targeted pathogens. Among those pathogens, *A. phagocytophilum* had the highest infection rate, with 44% of 209 screened ticks. The second most prevalent pathogen was *Theileria* spp. (36%), followed by *A. marginale* Theiler, 1910 with a 14% prevalence. All the other pathogens occurred in less than 10% of ticks. Twenty-three of the 38 pathogens investigated were not found in any ticks. Particularly, only four out of eight tested *Borrelia* geno-species occurred in seven individual ticks, but an additional seven ticks were infected with *Borrelia* spp. not belonging to the tested genotypes. Only *A. marginale* and *A. phagocytophilum* from seven tested *Anaplasma* species were present in ticks. Two out of the six *Rickettsia* species were absent but one individual was infected by an unknown species. Interestingly, only one tick was infected by a *B. divergens* species out of the seven. Among the infected ticks, we detected single (n=65), dual (n=56), and even triple infections (n=13).

Infection rate of adult ticks according to host and locality

The infection rate of adult ticks for the pathogens considered in different localities and hosts was highly variable (Table 2). In seven out of 12 localities, the infection rate was higher than 70%, even reaching 88% in Haghardzin, which also had the highest prevalence of dual infections (63%). The lowest prevalence was detected in Zikatar (27%).

Table 2: Infection rate of adult ticks for different groups of pathogens according to host and locality

Locality	*Percent*												
	Borrelia	*Anaplasma*	*Ehrlichia*	*N. mikuren-sis*	*Rickettsia*	*Francisella*	*Babesia*	*Theileria*	*Infected*	*Multiple*	*Single infec-tion*	*Dual infec-tion*	*Triple Infec-tion*
Artanish	5	60	0	0	5	0	1	51	72	44	28	37	7
Aygut	7	47	0	0	0	0	0	73	73	47	27	40	7
Gavar	6	59	6	6	12	0	0	0	71	12	59	6	6
Getap	0	25	20	0	0	0	0	30	45	25	20	20	5
Haghartsin	0	38	0	0	63	25	0	38	88	63	25	50	13
Hayrivank	0	25	0	0	25	0	0	0	50	0	50	0	0
Hovk	33	25	8	0	8	0	0	8	42	25	17	8	17
Ijevan	25	50	0	0	25	0	0	25	75	50	25	50	0
Jil	0	56	0	6	0	0	0	28	78	11	67	11	0
Marmarik	7	47	0	0	0	0	0	20	47	20	27	13	7
Pyunik	20	50	0	0	0	0	0	50	70	50	20	50	0
Zikatar	0	27	0	0	9	0	0	18	27	18	9	9	9
Host													
Calf	15	25	10	5	35	10	0	15	60	40	20	25	15
Human cloth	25	25	0	0	0	0	0	25	25	25	0	0	25
Cow	9	49	0	0	9	0	0	32	67	28	39	23	4
Dog	0	29	0	0	7	0	0	21	29	21	7	14	7
Goat	7	47	0	0	0	0	0	20	47	20	27	13	7
Sheep	3	57	5	1	1	0	1	49	74	40	33	36	5
Total	7	48	3	1	7	1	0	36	64	33	31	27	6

The infection rate of adult ticks for *Rickettsia* ($P< 0.01$), *Francisella* ($P< 0.01$), and *Theileria* ($P= 0.01$) are significantly different according to the host species.

Moreover, only the *Anaplasma* ($P= 0.16$), *N. mikurensis* ($P= 0.53$), and *Babesia* ($P= 0.99$) infection rate in adult ticks did not differ between localities. All the other pathogen infection rates in adult ticks were significantly associated with the locality.

Environmental drivers of prevalence

The results of the evaluation of the influence of environmental components on the infection rate of adult ticks for the different pathogens considered are observed. The tick infection rate for *Anaplasma* spp. was significantly associated with NDVI and slope single infections appeared to be governed by slope, elevation and temperature.

Co-infections and associations between pathogens

Out of all the collected ticks, 33% were co-infected by at least two pathogen groups. The association screening approach (29) showed that the association between *Rickettsia massiliae* Beati et. Raoult, 1993 and *Francisella*-like endosymbionts (observation = 2; min expected = 0; max expected = 1) was overrepresented in our sample compared to a random distribution.

Discussion

Our study describes the potential influence of the environment, hosts and the pathogen community in the diversity and distribution of tick-borne diseases among ticks from Armenia.

Tick-borne pathogens in Armenia

This is the first large-scale study of bacterial pathogens in the Transcaucasia region. However, studies on tick pathogens in Armenia began in the 1930s, when Galuzo conducted a survey to detect *Theileria annulata*. They found that all the infected ticks belonged to the genus *Hyalomma* (26). Then in 1960, Airapetyan and colleagues summarized the records of the Armenian Veterinary Research Institute and reported on a set of veterinary diseases that occurred in farm animals between 1930 and 1955 (27). In the 1970s, Tarasevit et al. conducted a large-scale survey for rickettsioses (28). They found *rickettsiae* or *Rickettsia*-like organisms in 163 (18.2%) ticks: 23 (2.6%) contained *Coxiella burnetii* and 105 (11.7%) spotted fever infection (28). The most recent surveys date back to 1994 and 1995 (18, 19). All the tested rickettsial spotted fever group Armenian isolates from *Dermacentor marginatus* were identified as *R. slovaca* (19). Additionally, Eremeeva et al provided evidence for a bigger role of *Rhipicephalus sanguineus* ticks in the epidemiology of rickettsial diseases (18). Finally, Gevorgyan and colleagues conducted a survey to detect Crimean Congo Hemorrhagic Fever virus antigens, and found in several tick species (17).

It is also important to mention that in 2010, six human cases of Lyme disease were registered in Armenia (29).

Infection rate of different pathogens in ticks

In a similar study in Turkey, Orkun and colleagues investigated the infection rates of *Babesia* spp. (2.66%), *Borrelia* spp. (0.29%), *Rickettsia* spp. (12.26%), *Theileria* spp. (0.09%), *Hepatozoon* spp. (0.19%), and *Hemolivia* spp. (0.49%) in ticks (30). The prevalence of *A. phagocytophilum* in the genus *Ixodes* ranges from 0% to 67% depending on the geographic regions (31) as we showed too. A similar overlap we saw in the case of *Rickettsia* spp., which were found in 7.2% of ticks in our study and in *I. ricinus,* varies between 1% and 53% (32). Interestingly, Milutinovic and colleagues found that *B. burgdorferi sensu lato* had the highest prevalence (42.5%) in *I. ricinus* ticks from Serbia (33). These studies all state that other determinants beyond tick species also play a role in the diversity of pathogenic agents and their distribution.

Our results show that the prevalence of *Francisella*-like endosymbionts, *Rickettsia* spp. and *Theileria* spp. and overall infection significantly differs among ticks collected from different animals.

Influence of locality, environmental variables and host on tick infection rates

According to our results the prevalence of *Borrelia* spp., *Ehrlichia* spp., *Rickettsia* spp., *Francisella* spp., and *Theileria* spp. was significantly different in ticks collected from different study sites. In the case of *Anaplasma* spp., it is observed to occur less regularly in temperate climate zones. In the United States and other countries, instances of the disease have been reported outside regions infested with ticks. Additionally, the geographic distribution in Europe has been progressively expanding northward in recent years, with isolated cases noted in France, Switzerland, the Netherlands, Hungary, and Austria (34). Our analyses of climatic variables driving tick infection prevalence showed that overall infection is significantly associated with slope, elevation, and the average yearly temperature of the study site. Perez et al discovered that the prevalence of *A. phagocytophilum* rises in tandem with wooded habitats (0-500 m), presumably due to host population size, and shows a slight increase with the abundance of bank voles, which had a higher reservoir capability compared to wood mice. On the other hand, the prevalence of *B. burgdorferi* s.l. increases in wooded-grassland ecotones, but only at local scales (50-100 m) (35). Additionally, bacterial infection status was influenced by tick characteristics and forest fragmentation, vegetation, and habitat variables (36).

Our study provides more baseline information to consider when modeling environmental drivers of tickborne pathogen distribution worldwide.

Conclusion

We used high-throughput microfluidic single-cell real-time PCR to study the prevalence and distribution of 42 tick-borne bacterial and parasitic pathogens for the first time in Armenia. Among these pathogens, *A. phagocytophilum* had the highest infection rate, and the second most prevalent pathogen was *Theileria* spp., followed by *A. marginale*. There was found a relation between environmental factors and tick infection rates for *Anaplasma* spp. significantly associated with "NDVI" and "slop" variables.
The infection rate of adult ticks in different localities appeared to be highly variable. The highest infection rate was in samples collected from Haghardzin, which also had the highest prevalence of dual infections, followed by Jil and Ijevan. The lowest prevalence was detected in Zikatar.
The infection prevalence in adult ticks differs depending on host species as well. The highest rate of infection was detected in samples collected from cows and calf. Co-infection was revealed among 33% of collected samples by at least two pathogen groups.

Journalism Ethics considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflict of interest

The authors declare that there is no conflict of interest.

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