International Journal for Parasitology: Parasites and Wildlife 24 (2024) 100958

Contents lists available at ScienceDirect

International Journal for Parasitology: Parasites and Wildlife

journal homepage: www.elsevier.com/locate/ijppaw

Bartonella, *Blechomonas* and *Trypanosoma* in fleas from the long-tailed ground squirrel (*Spermophilus undulatus*) in northwestern China

Xiaoshuang Han [a,b,1], Shanshan Zhao [a,b,1], Ziheng Liu [a,b,1], Yujiang Zhang [c], Guoyu Zhao [c], Chunju Zhang [d], Lijuan Tang [e], Lin Cui [a,b,*], Yuanzhi Wang [a,b,*]

[a] *Key Laboratory for Prevention and Control of Emerging Infectious Diseases and Public Health Security, the XPCC, School of Medicine, Shihezi University, Shihezi City, Xinjiang Uygur Autonomous Region, 832002, People's Republic of China*

[b] *NHC Key Laboratory of Prevention and Treatment of Central Asia High Incidence Diseases, First Affiliated Hospital, School of Medicine, Shihezi University, Shihezi City, Xinjiang Uygur Autonomous Region, 832002, People's Republic of China*

[c] *Xinjiang Key Laboratory of Vector-borne Infectious Diseases, People's Republic of China*

[d] *Tumushuk City Centers for Disease Control and Prevention, 17 Qianhai East Street, Tumushuk City, Xinjiang Uygur Autonomous Region, 843806, People's Republic of China*

[e] *Bayingol Vocational and Technical College, People's Republic of China*

ARTICLE INFO

Keywords:
Spermophilus undulatus
Flea
Bartonella
Trypanosomatidae

ABSTRACT

Fleas are known to be vectors for a variety of pathogens in veterinary medicine. However, no information is available on the presence of *Bartonella* and Trypanosomatidae in fleas of the long-tailed ground squirrel (LTGR, *Spermophilus undulatus*). The present study shows detection of these pathogens in LTGR fleas. During 2022–2023, a total of 396 fleas were collected from 91 LTGRs in 4 alpine regions of Xinjiang Uygur Autonomous Region (northwestern China) and grouped into 54 flea pools. Flea species were identified according to morphological characteristics and molecular data. In addition, all flea samples were analyzed for *Bartonella* with amplification and sequencing of a 380-bp part of the *gltA* gene and Trypanosomatidae with targeting the *18S rRNA* (850-bp) and *gGAPDH* (820-bp) genes. The flea species included *Frontopsylla elatoides elatoides* (203), *Neopsylla mana* (49), and *Citellophilus tesquorum dzetysuensis* (144). Of 54 flea pools, seven (12.96%) tested positive for *Bartonella*, and three (5.56%) were positive for Trypanosomatidae. Based on BLASTn and phylogenetic analyses, i) *Bartonella washoensis* in *F. elatoides elatoides* and *C. tesquorum dzetysuensis*, and *Bartonella rochalimae* in *F. elatoides elatoides* were identified. Interestingly, a new haplotype within the species *Ba. washoensis* was discovered in *C. tesquorum dzetysuensis*; and ii) *Blechomonas luni* was confirmed in *C. tesquorum dzetysuensis* and *Trypanosoma otospermophili* in *F. elatoides elatoides*. Two *Bartonella* species and two Trypanosomatidae members were discovered for the first time in fleas from LTGRs. This study broadens our understanding of the geographic distribution and potential vectors for *Bartonella* and Trypanosomatidae.

1. Introduction

The long-tailed ground squirrel (LTGR, *Spermophilus undulatus*) has been on The IUCN Red List of Threatened Species in 2016 (The IUCN Red List of Threatened Species, 2016). This species is a medium-size ground-dwelling sciurid distributed across central Asia, including Kazakhstan, Mongolia, Russian Federation and northwestern China (Durden et al., 2019). Residing within the alpine meadow ecosystem, it is closely connected with various wildlife species, domestic animals and human populations (Zhao et al., 2019). According to previous reports, LTGRs are indeed reservoirs for some pathogens, such as *Yersinia pestis*, *Pomona leptospirae*, tick-borne encephalitis virus and *Hepacivirus* C (Anan'ina et al., 2011; Bazanova and Innokent'eva, 2012; Demina et al., 2017; Li et al., 2019).

Fleas (Insecta, Siphonaptera) are obligate hematophagous insects. They usually parasitize a wide range of mammals, especially rodents (Chouikha and Hinnebusch, 2012). The importance of fleas in animal and human health is generally related to their role in the transmission of flea-borne diseases. They probably act as vectors, reservoirs, and/or amplifiers of multiple pathogens that are considered epidemiologically

* Corresponding author.
E-mail addresses: 420710143@qq.com (L. Cui), wangyuanzhi621@126.com (Y. Wang).
[1] Xiaoshuang Han, Shanshan Zhao and Ziheng Liu contributed equally to this study.

https://doi.org/10.1016/j.ijppaw.2024.100958
Received 1 May 2024; Received in revised form 23 June 2024; Accepted 23 June 2024
Available online 25 June 2024

important (Eisen and Gage, 2012). Previously, the following bacteria were reported from fleas in Xinjiang Uygur Autonomous Region (XUAR), northwestern China: three *Wolbachia* endosymbionts in *Nosopsyllus laeviceps laeviceps*, *Xenopsylla* sp., *Paradoxopsyllus repandus*, Candidatus *Rickettsia barbariae* in *Vermipsylla alakurt*, and *Yersinia pestis* in *Oropsylla silantiewi* (Zhao et al., 2016, 2017; Yin et al., 2019). However, little information is available on the occurrence and prevalence of *Bartonella* species in fleas from the LTGR around the world.

There are at least 40 *Bartonella* species, most of which are flea-borne bacteria. They can infect a broad array of mammals, including rodents and humans (Gutiérrez et al., 2015). Trypanosomes, which are flagellated protozoa, reside in the extracellular space of the host's bloodstream and are transmitted to mammals through blood-sucking arthropods such as ticks, fleas, and flies (Eyasu et al., 2021). To date, the Trypanosomatidae family (Euglenozoa: Kinetoplastida) comprises 24 genera, which are divided into 19 dixenous and 5 monoxenous genera (Votýpka et al., 2015). Notably, members of the genus *Blechomonas* are believed to be exclusively flea-borne pathogens (Kaufer et al., 2017). In this study, we aimed to explore the presence of *Bartonella* and Trypanosomatidae in fleas found on LTGRs in China.

2. Materials and methods

2.1. Sample collection and identification

Between June 2022 and August 2023, 91 LTGRs were collected from alpine regions of Wusu City, Korla City, Altay City and Jinghe County (1200–2500 m above sea level; the latter two regions are adjacent to Kazakhstan), XUAR, northwestern China (Fig. 1). The LTGRs were captured by Sherman traps (30 cm × 16 cm × 16 cm wire mesh), which were placed near the entrances of occupied burrows, baited with peanuts. Each survey site included 150 traps that were checked twice a day. Each trap was removed before nightfall and replaced on the survey site the following day (Zhao et al., 2019). All captured rodents were identified through morphological characteristics by experienced zoologists as reported in our previous study (Zhao et al., 2019). All procedures performed in this study involving wild mammals were in accordance with the ethical standards of Animal Ethics Committee of Shihezi University (Approval No. A2022-029-01).

The fleas were obtained from individual rodents through gentle brushing of their fur. All flea samples were morphologically identified according to key characteristics (Liu, 2007), and a solution of 70% ethanol was used to preserve them. Subsequently, the samples were allocated into pools containing varying numbers of fleas from 3 to 15, depending on flea species and geographical distribution (Table 1).

2.2. Detection, sequencing and phylogenetic analysis

Prior to DNA extraction, a thorough washing procedure involving purified sterile water was conducted on the fleas for a duration of 3 min. TIANamp Genomic DNA Kit (TIANGEN, Beijing, China) was employed for extracting the complete genomic DNA content from each pool. To verify flea species, the cytochrome *c* oxidase subunit II (*COII*) gene was

Table 1
Information on collected fleas, including total number, pool number, flea hosts and their geographical location.

Flea species	Number of specimens (pools)	Host species (number)	Location (flea number)
Frontopsylla elatoides elatoides	203 (29)	*Spermophilus undulatus* (50)	Wusu (163), Jinghe (52)
Citellophilus tesquorum dzetysuensis	144 (18)	*Spermophilus undulatus* (33)	Korla (15), Altay (51), Jinghe (66)
Neopsylla mana	49 (7)	*Spermophilus undulatus* (8)	Jinghe (49)
Total	396 (54)	*Spermophilus undulatus* (91)	Wusu (163), Korla (15), Altay (51) and Jinghe (167)

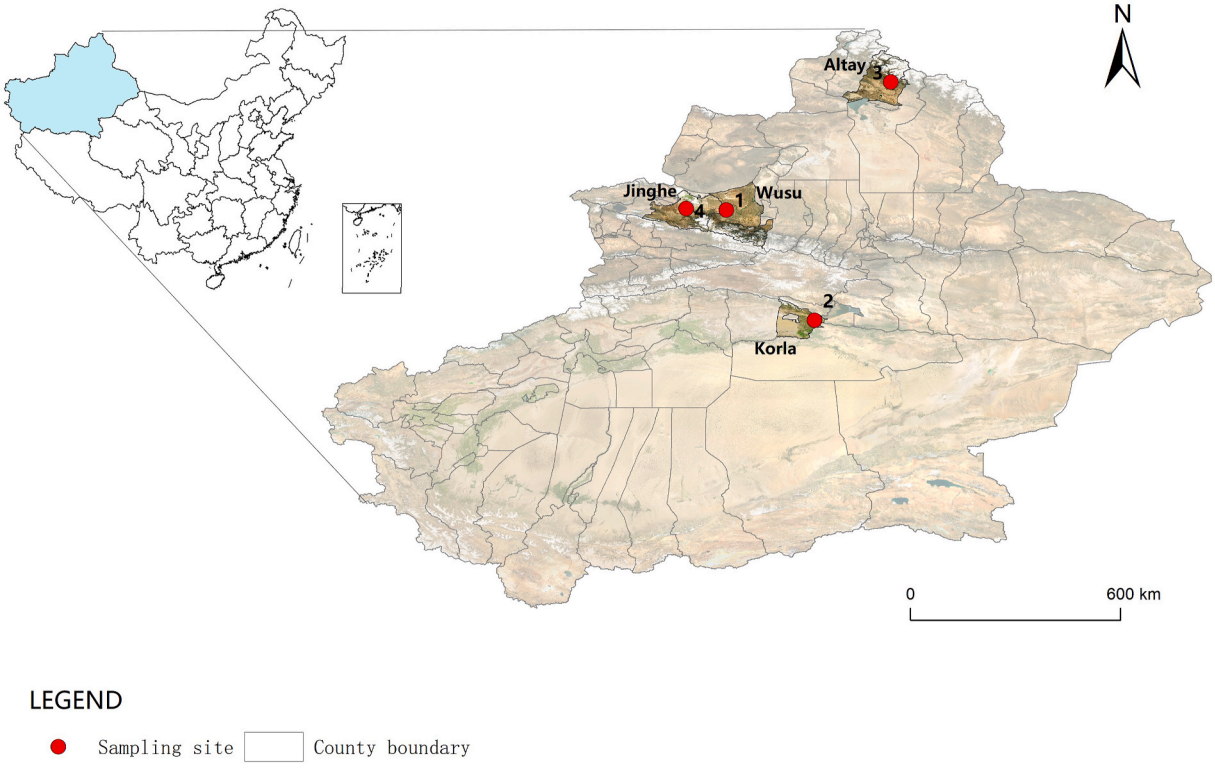
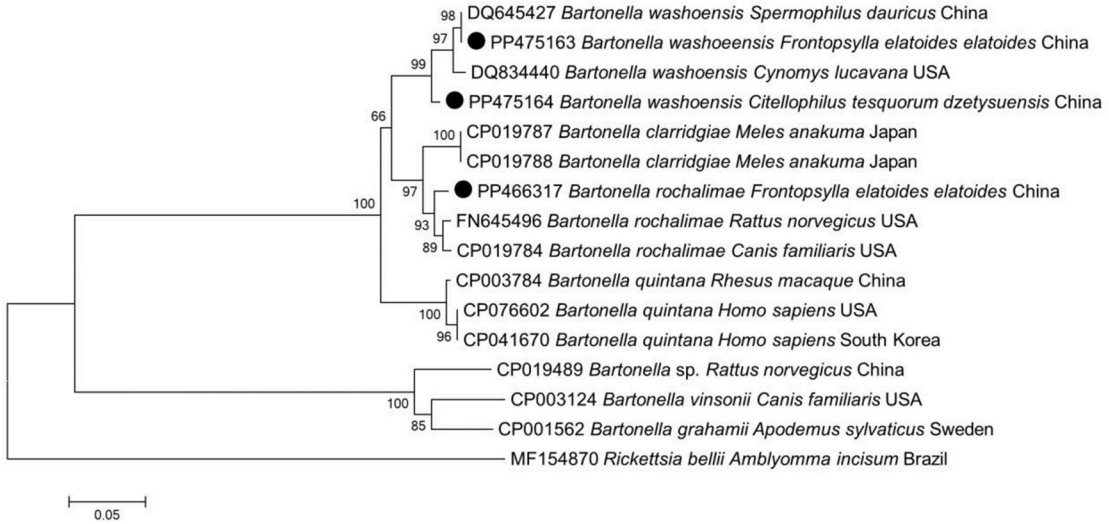


Fig. 1. Map of northwestern China showing sampling sites and coordinates.

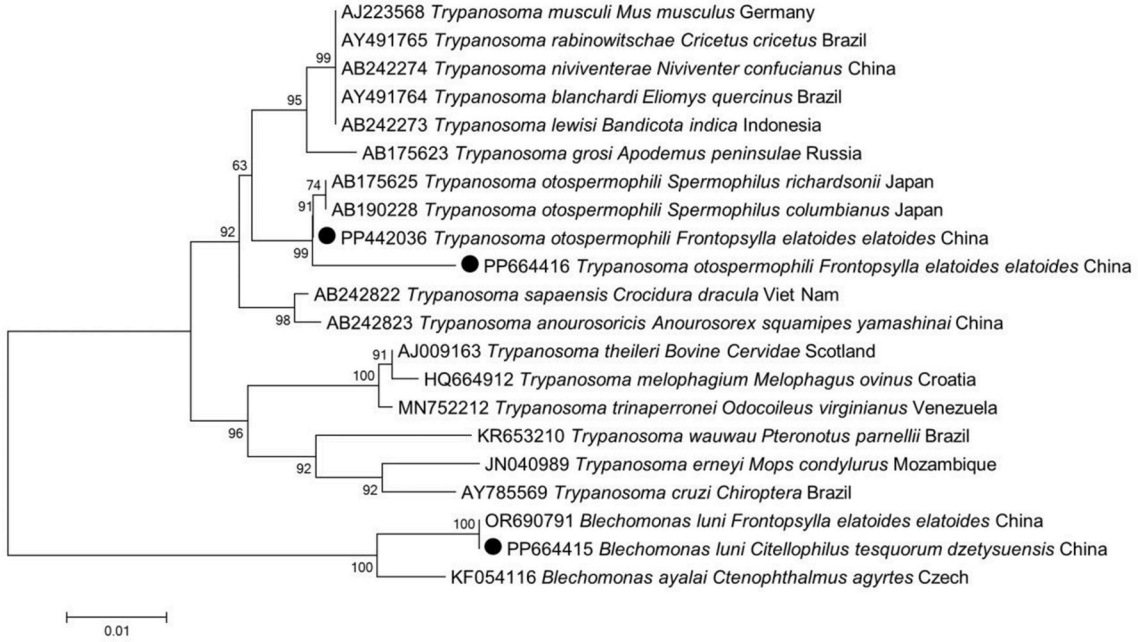
amplified and sequenced after morphological identification (Maekawa et al., 1999). Citrate synthase (*gltA*) was used to detect bartonellae. The *18S rRNA* gene was targeted to detect Trypanosomatidae, and glycosomal glyceraldehyde-3-phosphate dehydrogenase (*gGAPDH*) was used for confirmation (Mafie et al., 2019; Yin et al., 2019; Austen et al., 2020; Wang et al., 2024). The primers and PCR cycle conditions could be found in Table S1 and Table S2. Double distilled water served as the negative control. DNA extracts of *Bartonella* from fleas and of Trypanosomatidae from Mongolian pikas stored in our labs were used as the positive controls (Yin et al., 2019; Wang et al., 2024). Purification and sequencing of the PCR products followed previously described methods (Zhao et al., 2020). Phylogenetic trees were constructed using MEGA 7.0 software with the neighbor-joining methods. Bootstrap analyses with 1000 replicates were conducted to determine the relative support for the clades in the consensus trees.

3. Results

Out of the 396 fleas collected from 91 LTGRs, 3 different species were identified, i.e., *Frontopsylla elatoides elatoides* (203), *Neopsylla mana* (49), and *Citellophilus tesquorum dzetysuensis* (144). The average flea index was 4.35 (91 out of 396). Detailed information on the hosts, locations, flea pools, and flea species involved in this study are shown in Table 1. Among 54 pools of fleas, the following pathogens were detected: *Bartonella washoensis* (n = 5) in *F. elatoides elatoides* and *C. tesquorum dzetysuensis*, *Bartonella rochalimae* (n = 1) in *F. elatoides elatoides* and a novel haplotype (n = 1) within *Ba. washoensis* in *C. tesquorum dzetysuensis*, *Blechomonas luni* (n = 1) in *C. tesquorum dzetysuensis* and *Trypanosoma*



A



B

Fig. 2. Phylogenetic tree of (A) *Bartonella* (*gltA* gene) and (B) Trypanosomatidae (*18S rRNA* gene) from the LTGR fleas (NJ; bootstrap replicates: 1000). The new sequences provided in the present study are indicated by a black circle (followed by the accession number).

otospermophili (n = 2) in *F. elatoides elatoides*. Negative controls remained PCR negative in all tests. Results of the BLAST analysis are shown in Table S3.

Molecular analysis indicated that i) *Bartonella washoensis* detected in this study showed 100% (340/340) identity to *Ba. washoensis* reported in China from Daurian ground squirrel (*Spermophilus dauricus*) (GenBank accession number: DQ645427); ii) *Bartonella rochalimae* had 97.97% (338/345) identity to a conspecific isolate from domestic dog sampled in the USA (CP019784); iii) a novel haplotype within *Ba. washoensis* showed 97.03% (327/337) identity to *Ba. washoensis* reported from prairie dog in the USA (DQ834440) (Fig. 2A). The similarities between sequences of the *Ba. washoensis* and its novel haplotype available in GenBank were in the range of 97.03–97.64% and 97.32–97.35% according to nucleotides and amino acids, respectively (Supplementary Fig. 1); iv) *Bl. luni* showed 99.89% (881/882) identity to *Bl. luni* detected in China from flea (*F. elatoides elatoides*) of pikas (OR690791); and v) two different strains of *T. otospermophili* detected in this study showed 99.45% (900/905) and 98.14% (846/862) identities to this species from Columbian ground squirrel (*Urocitellus columbianus*) sampled in Japan (AB190228) (Fig. 2B).

4. Discussion

In this study, *Ba. washoensis* (including a novel haplotype) and *Ba. rochalimae* were identified in *F. elatoides elatoides* and *C. tesquorum dzetysuensis*, and two members of Trypanosomatidae in *C. tesquorum dzetysuensis* and *F. elatoides elatoides*. To our best knowledge, *Ba. washoensis*, *Ba. rochalimae*, *Bl. luni* and *T. otospermophili* were detected for the first time in LTGR fleas.

Bartonella infections have global distribution, characterized by their exceptional adaptation to rodents as their natural hosts and the integral role of fleas as the central vectors for transmission. (Angelakis and Raoult, 2014; Gutiérrez et al., 2015). Previously, *Ba. washoensis* was detected in *Ceratophyllus sciurorum* and *Oropsylla montana* fleas (Osikowicz et al., 2016; Lipatova et al., 2020). In addition, *Ba. rochalimae* was shown to be present in *Xenopsylla gerbilli minax* and *Xenopsylla conforms conforms* in XUAR (Yin et al., 2019). In this study, we detected for the first time *Ba. washoensis* in *F. elatoides elatoides* and *C. tesquorum dzetysuensis*, both fleas collected from LTGRs. Furthermore, this study revealed the presence of *Ba. rochalimae* in *F. elatoides elatoides*. In a previous study, *Bartonella* sp. has been detected in *Craneopsylla minerva minerva* and *Polygenis platensis* in Brazil (Schott et al., 2020). Our current investigation suggests that a new haplotype within *Ba. washoensis* was found in *C. tesquorum dzetysuensis*. Therefore, this work extends our understanding on potential vectors of bartonellae and their geographical distribution (Špitalská et al., 2022). This finding indicates the complex interaction among *Bartonella*, LTGR and flea vectors, which is crucial for understanding *Bartonella* infections and their geographical distribution.

Members of the family Trypanosomatidae are transmitted by various arthropods, such as ticks (Koual et al., 2023), fleas (Kaufer et al., 2017), flies (Mendoza-Roldan et al., 2021), bugs (Chimelli and Scaravilli, 1997) and other ectoparasites (Desquesnes et al., 2022). Several species have fleas as vectors, as exemplified by *Trypanosoma lewisi*, *Trypanosoma cruzi*, *Leptomonas tenua*, *Blechomonas maslovi*, *Blechomonas pulexsimulantis*, *Blechomonas ayala* and *Bl. luni* (Ortiz et al., 2018; Garcia et al., 2019). Previously, *Bl. luni* was reported in the fleas *Chaetopsylla globiceps*, *Chaetopsylla trichosa*, and *Archaeopsylla erinacei* (Votýpka et al., 2013). Alternatively, *Bl. luni*-like was shown to be present in *F. elatoides elatoides* fleas (Wang et al., 2024). Novel findings in this study include *Bl. luni* and *T. otospermophili* in the flea species *C. tesquorum dzetysuensis* and *F. elatoides elatoides*, respectively. With regard to *T. otospermophili*, it was considered as a parasite mainly in rodents from the USA (Sato et al., 2007). However, there has been limited documentation on its presence in fleas. In this current investigation, *T. otospermophili* was identified in *F. elatoides elatoides* found on LTGRs, sharing 98.14%–99.45% sequence identity with *T. otospermophili* from *Spermophilus*, thus suggesting that *T. otospermophili* has significant genetic diversity. To date, there are 44 species of the genus *Spermophilus* in the world, including about 6 species in China (Global Biodiversity Information Facility, 1825). In the future, it will be necessary to conduct detection of Trypanosomatidae in more flea species from hosts of the genus *Spermophilus*.

5. Conclusions

This study presents the first evidence of *Ba. washoensis* (including a novel haplotype), *Ba. rochalimae*, *Bl. luni* and *T. otospermophili* in flea species collected from LTGRs. These discoveries expand our knowledge on the geographical distribution and potential flea vectors of these pathogens.

Funding

This work was supported by the Natural Science Foundation of China [grant number 822260399]; the Natural Science Key Project of Xinjiang Uygur Autonomous Region [grant number 2022B03014]; the Key Scientific and Technological Projects in Key Areas of XPCC [grant number 2022AB014]; and the Technology Innovation Team for Local High-Incidence Tick-Borne Diseases [grant number bykj2023td-2].

Consent to participate

Informed verbal and written consent were obtained from each study participant.

Ethical approval

This study was approved by the Animal Ethics Committee of Shihezi University (Approval No. A2022-029-01).

Data availability

The sequences obtained and analyzed during the present study are deposited in the GenBank database [https://www.ncbi.nlm.nih.gov/nucleotide/] under the following accession numbers: PP475162, PP475165, PP475169, PP475171, PP475170, PP474966 (flea *COII* gene), PP466317, PP475163, PP475164 (*Bartonella gltA* gene), PP442036, PP664415 and PP664416 (Trypanosomatidae *18S rRNA* gene), as well as PP475166-PP475168 (Trypanosomatidae *gGAPDH* gene).

CRediT authorship contribution statement

Xiaoshuang Han: Writing – original draft, Methodology, Investigation, Conceptualization. **Shanshan Zhao:** Resources, Methodology, Investigation. **Ziheng Liu:** Writing – original draft, Investigation. **Yujiang Zhang:** Writing – review & editing, Formal analysis. **Guoyu Zhao:** Writing – review & editing, Conceptualization. **Chunju Zhang:** Methodology, Data curation. **Lijuan Tang:** Validation, Funding acquisition. **Lin Cui:** Writing – review & editing, Investigation, Conceptualization. **Yuanzhi Wang:** Writing – review & editing, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no conflict of interest.

Acknowledgments

The authors thank Prof. Sándor Hornok (Department of Parasitology and Zoology, University of Veterinary Medicine, Budapest, Hungary) for editing the text.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijppaw.2024.100958.

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