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Tick-borne infections and co-infections in patients with non-specific symptoms in Poland

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ABSTRACT

Aim: The aim of the study was the evaluation of the frequency of infections and co-infections among patients hospitalized because of non-specific symptoms after a tick bite.
Materials and methods: Whole blood, serum and cerebrospinal fluid samples from 118 patients hospitalised for non-specific symptoms up to 8 weeks after tick bite from 2010 to 2013 were examined for tick-borne infections. ELISA, Western blot and/or molecular biology (PCR; *fla* gene; *16S rRNA*; sequencing) and thin blood smears (MDD) were used. Control group included 50 healthy blood donors. All controls were tested with PCR and serology according to the same procedure as in patients.
Results: Out of 118 patients 85 (72%) experienced headaches, 15 (13%) vertigo, 32 (27%) nausea, 17 (14%) vomiting, 37 (31%) muscle pain, 73 (62%) fever and 26 (22%) meningeal signs. 47.5% were infected with at least one tick-borne pathogen. *Borrelia burgdorferi* sensu lato infection was confirmed with ELISA, Western blot in serum and/or (PCR (*fla* gene) in whole blood in 29.7% cases. In blood of 11.9% patients *Anaplasma phagocytophilum* DNA (*16S rRNA* gene) was detected; in 0.9% patients 1/118 *Babesia* spp. DNA (*18S rRNA* gene) was also detected. Co-infections were observed in 5.1% of patients with non-specific symptoms. *B. burgdorferi* s.l. – *A. phagocytophilum* co-infection (5/118; 4.2%) was most common. In 1/118 (0.8%) *A. phagocytophilum* – *Babesia* spp. co-infection was detected. All controls were negative for examined pathogens.
Conclusions: Non-specific symptoms after tick bite may be caused by uncommon pathogens or co-infection, therefore it should be considered in differential diagnosis after tick bite.

1. Introduction

In tick-endemic areas in Poland people are exposed to various pathogens transmitted by *Ixodes ricinus* ticks: *Borrelia burgdorferi* sensu lato, *Anaplasma phagocytophilum*, *Rickettsia* spp., *Bartonella* spp., *Francisella tularensis,* tick borne encephalitis virus (TBEV), or *Babesia* spp. New tick-borne pathogens, such as *Candidatus* Neoehrlichia mikurensis or *Borrelia miyamotoi* have been reported. In most cases only *B. burgdorferi* s.l. and TBEV infection are considered during the diagnostic process after tick bite. However, other pathogens might be the real cause of symptoms reported by patients. These pathogens are of special importance in immunodeficient patients [1–7].

Lyme borreliosis (LB) is the most commonly diagnosed tick-borne infection worldwide. It is a multi-system disorder, which may affect skin (erythema migrans (EM), acrodermatitis chronica atrophicans, borrelial lymphocytoma), nervous system (neuroborreliosis), joints (Lyme arthritis), heart (Lyme carditis) or eyes (ocular borreliosis). At the beginning of infection diverse nonspecific symptoms usually appear and they are then often the sign of infection. Different species of *B. burgdorferi* s.l.: *B. burgdorferi* sensu stricto, *B. afzelii* and *B. garinii* may lead to a different clinical picture [8–12].

Human granulocytic anaplasmosis (HGA) is caused by *A. phagocytophilum*. Several variants of *A. phagocytophilum* have been described but not all may cause a symptomatic infection in

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humans. *A. phagocytophilum*, formerly included in the *Ehrlichiaceae* family, now belongs to the *Anaplasmataceae* family (including *Ehrlichia*, *Aegyptianella*, *Neorickettsia*, *Wolbachia*, *Candidatus* Neoehrlichia and *Candidatus Xenohaliotis)*. In 2001, based on the similarity of *16S rDNA* gene sequences (99.1% homology) and *groESL* gene (100% homology), three previously distinct microorganisms: *Ehrlichia phagocytophila, Ehrlichia equi* and human granulocytic ehrlichiosis (HGE) were combined in *A. phagocytophilum* species, which may be pathogenic for humans [13].

In most cases it is an asymptomatic, self-limiting disorder or a mild infection with mild, non-specific flu-like symptoms. Severe HGA occurs occasionally and presents as intravascular coagulation syndrome, thrombosis or kidney failure, atypical pneumonia or gastroenteritis with GI bleeding. Leucopenia, thrombocytopenia, increased activity of aminotransferases are often observed [4,14–16].

Human babesiosis caused by protozoan *Babesia* spp.: *B. microti*, *B. divergens* and *B. venatorum* (all present in Poland) in immunocompetent patients is usually a self-limiting disorder. In most cases only non-specific flu-like symptoms appear. A severe clinical course is observed very rarely. It is linked with high parasitemia, usually in patients with immunological disorders [17,18]. Hepatomegaly, splenomegaly, hemolytic anemia, renal dysfunction with hemoglobinuria, acute respiratory distress syndrome (ARDS), cardiovascular problems or proteinuria may appear [16,19–21].

Various and non-specific symptoms such as headache, fever, sweats, vertigos, nausea, vomiting, muscle pain can appear after a tick bite. They might be a consequence of tick infestation, very early signs of various tick-borne infections, even in cases of suspected meningitis of unknown origin. Prolonged and intense non-specific symptoms may suggest co-infection with tick-borne pathogens, e g. TBEV and *B. burgdorferi* [4,22–26].

Coexistent chronic diseases can influence the severity of a tick-borne infection [25]. Mixed infections can have a more severe clinical course with long lasting sequelae; their diagnosis can be more difficult. *B. burgdorferi* s.l. – *Babesia* spp., *B. burgdorferi* s.l. – *Bartonella* spp., *B. burgdorferi* s.l. – *A. phagocytophilum* co-infections transmitted by ticks result in various intense symptoms and lead to a prolonged and severe LB [5,22–23,27,28].

The aim of the study was to evaluate the frequency of infections and co-infections in patients hospitalized for non-specific symptoms after a tick bite when TBE, EM or neuroborreliosis were not diagnosed. We compared the frequency of symptoms and various laboratory parameters between patients infected with tick-borne pathogens and those with no detectable infection.

2. Material and methods

2.1. Material

118 patients (53 women and 65 men) in mean age 42.11 +/−16.73 years old out of 549 hospitalized in The Department of Infectious Diseases and Neuroinfections of the Medical University of Bialystok from June 2010 to October 2013 with non-specific symptoms after a tick bite were included in the study. In all patients, tick-borne encephalitis (TBE), neuroborreliosis or EM were excluded based on serological serum and cerebrospinal fluid tests (TBE) and absence of intrathecal production of anti-B. *burgdorferi* antibodies (neuroborreliosis). Erythema migrans was excluded based on clinical criteria i.e. lack of typical skin lesion. Clinical information was obtained from patients' medical documentation and a written questionnaire designed for this study. Control group included 50 healthy blood donors.

EDTA-blood was used for May-Grünwald-Giemsa (MGG) smears for *A. phagocytophilum* and *Babesia* spp. detection, and for molecular detection by PCR of *B. burgdorferi* s.l., *A. phagocytophilum* and *Babesia* spp. Serum was used for immunoserological diagnostic (ELISA, Western blot) of *B. burgdorferi* s.l. or TBEV infection. Cerebrospinal fluid was collected from patients with suspected neuroinfection. It was used for a biochemical analysis, detection of anti TBEV antibodies by ELISA and for *B. burgdorferi* s.l. DNA detection.

In all patients laboratory parameters such as: C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), red blood cells count (RBC), hemoglobin (Hb), white blood cell count (WBC), platelet count (PLT), alanine and asparagine aminotransferase activity (ALAT, ASPAT), lactate dehydrogenase (LDH), creatinine, glucose, fibrinogen and bilirubin were measured.

The study was approved by the Bioethical Commission of the Medical University of Bialystok.

2.2. Methods – microscopic blood smear (MGG)

Fresh whole blood was collected in EDTA bottles. Thin blood smears were stained using May-Grünwald-Giemsa method and examined for *A. phagocytophilum* morulae in neutrophils and *Babesia* spp. piroplasms in erythrocytes. Thin blood smear was counted up to 200 white blood cells and at least 500 red blood cells [27,29].

2.3. Methods – DNA extraction

DNA was extracted using the Qiagen DNAeasy Blood and Tissue Mini kit. 200 μl of cerebrospinal fluid was centrifuged for 20 min at 8000 rpm; 200 μl of whole blood was gently mixed before extraction (of each). Purified DNA isolates were frozen at −20 °C.

2.4. Methods – A. phagocytophilum *PCR*

To detect *A. phagocytophilum* DNA in vitro, a PCR targeted the *16S rDNA* gene fragment encoding small ribosomal *16S rRNA* subunit was used. Amplification was performed with the *Anaplasma* PCR kit (Blirt-DNA Gdańsk, Poland) in DNA isolates from whole blood. Analysis was conducted according to the manufacturer's instruction (single PCR 2010–2011; nested PCR 2012–2013). Samples collected between 2010 and 2011 were re-analysed using nested PCR. Results confirmed the previous data. Positive results were 227 bp long fragments of the *16S rDNA* gene fragment in single reaction, and in nested PCR the *16S rDNA* gene fragments: 932 bp long in PCR-OUT and 546 bp long in PCR-IN [27]. Positive and negative controls from the kits were used.

At the Laboratory Centre in Kalmar, Sweden *Anaplasma* spp. was detected using primers that amplify a 257 bp long fragment of the *16S rRNA* gene of *Anaplasma* spp., *Ehrlichia* spp. as well as *Neoehrlichia* spp. [30]. The real-time PCR reaction was performed in Light Cycler 480 (Roche, Switzerland), using iQ™ SYBR® Green Supermix (Bio-Rad Laboratories, USA). The primers sequence were: forward 5′-GGGGATGATGTCAARTCAGCAY-3′ and reverse: 5′-CACCAGCTTCGAGTTAAGCCAAT-3′ [30].

Thermal cycling conditions for amplification of *Anaplasma* spp. included an initial denaturation step at 95 °C for 3 min, followed by 45 cycles of 95 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s. In addition, a melting curve analysis of the amplified products was performed between 60 °C and 95 °C. Samples with a crossing point (Cp) value lower than 40 were considered positive. All positive amplicons were purified with the Wizard® SV Gel and PCR Clean-Up System (Promega, USA) and subjected to Sanger sequencing at a commercial facility (Macrogen Europe, The Netherlands) [30]. All amplification courses were performed on the SensoQuest Lab-Cycler (SensoQuest, Germany).

2.5. Methods – B. burgdorferi *s.l. PCR*

For *B. burgdorferi* s.l. molecular detection in whole blood and in cerebrospinal fluid the *Borrelia burgdorferi* PCR kit (GeneProof, Czech Republic) was used. In "one tube" nested PCR a 276 bp fragment of the *fla* gene encoding flagellin was amplified. The PCR inhibition possibility was controlled by 420 bp long fragments of internal standard. Additionally, amplification for *16S rRNA* fragments (120 bp) encoding small ribosomal subunit (168 bp) internal standard was used. Amplifications were performed according to *GeneProof's manual* with own modifications [27]. Positive and negative controls from the kits were used.

To further confirm the results PCR amplification was performed with a real-time PCR assay targeting the *16S rRNA* gene as previously described [31]. The primers sequence were: forward 5′-GGTCAAGACTGACGCTGAGTCA-3′, reverse 5′-GGCGGCCAC-TTAACACGTTAG-3′ [31]. For species identification all positive samples in the real-time PCR assay was subjected to sequencing with a nested assay targeting the *clpA*-gene, which is a housekeeping loci part of the published Multilocus Sequence Analysis (MLST) assay for *Borrelia burgdorferi* sensu lato [32]. PCR was performed using the Platinum® *Taq* DNA Polymerase (Invitrogen™) and cycling conditions as previously described. Obtained amplicons were manually edited in Geneious 8.1.9 (Biomatters Ltd), aligned with published *Borrelia* spp. sequences, and subjected to BLAST-searches on the NCBI-homepage. All amplification courses were performed on the SensoQuest LabCycler (SensoQuest, Germany).

2.6. Methods – Babesia *spp. PCR*

For *Babesia* spp. molecular detection a fragment of *18S rDNA* gene, encoding a small ribosomal subunit, localised on highly conservative V4 region was used. For conventional PCR a pair of specific primers: *18S rDNA* BAB-F2 sens 5′ – GAC ACA GGG AGG TAG TGA CAA G – 3′ and *18S rDNA* BAB-R2 antisens 5′ – CTA AGA ATT TCA CCT CTG ACA GT – 3′ was used (Sigma-Aldrich, Germany) [27,33].

Amplification was performed by using *Taq PCR Core Kit* (Qiagen, Germany). DNA extracted from whole blood was added to a mixture containing: Buffor ×10, 25 mM $MgCl_2$, 10 mM dNTPs, 20 μM particular primers and 5U/μl *Taq* polymerase. Conditions of PCR were constructed experimentally [33]. Amplification was performed in the following program: initial denaturation (94 °C 3 min), 40 cycles: denaturation (94 °C 40 s), annealing (58 °C 60 s), extension (72 °C 60 s), and final extension (72 °C 10 min). As a positive control extracted DNA from a deer (*Cervus elaphus*) spleen infected with *Babesia* spp. was used. Positive results (approximately 420 bp long fragments of the *18S rDNA* fragment) were confirmed by sequencing.

All amplification courses were performed on the SensoQuest LabCycler (SensoQuest, Germany). Amplicons separation was done on 2% agarose gel (Sigma, Germany) stained with ethidium bromide (5 μg/1 ml; Syngen, USA) during electrophoresis at 80 V. Molecular weight markers (M100-M500, M100-1000-Blirt S.A. Poland) were used. Results were viewed under UV light in Gel Logic System 100 (Kodak Imaging System, Inc., USA). Positive and negative controls were included.

Amplification products obtained in conventional PCR for *Babesia* spp. *18S rDNA* gene fragment were cleaned using QiaAmp PCR purification Kit (Qiagen, Germany). Under the same conditions and with the same primers as in qualitative PCR sequencing reactions from both strands were performed using Big Dye Terminator Cycle Sequencing Kit (Applied Biosystem, USA) with ABI3500 Automated Sequencer (Applied Biosystem, USA). Sequences obtained were compared with those in the NCBI database [33].

2.7. Methods – *ELISA* Borrelia burgdorferi *s.l*

Specific anti -*B. burgdorferi* IgM and IgG antibodies were measured in serum and/or in cerebrospinal fluid using Borrelia burgdorferi IgG/IgM ELISA Kit (DRG, Germany) or *Borrelia* IgM/IgG ELISA Recombinant Antigen (Biomedica, Austria).

2.8. Methods – *ELISA TBEV*

Specific anti-TBEV IgM and/or IgG were measured in serum and/or in cerebrospinal fluid sing *SERION ELISA classic TBE Virus IgG/IgM kit* (Institut Virion/Serion GmbH, Germany).

2.9. Methods – *statistical analysis*

Statistical analysis was performed with STATISTICA Data Miner + QC. Arithmetic mean, standard deviation and median were calculated for measureable features. Percentage was calculated for immeasurable features. Independent groups were compared using U Mann-Whitney test for quantity parameters and by Pearson's chi-squared test for quality features. P value <0.05 was considered statistically significant.

3. Results

A hundred and eighteen patients (21.5%) included to the study were hospitalized because of a suspicion of meningitis, fever, flu-like symptoms, anemia, thrombocytopenia, leukopenia. We excluded patients with EM, TBE and neuroborreliosis.

Among patients admitted between 3 and 56 days after a tick bite there were 65 (55.1%) men (age 18–81, mean – 40,6+−16,7) and 53 (44.9%) women (age 19–89, mean – 43,9 ± 19,6). Most lived in cities [72 (61.9%)]. They sought medical attention 18.71 ± 11.52 days after a tick bite (3 to 56 days; median 14 days). The majority of patients [69 (58.5%)] reported multiple tick bites (more than 1). Only 12 (10.2%) patients were bitten only once, just before admission but never before. There was a significant difference between the presence of infection and patients' sex, but no significant differences between the presence of infection and place of living. Women (31/53; 58.49%) were more often infected than men (25/65; 38.46%) ($p = 0.03$). There was no significant differences between presence of infection, time of tick bite ($p = 0.594$) and duration of hospital stay ($p = 0.242$).

The time between tick bite and symptoms ranged from 4 to 56 days, mean 17.93 ± 10.94, in subjects with an unidefined infection and 3 to 56 days, mean 19.67 ± 12,26 days in patients with a detected monoinfection and co-infection. For *B. burgorferi* this time was the same as for *A. phagocytophilum* or *Babesia* spp.

The most common non-specific symptoms in the study group ($n = 118$) were headaches (72%), fever (62%), muscle pain (31%), joint pain (28%), nausea (27%). The physical examination revealed neck stiffness in 25% of patients, other meningeal signs were present in 22% of patients (Table 1).

Comparison of symptom frequency between patients with no detectable infection and those with a proven presence of at least one pathogen showed no statistically significant differences. Accompanying fever lasted from 1 to 15 days, mean 4.21 ± 2.88 days (median 3 days) with temperature from 37 °C to 40 °C, mean 37.96 ± 0.82 °C.

There were no statistically significant differences in laboratory parameters such as: erythrocyte sedimentation rate (ESR), red blood cells count (RBC), hemoglobin (Hb), white blood cell count (WBC), platelet count (PLT), alanine and asparagine aminotransferase activity (ALAT, ASPAT), lactate dehydrogenase (LDH), creatinine, glucose, fibrinogen and bilirubin between patients with detected infections and those with lack of infection after a tick

Table 1
Frequency of non-specific symptoms after tick bite in patients with undefined infection or with at least one pathogen detected.

Symptoms	Undefined infection n = 62		Monoinfection + Co-infection n = 56		p
	n		n		
Headache	44	70.97%	41	73.21%	0.786
Vertigo	7	11.29%	8	14.29%	0.626
Nausea	13	20.97%	19	33.93%	0.114
Vomits	10	16.13%	7	12.5%	0.575
Muscle pain	17	27.42%	20	35.71%	0.332
Joint pain	14	22.58%	19	33.93%	0.170
Fever	34	54.84%	39	69.64%	0.098
Meningal signs	16	25.81%	10	17.86%	0.298
Neck stiffness	17	27.42%	13	23.21%	0.600

bite ($p < 0.05$). The concentration of CRP was statistically higher in patients infected with at least one pathogen in comparison to those with no infection.

The treatment depended on the clinical picture and laboratory tests results. Anaplasmosis was treated with doxycycline, Lyme borreliosis with doxycycline or ceftriaxone. Infection with *Babesia microti* was not treated as no typical clinical symptoms were present and patients improved without anti – *Babesia* treatment.

3.1. Monoinfections

Anaplasma phagocytophilum morules in neutrophils and *Babesia* spp. piroplasms in erythrocytes were not detected in any of the blood smears. TBE infection was excluded by the absence of specific anti-TBEV antibodies in serum and/or in cerebrospinal fluid.

In the study group 56 (47.5%) patients were infected with at least one tick-borne pathogen. Single *B. burgdorferi* s.l. infection was confirmed in 35 (29.7%) cases using immunoserological and/or molecular biology methods. Specific serum anti-*Borrelia* IgM antibodies were detected in 16 (13.6%) patients; specific fragment of *B. burgdorferi* s.l. *fla* gene was detected by nested end-point PCR in the blood of 8 (6.8%); in 11 (9.3%) *B. burgdorferi* s.l. infection was confirmed simultaneously using immunoserological (ELISA, Western blot) and molecular methods (PCR). In the blood of 14 (11.8%) patients only *A. phagocytophilum* DNA (*16S rDNA* gene) was detected; in 1 (0.8%) *Babesia* spp. DNA (*18S rDNA* gene) was detected (Table 2).

3.2. Co-infections

Co-infections were observed in 6 (5.1%) of patients with non-specific symptoms. Multiple infection of *B. burgdorferi* s.l. – *A. phagocytophilum* [5 (4.2%)] dominated; *A. phagocytophilum* – *Babesia* spp. co-infection was detected in one case (0.8%) (Table 2).

3.3. Sequence analysis

Anaplasma sequence showed 100% similarity with the *A. phagocytophilum* strain Webster (188/188 bp).

Sequencing of *Borrelia burgdorferi* was successful in 19 cases. *B. garnii* sequences (15 cases) showed a 97.5–100% pairwise similarity to the *B. garinii* sequences in the GenBank database (accession numbers JN828689.1, HM345904, KF8994056, KF422855, KY606907 and KY606908).

B. afzelii sequences in four cases showed a 99.6-100% pairwise similarity to the previously published *B. afzelii* sequences (accession numbers KY606909-KY606913).

Amplification products of *18S rDNA* gene fragment sequences for *Babesia* spp. revealed a 99%–100% homology with the sequences of *B. microti 18S rRNA* from Eurasian present in Gen Bank NCBI in positions: KC581934.1, KC470049, AY789075, AY943958, AB071177.1, which indicated *Babesia microti*. In one patient a new sequence of the *18SrRNA* gene was reported to the NCBI Gene Bank and deposited under number KT429729 [31].

3.4. Other results

Results of all performed tests in control group were negative.

4. Discussion

Tick-borne pathogens, such as *Borrelia burgdorferi*, *Anaplasma phagocytophilum*, *Babesia* spp, *Rickettsia* spp., *Francisella tularensis*, *Bartonella* spp. may cause non-specific symptoms. Most frequent symptoms after contact with a vector include fever, sweats, headache, vertigo, nausea, vomiting, muscle and/or joint pain and malaise/fatigue [22–24,34].

In our study there was no significant difference in the reported symptoms between patients infected with at least one pathogen and those with no confirmed infection. It might suggest that other new pathogens, not included in this study, could be the real cause of the non-specific symptoms in large number of people after a tick bite. Various non-specific symptoms after a tick bite may suggest a more severe course of infection and probability of co-infection, as reported previously [1,4–6,24].

Lindblom et al. emphasized that patients with confirmed *B. burgdorferi* s.l. or *Rickettsia* spp. infections presented with very similar symptoms. Therefore, identification of the etiologic factor of tick-borne diseases is often impossible if solely based on the clinical presentation [34]. Comparable results were observed in our study where the frequency of non-specific symptoms was similar and did not depend on the causative agent of the tick-borne diseases [34].

Table 2
Presence of infection in patients after tick bite.

Type of infection		n	
Undefined infection		62	52.54%
Monoinfection			
	TBEV	0	0%
	B. burgdorferi sl[a]	35	29.66%
	A. phagocytophilum	14	11.86%
	Babesia spp.	1	0.85%
Co-infection			
	B. burgdorferi sl + *A. phagocytophilum*	5	4.24%
	Babesia spp. + *A. phagocytophilum*	1	0.85%
Total		118	100%

[a] Specific serum anti-*Borrelia* IgM antibodies were detected in 16 (13.6%) patients; specific fragment of *B. burgdorferi* s.l. *fla* gene was detected by nested end-point PCR in the blood of 8 (6.8%); in 11 (9.3%) *B. burgdorferi* s.l. infection was confirmed simultaneously using immunoserological (ELISA, Western blot) and molecular methods (PCR).

Nordberg analyzed 32 patients with non-specific symptoms after a tick bite [24]. In 28.13% of these patients specific anti-*Borrelia* antibodies and in 9.4% cases specific anti-*A. phagocytophilum* antibodies were detected [24]. Similar results were obtained in our study. In patients with non-specific symptoms after a tick bite with no features of TBE or EM, *B. burgdorferi* s.l. infection was detected in 29.66% and *A. phagocytophilum* in 11.86% cases. In our study *B. burgdorferi* s.l. – *A. phagocytophilum* co-infection was present in 5.09% patients, while in Nordberg's in 6.25% cases. In Swedish and Polish cohorts in 50% and 52.64% of patients with non-characteristic symptoms after a tick bite, respectively, etiologic agents of infection were not detected. This might suggest new pathogenic microorganisms transmitted by ticks [24].

Since we and other authors detected co-infections in humans, this suggests possible co-infections in ticks. In Poland, the rate of co-infection with *B. burgdorferi* s.l., *A. phagocytophilum* and *Babesia* spp, in ticks ranges from 0% to 3.96% [35–37]. In Pomerania it was 10.6% (32/303) [36]. In the south-eastern Poland, 1620 ticks were examined and in 217 cases (217/1620, 13.4%) pathogens were found. Co-infection was demonstrated in 2.16% of ticks (35/1620) [37]. In the north-east part of the country in 1200 ticks tested, the co-infection rate was 0.67% (8/1200) [38].

Clinical manifestations after a tick bite may resemble other diseases. This might lead to misdiagnosis of the real cause of symptoms or possible co-infection. The presence of different tick-borne pathogens at the same time lead to a more severe clinical course vs. monoinfection. Nyarko et al. suggested that *A. phagocytophilum* infection supported *B. burgdorferi* s.l. penetration into the internal organs and worsened the clinical course of Lyme borreliosis [25]. *Anaplasma phagocytophilum* – *B. burgdorferi* s.l. co-infection resulted in more severe and persistent clinical manifestation of tick-borne diseases[4].

Studies in mice confirmed the influence of tick-borne co-infection on a more severe disease course. Grab et al. and Zeidner et al. reported that *B.burgdorferi* s.l. – *A. phagocytophilum* Webster strain co-infection reduced the concentration of IL-4, IL-12, IFN-γ, TNF-α and increased IL-2, IL-6, IL-8, IL -10 and metalloproteases [35,36]. Additionally, *A. phagocytophilum* involved neutrophils during infection; this was why they might not be present in early phase of the response against *B. burgdorferi* s.l. It makes infection spread easier and Lyme borreliosis more severe [4,39–40]. Similar results were shown in a mouse model of *B. burgdorferi* s.l.- *Babesia* spp. co-infection, where this protozoan invasion caused more severe *Lyme arthritis* [19].

It is interesting that in our study more female than male were infected with tick-borne pathogens. In a previous study of 703 TBEV and 13606 LB cases 68% male dominance was found in TBEV infection, while 53% woman majority in LB cases [41].

Immunoassays are the most common methods used to identify specific antibodies against particular pathogens. However, it is also increasingly important to detect the genetic material of tick-borne pathogens. Blood smears under the microscope are also useful for detection of pathogens such as *A. phagocytophilum* and *Babesia* spp. Using as many tests as possible allows the patient to be fully diagnosed after a bite, because there is not standardized diagnostic algorithm, especially in cases with not clear clinical course of the disease after tick bite. It should be kept in mind that there is no uniform diagnostic pathway for people with suspected tick-borne diseases, primarily due to differences in geographic distribution [4,5,34,42,43]. The necessity of using different methods is also due to the constraints associated with the techniques used. Immunoserological methods can only be used if the level of antibody produced by the patient is detectable, and the result often depends on different cut-offs. An additional problem may be cross-reactions with other microorganisms, which seems to be of particular importance in the case of co-infection [4,22].

In this study we used a PCR method, which seems to be useful in the diagnosis of tick-borne diseases, especially in the early phase of infection, when specific antibodies might not be detectable with available laboratory methods. PCR has higher sensitivity particularly in skin, joint fluid, cerebrospinal fluid. However in blood it may also bring important information, when other methods failed, for example immunoserology, when patient has immunoserological disorder, or when samples are taken too early [27].

We detected *B. burgdorferi* in 13.56% of patients by ELISA, 6.78% by PCR and 9.32% by both methods. It emphasizes the necessity of using various techniques to make the diagnostic process more reliable. Precautionary measures including standards were fulfilled according to the European laboratory norms.

The limitation of our study was the fact that patients with non-specific complaints reported subjective symptoms which may suggest other non-tick-borne diseases. Moreover the description of the symptoms was often imprecise. Another limitation was that other tick-borne pathogens as *Rickettsia* spp., *Bartonella* spp. were not tested.

In 52.4% of our patients no infections were detected. We postulate that new unknown and rare pathogens could be responsible for the symptoms. It is also imaginable that the symptoms were not linked to the reported tick bites at all and instead were due to a non-tick-borne infection, such as internal diseases. Further studies are needed.

5. Conclusions

Non-specific symptoms after tick bite may be caused by rarely detected pathogens, such as *A. phagocytophilum* or *Babesia* spp. or co-infection, therefore it should be considered in the differential diagnosis after tick bite.

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Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

Study was approved by Ethical Committee at Medical University of Bialystok.

Informed consent

All patients signed informed consent at the admission to the hospital.

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