



First report of *Bartonella* sp. isolated from *Hippobosca equina* L. (Hippoboscidae: *Hippobosca*) in Lublin Province, south-eastern Poland

Weronika Maślanko^{1,A-F}✉, Marek Asman^{2,B,F}, Katarzyna Bartosik^{3,E-F}, Magdalena Świsłocka^{4,C,F}

¹ Department of Animal Ethology and Wildlife Management, University of Life Sciences, Lublin, Poland

² Faculty of Medical Sciences in Zabrze, Department of Medical and Molecular Biology, Medical University of Silesia, Katowice, Poland

³ Department of Biology and Parasitology, Faculty of Health Sciences, Medical University, Lublin, Poland

⁴ Department of Zoology and Genetics, Faculty of Biology, University of Białystok, Poland

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Abstract

Introduction and Objective. *Hippobosca equina* (Diptera: Hippoboscidae), is a widespread blood-feeding ectoparasite associated with the forest ecosystem. The insect is characterized by a wide host range and low host specificity, which increases the risk of feeding on animals that constitute a reservoir of transmissible pathogens, including *Bartonella* spp.

Materials and Method. *Hippobosca equina* adults were collected from humans and companion animals within a continental mesotrophic oak-pine mixed forest in eastern Poland. DNA was isolated by the ammonia method, and isolates obtained from single individuals were tested by PCR method for the presence of 5 vector-borne pathogens. In case of the positive results, the amplicons were sequenced and examined by a BLAST search.

Results. The PCR analysis of DNA isolates obtained from 100 *H. equina* specimens revealed the presence of the RNA polymerase beta-subunit gene (*rpoB*) of the genus *Bartonella*, in 1% of the studied insects, i.e. one *H. equina* female. The *rpoB* gene haplotype of *Bartonella* sp. reported in this study, was identical to a *Bartonella* sp. sequence obtained from deer keds in Lithuania, and very closely related to strains with zoonotic potential. None of the *H. equina* specimens studied was positive for the presence of *B. burgdorferi* s.l., *Anaplasma phagocytophilum*, *Babesia* spp., and *Coxiella burnetii*.

Conclusions. The study indicates the need to screen the occurrence of *Bartonella* spp., both in potential vectors and reservoirs of this pathogen in various habitats.

Key words

ectoparasite, *Bartonella* sp., *Hippobosca equina*, Hippoboscidae, forest fly, ked, louse fly

INTRODUCTION AND OBJECTIVE

The genus *Bartonella* (fastidious hemotropic Gram-negative facultative intracellular microorganisms) belongs to the α -subgroup of the proteobacteria. Infection of erythrocytes and endothelial cells of a vertebrate host manifests itself as a polymorphic clinical disease with accompanying bacteremia [1]. Clinical manifestations in humans include bacteremia, local lymphadenopathy, endocarditis, as well as ocular, and cutaneous manifestations [2–9]. Symptoms are related to pathogen species and correspond with the patient's immune status [6, 10, 11]. A host can be infected by *Bartonella* spp. via transfusions or organ transplants, arthropod saliva (fleas, sandflies, lice), or flea faeces in the case of Cat-Scratch Disease (CSD) [12, 13]. It is noteworthy that there is still a debate about the vector potential of ticks in relation to *Bartonella* spp. [14, 15]. The documented primary reservoirs of those pathogens, whose range covers the forest habitats of the study area in eastern Poland, include wild species, e.g. brown rat

(*Rattus norvegicus*), bank vole (*Clethrionomys glareolus*), and yellow-necked mouse (*Apodemus flavicollis*), as well as farm animals, e.g. domestic sheep (*Ovis aries*) and companion animals: dog (*Canis lupus familiaris*) and domestic cat (*Felis catus*). In these animals, persistent bacteremia promotes the transmission of the pathogen by arthropod vectors [16].

Hippobosca equina Linnaeus, 1758 (Diptera: Hippoboscidae) is a blood-feeding ectoparasite associated with the forest ecosystem. This cosmopolitan species is characterized by a wide host range and low host specificity, which increases the risk of feeding on animals that constitute a reservoir of pathogens, including *Bartonella* spp. [17, 18]. Studies conducted by de Bruin et al. [19] on vertical transmission of *Bartonella schoenbuchensis* in *Lipoptena cervi* showed that it is highly probable that deer keds (Hippoboscidae) are involved in the transmission of this pathogen in nature. Both *L. cervi* and *H. equina* occur in similar types of habitats, infest the same host species, and share the same feeding pattern. As the interactions between *Bartonella* spp. and arthropod vectors in natural habitats are still poorly understood, more survey is needed to gain a deeper understanding of these interactions and their impact on the ecosystem. The observations made by our research

✉ Address for correspondence: Weronika Maślanko, Department of Animal Ethology and Wildlife Management, University of Life Sciences, Lublin, Poland
E-mail: weronika.maslanko@up.lublin.pl

team in recent years regarding Hippoboscidae parasitizing wild and domestic animals, as well as humans [18, 20, 21], inspired us to investigate the prevalence of vector-borne pathogens in *H. equina* adults collected from humans and companion animals in eastern Poland.

MATERIALS AND METHOD

Study area. The Puławy Forest District is an organizational unit of the State Forests with an area of 1,511.76 km² located in eastern Poland (Fig. 1).

Habitats in a natural and close to natural state occupy approximately 69.10% of the area of the study, disturbed habitats account for approximately 22.06%, and degraded habitats – 8.84%. In general, coniferous forest covers 31.59%, mixed coniferous forest – 20.77%, mixed forest – 24.83%, and deciduous forest – 22.81%. In the Puławy Forest District, 14 forest habitat types have been distinguished. The area of the Puławy Forest District is dominated by the following habitat types: fresh coniferous forest (29.79%), fresh mixed forest (22.88%), fresh deciduous forest (19.19%), fresh mixed

coniferous forest (18.61%), alder carr stands (3.05%), and mixed humid forest (2.14%). Pine (73.0%) is the dominant species in the forests, with oak (16.0%), birch (5.0%), and alder (5.0%) in their composition. Depending on the habitat, the understory is composed of hornbeam, oak, linden, spruce, hawthorn, rowan, hazel, black elderberry, red elderberry, buckthorn, spindle tree, viburnum, currant, juniper, black cherry, and many others [22].

The arthropods studied in the research were collected on 2.5 ha grasslands forming an open island within a continental mesotrophic oak-pine mixed forest (*Pino-Quercetum* auct. polon = *Quercus roboris-Pinetum* + *Serratulo-Pinetum*) [23]. This mixed forest is inhabited by numerous species of wild Artiodactyla, e.g. deer (*Cervus elaphus* L.), roe deer (*Capreolus capreolus* L.), and moose (*Alces alces* L.), and their numbers affecting the abundance of arthropod vectors are increasing [24, 25].

Sampling and species identification. *Hippobosca equina* adults were collected from mid-May – end of July in 2021, a total of 100 individuals (n=77 specimens), and 2022 (n=23). The insects were collected from 3 humans and 3 dogs, and

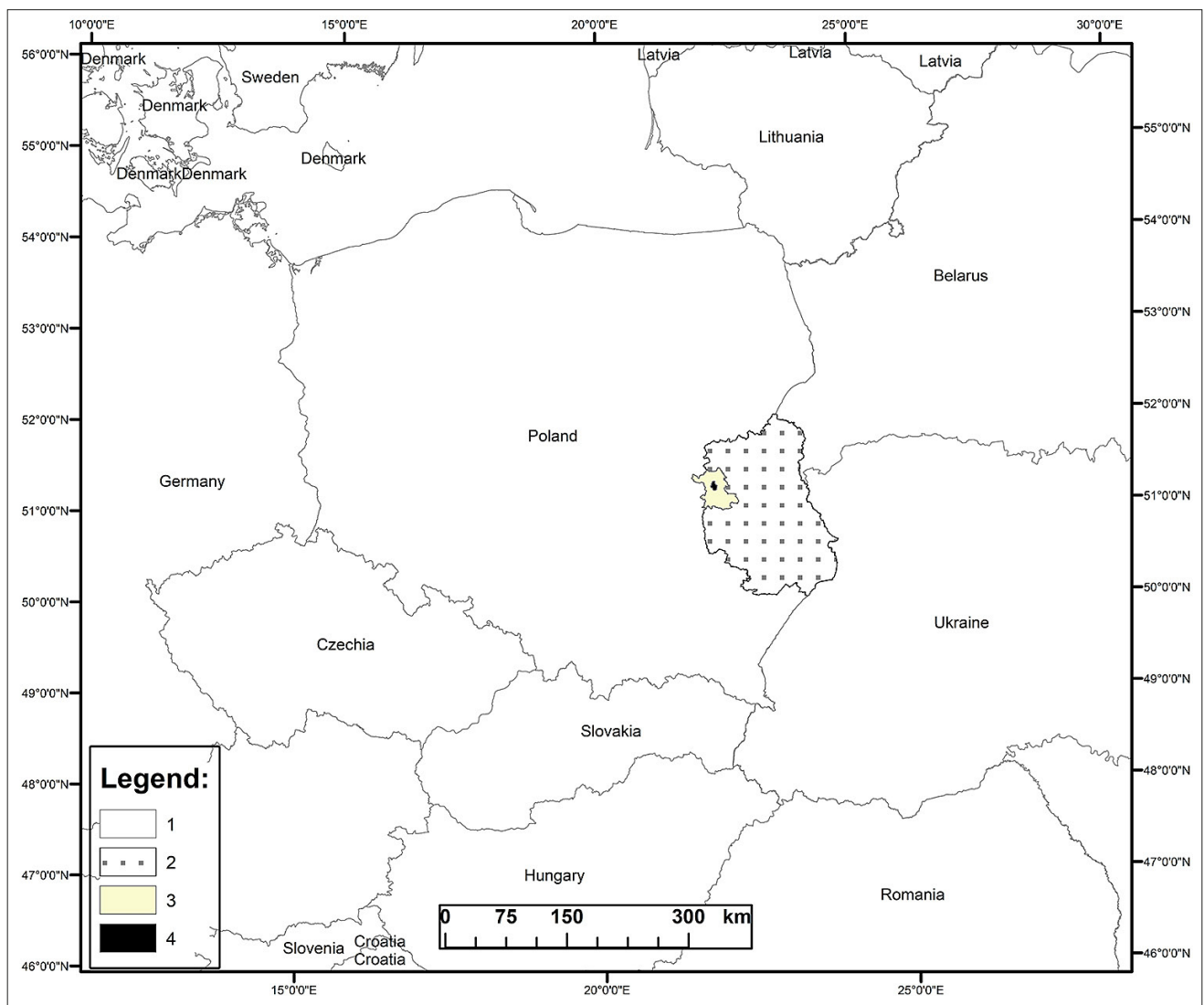


Figure 1. Localization of study area.

1 – European countries; 2 – Lublin Province; 3 – Puławy Forest District; 4 – Zagórki Forestry (prepared by Weronika Maślanko in ArcGIS 10.3 software)

Table 1. Oligonucleotide primers used to detect *Borrelia burgdorferi* sensu lato, *Anaplasma phagocytophilum*, *Bartonella* spp., *Babesia* spp., *Coxiella burnetii*, and PCR conditions

Species	Primer	Sequence (5'-3')	Gene detected	Size of amplification product [bp]	PCR conditions [°C/s]			No. of cycles
					Denaturation	Annealing	Extension	
<i>Borrelia burgdorferi</i> sensu lato	Fla1	AGAGCAACTTACAGACGAAATTAAT	<i>fla</i>	482	94/30	54/45	72/45	35
	Fla2	CAAGTCTATTTGGAAAGCACCTAA						
<i>Anaplasma phagocytophilum</i>	Ehr521	TGTAGGCGTTTCGTAAGTTAAAG	16S rRNA	274	94/45	60/45	72/45	40
	Ehr747	GCACTCATC GTTTACAGCGTG						
<i>Bartonella</i> spp.	1400F	CGCATTGGCTTACTTCGTATG	<i>rpoB</i>	825	94/30	53/30	72/45	35
	2300R	GTAGACTGATTAGAACGCTG						
<i>Babesia</i> spp.	Babfor	GACTAGGGATTGGAGTGC	18S rRNA	620	94/60	53/45	72/90	35
	Babrev	GAATAATTCACGGATCACTC						
<i>Coxiella burnetii</i>	OMP1	AGTAGAAGCATCCCAAGCATTG	<i>com-1</i>	501	94/60	56/60	72/60	36
	OMP2	TGCCTGCTAGCTGTAACGATTG						

placed in sterile polypropylene test tubes with 70% ethanol for further investigations. Identification of the species and gender of the adult insects was carried out in the laboratory at the Department of Biology and Parasitology of the Medical University in Lublin, eastern Poland, using an OLYMPUS SZX16 (Olympus, Tokyo, Japan) stereoscopic microscope, and the key for identification of arthropod species by Oboňa [26].

DNA extraction, amplification and sequencing. The DNA from single individuals was isolated by the ammonia method [27] and the concentration measured spectrophotometrically in a nano spectrophotometer PEARL (Implen, Munich, Germany) at the 260/280 nm wavelength. Selected transmissible pathogens in the insects were detected with the PCR method. To detect *B. burgdorferi* s.l., a pair of primers specific to the flagellin gene was used [28]. *Anaplasma phagocytophilum* and *Babesia* spp., were detected with the use of primers specific to the 16S rRNA and 18S rRNA genes, respectively [29, 30]. In turn, primers specific to the *rpoB* and *com1* genes were used to detect *Bartonella* spp. and *Coxiella burnetii* in the insects [31, 32]. The PCR products were separated electrophoretically in 2% ethidium bromide-stained agarose gels, and visualized under ultraviolet light in a gel visualization device (Vilber Lourmat, Collégien, France). The presence of amplification products with a size of 482 base pairs [bp] for *B. burgdorferi* s.l., 274 bp for *A. phagocytophilum*, 620 bp for *Babesia* spp., 825 bp for *Bartonella* spp., and 501 bp for *Coxiella burnetii*, was recognized as positive. Oligonucleotide primers used to detect vector-borne pathogens as well as PCR conditions are listed in Table 1.

Statistical and phylogenetic analyses. The results of sequencing of the *rpoB* gene coding for the RNA polymerase beta subunit were aligned manually using BioEdit v.7.0.5.3 [33], and compared with the GenBank references by BLAST (<http://www.ncbi.nlm.nih.gov>/accessed on 20 September 2023) to determine the pathogen species. To test the phylogenetic relationships among the *rpoB* gene haplotype derived in this study and the sequences downloaded from GenBank, a phylogenetic tree was constructed using a maximum-likelihood (ML) algorithm in Mega v.11 software [34], with 1,000 bootstrap replicates used to assess the support for the tree nodes. In the phylogenetic analyses, a nucleotide

substitution model (GTR+I) was determined under the Akaike information criterion (Akaike 1973) implemented in Model Test v.0.1.1 [35]. Pairwise distance was calculated using the *p-distance* method between the newly-obtained sequence and variants of the *rpoB* gene with which it clustered together on the phylogenetic tree.

RESULTS

During the field studies, 100 *H. equina* specimens were collected (50 males and 50 females). Among parasites secured for further examination, 59 individuals of forest fly (33 females and 26 males) were captured on humans, and 41 individuals (17 females and 24 males) collected from dogs. On average, 19.6 *H. equina* specimens (range: 13–30) were collected from humans, while 13.6 insects (rang: 9–21) were captured from dogs. PCR analysis revealed the presence of the RNA polymerase beta-subunit gene (*rpoB*) of the genus *Bartonella*, which was detected in 1% of the studied insects, i.e. one *H. equina* female collected on a human. The other tested pathogens were not found in the studied material.

The obtained sequence with a length of 825 base pairs representing the *Bartonella* sp., *rpoB* gene coding for the RNA polymerase beta subunit, was identical to a *Bartonella* sp., sequence obtained from deer keds in Lithuania (*Lipoptena cervi*; GenBank Accession No. MT876352, data unpublished). The haplotype of *Bartonella* sp., revealed in this study showed 99.59% similarity (pairwise distance: 0.00413) with a sequence of *Bartonella* sp., from the Czech Republic (GenBank Accession No. MK301299; isolated from *Anopheles maculipennis* complex), 99.45% similarity (pairwise distance: 0.00551) with *Bartonella* sp., from Poland (GenBank Accession No. MF580662; isolated from *L. cervi*), and 99.31% similarity (pairwise distance: 0.00689) with a sequence from the USA (GenBank Accession No. AY805112; isolated from tick cell culture of whole blood from white-tailed deer).

DISCUSSION

In the sparse literature data available on *Hippobosca equina*, faunistic and molecular analyses have been carried out on limited groups of specimens, probably due to difficulties in

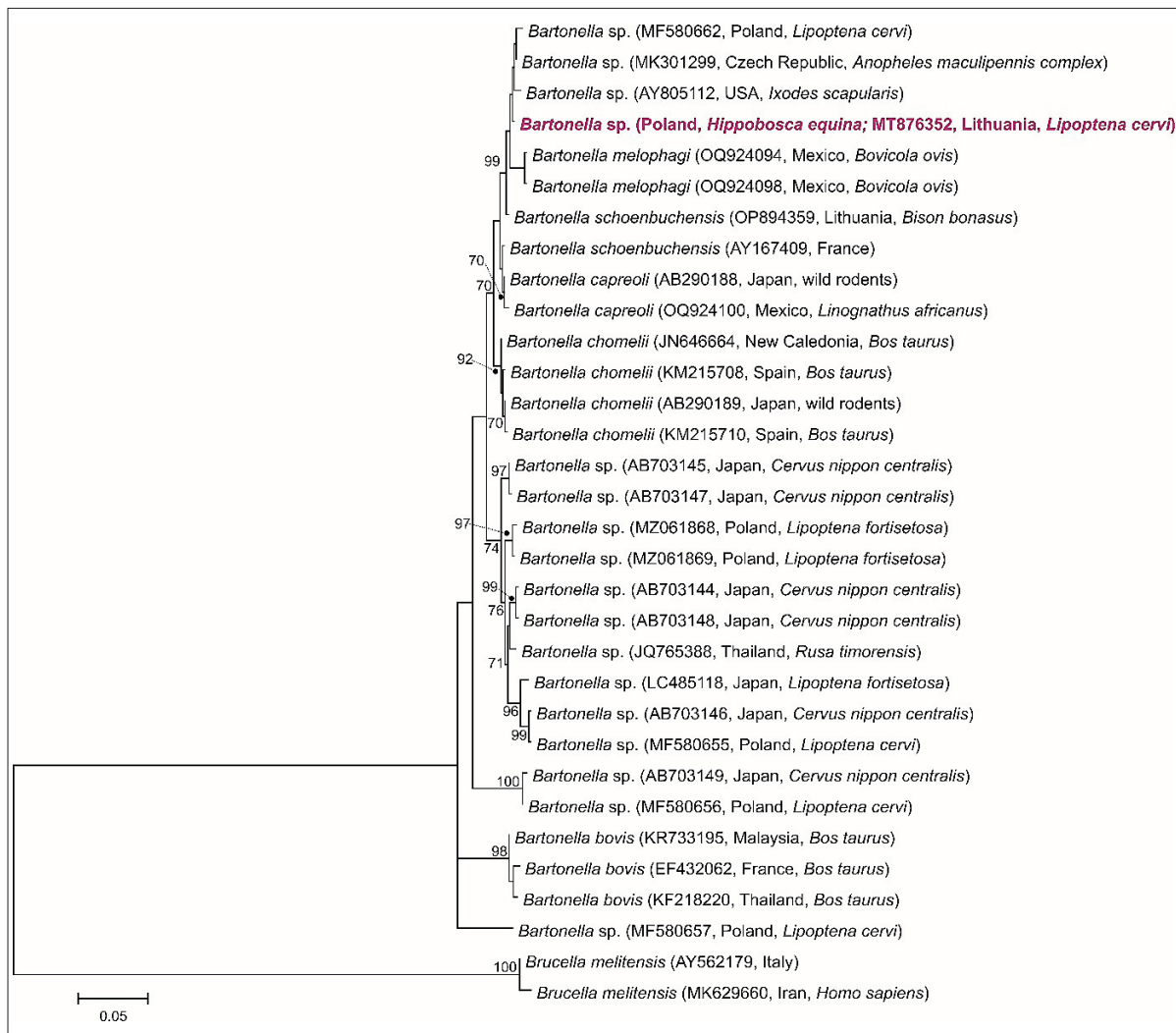


Figure 2. Phylogenetic analysis of *Bartonella* sp., haplotypes of the *rpoB* gene coding for the RNA polymerase beta subunit identified with those available and downloaded from the GenBank database. Maximum-likelihood tree computed with the GTR+I model of substitution evolution. Numbers listed at the nodes represent the percent support for the node from 1,000 bootstrap replicates. The haplotype obtained in this study is marked in violet. The ML tree was rooted with sequences of *Brucella melitensis*, a microorganism belonging together with *Bartonella* sp. to the order Hyphomicrobiales

obtaining specimens of this species. Analysis of scientific data regarding the occurrence of *Bartonella* spp. DNA in *H. equina* shows that the percentage of infected individuals could be relatively high, e.g. in Saxony, Germany, 82% of 11 *H. equina* specimens studied were positive for *Bartonella* spp. [36], and positive results were obtained in 75.86% of 22 insects examined in Algeria, North Africa [37]. Halos et al. [38] detected the DNA of *Bartonella chomelii* and/or *Bartonella schoenbuchensis* in all 12 *H. equina* specimens collected from cattle in different parts of France, but none of the 6 *H. equina* specimens collected from horses were positive.

Peña-Espinoza et al. [39] found that 19.3% of 62 studied *H. equina* specimens collected from cattle in Austria were positive to *Bartonella* spp.

The ML phylogenetic reconstructions included in this study revealed that the identified sequence of the *rpoB* gene and the sequences downloaded from GenBank created a new phylogenetic lineage of *Bartonella*, together with identical sequences from Lithuania and Poland (GenBank Accession No. MF580662), the Czech Republic (GenBank Accession No. MK301299), and the USA (GenBank Accession No.

AY805112) (Fig. 2). The variant of the *rpoB* gene obtained in this study showed the highest similarity to the haplotype described in the Czech Republic [40] and Poland [41]. These two aforementioned sequences demonstrated about 99% homology with *Bartonella* species, namely, *B. schoenbuchensis* and *B. chomelii*. *Bartonella schoenbuchensis* was found in such vectors as *L. cervi* or *L. mazamae* and in reservoir hosts, i.e. cattle, wild ruminants, and humans [41]. In Massachusetts, USA, a *Bartonella* sp., similar to *B. schoenbuchensis* was described in *Lipoptena mazamae* [42], which suggests that *Lipoptena* species can extend the range of *B. schoenbuchensis*. The *rpoB* gene haplotype of *Bartonella* sp. reported in this study is very closely related to strains with zoonotic potential (99% of homology to *B. schoenbuchensis*). However, future work should analyze other genes, including *gltA* and *ftsZ*, to determine whether this is a new *Bartonella* species and to clarify its relationship to *B. schoenbuchensis*.

To the best of the knowledge of the authors, this is the first report on the detection and characterization of the *Bartonella* haplotype in *H. equina* in Poland. The presence of *Bartonella* sp. in the forest fly prompts further research

on its role in the circulation of these microorganisms in the host-parasite system.

Although there is no empirical data that would indicate the role of *H. equina* in the transmission of *Bartonella* spp. to the vertebrate host, the frequency of occurrence of these pathogens in the forest fly in the light of literature data may be a cause of their participation in the persistence of these microorganisms within natural foci [36–39]. This issue, however, requires further research, taking into account the primary reservoir capacity of *H. equina* hosts. It has been observed that individuals working in forestry have an increased likelihood of being exposed to *Bartonella* infection. As shown in a study conducted by Jurke et al. [43] covering 722 forestry workers in the German state of North Rhine-Westphalia, as many as 41.2% of participants were seropositive for anti-*Bartonella* IgG. This poses a potential occupational health hazard and necessitates the implementation of appropriate safety measures.

The prevalence of zoonotic diseases emerging at the interface of human and animal environments increases with the growth of co-occurring human and animal populations. Hence, following the One Health approach, monitoring the occurrence of vector-borne pathogens, is highly important [16, 44–49].

In the natural environment, people are exposed to direct and indirect effects of insect parasitism, including *Simulium* spp., *Lipoptena* spp., and *Hippobosca equina* [18, 20, 21, 50–54]. However, many of hematophagous insects have a relatively short feeding period, which can make it challenging to secure them for identification purposes. On the other hand, knowledge about the morphology of parasitic arthropods is not common and should be disseminated among people who are particularly vulnerable to their attacks.

CONCLUSIONS

The obtained results reveal frequent human and animal exposure to *H. equina* bites. This indicates the need to screen these parasitic arthropods for the presence of vector-borne pathogens across different habitats.

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