BIODIVERSITAS
Volume 24, Number 12, December 2023
Pages: 6852-6857

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d241246

Molecular detection of *Borrelia* spp. (Spirochaetales: Borreliaceae) in ticks (Acari: Ixodidae) collected from tortoises in Java, Indonesia

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Manuscript received: 19 October 2023. Revision accepted: 28 December 2023.

Abstract. *Sophia HF, Supriyono, Soviana S, Novianto D, Hadi UK. 2023. Molecular detection of* Borrelia *spp. (Spirochaetales: Borreliaceae) in ticks (Acari: Ixodidae) collected from tortoises in Java, Indonesia. Biodiveritas 24: 6852-6857.* Reptiles are known hosts of various tick species, many of which are zoonotic and pose veterinary and public health risks. *Amblyomma* ticks frequently parasitize reptiles and are potential vectors of zoonoses. This study aimed to investigate the prevalence of tick infestations and to identify *Borrelia* species infections in ticks found on tortoises. One hundred six tortoise hosts were sampled for ticks in three provinces in Indonesia. DNA extraction and *Borrelia* species were detected using conventional PCR techniques. The results revealed the presence of nineteen hard-bodied ticks on *Chelonoidis carbonarius* Spix, 1824, *Chelonoidis denticulatus* Linnaeus, 1766, and *Geochelone sulcata* Miller, 1779. All the ticks collected were identified as *Amblyomma sparsum* Neumann 1899, and the prevalence of *A. sparsum* infestations on tortoises in this study was 7.55%. *Borrelia* spp. was detected in some of the *A. sparsum* ticks infesting tortoises. Subsequent sequencing unveiled two distinct reptile-associated (REP) borrelia strains. This study sheds light on the tick infestation patterns and the potential transmission of *Borrelia* species among tortoises kept as exotic pets in urban areas by *A. sparsum* ticks. The findings provide valuable insights into the bioecology of ticks and the associated health risks they pose to reptiles, thereby contributing to our understanding of zoonotic disease dynamics in the region.

Keywords: *Amblyomma sparsum*, PCR, reptile-associated borrelia, tick infestations, tortoises

Abbreviations: LD: Lyme Disease; REP: Reptile-associated Borreliae; RF: Relapsing Fever; TBD: Tick-borne Disease; TBP: Tick-borne Pathogen

INTRODUCTION

Global demand for exotic pets has surged exponentially in recent years. Reptiles are the most sought-after in Indonesia, with chelonians and snakes most frequently traded (Putranto et al. 2016). This demand may be one factor in the recent increase in zoonoses and spillover from wildlife reservoirs. Zoonosis, defined as any disease transmitted from animals to humans or vice versa (Suardana 2015), accounts for most zoonotic diseases; 71.8% of zoonoses originate from wild animals (Hilderink and de Winter 2021). Many factors, including human population expansion, wildlife habitat encroachment, climate change affecting wildlife and vector distribution, and increased ownership of exotic pets, contribute to the rise in zoonosis exposure and spillover from wildlife reservoirs (Hilderink and de Winter 2021).

Ticks are hematophagous ectoparasites of veterinary and medical importance. They are found worldwide and are second only to mosquitoes regarding their public health risks (Luo et al. 2019; Nicholson et al. 2019). Ticks are obligate parasites, common in tropical and subtropical regions, and vary significantly in host preference depending on the species and the life stage (Bizhga et al. 2022). At one or more developmental stages, ticks feed on the blood of mammals, birds, reptiles, and amphibians (Dantas-Torres et al. 2019). In the process of feeding on the host, ticks not only weaken the host by sucking their blood but can also transmit numerous disease agents, causing significant morbidity and mortality in domestic animals, wildlife, and humans (Dantas-Torres and Otranto 2016; Dantas-Torres et al. 2019). Tortoises are commonly infested with Ixodidae ticks, such as *Amblyomma* spp. and *Hyalomma* spp. (Andoh et al. 2015; Kaenkan et al. 2020).

Among the most common zoonotic cases reported are tick-borne diseases (TBDs) caused by infectious agents transmitted by tick bites. Globally, some of the most severe emerging infectious diseases are caused by tick-borne pathogens (TBPs). Ticks are also mechanical and biological vectors of many Gram-positive and Gram-negative bacteria. Cases of TBDs worldwide include anaplasmosis (*Anaplasma* spp.), babesiosis (*Babesia* spp.), ehrlichiosis (*Ehrlichia* spp.), tick-borne encephalitis (tick-borne encephalitis virus), tick paralysis, and infections by *Borrelia* spp. bacteria (Nicholson et al. 2019; Krčmar et al. 2022).

Borrelia clusters into distinct evolutionary lineages: Lyme disease, relapsing fever, and, more recently, the reptile-associated (REP) borrelia clades. Lyme disease in humans is caused by the *Borrelia burgdorferi sensu lato* complex and is primarily transmitted by *Ixodes* spp. (Kireçci et al. 2013). Relapsing fever has more than 20 etiological agents. The main vectors of relapsing fever are the argasid ticks of the genus *Ornithodoros*; however, *Borrelia lonestari* is transmitted by *Amblyomma* spp., *Borrelia miyamotoi* by *Ixodes* spp., and *Borrelia theileri* by *Rhipicephalus* spp. (Wang 2015). The third group of *Borrelia* spp. is the reptile-associated (REP) borreliae, which includes several species detected in ticks associated with various reptiles (Trinachartvanit et al. 2016; Panetta et al. 2017; Supriyono et al. 2019; Kaenkan et al. 2020; Morales-Diaz et al. 2020; Colunga-Salas et al. 2021; Gofton et al. 2023). *Borrelia turcica* was first discovered in *Hyalomma aegyptium* Linnaeus 1758 ticks collected from tortoises in Turkey (Güner et al. 2004) and is phylogenetically separate from Lyme disease and relapsing fever borreliae.

Numerous studies have pointed to reptiles and the ticks associated with them as potential reservoirs in the transmission cycles of TBPs (Halajian et al. 2016; Omondi et al. 2017; Santodomingo et al. 2018). This is a matter of significant concern for TBD control strategies, as these ectotherms are typically asymptomatic carriers that could serve as infection reservoirs. Despite the increasing emergence of various TBPs worldwide with documented impacts on the health of humans and wildlife, research on their diversity and specific interactions with ticks and the reptilian host species in Indonesia is lacking. The presence and diversity of bacteria associated with ticks of reptiles, particularly those species that infest reptiles and are close to human settlements, have not been extensively studied in Indonesia. This lack of information on tick-borne bacteria of reptilian ticks in Indonesia warrants novel research in this area. Therefore, this study aimed to investigate tortoise tick infestations and to detect *Borrelia* spp. infections in ticks found on tortoises in Indonesia. Understanding the potential for these ticks to serve as vectors for zoonotic diseases is essential for public health and wildlife conservation efforts.

MATERIALS AND METHODS

Sample collection

One hundred six tortoises (Testudines: Testudinidae) were sampled and examined for ticks between December 2021 and January 2023 in three provinces of Indonesia. Tortoises were sampled in Bekasi, Bogor, and Cibinong (West Java Province), East Jakarta (the Special Capital Region of Jakarta), and Tangerang (Banten Province). Captive-bred and wild caught tortoises were sampled from pet shops, reptile breeding facilities, and homes. Tortoises were restrained by hand whilst ticks were collected in situ. Among these, seven tortoises from Bekasi (-6° 15’ N., 106° 55’ E.) and one tortoise from East Jakarta (-6° 18’ N., 106° 54’ E.) were infested with hard ticks. All the infested female *Geochelone sulcata* Miller, 1779 from the Bekasi site and the male *G. sulcata* from the East Jakarta site were captive-bred in Indonesia. The male *G. sulcata* from the Bekasi site was imported from an overseas breeding facility. The infested *Chelonoidis carbonarius* Spix, 1824 and the *Chelonoidis denticulatus* Linnaeus, 1766 at the Bekasi site were wild caught. However, further information on the location of the male *G. sulcata* overseas breeding facility and the collection location of *C. carbonarius* and *C. denticulatus* was unavailable. These ticks were preserved in 70% ethanol until they were transported to the Laboratory of Medical Entomology, where they underwent species identification using established morphological keys (Horak et al. 2018).

DNA extraction and amplification

Nineteen ticks were separated into 12 pools for DNA extraction. Ticks were dissected at the posterior ⅓ of the idiosome, and their gut and salivary glands were collected. Genomic tick DNA from the gut and salivary glands was extracted using Geneaid® Genomic DNA Mini Kit (Tissue) (Geneaid Biotech Ltd., Taiwan) per the manufacturer’s instructions.

To confirm the presence of *Borrelia* spp., a conventional PCR assay targeting the *flaB* gene was conducted (Supriyono et al. 2019). PCR was conducted with a primer set of PAD (5’-GATCARGCWCAAYATAACCAWATGCA-3’) and PDU (5’- AGATTCAAGTCTGTTTTGGAAAGC-3’). Each PCR included 10 µL of HotStarTaq® Plus Master Mix (Qiagen GmbH, Germany), 1 µL of PAD primer and 1 µL of PDU primer (Integrated DNA Technologies, United States), 2 µL of coral load, 3 µL of ddH_2O, and 3 µL of DNA template. The thermal cycling parameters for the PCR were as follows: initial denaturation at 95°C for 2 min, followed by 35 cycles of 94°C for 45 s, 50°C for 30 s, 72°C for 45 s, 72°C for 10 min, and holding at 20°C. PCR products were visualized using electrophoresis on 1.2% agarose gel stained with ethidium bromide in 1X TAE buffer. Electrophoresis was carried out at 100 V for 25 minutes.

Phylogenetic analysis

The DNA of positive samples was compared with sequences in the NCBI GenBank database by nucleotide BLAST. Sequence data obtained in this study were deposited in the GenBank database under accession numbers PP145307–PP145308. Phylogenetic analyses were performed using the MEGA11 software. The phylogenetic trees were constructed by the neighbor-joining method, and bootstrap analyses (1000 replicates) were carried out according to the Kimura 2-parameter model.

RESULTS AND DISCUSSION

Tortoise examination, tick collection, and identification

This study collected ixodid ticks from *C. carbonarius*, *C. denticulatus*, and *G. sulcata* (Figure 1). The distribution of tick-infested tortoises in this study is detailed in Table 1. At the Bekasi site, infested tortoises included *G. sulcata* (one male approximately 25 years old and four females ranging from approximately eight to 20 years old), *C. carbonarius* (one female approximately 10 years old), and *C. denticulatus* (one male approximately 10 years old). At the East Jakarta site, the infested tortoise was a *G. sulcata* (an adult male of unknown age). The prevalence of hard tick infestation on tortoise hosts in this study between December 2021 and January 2023 was 7.55% (8/106). Nineteen adult tick specimens were collected from the 8 tortoise hosts: 10 were male (52.63%) and 9 were female (47.37%). No nymphs were found on any of the tortoise hosts examined. Tick species, stage, and sex were identified based on morphologic features following taxonomic keys. The collected tick specimens were identified as *Amblyomma sparsum* Neumann, 1899. Dorsal and ventral views of male and female *A. sparsum* collected in this study are detailed in Figure 2.

All the tortoises at the Bekasi site were housed together in one enclosure. The tortoises had access to a 10 × 2 m concrete indoor area and an adjacent enclosed yard with full access to sunlight. The indoor area featured a basking area with a heated lamp, a shallow pool for swimming and drinking, and a sandy area for enrichment and breeding. The tortoises were primarily fed leafy greens, such as caisim (*Brassica chinensis* var. *parachinensis* (L.H.Bailey) Sinskaya), and had access to water ad libitum. The tortoise enclosure was situated near iguana and chicken enclosures. An on-site veterinarian conducted routine medical examinations. The tortoise from the East Jakarta site was housed alone in an outdoor enclosure for most of its time. The owner reported observing ticks on its body for some time before seeking examination by an exotic veterinarian in a private clinic.

Table 1. The number and percentage (%) of collected ticks from tortoise hosts in Indonesia

Collection sites	Tortoise		Tick
	N	% of Tick infestation	N of collected ticks
Bekasi	7	100	17
Bogor	15	0	0
Cibinong	82	0	0
Jakarta	1	100	2
Tangerang	1	0	0
Total	106	7.55	19

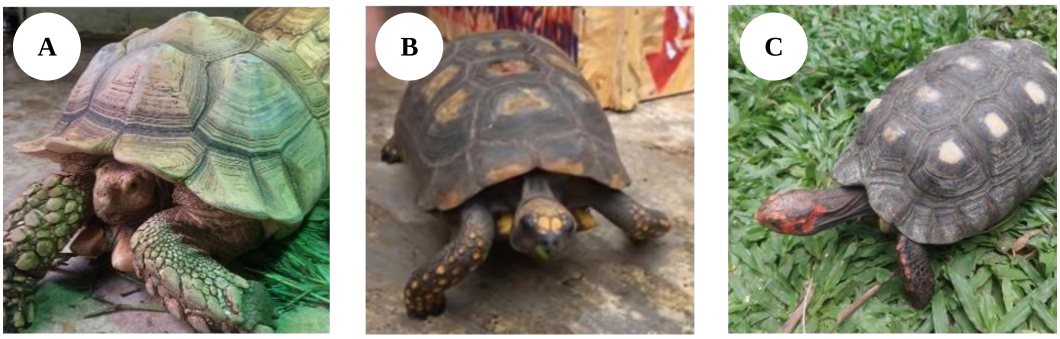

Figure 1. Tortoise hosts infested with *A. sparsum* in this study. A. *G. sulcata;* B. *C. carbonarius*; C. *C. denticulatus*

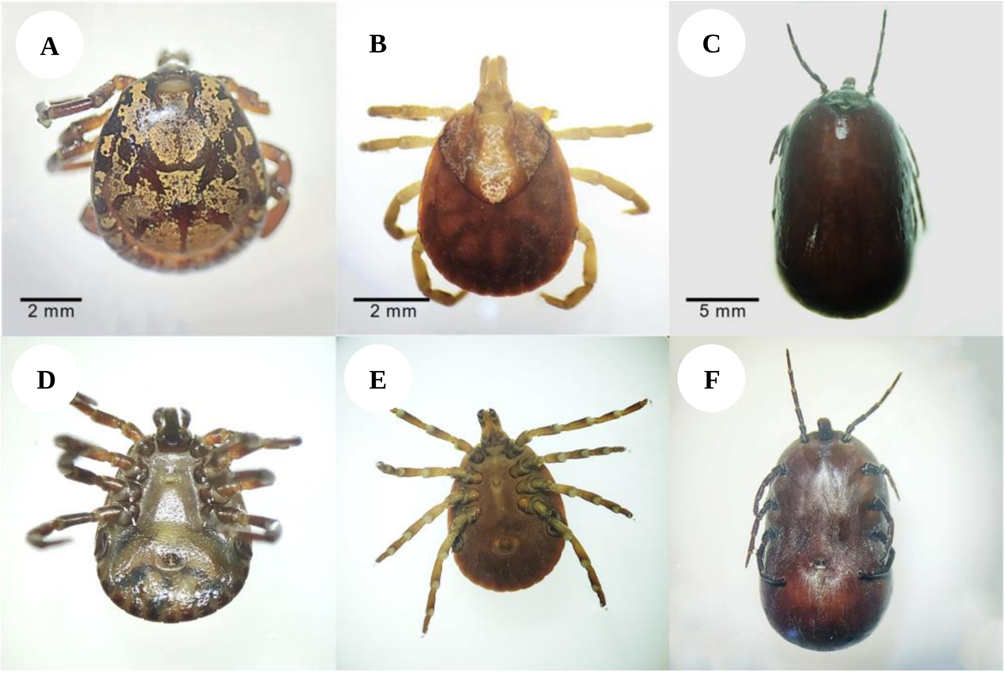

Figure 2. Dorsal and ventral views of the *A. sparsum* collected in this study. A. Male, dorsal; B. Unengorged female, dorsal; C. Engorged female, dorsal; D. Male, ventral; E. Unengorged female, ventral; and F. Engorged female, ventral

Detection of *Borrelia* spp.

Nineteen *A. sparsum* ticks were pooled into 12 pools for genomic DNA extraction. The presence of *Borrelia* spp. in the *A. sparsum* samples was confirmed by observing bands of 350 bp, indicative of successful *flaB* gene amplification. Pools 3, 4, 5, 8, and 12 were positive for *Borrelia* spp. Among these *Borrelia*-positive ticks, 67% were male (*n* = 6), and 33% were female (n = 3). All positive tick specimens originated from a single reptile facility in Bekasi and all the infested tortoise hosts were *G. sulcata*. Sanger sequencing of *flaB* was performed on Pool 4 and Pool 5. Pool 4 was extracted from adult female ticks infested on *G. sulcata*, while Pool 5 was extracted from an adult male tick infested on *G. sulcata*.

Phylogenetic analysis

Phylogenetic analyses conducted in MEGA11 based on *flaB* revealed that the *Borrelia* strains detected in this study are separate from the relapsing fever and Lyme disease borrelia groups and fall under the third recently identified group-the reptile-associated (REP) borrelia. The phylogenetic analyses suggested that the two detected *Borrelia*, *Borrelia* sp. IR2 and *Borrelia* sp. IR3, formed two monophyletic clades (Figure 3); *Borrelia* sp. IR2 from Pool 4 was closely related to uncultured *Borrelia* sp. (Accession number OP082443; 98.92% similarity) detected in *Amblyomma marmoreum* collected from Bell's hinge-back tortoises (*Kinixys zombensis* Hewitt, 1931). *Borrelia* sp. IR3 from Pool 5 was closest related to *Borrelia* sp. tAG66M (Accession number AB529322; 94.12% similarity) detected in *Amblyomma geoemydae* Cantor, 1847 collected from Ryukyu Yellow-Margined box turtles (*Cuora flavomarginata* subsp. *evelynae* Ernst & Lovich, 1990).

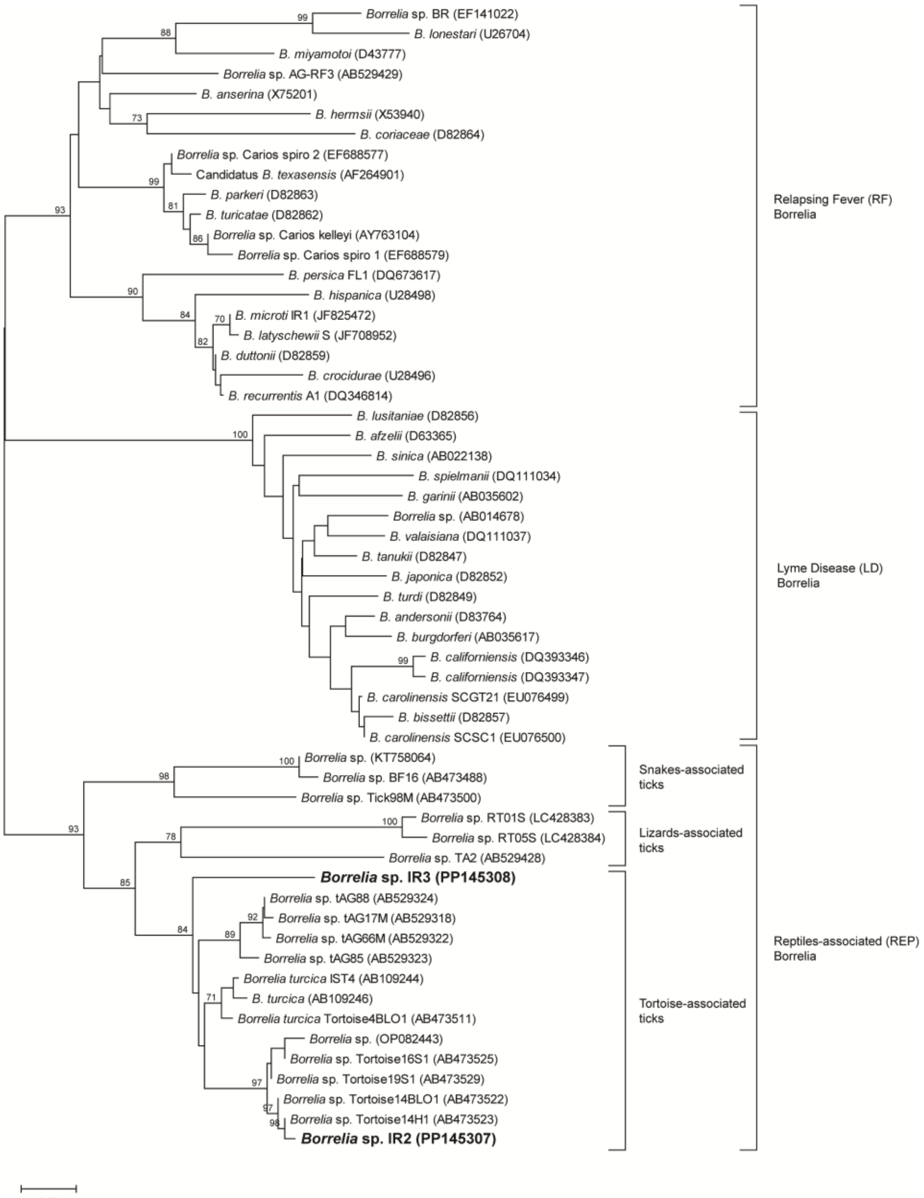

Figure 3. Phylogenetic tree analysis of *flaB* of the detected *Borrelia* spp. The phylogenetic branches were supported in >70% by the bootstrap analysis. The bar indicates the percentage of sequence divergence. Bold letters indicate the bacteria analyzed in this study

Discussion

Hard-bodied ticks of the genus *Amblyomma* are distributed globally across tropical and subtropical regions. The Neotropical Faunal Region has the highest number of *Amblyomma* species but can also be found in other regions such as Africa, Asia, Australia, and the Pacific Islands (Guglielmone et al. 2014). In Indonesia, *Amblyomma* infesting reptiles has been reported in several studies (Katmono et al. 2019; Supriyono et al. 2019).

In previous studies, African reptiles and large mammals have been identified as hosts for adult *A. sparsum* (Omondi et al. 2017). Monitor lizards (*Varanus* spp.) are commonly parasitized by *A. sparsum*, but other reptiles such as *Agama* spp., *Python sebae* Gmelin, 1789, *Bitis arietans* Merrem, 1820, *Kinixys belliana* Gray, 1831, and *Geochelone pardalis* Bell, 1828 may also be infested (Walker 1991). This study found ticks attached to the axillae and caudal folds of *G. sulcata*. This study is the first record of *A. sparsum* parasitizing afrotropical tortoises of the species *G. sulcata* and neotropical tortoises of the species *C. carbonarius* and *C. denticulatus* in Indonesia.

Amblyomma ticks are potential vectors and have been reported to carry several TBPs and endosymbionts, including *Rickettsia*, *Anaplasma*, *Hepatozoon*, *Ehrlichia*, *Francisella*, and *Borrelia* spp. (Sumrandee et al. 2014; Kho et al. 2015; Trinachartvanit et al. 2016; Ogrzewalska et al. 2019). *Borrelia* spp. are infectious agents carried by arthropods that can infect vertebrates. *Borrelia* is traditionally classified into two main genetic groups: relapsing fever (RF) borrelia and Lyme disease (LD) borrelia. LD borrelia is further divided into *B. burgdorferi sensu stricto* (s. s.) and *B. burgdorferi sensu lato* (s. l.). Both can cause disease in humans and animals, but *B. burgdorferi* (s. l.) primarily affects humans. The etiological agents of Lyme disease are mainly *B. burgdorferi* (s. s.), *Borrelia garinii*, and *Borrelia afzelii*, while *Borrelia hermsii*, *Borrelia turicatae*, and *Borrelia parkeri* may cause tick-borne relapsing fever. Erythema migrans is the earliest and hallmark clinical symptom of Lyme disease. Tick-borne relapsing fever is characterized by several days of high fever (e.g., 39°C) interspersed with afebrile periods. Without antibiotic treatment, the cycle may repeat several times. Recent research has identified a third group, the reptile-associated (REP) borrelia, though a *Borrelia* sp. of this group is associated with echidna (Panetta et al. 2017). REP borrelia appears to have evolved from a common ancestor with RF borrelia. Although LD and RF borreliae are thought to have co-evolved with their vector ticks (Parola and Raoult 2001), REP borrelia may be transmitted by hard-bodied ticks despite their divergent evolutionary history.

Borrelia has a unique life cycle that alternates between hematophagous arthropod vectors and vertebrate hosts and is not found anywhere else in the environment. Hard-bodied ticks (such as *Ixodes* spp.) are vectors of Lyme disease borrelia. In contrast, relapsing fever borrelia is transmitted through soft-bodied (argasid) ticks, except for *B. recurrentis*, which is transmitted by the *Pediculus humanus* louse (Takano et al. 2011). REP borrelia is believed to be transmitted through hard-bodied ticks of the genera *Amblyomma* or *Hyalomma* and has been detected in the blood and tissue of reptiles and in *Amblyomma* and *Bothriocroton* ticks that were infesting various reptiles (Panetta et al. 2017; Supriyono et al. 2019). Infected ticks contract *Borrelia* by feeding on the vertebrate reservoir. Once the infected tick molts, the competent *Borrelia* remains in the tick. This process is known as trans-stadial transmission. Takano et al. (2011) investigated the presence and transmission of REP borrelia in *Amblyomma geoemydae* feeding on Ryukyu Yellow-Margined box turtles and an Asian Yellow Pond Turtle. Their results showed that post-transstadial transmission of *Borrelia* sp. tAG was established in *A. geoemydae* midgut. However, *Borrelia* sp. tAG had not colonized the salivary glands in molted ticks. This characteristic is typical of LD borrelia, in which transmigration from the midgut to the salivary gland occurs after the attachment of a tick to a host. Further studies are needed to establish when REP borrelia begins transmigration from the midgut to the salivary gland. Takano et al. (2011) hypothesize that it occurs during feeding, as is seen in LD borrelia.

The present study is the first record of *Borrelia* detected in ticks infesting testudines in Indonesia, expanding the database of tick infestations on reptiles in Indonesia. Previously, *Borrelia* was also detected in ticks infesting varanids in Indonesia (Supriyono et al. 2019; Gofton et al. 2023). Numerous reptile species have been increasingly traded and maintained as exotic pets in various countries worldwide. Consequently, there is a potential concern regarding the health risks associated with tick-borne pathogens and potential zoonoses. Therefore, it is crucial to understand the patterns of circulation of tick-borne bacteria in the environment to evaluate the risk of infection for wildlife and humans. Hard-bodied ticks are the most common ectoparasite of tortoises, and studies have shown that they may carry REP borrelia bacteria, which can infect tortoises (Mofokeng et al. 2022). While there is currently no evidence that these bacteria can be transmitted from ticks to mammals, it remains a possibility that cannot be ignored. *Borrelia* bacterial infections can cause serious health problems in humans and animals, making it crucial to conduct epidemiological surveillance to identify emerging infectious diseases. Tick and TBP surveillance, disease detection, and vector control are critical for mitigation, control, and early warning response strategies in disease outbreak cases (Omondi et al. 2017).

This study has provided substantial insights into tick infestations and *Borrelia* spp. infections among hard-bodied ticks of the genus *Amblyomma*, particularly those parasitizing tortoises in Indonesia. The findings revealed a prevalence rate of 7.55% for *A. sparsum* ticks on tortoises. Furthermore, *Borrelia* spp. was detected in several instances of *A. sparsum* infestations, and subsequent sequencing unveiled two separate reptile-associated (REP) borrelia strains. These results indicate that tick-infested tortoises harbor *Borrelia* spp., potentially posing a threat of infection to reptiles. This underscores the importance of monitoring tick infestations and *Borrelia* infections in tortoises and other reptiles in Indonesia.

ACKNOWLEDGEMENTS

We thank drh. Hammada Raudlowi (deHakims) and drh. Muhammad Piter Kombo (AVES Jakarta) for their assistance in collecting research samples. We also thank the Laboratory of Medical Entomology, School of Veterinary Medicine and Biomedical Sciences, Institut Pertanian Bogor, Indonesia.

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