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QUANTITATIVE ANALYSIS OF BACTERIAL LOAD OF *BORRELIA* SPP. AND *RICKETTSIA* SPP. IN IXODES RICINUS TICKS COLLECTED FROM BIRDS IN DIFFERENT SITES OF REPUBLIC OF MOLDOVA

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Abstract
Epidemiological knowledge on quantity data of pathogens loads in ticks scarce. Quantitative studies of bacterial loads in ticks collected from birds is even more rarely described. To reduce this gap of information, a 192 Ixodes ricinus nymphs collected from birds in Republic of Moldova were examined. Nucleic acids were extracted from individual ticks, followed by PCR amplification specifically targeting Rickettsia spp. and Borrelia spp. The study found that the average bacterial load for ticks with only one type of detected bacteria was 3.53 × 10^3 for Borrelia and 1.23 × 10^5 Rickettsia. In ticks where DNA from both pathogens was detected, the average bacterial load was 9.47 × 10^4, with specific loads of 2.81 × 10^3 for Borrelia and 9.17 × 10^4 for Rickettsia per tick. Ticks testing positive for Rickettsia exhibited a significantly higher bacterial load compared to those only positive for Borrelia DNA. The highest average bacterial loads were observed in nymphs collected from blackbirds, followed by those from starlings. The study also noted no statistically significant differences in bacterial loads between ticks with mono-detection of pathogens and those with co-detection of Borrelia and Rickettsia.

Keywords: quantitative analysis, ticks, birds, Borrelia, Rickettsia

1. INTRODUCTION

A borreliosis-rickettsial mixed infections, has been recorded in many ticks of the genus *Dermacentor*, *Ixodes*, *Haemaphysalis*, *Hyalomma* in whose hemolymph *Rickettsiae* of the group of spontaneous fevers were found, and in the intestine - *Borrelia* (Norte et al 2013). In recent years, as is known, new data have been obtained in Europe on the role in infectious pathology in co-infection with spirochetes of the genus *Borrelia* (Hildebrandt et al 2010). There are other evidences, which are not quite clear from the standpoint of modern microbiology, of the simultaneous infection of ticks of those species with *Borrelia* and *Rickettsiae* (Magnarelli et al 1995). According to American researchers, the level of borreliosis- rickettsial mixed infection can reach up to 14% (Salman et al., 2012). It is very likely that pathogenic rickettsia are also common in Moldova; therefore, various variants of mixed infection with the presence of rickettsia can be found in ticks Republic of Moldova. Accurate data on the presence of ticks and tick-borne pathogens is crucial for estimating the risk of related diseases in humans and animals, and for implementing appropriate control measures when needed.

To enhance the limited epidemiological data available, birds were captured in Moldova. Ticks were then collected from these birds, identified, and analyzed using PCR to detect bacterial pathogens.

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This study aims to perform a quantitative analysis of the bacterial load in ticks collected from birds within the Republic of Moldova.

2. MATERIALS AND METHODS

Field material was gathered during the 2014-2015 field season from several locations including the Yagorlyk Reserve, Prutul de Jos Reserve, Codrii Reserve, Plaiul Fagului Reserve, the areas surrounding the city of Chisinau, and the village of Badraji Vechi.

During collecting, birds were captured using specialized nylon nets. Nine black nylon nets were utilized, crafted in-house by modifying four nets purchased from Ecotone Inc. (Gdynia, Poland). The original Ecotone Mist Net 716/12 nets were adapted from five pockets to three, shortened from 12 meters to 6 meters in length, and reduced in height from 2.5 meters to 1.6 meters.

The captured birds after the inspection were immediately released. Ticks were collected from the birds using specialized tools and stored in 70% ethanol, with each bird's ticks placed in a separate 1.5 ml Eppendorf tube. In the laboratory, ticks were identified under a stereomicroscope using identification keys from Nosek (1972), Filippova (1979), and Apanaskevich (2010)..

DNA was individually extracted from each tick using the QIAGEN DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany), following the manufacturer's guidelines.

For the amplification of B. burgdorferi sensu lato DNA, the 5S-23S rDNA intergenic spacer region was targeted using PCR. The primers used were rrfA and rrlB, which typically produce a PCR product size of 198 base pairs (bp). The protocol was outlined by Richter et al. (2006). Visualization of the PCR bands was achieved through electrophoresis on 2% agarose gels in Tris-acetate-EDTA buffer (TAE) and staining with ethidium bromide (Sigma-Aldrich, Hamburg, Germany). Positive samples were further verified using a second set of primers targeting the Borrelia-specific flaB flagellin gene, as described by Assous et al. (2006). This confirmation also involved electrophoresis on 1.2% agarose gels in TAE followed by ethidium bromide staining.

Species from the Rickettsia spotted fever group were identified using a hybridization probe-based real-time TaqMan PCR assay, which targets a 74-bp fragment of the gltA gene, as described by Stenos et al. (2005). The assay was performed on RotorGene Q cyclers (Qiagen, Hilden, Germany). For samples that tested positive, two additional conventional PCR tests were conducted. Initially, a PCR using primers 120-M59 and 120-807 was employed to amplify a 764-bp fragment of the rickettsial 135-kDa outer membrane protein B gene (ompB), following the method outlined by Roux et al. (2000). If a correctly sized amplicon was produced, further verification was carried out using a protocol described by Mediannikov et al. (2006), employing the CS1d and CS2d primers to amplify 1254 bp of the gltA gene. PCR products were visualized by electrophoresis on 1% agarose gels in TAE, stained with ethidium bromide.

To measure amount of bacteria in the tick organism standards were used with a known concentration ($2x10^5$ for Rickettsia (DNA of *Rickettsia prowazekii*) and $2x10^6$ for Borrelia (DNA of *Borrelia burgdorferi sensu lato*). A calibration line for real-time PCR was obtained by four consecutive 10-fold dilutions of available standards.

3. RESULTS AND DISCUSSIONS

In total, out of 192 *Ixodes ricinus* engorged nymphs, 44 cases of *Borelia* spp, DNA and 23 cases *Rickettsia* spp. DNA were detected. Among these cases, 4 cases of code detection were found.

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The overall mean bacterial load found was 3.53×10^3 for *Borrelia* and 1.23×10^5 *Rickettsia* for mono-detection (Figure 1).
Ticks in which DNA from both pathogens was co-detected exhibited an average of 9.47×10^4 bacteria, in proportion 2.81×10^3 for *Borrelia* and 9.17×10^4 *Rickettsia* genome equivalent per tick. Ticks testing positive for Rickettsia ($P<0.01$) and those with co-detected DNA of both bacterial agents ($P<0.1$) exhibited significantly higher bacterial loads compared to ticks that only tested positive for Borrelia DNA. The highest average bacterial loads were found in nymphs collected from blackbirds (*Borrelia* DNA mono-detected: 7.56×10^3; *Rickettsia* DNA mono-detected: 4.87×10^5; Borrelia co-detected: 7.19×10^3; Ricketsia co-detected: 3.12×10^5; *Borrelia-Rickettsia* DNA co-detected: 3.21×10^5), and then in nymphs collected from starlings (*Borrelia* mono-detected: 5.56×10^3; Rickettsia mono- detected: 2.09×10^5; *Borrelia* co-detected: 5.54×10^3; *Rickettsia* co-detected: 3.03×10^5; *Borrelia-Rickettsia* co-detected: 3.03×10^5) (Table 1).
No statistically significant differences were found when comparing the level of the bacterial load of *Borrelia* and *Rickettsia* in cases of mono-detection with the bacterial loads of *Borrelia* and *Rickettsia* in ticks where both pathogens were detected.
The Shannon index for ticks collected from birds reached values of 1.39 ± 0.44. There was no significant correlation between the percentage of infested birds and biodiversity scores.

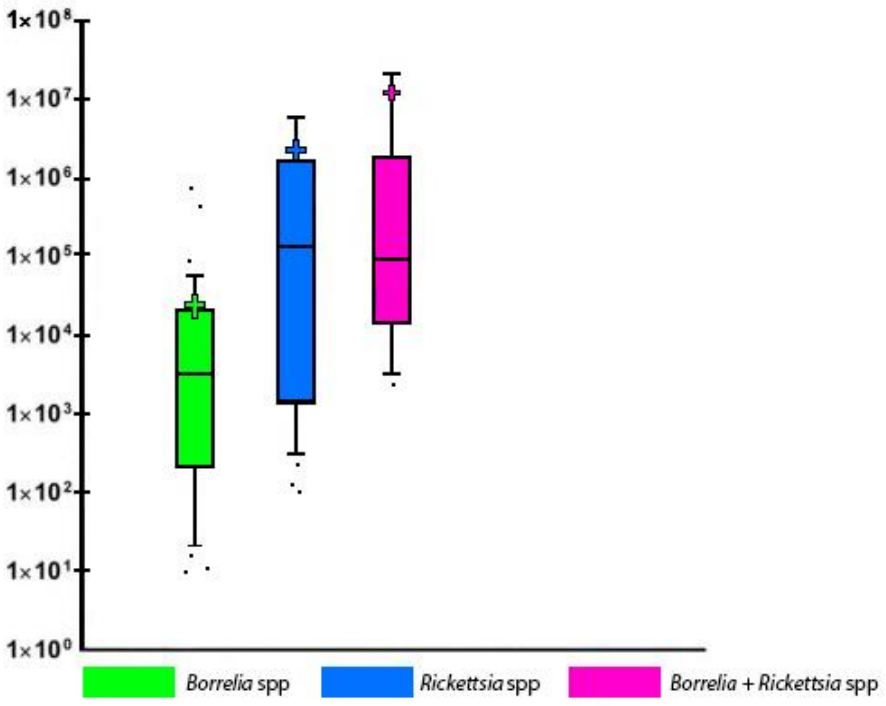

Figure 1. Average level of bacterial load in ticks (number of bacterial genomic equivalents)

When treating a patient diagnosed with Lyme disease who exhibits more severe symptoms than typically expected, or who does not respond adequately to standard antibiotic therapy, it is advisable to consider the possibility of concurrent rickettsiosis (Wormser et al., 2006). Antibiotics effective against Lyme disease do not treat babesiosis, making early diagnosis of coinfection particularly

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critical, especially in immunocompromised patients (Vannier et al., 2012). Patients with Lyme disease who also have concomitant Hemolytic Streptococcal Anemia (HSA) generally experience more symptoms for a longer duration compared to those with Lyme disease alone, as noted by Koenen et al. (2013). However, a study by Belongia et al. (1999) found no significant difference in symptom duration or severity between the two groups. The delay in diagnosing Human Granulocytic Anaplasmosis (HGA) is less concerning because doxycycline, commonly prescribed for Lyme disease, is also effective against HGA, according to Koenen et al. (2013).

Tab.1. Quantitative analysis of bacterial load of Borrelia spp. and Rickettsia spp. in ticks collected from birds in different sites of Republic of Moldova

Collection point	Number of ticks tested	Monodetection *B.burgdorferi s.l.*				Monodetection *Rickettsia* spp			
		Number of samples with *Borrelia* DNA	Ct_{min}-Ct_{max} (limit values for cycles)	Average Ct	Average number of genomic equivalents per tick	Number of samples with *Rickettsia* DNA	Ct_{min}-Ct_{max} (limit values for cycles)	Average Ct	Average number of genomic equivalents per tick
Yagorlyk	95	16	22.1-33.2	28.5±1.1	9.3×10^2	12	23.1-35.1	27.8±1.7	1.18×10^5
Chisinau city	42	12	20-34.4	27.4±1.9	1.3×10^4	9	23.4-33.1	26.4±2.1	2.76×10^5
Plaiul Fagului	19	6	24.5-32.3	29.6±3.8	3.7×10^3	1	28.1		7.9×10^4
Codri	21	7	24.4-29.6	26.5±3.9	2.9×10^3	1	32.9		7.81×10^2
Prutul de jos	10	2	28.1-32.7	30.4±2.5	1.61×10^2	0			
v. Bedrajiy Vechi	5	1	27.6		8.94×10^2	0			
Avarage			**20-34.5**	**30.1±2.1**	**$3{,}53\times 10^3$**		**23.1-35.1**	**28.2±4.2**	**1.23×10^5**

Additionally, no studies have demonstrated that co-infection with *Babesia microti* or *Anaplasma phagocytophilum* leads to long-term complications associated with Lyme disease, as reported by Krause et al. (2013) and Wang et al. (1997).
For other co-infections involving moderate to severe disease, including cases with three pathogens, instances have been described by Platonov et al. (2011). However, such cases are too rare to definitively conclude an association between pathogen interaction and disease severity in these scenarios.

4. CONCLUSIONS

The analysis presented indicates an association between Borrelia spp. and Rickettsia spp. in ixodid ticks. Birds serve as significant reservoirs for tick-borne pathogens, with ticks acting as crucial vectors for both bacterial species. Ticks testing positive for Rickettsia and those with co-detected DNA from both bacterial agents exhibited a significantly higher bacterial load compared to ticks that were only positive for Borrelia DNA.

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