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EDITOR'S NOTE: Establishing the Journal of Tropical One Health

The Journal of Tropical One Health is a new scholarly initiative spearheaded by the Tropical Infectious Diseases Research & Education Centre (TIDREC) at the Universiti Malaya, Kuala Lumpur, Malaysia to address the urgent need for a high-quality, dedicated publication platform for One Health research in tropical regions. As health threats become increasingly complex, driven by environmental changes, globalization, and zoonoses - a journal centred on interdisciplinary tropical health science is both timely and essential.

Our vision is to develop this journal into a respected, internationally indexed publication (Scopus, ESCI, and eventually Web of Science), contributing to the global One Health discourse while showcasing high-impact research from the tropics and beyond.

Key Features:

- Open-access model to maximize visibility and impact
- Open Journal Systems (OJS)-based publishing platform hosted by Universiti Malaya
- Editorial board featuring local and international experts in medicine, veterinary sciences, environmental health, and public policy
- Strong emphasis on ethical publishing, transparency, and rigorous peer review

Mission

Journal of Tropical One Health is dedicated to advancing knowledge at the intersection of human, animal, and environmental health, with a focus on tropical regions. Our mission is to foster interdisciplinary research and dialogue that supports the One Health approach to address emerging health challenges in a globally interconnected world

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Scope

Journal of Tropical One Health publishes high-quality, peer-reviewed research that contributes to the understanding and management of health issues arising from the complex interactions between humans, animals, and the environment in tropical regions.

We welcome original research articles, reviews, short communications, and case reports in (but not limited to) the following areas:

- Zoonotic and vector-borne diseases
- Vector biology and control
- Vaccines and therapeutics
- Surveillance, diagnostics, and outbreak response
- Antimicrobial resistance and stewardship
- Tropical public health and veterinary medicine
- Wildlife, livestock, and companion animal health
- Food safety, food security, and sustainable agriculture
- Environmental and climate-linked health threats
- One Health policies, systems, and practices
- Community-based health interventions and cross-sectoral collaborations

Submissions from low- and middle-income tropical countries and interdisciplinary teams are especially encouraged.

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JOURNAL OF TROPICAL One Health
EDITORIAL

Journal of Tropical One Health – A platform for advancing One Health in the tropics

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ABSTRACT

The Journal of Tropical One Health (JTOH) serves as a multidisciplinary platform dedicated to the interconnectedness of human, animal, and environmental health in the tropical region. The tropical world continues to face intersecting health threats, including zoonotic and vector-borne diseases, antimicrobial resistance, and climate and environmental health threats. These challenges, compounded by gaps in surveillance, vaccines, therapeutics, and food security, highlight the critical need for One Health approaches that integrate human, animal, and environmental health systems. By publishing high-quality research, reviews, case reports, short communications, and perspectives, JTOH aims to bridge knowledge gaps and support informed decision-making for sustainable One Health solutions in the tropics. With this inaugural issue, we invite the global One Health community to contribute their expertise and innovations to advance integrated approaches that improve health, equity, and resilience across tropical ecosystems.

Keywords: zoonotic and vector-borne diseases, antimicrobial resistance, environmental health, climate change, food safety and security

Introduction

In recent decades, successive pandemics have reminded us that the next global health crisis often begins where humans, animals, and ecosystems converge. From outbreaks and epidemics of SARS and avian influenza to Ebola, Zika, Nipah, and the COVID-19 pandemic, each event has exposed vulnerabilities in our preparedness and the dangers of addressing health in isolation. The One Health approach provides a way forward by bringing together diverse disciplines to predict, prevent, respond and mitigate outbreaks, epidemics, and pandemics more effectively. Nowhere is this more urgent than in the tropical regions, where biodiversity, dense populations, and rapid urbanization create both vulnerability and opportunity for proactive One Health action.

As the first Editor-in-Chief, I am honoured to introduce this journal as a dedicated platform for advancing the One Health agenda, particularly within tropical settings where the convergence of humans, animals, and ecosystems creates both opportunities and challenges for disease control and health equity.

While several international journals address One Health broadly, including One Health, One Health Outlook, One Health Advances, and IJID One Health, there remains a critical need for a journal dedicated to the tropical One Health perspective. Tropical regions face distinct challenges, including high biodiversity, dense human and animal populations, environmental pressures, emerging infectious diseases, and interconnected issues such as food insecurity and livelihood vulnerabilities that require context-specific research, surveillance, and policy solutions. JTOH distinguishes itself by focusing specifically on these epidemiological, ecological, and socioeconomic realities, highlighting research that addresses not only emerging and neglected diseases but also the broader health, environmental, and societal challenges unique to tropical settings.

Tropical nations, many of which are low- and middle-income experience the greatest impact of infectious and neglected tropical diseases, zoonoses, and vector-borne illnesses. Furthermore, these regions face accelerating environmental changes, including deforestation, climate variability, and agricultural

expansion, which heighten the risk of pathogen spillover. The One Health framework offers a powerful lens through which these complex problems can be studied and addressed collaboratively.

In Southeast Asia, for example, regional initiatives have strengthened surveillance and response to zoonotic diseases through multi-country collaboration. Yet, local capacities remain constrained by inadequate laboratory infrastructure and limited mechanisms for timely and transparent data sharing across human, animal, and environmental health sectors, which hampers early detection of outbreaks, slows coordinated responses, and reduces the effectiveness of regional surveillance networks.

Human-driven environmental changes such as expanding human population, urbanization, globalization, climate change, deforestation, and wildlife trade and consumption continue to disrupt ecosystems and contribute to the emergence of diseases (Spernovasilis et al., 2022). The rise of simian malaria, particularly Plasmodium knowlesi and P. cynomolgi, illustrates how such disturbances can alter vector and host interactions, enabling the spillover of pathogens from wildlife to humans. While these infections are endemic to forested regions of Southeast Asia, imported cases have now been reported in multiple countries among travellers visiting the region (Jeyaprakasam et al, 2020), highlighting their global relevance. These patterns underscore how local environmental changes can have far-reaching public health implications.

In addition to simian malaria, tick-borne diseases provide another important example of emerging health threats in tropical regions. Many of these pathogens, including Rickettsia, Bartonella, and Borrelia species, are endemic to Asia and display considerable diversity across different ecological zones (Yean et al., 2024). Their varied distribution and clinical presentations pose challenges for accurate diagnosis, as standard diagnostic tools may not detect all regional strains (Low et al., 2020). Moreover, effective treatment and management strategies often need to be tailored to the specific pathogens and local epidemiology. Given the increasing human–animal–environment interactions in the tropics, heightened surveillance, region-specific diagnostic approaches, and targeted therapeutic interventions are essential to mitigate the public health impact of these neglected but potentially serious infections.

Food insecurity remains a major One Health concern in many tropical regions. In parts of Africa and Western Asia, high food price inflation has made nutritious diets unaffordable for billions, disproportionately increasing child malnutrition (FAO et al., 2025). This worsening crisis, driven by climate change, conflict, and economic instability, not only threatens human health directly but also increases the risk of infections among affected communities. For example, outbreaks of Avian influenza have caused the loss of hundreds of millions of poultry worldwide, often requiring large-scale culling to control transmission. Such outbreaks disrupt poultry production systems, increase the price of meat and eggs, and reduce the availability of affordable animal protein, particularly affecting vulnerable populations that rely heavily on poultry for nutrition and income (Xie et al., 2024; FAO, 2025;). These impacts highlight how animal disease outbreaks can directly affect food supply, livelihoods, and nutrition, demonstrating the interconnected nature of human, animal, and environmental health within the One Health framework.

Given the growing complexity and diversity of One Health challenges in tropical regions, JTOH publishes original research articles, reviews, short communications, and perspectives that address One Health issues within tropical regions. The journal welcomes contributions from diverse disciplines, including but not limited to:

i. **Zoonotic, vector-borne, and emerging infectious diseases**
   - Epidemiology, surveillance, prevention, and control
   - Outbreak, epidemic, and pandemic preparedness
   - Vector biology and control

ii. **Neglected tropical diseases and antimicrobial resistance (AMR)**
   - AMR in human, animal, and environmental contexts
   - Stewardship and integrated mitigation strategies

iii. **Animal health**
   - Livestock, companion animals, and wildlife in tropical ecosystems
   - Disease monitoring, diagnostics, and interventions

iv. **Vaccines, therapeutics, and diagnostics**
   - Development, evaluation, and implementation in tropical health contexts

v. **Environmental and climate-linked health threats**
   - Impact of climate change, deforestation, land-use changes, and pollution on disease transmission

vi. **Food safety, food security, and sustainable agriculture**
- Impacts of climate change and infectious diseases on agricultural productivity and food systems

vii. **Public health policy, governance, and One Health systems**
- Cross-sectoral collaborations, interdisciplinary approaches, and health education
- Socioeconomic and cultural determinants influencing One Health implementation
- Community-based interventions and stakeholder engagement

Conclusion

In conclusion, the journal aims to serve as a leading platform for evidence and dialogue across human, animal, and environmental health in the tropics. We hope JTOH will inspire researchers, practitioners, and stakeholders to share their expertise, increase the visibility of tropical One Health research, and establish the journal as a recognized and influential voice in addressing emerging infectious diseases, zoonoses, antimicrobial resistance, and other critical health challenges. Looking ahead, JTOH seeks to influence policy, promote equity in research and practice, and advance sustainable, integrated One Health solutions across tropical ecosystems.

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SHORT COMMUNICATION

Discovery of a Japanese Encephalitis Virus Nakayama Substrain Highlights the Dual Lessons of Molecular Characterization and Laboratory Biosafety

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ABSTRACT

Japanese encephalitis virus (JEV) remains an important cause of viral encephalitis in Asia, with high mortality and neurological sequelae. During a routine inventory at a Malaysian research institution, an unregistered vial labeled "JEV" was recovered from an unsecured −80 °C freezer. The sample, designated MY8662, was investigated under biosafety level 3 conditions. Inoculation of Vero cells produced cytopathic effects, and real-time RT-PCR confirmed JEV identity. Full-genome sequencing revealed 99.68% nucleotide and 99.88% amino acid similarity to the Nakayama vaccine strain, with 35 nucleotide substitutions resulting in four amino acid changes, including novel substitutions in the envelope and NS4A proteins. Phylogenetic analysis clustered MY8662 closely with Nakayama, suggesting a substrain relationship. Replication kinetics in Vero cells showed exponential viral RNA increase with a doubling time of 2.6 hours, comparable to other JEV strains. The incident also revealed biosafety concerns associated with obscure inventories, prompting corrective measures including access-controlled biorepositories and barcode-based inventory systems. This case illustrates the dual significance of forgotten laboratory materials, providing insights into JEV genomic diversity while underscoring the importance of sustainable biosafety and biosecurity practices. Strengthened institutional oversight and risk-based management are essential to prevent accidental exposures or loss of infectious agents.

Key words: Malaysia, arbovirus, Flaviviridae, zoonosis, biosafety, biosecurity

Introduction

Japanese encephalitis (JE) is the most important viral encephalitis in Asia, with an estimated 67,000 cases annually and significant morbidity and mortality (Campbell et al., 2011). The Japanese encephalitis virus (JEV), a mosquito-borne flavivirus, circulates between birds and Culex mosquitoes, with pigs serving as amplifying hosts. Although humans are dead-end hosts, outbreaks continue to occur across Southeast Asia, including Malaysia (Khor et al., 2020). The Nakayama strain, isolated in 1935, remains a prototype genotype III (GIII) JEV strain and the basis of inactivated vaccines. JEV comprises five genotypes with up to 19% nucleotide divergence; Nakayama's position as a GIII prototype makes it a useful benchmark for molecular comparison (Mansfield et al., 2025). Understanding substrain diversity has implications for virulence and immunity, but equally important is the safe handling of pathogens. Global inconsistencies in dengue virus (DENV) biosafety classification highlight the risks of poor harmonization and the need for transparent, evidence-based risk assessment when managing flaviviruses (Le & Blacksell, 2025). The World Health Organization emphasizes sustainable biosafety practices based on local risk assessments (WHO, 2020). Historical lapses, such as the discovery of forgotten smallpox vials at the US NIH in 2014, highlight the risks posed by inadequate inventory control (CDC, 2014). Here, we describe the recovery and molecular characterization of an undocumented JEV specimen in Malaysia and discuss both its genetic features and biosafety implications.

Materials and methods

In 2009, during a routine inventory reconciliation, an undocumented vial labeled 'JEV' was discovered in an

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unsecured -80 °C freezer. The vial was presumed to have been deposited prior to 2007, as laboratory biosafety protocols had been strengthened following a 2007 incident involving a *Bacillus* species initially suspected to be *Bacillus anthracis*, which required investigation under biosafety level 3 conditions (Loong et al., 2017). Despite these earlier corrective measures, the presence of this forgotten JEV vial highlighted persistent gaps in historical inventory control. The sample was immediately transferred to a biosafety level 3 facility for characterization. A biorisk assessment was conducted, emphasizing that biological safety levels should be set based on procedure-specific risks rather than nominal risk groups (Artika & Ma'roef, 2017). This assessment prioritized controls for aerosol-generating procedures, mandated sealed centrifuge rotors and certified Class II biosafety cabinets for manipulations, and refreshed sharps safety training. Vero cells were inoculated with the specimen and observed for cytopathic effects. Viral RNA was extracted and subjected to SYBR Green I-based real-time RT-PCR targeting the NS3 region (sense-direction: 5'-AGA GCG GGG AAA AAG GTC AT-3' and antisense-direction: 5'-CTT CAC GCT CTT CCT ACA GT-3', producing a 162-bp fragment) to confirm JEV identity. To obtain the complete genome, eleven pairs of overlapping primers were used, and bidirectional sequencing was performed (Supplementary Table S1). Sequences were assembled and aligned with 23 reference JEV strains available in GenBank. Phylogenetic analysis was performed using the maximum likelihood method with 1,000 bootstrap replications. Replication kinetics were determined by sampling infected culture supernatants at eight-hour intervals, quantifying viral RNA copy numbers using RT-PCR against a synthetic RNA standard. Given recommendations to integrate whole genome sequencing into JEV preparedness frameworks, sequencing was used to verify lineage and assess divergence from reference Nakayama sequences (Mansfield et al., 2025). A biorisk assessment of the unregistered vial prompted corrective actions, including enhanced access control, barcode-based inventories, and reconciliation of historical stocks.

Results and discussion

Inoculated Vero cells exhibited cytopathic effects by day four, including cell rounding and detachment, and real-time RT-PCR confirmed JEV identity. The isolate was designated MY8662. Genome sequencing revealed 10,977 nucleotides comprising a 95-nucleotide 5'-UTR, a 10,296-nucleotide open reading frame, and a 586-nucleotide 3'-UTR (Table 1). Pairwise comparison showed 99.68% nucleotide and 99.88% amino acid identity with the Nakayama strain. Thirty-five nucleotide differences produced four amino acid substitutions: prM-Lys16Asn, E-Glu83Lys, E-Ile176Thr, and NS4A-Phe260Val (Table 1). Phylogenetic analysis grouped MY8662 with Nakayama in a distinct branch with strong bootstrap support, suggesting a substrain relationship (Figure 1). Here we use 'substrain' to denote a variant of the prototype Nakayama strain with >99.5% nucleotide identity but consistent, reproducible sequence differences. Replication kinetics were assessed to determine whether the four amino acid substitutions in MY8662 affected viral growth efficiency. RNA copies rose exponentially with a doubling time of 2.6 hours (Supplementary Figure S1), comparable to other JEV strains (Yamaguchi et al., 2011), suggesting that these substitutions do not confer a replication advantage or disadvantage in Vero cells.

Table 1. Comparison of the complete nucleotide and deduced amino acid sequences between MY8662 and Nakayama strain.

Genome segment	Size		Nucleotide substitution		Amino acid substitution	
	Nucleotide	Amino acid	No. of substitution	% substitution	No. of substitution	% substitution
5' UTR	95	0	3	3.16	0	0.00
Capsid	381	127	1	0.26	0	0.00
Membrane	501	167	3	0.60	1	0.60
Envelope	1500	500	8	0.53	2	0.40
NS1	1245	415	5	0.40	0	0.00
NS2A	492	164	0	0.00	0	0.00
NS2B	393	131	1	0.76	0	0.00
NS3	1857	619	2	0.11	0	0.00
NS4A	801	267	3	1.12	1	0.37
NS4B	411	137	1	0.73	0	0.00
NS5	2715	905	3	0.11	0	0.00
3' UTR	586	0	5	0.85	0	0.00
Complete	10977	3432	35	0.32	4	0.12

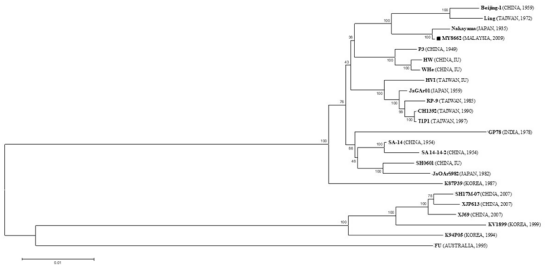

Figure 1. Phylogenetic tree based on complete nucleotide sequences of MY8662 (indicated by ■) and other selected 23 other JEV strains. The scale bar represents the number of nucleotide substitutions per site. Bootstrap confidence limits are shown at each node. The strain name is followed by country and year of isolation. IU – information unknown.

The amino acid substitutions warrant consideration. The prM-Lys16Asn change occurs at a conserved glycosylation site important for virion maturation (Bause, 1983; Kim et al., 2008). The E-Glu83Lys substitution may affect immune recognition, while E-Ile176Thr lies at a position associated with neurovirulence (Arroyo et al., 2001; Singha et al.,

2013). The NS4A-Phe260Val substitution is conservative and likely minimal in effect. These findings support MY8662 as a Nakayama substrain, but functional studies are required to clarify phenotypic significance. Beyond molecular data, the discovery emphasized biosafety challenges. Arbovirus laboratory acquired infections, including dengue, have been reported from aerosol-generating steps and sharps accidents (Artika & Ma'roef, 2017), reinforcing the importance of enhanced containment and sharps safety. The absence of inventory control in this case posed risks similar to those noted in high-profile incidents involving forgotten pathogens (CDC, 2014). Immediate implementation of access-controlled storage, digital inventory systems, and annual training refreshers addressed these hazards. Immediate implementation of access-controlled storage, digital inventory systems, and periodic training refreshers addressed these hazards. These corrective actions exemplify the WHO Laboratory Biosafety Manual (4th ed.) core principles of biorisk management, particularly the hierarchy of controls (e.g., substitution of unsafe practices with engineering controls) and the institutional commitment to sustainable biosafety (WHO, 2020). This also echoes preparedness frameworks recommending harmonized diagnostics and whole-genome sequencing for JEV (Mansfield et al., 2025). In summary, MY8662 demonstrates close genetic similarity to the Nakayama strain with minor substitutions, while its discovery underscores the critical biosafety lesson that undocumented stocks pose dual scientific and safety challenges.

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Conflict of interest statement

The authors declare no conflicts of interest.

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JOURNAL OF TROPICAL One Health
SHORT COMMUNICATION

Absence of *Borrelia* spp. in Urban Pest Animals from Peninsular Malaysia

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ABSTRACT

Urbanization is rapidly influencing zoonotic disease dynamics, yet the presence of *Borrelia* spp., which are the causative agents of Lyme disease and relapsing fever, within Malaysia's high-density urban environments remains poorly characterized. This study provides the first molecular investigation of *Borrelia* prevalence among small mammal hosts in two major urban locations, namely Kuala Lumpur and Penang. Archived spleen DNA samples from 120 selected rodents and shrews were screened using an optimized nested PCR targeting the *flaB* gene, a highly sensitive and specific genetic marker for *Borrelia* identification. Despite the validated efficacy of the assay, all 120 screened samples yielded negative results, confirming an overall prevalence of 0% in the sampled populations. This non-detection contrasts sharply with reports from rural and forested regions of Malaysia, suggesting that urbanization may disrupt the vector-host transmission cycle through altered habitat and microclimate circumstances. These findings provide critical urban baseline data for *Borrelia* surveillance in Peninsular Malaysia and emphasize the need for integrated, One Health approaches, incorporating serological screening and tick vector assessments, to monitor potential shifts in *Borrelia* ecology within Malaysia's expanding urban landscapes.

Keywords: *Borrelia* spp., urban pest animals, urban ecology, zoonotic surveillance

Introduction

Borrelia spp. are tick-borne zoonotic spirochetes that cause infections in humans, specifically Lyme disease (LD) and relapsing fever (RF) (Steere et al., 2016; Madison-Anternucci et al., 2020). These bacteria are adapted to have flagella, and the core filament of the flagella is encoded by flagellin B (*flaB*) gene, which serves as a reliable molecular marker for their identification and characterization (Motaleb et al., 2000; Wodecka, 2011). Southeast Asia has been recognized as a hotspot for emerging zoonotic infections, with recent studies in Malaysia having detected *Borrelia* strains, including those related to LD and RF, in small mammals and ticks collected from rural areas, such as indigenous communities, forests, and oil palm plantations (Khoo et al., 2018; Lau et al., 2020; Mohd-Azami et al., 2023). These findings highlight the continuous prevalence of *Borrelia* spp. in small mammals, including rodents and shrews, as well as the increasing diversity of emerging *Borrelia* species across non-urban Malaysia.

Despite evidence of circulation in rural Malaysia, a significant knowledge gap remains regarding the prevalence and diversity of *Borrelia* in urban environments. Urbanization, which is associated with increased human-animal interactions, creates favorable conditions for urban pests to transmit zoonotic diseases, as these animals frequently live in close proximity to humans (Blasdell et al., 2022). This lack of understanding regarding the presence and transmissibility of *Borrelia* spp. by these animals in urban environments, combined with the possibility of underreporting and underdiagnosis of human *Borrelia* infection, complicates effective disease surveillance, diagnosis, and prevention strategies. Consequently, this poses a serious, unquantified public health threat.

Materials and methods

To address this knowledge gap, we screened archived spleen DNA from rodents and shrews collected in Kuala Lumpur and Penang, the two major urban areas in Peninsular Malaysia, using nested PCR targeting the flaB gene of *Borrelia* spp. The samples, obtained from the Tropical Infectious Diseases Research & Education Centre (TIDREC), Universiti Malaya, were collected under ethical approval from the Institutional Animal Care & Use Committee (IACUC), Universiti Malaya (G8/23122019/11102019-01-R).

Sampling was conducted within Kuala Lumpur (3° 08' 03.2"N, 101° 42' 55.9"E) and Penang (5° 25' 50.8"N, 100° 18' 42.3"E). Between January and October 2023, small mammals (rodents and shrews) were captured from urban habitats, including wet markets, residential and recreational areas, and green patches. Host species identification was confirmed using standard morphometric measurements and conventional PCR targeting the mitochondrial cytochrome *c* oxidase subunit 1 (*CO*1) gene (Robins et al., 2007). Genomic DNA was extracted from spleen tissues using a commercial kit (Nucleospin® Tissue Extraction Kit, Machery-Nagel, Düren, Germany) and stored at -20°C. A total of 120 selected DNA samples (70 from Kuala Lumpur and 50 from Penang) were screened for *Borrelia* species by amplifying a fragment of the *flaB* gene, which was selected due to its high sensitivity and specificity in *Borrelia* detection. The assay employed a two-step nested PCR approach with well-established primer pairs, originally described by Takano et al. (2010). Amplified products were visualized by agarose gel electrophoresis, with 1% agarose in 1x Tris-acetate-EDTA (TAE) buffer stained with SYBR® Safe DNA Gel Stain (Invitrogen). The gels were run at 85 volts (V) for 35 minutes. Successful amplification of the target *flaB* gene was confirmed by the presence of a band at the expected size of 345 bp under ultraviolet (UV) light.

Results and discussion

The host species composition was diverse, with seven different species identified through *CO*1 gene analysis (Table 1). The most abundant species was *Rattus rattus diardii* (n=115; 48.73%), followed by *Rattus norvegicus* (n=57; 24.15%) and *Rattus tanezumi* (n=47; 19.92%). Table 1 provides the full breakdown of host species composition, sampling effort, and molecular detection results by location.

Due to funding limitations, the nested PCR assay targeting the *flaB* gene was performed on randomly selected 120 archived DNA samples from Kuala Lumpur (n=70) and Penang (n=50) using the optimized protocol (Mohd-Azami et al., 2023). The assay was validated in every run, in which the *Borrelia*-positive control consistently yielded the expected ~345 bp amplicon, and the negative control showed no specific amplification. Despite the demonstrated efficacy of the assay, all 120 spleen DNA samples yielded negative results for *Borrelia flaB* gene sequences. The overall prevalence of *Borrelia* species across all screened small mammal populations was 0% (0/120). Representative gel electrophoresis results confirming the non-detection are presented in **Figure 1 (a)** and **(b)**. The current study is one of the first molecular investigations on the prevalence of *Borrelia* spp. in small mammal populations from the major urban centers of Kuala Lumpur and Penang, in Peninsular Malaysia. Despite utilizing a highly sensitive and optimized nested PCR targeting the *flaB* gene, all 120 screened spleen DNA samples yielded negative results, although both positive and negative controls performed as expected. This 0% prevalence is a significant epidemiological finding, indicating that *Borrelia* species associated with small mammal reservoirs may be absent or circulating at a prevalence below the detection limit of our assay in these specific urban areas.

This result contrasts sharply with recent *Borrelia* surveillance in Malaysia. Previous research has consistently confirmed the pathogen's presence in rodents and ticks collected from rural, semi-urban, and forested habitats across Selangor, Sarawak, Johor, and Perak, which includes strains related to both Lyme disease (*B. burgdorferi* s.s.) and relapsing fever (*B. miyamotoi*, *B. theileri*, *B. lonestari*) (Khoo et al., 2018; Lau et al., 2020; Mohd-Azami et al., 2023). The discrepancy between the positive findings in rural areas, forested areas, and natural settings, compared to the non-detection in urban environments such as Kuala Lumpur and Penang, suggests that *Borrelia* has a distinct non-urban epidemiological cycle in Malaysia. The absence of detection is most likely influenced by several ecological factors driven by urbanization. Firstly, habitat fragmentation and the lack of landscape connectivity in dense urban environments may restrict the migration of infected reservoir hosts and tick vectors from forested source areas (Shaw et al., 2024). Secondly, the altered urban microclimate, which is characterized by higher temperatures and lower humidity, is often detrimental to the survival and establishment of *Ixodes* ticks, the primary vectors for *Borrelia* (Heylen et al., 2019). The restricted or complete absence of tick vectors in these highly modified urban environments may therefore inhibit the maintenance of the *Borrelia* transmission cycle. The non-detection must be interpreted in consideration of the study's methodological limitations. Specifically, the temporal snapshot of sampling and the exclusive reliance on spleen tissue may have overlooked the low-level infections that were preferentially sequestered in other organs such as the bladder or skin (Bockenstedt et al., 2020).

Table 1: Host species composition and molecular detection summary for *Borrelia flaB* gene in small mammals from urban Kuala Lumpur and Penang

Location	Host species (n)							Total collected (n)	Total processed (n)	*Borrelia* positive (n)
	Rattus rattus diardii	*Rattus norvegicus*	*Rattus tanezumi*	*Suncus murinus*	*Tupaia glis*	*Rattus tiomanicus*	*Rattus argentiventer*			
Kuala Lumpur	81	2	45	3	6	2	0	139	70	0
Penang	34	55	2	5	0	0	1	97	50	0

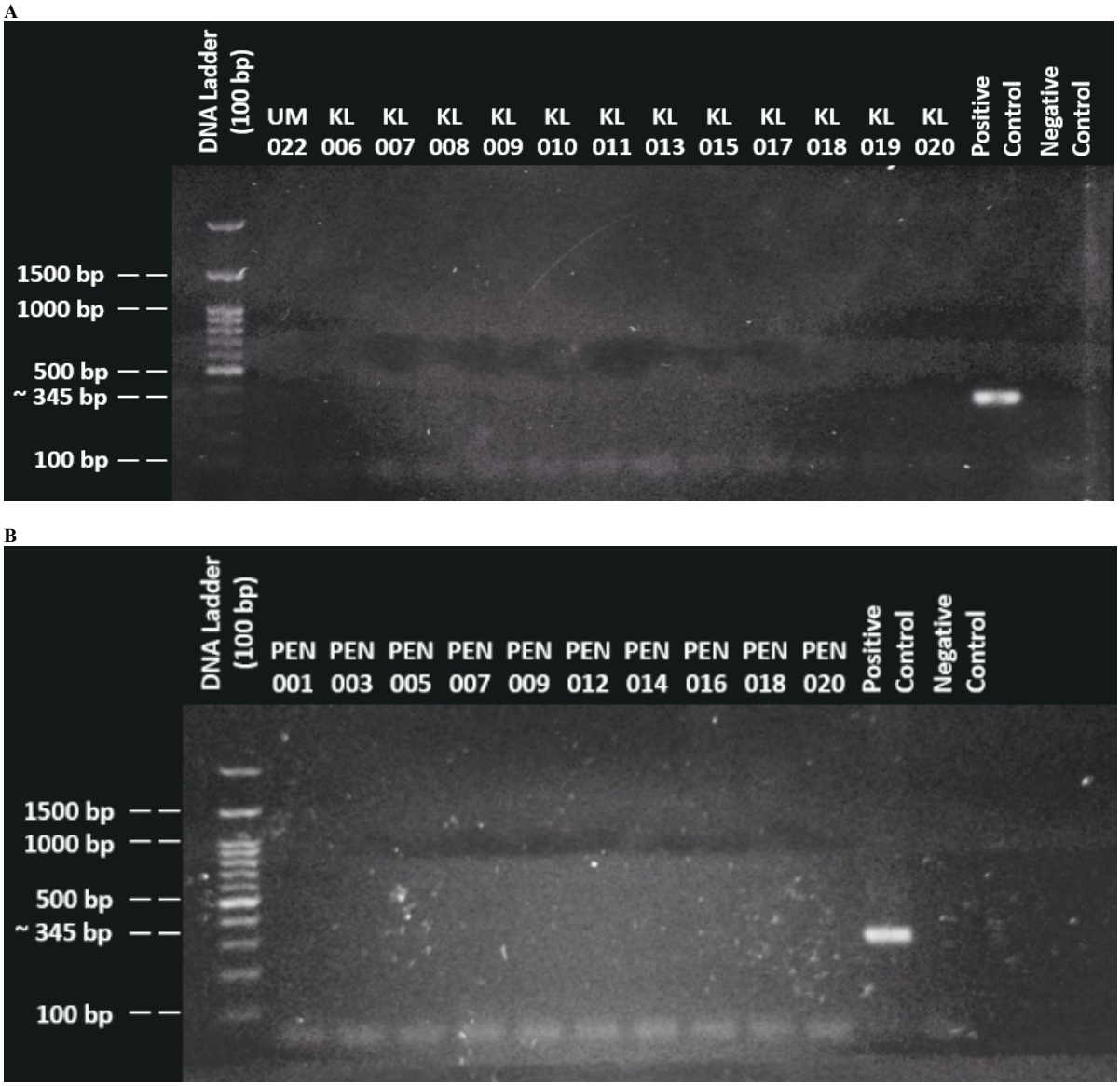

Figure 1 Amplification of the *Borrelia flaB* gene. (A): Representative gel electrophoresis result for nested PCR amplification of *Borrelia flaB* gene from spleen samples collected in Kuala Lumpur. (B): Representative gel electrophoresis result for nested PCR amplification of *Borrelia flaB* gene from spleen samples collected in Penang.

Conclusion

This study establishes baseline data on Borrelia prevalence in urban Malaysia, showing a 0% detection rate in small mammals from Kuala Lumpur and Penang, suggesting that Borrelia may not currently pose an immediate, high-priority public health concern in these urban environments compared to rural or semi-urban areas. This knowledge is essential for directing targeted public health efforts and optimizing resource allocation for zoonotic disease control, while emphasizing the importance of a One Health approach to monitor for the potential shifts in the urban *Borrelia* epidemiology (Mackenzie & Jeggo, 2019). Therefore, future research must adopt an enhanced strategy, including screening substantially larger sample sizes, expanding geographical scope to include peri-urban and rural areas for comparison, and analyzing multiple tissue types to increase sensitivity. Importantly, integrated surveillance must incorporate comprehensive tick collection and serological assays to provide a complete picture of *Borrelia* ecology and risk in Malaysia's rapidly evolving urban landscapes.

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Conflict of interest statement

The authors declare no conflict of interest

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SHORT COMMUNICATION

Modification of the rPLU5 Primer to Address Primer-Template Mismatch in a *Plasmodium* 2 Genus-Specific Nested PCR Assay

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ABSTRACT

A primer-template mismatch was identified at the second nucleotide from the 3′ end of the nest 1 reverse primer (rPLU5) used in the *Plasmodium* genus-specific nested PCR assay developed by Singh et al. (1999). The mismatches near the 3′ end of primer have a strong destabilizing effect on primer annealing. Therefore, rPLU5 was modified (C→T substitution) and nested PCR was optimized using annealing temperature gradients: 55–65 °C for nest 1 and 60–70 °C for nest 2. Optimal amplification was observed at 59–63 °C for nest 1 and 60–66 °C for nest 2, with the lowest temperatures recommended to maximize sensitivity and specificity. Furthermore, the performance of original and modified rPLU5 was compared using *Plasmodium*-positive DNA samples extracted from human clinical specimen and macaque specimen. The original rPLU5 amplified only the macaque sample, whereas the modified rPLU5 successfully amplified both samples, indicating improved detection. However, given the limited number of samples and absence of species identification, this modification should be interpreted as a precautionary optimization to enhance assay robustness rather than a performance limitation of the original rPLU5. Overall, this work presents an optimized nested PCR assay incorporating an improved primer design that minimizes mismatch-related amplification issues.

Keywords: malaria, mosquito, parasite, annealing temperature

Introduction

Plasmodium is a genus of parasitic protozoa that causes malaria in humans and other animals. Transmission occurs through the bite of an infected female *Anopheles* mosquito. The diagnosis of malaria can be based on the parasite detection using microscopy, antigen detection using rapid diagnostic tests or DNA detection using molecular methods (Sato, 2021). Singh et al. (1999) developed a nested PCR assay for detecting *Plasmodium* DNA. Nested PCR involves two sequential PCR amplifications using two primer sets: 1) nest 1 (first PCR amplification) primers amplify a larger region of the target DNA, and 2) nest 2 (second PCR amplification) primers bind within the first amplicon and amplify a smaller internal fragment. This two-step approach enhances sensitivity and specificity by allowing detection of low DNA level and minimizing non-specific products (Green & Sambrook, 2019; Singh et al.). We performed an *in silico* analysis of the *Plasmodium* genus-specific primer sets (Singh et al., 1999) to assess primer-template mismatches against nine *Plasmodium* species. A primer-template mismatch was identified at the second nucleotide from the 3′ end of the nest 1 reverse primer (rPLU5). Previous studies showed that mismatches near the 3′ end of primer have a strong destabilizing effect on primer annealing (Klungthong et al., 2010; Stadhouders et al., 2010). Therefore, we modified the rPLU5 primer, and subsequently optimized the nested PCR setup using the revised primer.

Materials and methods

Small subunit ribosomal RNA (ssrRNA) gene sequences of nine *Plasmodium* species, including *P. coatneyi* (NC_033560), *P. vivax* (NC_009910), *P. cynomolgi* (NC_020397), *P. fieldi* (AB287282), *P. inui* (EU400386), *P. knowlesi* (NC_041784), *P. falciparum* (NC_004326), *P. malariae* (NC_041784) and *P. ovale* (L48987) were retrieved from NCBI database. Multiple sequence alignment of these reference

sequences was generated using ClustalX2 and visualized in GeneDoc. *In silico* analysis revealed a primer-template mismatch at the second nucleotide from the 3′ end of rPLU5 primer, where the cytosine (C) was non-complementary to the adenosine (A) in all *Plasmodium* species (Figure 1). The original rPLU5 primer (5'-CCTGTTGTTGCCTTAAACTCC-3') was thereby modified by substituting the mismatched C with thymine (T). For nest 1 PCR, rPLU1 forward primer sequence was 5'-TCAAAGATTAAGCCATGCAAGTGA-3' (24 bp), and the modified rPLU5 reverse primer sequence is 5'-CCTGTTGTTGCCTTAAACTTC-3' (21 bp), with an expected product size of 1,627–1,720 bp. The C→T substitution in the rPLU5 was underlined. For nest 2 PCR, rPLU3 forward primer sequence was 5'-TTTTTATAAGGATAACTACGGAAAAGCTGT-3' (30 bp), and rPLU4 reverse primer sequence was 5'-TACCCGTCATAGCCATGTTAGGCCAATACC-3' (30 bp), with an expected product size of 233–243 bp. MyTaq Red Mix (Meridian, USA) was used in place of the PCR kit originally referenced by Singh et al. (1999). The nested PCR setup was adapted from Singh et al. (1999) with slight modifications based on the manufacturer's protocol of MyTaq Red Mix kit. The nest 1 PCR reaction mixture with a final volume of 20 µL comprised: 10 µL of 2× MyTaq Red Mix, 0.5 µL of 10 µM rPLU1, 0.5 µL of 10 µM modified rPLU5, 2 µL template DNA (*Plasmodium*-positive DNA sample spiked into human serum) and 7 µL of nuclease-free water. Thermocycling conditions were as follow: an initial denaturation at 95 °C for 4 min; 35 cycles of amplification consisting of denaturation at 95 °C for 30 s, annealing at 55–65 °C for 1 min (55, 57, 59, 61, 63 and 65 °C were tested), and extension at 72 °C for 1 min; and a final extension at 72 °C for 4 min. The nest 1 amplicons were then analyzed by agarose gel electrophoresis, and the amplicon showing a clear target band of expected size (1,627–1,720 bp) was used as DNA template for nest 2 PCR. The nest 2 PCR reaction mixture with a final volume of 20 µL comprised: 10 µL of 2× MyTaq Red Mix, 0.5 µL of 10 µM rPLU3, 0.5 µL of 10 µM rPLU4, 2 µL of DNA template (nest 1 amplicon) and 7 µL of nuclease-free water. Thermocycling conditions were as follows: an initial denaturation at 95 °C for 1 min; 35 cycles of amplification consisting of denaturation at 95 °C for 15 s, annealing at 60–70 °C for 15 s (60, 62, 64, 66, 68 and 70 °C were tested), and extension at 72 °C for 10 s. The nest 2 amplicons were then analyzed by agarose gel electrophoresis. Furthermore, the performance of original and modified rPLU5 primers were compared using *Plasmodium*-positive DNA samples extracted from a human clinical specimen and macaque specimen.

Results and discussion

The presence of single band of expected size (1,627–1,720 bp for nest 1 and 233–243 bp for nest 2) in the gel confirmed successful amplification of *Plasmodium* DNA. The annealing temperature of nest 1 PCR was evaluated at 55, 57, 59, 61, 63 and 65 °C. The amplicon at 55 °C showed a non-specific band (400 bp). A faint target band (1,500–2,000 bp) appeared at 57 °C, accompanied by a non-specific band similar to that observed at 55 °C. From 59 to 63 °C, clear target bands were observed, whereas no amplification was detected at 65 °C (Figure 2A). Sub-optimal annealing temperature results in non-specific amplification, whereas excessive stringency of super-optimal temperature leads to weak or no amplification (Rychlik, Spencer & Rhoads, 1990). Accordingly, the nest 1 amplicons at 59 and 63 °C were selected as templates for subsequent nest 2 PCR. The resulting nest 2 amplicons from both templates revealed similar amplification pattern across all annealing temperatures evaluated (60, 62, 64, 66, 68, and 70 °C). Nest 2 target bands (200–300 bp) were consistently observed from 60 to 68 °C, with a weaker intensity at 68 °C, whereas a non-specific band (400 bp) appeared at 70 °C (Figure 2B). Based on the results, the annealing temperatures of 59–63 °C for nest 1 PCR and 60–66 °C for nest 2 PCR were appropriate. The lowest annealing temperature within range was recommended as it maintains specific amplification without non-specific product, thereby maximizing sensitivity and specificity.

Two *Plasmodium*-positive DNA samples, one from a human clinical specimen and one from a macaque specimen, were tested to compare the performance of the original and modified rPLU5 primers. The comparison was performed using the annealing temperature described by Singh et al. (1999). As the nest 1 PCR produced weak or non-visible band, *Plasmodium* detection was determined based on the presence of target band in the nest 2 PCR. The original rPLU5 successfully amplified the macaque sample but failed to detect the human sample, whereas the modified rPLU5 successfully amplified both samples (Figure 3). This indicates that the primer modification improved detection in the tested samples. However, given the limited number of *Plasmodium*-positive DNA samples and absence of species identification, this modification should be interpreted as a precautionary optimization to enhance assay robustness rather than a performance limitation of the original rPLU5. Further validation using a larger panel of well-characterized *Plasmodium* species is required to confirm its impact on sensitivity and specificity. Overall, this work presents an optimized nested PCR assay incorporating a primer design corrected to reduce potential mismatch issues, thereby supporting more reliable *Plasmodium* detection.

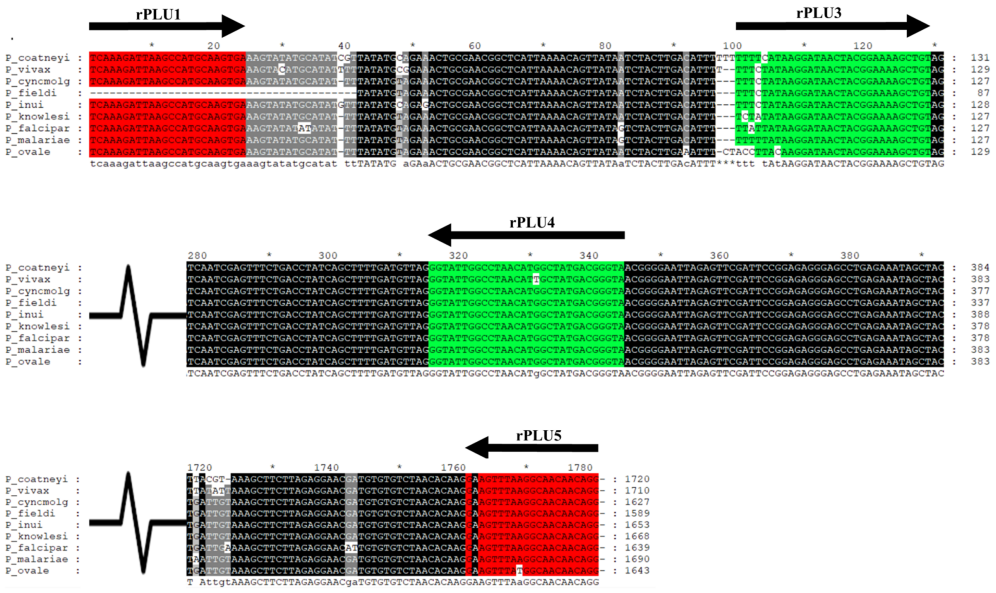

Figure 1. Multiple sequence alignment of nine *Plasmodium* species (*P. coatneyi, P. vivax, P. cynomolgi, P. fieldi, P. inui, P. knowlesi, P. falciparum, P. malariae* and *P. ovale*).
The arrows indicate orientation (5'→3') of nest 1 primers (rPLU1 and rPLU5) and nest 2 primers (rPLU3 and rPLU4). Highlighted nucleotides at the primer binding region indicate primer-template matches, whereas unhighlighted nucleotides indicate primer-template mismatches. The adenosine (A) at alignment position 1,762 in all *Plasmodium* species is mismatched with the 3' end of rPLU5 primer.

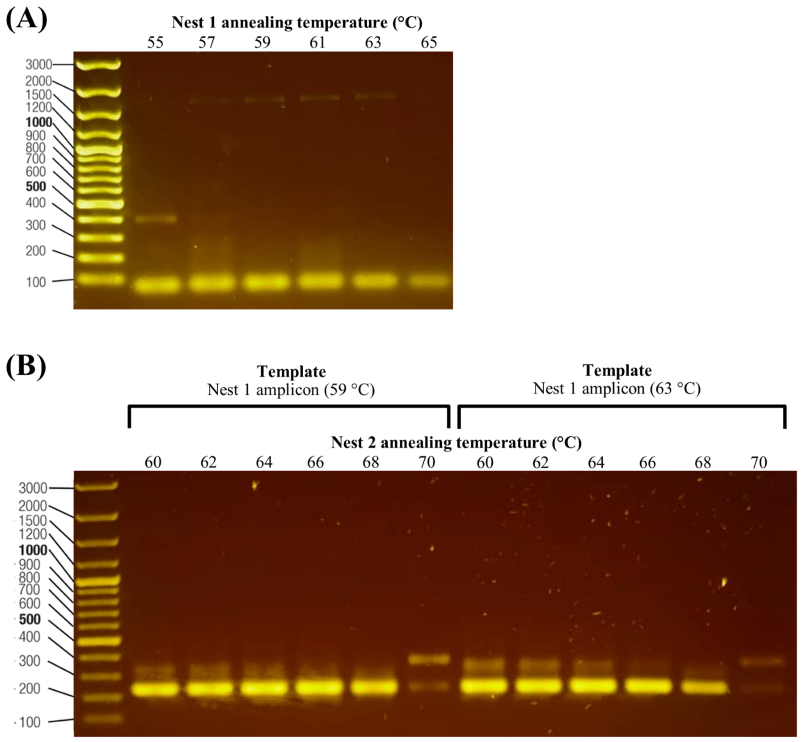

Figure 2. Optimization of annealing temperatures for (A) nest 1 and (B) nest 2 PCR.
(A) Nest 1 amplicons amplified at annealing temperatures of 55, 57, 59, 61, 63 and 65 °C are shown. Clear target bands (1,500–2,000 bp) are observed from 59 to 63 °C, while non-specific bands (400 bp) are visible at 55 and 57 °C. No amplification is detected at 65 °C.
(B) Nest 2 amplicons amplified at annealing temperatures of 60, 62, 64, 66, 68, and 70 °C, using nest 1 amplicons amplified at 59 °C and 63 °C as templates, are shown. Clear target bands (200–300 bp) are observed from 60 to 68 °C, with weaker intensity at 68 °C, while a non-specific band (400 bp) appears at 70 °C.

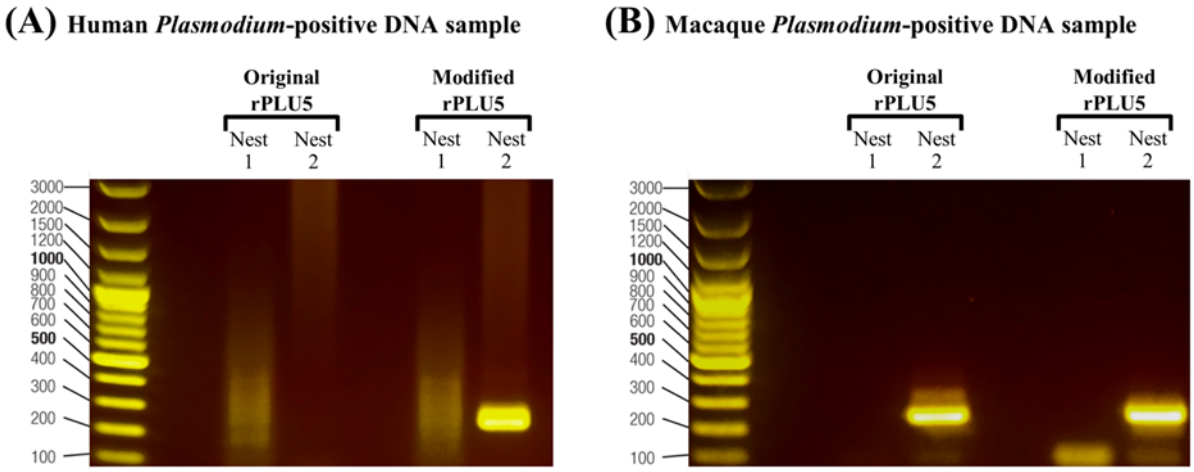

Figure 3. Comparison of performance between original and modified rPLU5 primers.
Nested PCR was performed using *Plasmodium*-positive DNA samples extracted from (A) a human clinical specimen and (B) a macaque specimen. Nest 2 target bands (200–300 bp) are observed in both samples using modified rPLU5 whereas no amplification was detected for human sample using original rPLU5.

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Conflicts of interest statement

The authors declared no conflicts of interest.

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SHORT COMMUNICATION

Immunoinformatics Analysis of Mojiang Virus G Protein Epitopes

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ABSTRACT

Mojiang virus (MojV), a rodent-borne parahenipavirus, is closely related to Nipah and Hendra viruses. Located in the tropical–subtropical Asia emerging-disease hotspot, MojV highlights One Health risks at the wildlife–human interface amid land-use change and climate-driven reservoir shifts. With no vaccines or treatments available, we used immunoinformatics to identify conserved B- and T-cell epitopes in the attachment glycoprotein (G). Four high-scoring, non-toxic, non-allergenic epitopes were prioritised: B-cell epitope LGTGGGGYQVL (385–395), CTL epitope DTTIKPIEY (174–182), CTL/HTL epitope LRFGITPDISVRSTT (465–479), and HTL epitope KDEIWCIAITEGKKQ (572–586). These epitopes offer promising candidates for multiepitope vaccines, improved diagnostics, and cross-henipavirus research. This study provides a framework to support vaccine preparedness against understudied zoonotic threats in tropical and subtropical regions.

Keywords: paramyxovirus, immunoinformatics, emerging zoonotic virus

Introduction

Mojiang virus (MojV) is a zoonotic paramyxovirus first identified in 2012 from *Rattus flavipectus* rats after a cluster of three fatal pneumonia cases among miners in Mojiang, Yunnan Province, China (Wu et al., 2014). Phylogenetically related to the Henipavirus genus within the Paramyxoviridae family, MojV possesses a ~18 kb negative-sense single-stranded RNA genome encoding structural and nonstructural proteins, including nucleocapsid (N), phosphoproteins (P/V/W/C), matrix (M), fusion (F), attachment (G), and large (L) proteins (Wu et al., 2014). Like other parahenipaviruses, MojV entry is mediated by G and F glycoproteins, where G binds host receptors to trigger F-mediated membrane fusion (Da Silva et al., 2021). Located at the tropical–subtropical Asia interface, an established hotspot for emerging zoonoses driven by intensive human–wildlife contact, land-use change, mining activities, and climate-mediated reservoir expansion, MojV exemplifies critical One Health challenges at the animal–human–environment interface. Despite its unclear human pathogenic potential, the virus underscores the growing threat of rodent-associated paramyxoviruses in rapidly changing ecosystems of Southeast and South Asia. Currently, no vaccines, therapeutics, or specific diagnostic tools exist for MojV or related rodent-borne parahenipaviruses. In this context, understanding conserved immunogenic epitopes on the G glycoprotein, the primary target of neutralising antibodies and T-cell responses in parahenipaviruses is essential for advancing serological surveillance, risk assessment, and regional preparedness. Here, we employed immunoinformatics to predict and prioritise safe, antigenic, and conserved B- and T-cell epitopes in the MojV G protein, providing a foundational framework for monitoring and mitigating spillover risks from understudied wildlife-associated viruses in tropical and subtropical emerging-disease hotspots.

Materials and methods

The complete amino acid sequences of Mojiang virus attachment glycoprotein (G) (GenBank accessions: YP_009094095.1, AHM23777.1, and PDB: 5NOP chains A and B) were retrieved from NCBI and PDB databases. Multiple sequence alignment was performed using Clustal Omega. Prediction, screening, and ranking of linear B-cell epitopes, cytotoxic T-lymphocyte (CTL) epitopes, and helper T-lymphocyte (HTL) epitopes were carried out using an immunoinformatics pipeline integrating tools for antigenicity (VaxiJen v2.0), allergenicity (AllerTOP v2.0), toxicity (ToxinPred), immunogenicity (IEDB MHC I Immunogenicity Tool), population coverage

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(IEDB Population Coverage Tool), surface accessibility and topology (TMHMM v2.0), and cross-conservation within Paramyxoviridae (IEDB Conservancy Tool). All analyses followed the workflow described by Ting et al. (2025), with default threshold parameters unless otherwise specified. The final selection of potential epitopes was based on combined high scores, non-allergenicity, non-toxicity, surface exposure, absence of human homology, and conservation across the analysed sequences.

Results and discussion

Multiple sequence alignment of the four available Mojiang virus (MojV) attachment glycoprotein (G) sequences (YP_009094095.1, AHM23777.1, and PDB 5NOP chains A and B) using Clustal Omega revealed 72.4% overall identity, with the highest conservation in the globular head domain and greatest variability in the N-terminal signal peptide and stalk region around residues 164–165 (Figure 1). This pattern is typical of henipaviruses, where the receptor-binding head domain is under strong evolutionary constraint to preserve viral entry function, making it an ideal source of stable, broadly reactive epitopes (Larsen et al., 2025). After selection for toxicity, antigenicity (VaxiJen ≥0.4), allergenicity, immunogenicity, surface accessibility, and conservation within Paramyxoviridae, only 19 epitopes remained safe and immunogenic. From these, four potential candidates were prioritised (Table 1): the dominant helper T-cell epitope KDEIWCIAITEGKKQ (572–586; VaxiJen 1.8937), overlapping CTL/HTL epitope LRFGITPDISVRSTT (465–479; VaxiJen 1.4636), CTL epitope DTTIKPIEY (174–182; VaxiJen 1.3147), and linear B-cell epitope LGTGGGGYQVL (385–395; VaxiJen 0.5193), located in a solvent-exposed loop of the receptor-binding domain and thus promising for neutralising antibodies. All four candidate epitopes were non-toxic, non-allergenic, surface-exposed, and highly conserved, with overlapping CTL/HTL regions likely to drive robust, long-lasting immunity. These epitopes represent critical targets for intervention in the tropical–subtropical Asia hotspot, where mining, deforestation, and climate-driven expansion of Rattus species are increasing the frequency of human–rodent contact (Blasdell et al., 2022; Bai et al., 2023). The 2012 MojV spillover in an abandoned mine highlights a distinct rodent-to-human transmission pathway that complements the bat-driven route of Nipah and Hendra viruses. The identified epitopes, therefore, provide useful markers for (i) broad serological surveillance in at-risk occupational and rural populations, (ii) improved diagnostic differentiation of paramyxovirus-like illness, and (iii) potential cross-protective antigen design, which are critical steps for integrated human–animal–environmental health strategies in dynamic ecosystems. The current study demonstrates that immunoinformatics can deliver rapid, cost-effective solutions for understudied zoonotic threats, supporting proactive regional preparedness against future parahenipavirus spillovers. The findings from this study provide the basis for experimental validation of peptide-MHC binding and immunogenicity assays in the future.

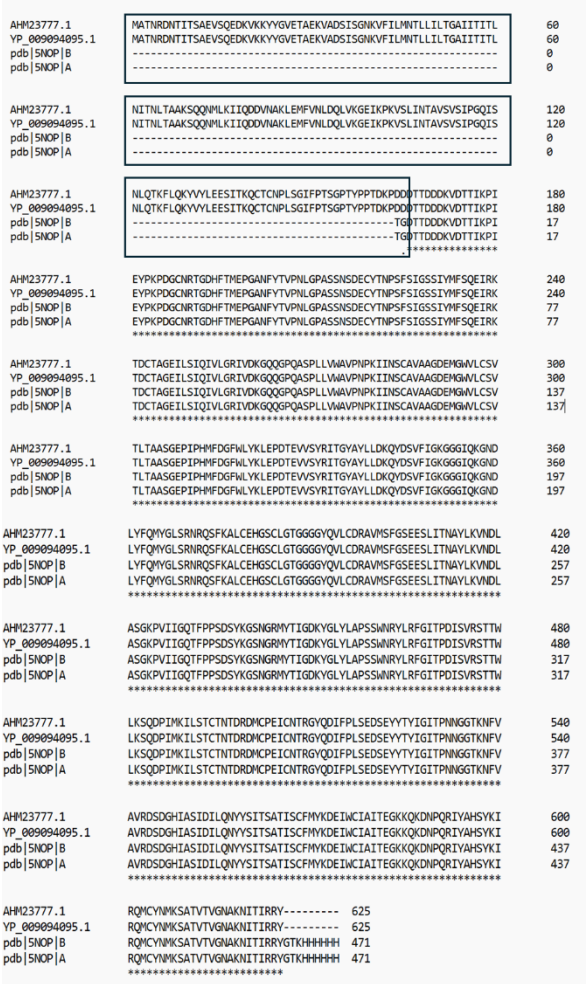

Figure 1. Multiple sequence alignment of the four available Mojiang virus (MojV) attachment glycoprotein (G) sequences (YP_009094095.1, AHM23777.1, and PDB 5NOP chains A and B). The region with the greatest variability in the N-terminal is indicated by a thick black frame, while the other regions are highly conserved.

Table 1. Predicted linear epitopes of MojV G protein and their antigenicity, allergenicity, toxicity, immunogenicity, conservancy and surface accessibility.

	Sequence	Position	Antigenicity	Allergenicity	Toxicity	Immunogenic	Conservancy (≥90%)	Surface accessibility
HTL	KDEIWCIAITEGKKQ	572–586	1.8937	Non-allergen	Non-toxin	Yes	Yes	Outside
CTL/HTL	LRFGITPDISVRSTT	465–479	1.4636	Non-allergen	Non-toxin	Yes	Yes	Outside
CTL	DTTIKPIEY	174–182	1.3147	Non-allergen	Non-toxin	Yes	Yes	Outside
BCL	LGTGGGGYQVL	385–395	0.5193	Non-allergen	Non-toxin	Yes	Yes	Outside

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Conflict of interest statement

The authors declare no conflicts of interest.

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SHORT COMMUNICATION

Gastrointestinal Parasites in Wild Boars from an Indigenous Community Settlement in Sarikei, Sarawak

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ABSTRACT

Wild boars (*Sus scrofa*) serve as significant reservoirs for diverse gastrointestinal parasites capable of infecting both wildlife and domestic pigs and may cause economic and production losses in pig and wild-boar farming.The present study focused on gastrointestinal helminths and protozoa in 23 free-ranging wild boars kept by the indigenous community in Sarikei, Sarawak.This investigation assessed the occurrence of helminth infections in wild boars from an indigenous community settlement in Sarikei, Sarawak, employing both microscopic analysis and molecular using multiplex PCR. All samples exhibited polyparasitism. Strongyles represented the highest percentage of the parasites at 87%, followed by *Eimeria* spp. 65.2%, *Entamoeba* spp. 47.8%, *Ascaris* spp. 34.8%, *Strongyloides* spp. 26.1%, and *Metastrongylus* spp. 8.7%. Multiplex PCR specifically targeting *Oesophagostomum* spp. detected the 330 bp amplicon corresponding to *Oesophagostomum quadrispinulatum* in 11 of the 23 samples (47.8%). These findings demonstrate a substantial gastrointestinal parasite burden and frequent polyparasitism in wild boars kept under free-ranging conditions and may cause economic and production losses in small-scale wild-boar husbandry. *Ascaris* spp. and *Entamoeba* spp., which have potential zoonotic risk, were detected in this study, indicating the need for further investigation in people and domestic livestock in the surrounding community. Enhanced monitoring and an integrated health approach are essential to mitigate transmission risks.

Keywords: Microscopy, PCR, formal-ether concentration technique, wild boar, gastrointestinal parasites

Introduction

Intestinal parasitic infections (IPIs) are widespread among humans and domestic animals, particularly in rural areas of Southeast Asia where sanitation, water quality, and health literacy are often inadequate (Sangaré et al., 2015; Sitotaw et al., 2020). Numerous IPIs are acquired via contaminated food, water or soil and many remain asymptomatic or present with nonspecific symptoms, contributing to underdiagnosis (Hotez et al., 2014).

In Malaysia, numerous indigenous communities reside in secluded rural regions and practice free-range livestock management, allowing wild boars and other animals to roam around houses and waterways. Limited sanitation and proper sewage systems exacerbates environmental contamination and heightens the risk of exposure to parasites (Khor and Zalilah, 2008). Wild boars (*Sus scrofa*), distributed worldwide, serve as notable reservoirs of parasites capable of infecting wild and domestic animals and, for some species, humans (Meng et al., 2009).

Gastrointestinal nematodes, including strongyle-type worms and nodular worms such as *Oesophagostomum* spp. are associated with intestinal pathology, reduced growth and lower productivity in pigs which may cause economic and production losses (Pattison et al., 1979). In Malaysian Borneo, wild boar hunting and small-scale husbandry are longstanding practices among non-Muslim indigenous communities, for whom wild boar meat represents a culturally significant and nutritionally important source of protein (Kurz et al., 2021). Wild boars are commonly encountered near indigenous settlements, where they may be temporarily kept or maintained under semi-captive free-ranging

conditions prior to consumption. This form of free-ranging management, characterised by minimal biosecurity and unrestricted contact between animals and the surrounding environment, creates conditions favourable for parasite acquisition and transmission at the wildlife–human interface (Meng et al., 2009). In rural indigenous communities where wild boars are kept under free-ranging conditions as a source of meat and potential income, heavy parasite burdens in these animals may consequently affect animal health, household-level production and food security.

Accurate identification of these parasites is essential for understanding their epidemiology. Eggs of *Oesophagostomum* spp. are morphologically indistinguishable from those of other strongylids by light microscopy, hence molecular tools are required for species-level identification (Lin et al., 2008). A validated multiplex PCR assay permits the specific detection of *O. dentatum* and *O. quadrispinulatum*, the two principal nodular worms of pigs and allows more precise characterisation of parasite fauna (Lin et al., 2008; Lin et al., 2012). Although these species are not currently recognised as human pathogens, documenting their occurrence in free-ranging wild boars kept as backyard livestock is relevant for veterinary health, small-scale production and baseline description of parasite communities in Malaysia. In addition, parasites such as *Ascaris* spp. and *Entamoeba* spp., which include species of recognised zoonotic concern, may also be present in this setting and warrant further investigation in local human and livestock populations (Hotez et al., 2014; Mohammadi et al., 2004).

Despite the likely importance of these infections, data on gastrointestinal parasitism in Malaysian wild boars remain scarce. This study aimed to characterise the occurrence and coinfection patterns of gastrointestinal helminths and protozoa in wild boars raised by an indigenous community in Sarikei, Sarawak, using microscopy and species-specific multiplex PCR for *Oesophagostomum* spp. and to provide baseline information relevant to animal health and small-scale wild-boar production in this setting.

Materials and methods

Sampling Procedure

A total of 23 stool samples from free range wild boars were collected in an indigenous people village at Sarikei, Sarawak. The collected stool samples were transferred in a sterilized stool container and were stored in 4◦C freezer upon reaching the laboratory until processed. Following collection, samples were aliquoted into 1.5 ml microcentrifuge tubes at Sarikei Hospital, Sarawak. The aliquoted samples were subsequently transported to Kuala Lumpur via domestic flight for laboratory processing and molecular analysis.

Microscopic Screening

The samples were processed using the formalin-ether concentration method (Young et al., 1979), subsequently followed by microscopic examination with Lugol's iodine stain. Parasites were identified by examining their morphological characteristics under 10X and 40X magnification (Otranto & Wall, 2024).

DNA Extraction and PCR Amplification

Genomic DNA was extracted from each stool sample using the NucleoSpin® Soil DNA extraction kit (Macherey-Nagel, Germany) according to the manufacturer's instructions, eluted in 50 µl and stored at −20°C until PCR.

Species-specific multiplex PCR was performed using the primer described by Lin et al. (2008) for the simultaneous identification of *O. dentatum* and *O. quadrispinulatum*. Two primer pairs were combined in a single reaction: OdspF (5′-GCAACAGGTACCTT AGAGCTA-3′) and OdspR2 (5′-TTGCAAAT GACATGAAACTAC-3′), which amplified a 130 bp fragment of the partial ITS-2 of *O. dentatum*, and OqspF (5′-ACTAACGTTTTACATTTGGGA-3′) and OqspR (5′-CATTCGTGTACCTTAGACGTA-3′), which amplified a 330 bp fragment encompassing partial ITS-1, the complete 5.8S rRNA gene and partial ITS-2 of *O. quadrispinulatum*.

Multiplex PCRs were set up in 25 µl reactions with 2× ExPrime Taq Master Mix (GENETBIO Inc., Daejeon, South Korea), with each reaction containing 12.5 µl of 2× Master Mix, 50 pmol of each primer (OdspF, OdspR2, OqspF and OqspR), all four primers and 1 µl of DNA template, and run under the cycling conditions of Lin et al. (2008). PCR products were separated on 2% agarose stained with SYBR Safe DNA Gel Stain (Thermo Fisher Scientific, USA) and visualized under ultraviolet illumination; bands of 130 bp and 330 bp were interpreted as positive for *O. dentatum* and *O. quadrispinulatum* respectively.

Data Analysis

A table was tabulated to summarize the occurrence of the detected parasites. The 95% confidence intervals (CI) for infection estimates were computed using the Wilson score interval, which is appropriate for small sample sizes. Co-infection patterns were categorized into helminth–protozoan, helminth–helminth, and protozoan–protozoan combinations.

Results and discussion

A total of 23 stool samples from wild boar were examined (Table 1). Among all the samples, gastrointestinal nematodes (95.65%) recorded the highest infection rate followed by protozoan (82.61%). According to the species, strongyles was found to be the most abundant parasites in the wild boar, having an infection of 87% (20 out of 23), followed by *Eimeria* spp. 65.2% (15 out of 23), *Entamoeba* spp. 47.8% (11

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out of 23), *Ascaris* spp. 34.8% (8 out of 23), *Strongyloides* spp. 26.1% (6 out of 23) and *Metastrongylus* spp. 8.7% (2 out of 23) (Table 2).
Out of 23 stool samples examined, all samples harbored more than one parasitic nematodes and protozoan. There were no monoparasitism found in this study. Polyparasitism observed can be divided into three categories which are Helminth + Helminth (H+H), Helminth + Protozoa (H+P) and Protozoa + Protozoa (P+P) (Table 2). The polyparasitism between H+P showed the highest infection rate (78.26%) followed by H+H (17.39%) and P+P (4.35%).

Double infection comprised of 5 different pairs of the above mentioned parasites, triple infection included 6 different combinations and quadruple infection comprised of 4 different combinations of parasites. The combination of *Eimeria* spp. and strongyles demonstrated the highest infection (21.7% or 5 out of 23) followed by the co-infection between *Strongyloides* spp. with strongyles (13% or 3 out of 23). There were two combinations which demonstrated 8.7% (2 out of 23) of the studied samples that were the combination infections of *Eimeria* spp., strongyles with *Ascaris* spp. and co-infection between *Eimeria* spp., strongyles, *Ascaris* spp. *with Entamoeba* spp. All the other infection of combinations infection each exhibited 4.4% (1 out of 23) from the entire studied sample.

Species-specific multiplex PCR targeting *O. dentatum* (130 bp) and *O. quadrispinulatum* (330 bp) detected only the 330 bp amplicon, corresponding to *O. quadrispinulatum*, in 11 of the 23 samples (47.8%). No amplification at 130 bp was observed.
This study addressed the limited information on gastrointestinal parasites in Malaysian wild boars and showed a high parasitic burden dominated by strongyle-type nematodes, with four nematode and two protozoan species identified. The significant occurrence of parasitic infection are likely related to free-ranging management system, absence of anthelmintic treatment and inadequate hygiene practices among the indigenous community settlement, which favour environmental contamination and continual exposure of animals to infective stages (Khor and Zalilah, 2008; Hajare et al., 2022).

The overall helminth infection (95.65%) in this study exceeded reports from Russia (25%), Iran (88%) and Thailand (82.41%) (Mansouri et al., 2016; Tabakaeva et al., 2024; Thanasuwan et al., 2024). Conversely, the protozoan infection (82.61%) was also higher than values from Western Iran (67%) and Russia (33.3%) (Mohammadi et al., 2004; Belov et al., 2022). Co-infections were common, with helminth-protozoan combinations most frequent (78.26%), followed by helminth-helminth (17.39%) and protozoan-protozoan (4.35%) co-infections. Such polyparasitism can exacerbate subclinical gastrointestinal disease and may further compromise growth and productivity in wild boars kept for meat (Pattison et al., 1979; Hale et al., 1981).

Species-specific multiplex PCR demonstrated that nearly half of the wild boars were infected with *O. quadrispinulatum*, whereas *O. dentatum* was not detected. *Oesophagostomum quadrispinulatum* is a recognised nodular worm of pigs associated with intestinal lesions and reduced performance in pigs, suggesting that it may also contribute to gastrointestinal morbidity in these free-ranging wild boars (Lin et al., 2012; Pattison et al., 1979). However, not all samples with strongyle-type eggs were positive in the *Oesophagostomum* multiplex PCR, which is expected because the assay targets only *O. quadrispinulatum* and *O. dentatum*, while other strongyle-positive samples may therefore be infected with different strongyle nematodes of wild boars. The strongyle-type infections in PCR-negative samples therefore likely include a mixture of other strongyle nematodes and broader molecular methods would be needed to identify all strongyle species present in these wild boars.

Potential zoonotic parasites were also detected, notably *Ascaris* spp. and *Entamoeba* spp., which include species of zoonotic concern that may be transmitted to humans (Hotez et al., 2014; Mohammadi et al., 2004). Although this study did not assess infections in people or domestic livestock, their presence in wild boar faeces indicates a need for further investigation in the surrounding community and in co-habiting animals. Overall, these findings provide valuable preliminary information on parasite diversity and co-infection patterns in Malaysian wild boars and support the need for expanded sampling, molecular characterisation and integrated parasite control strategies in similar rural settings.

Table 1. Overall infection of gastrointestinal parasites in wild boar

Parasitic infection	**Microscopy**		
	N	**%**	**95% CI**
Helminth	**22**	**95.65**	**87.32-100**
Strongyles	20	86.97	73.71-100
Ascaris spp.	8	34.78	15.61-53.95
Strongyloides spp.	6	26.09	8.59-43.59
Metastrongylus spp.	2	8.70	0.00-20.59
Oesophagostomum quadrispinulatum (PCR)*	11	47.83	27.47-68.19
Protozoa	**19**	**82.61**	**67.12-98.10**
Eimeria spp.	15	65.22	45.75-84.69
Entamoeba spp.	11	47.83	27.47-68.19
Overall infection	**23**	**100**	**-**

Table 2. Types of gastrointestinal parasites in wild boar with polyparasitism

Type of Parasitsm	n	%	95% CI
Helminth+Helminth	4	17.39	6.75-36.7
Double Infection	4	17.39	6.75-36.7
Strongyles + *Metastrongylus* spp.	1	4.35	0.67-20.50
Strongyles +*Strongyloides spp.*	3	13.04	4.52-32.08
Helminth + Protozoa	18	78.26	57.82-90.17
Double infection	6	26.09	12.49-46.38
Eimeria spp. + *Strongyle*s	5	21.74	9.83-42.18
Ascaris spp. + *Entamoeba* spp.	1	4.35	0.67-20.50
Triple infection	7	30.43	15.29-50.43
Eimeria spp. +*Strongyles* + *Strongyloides* spp.	1	4.35	0.67-20.50
Eimeria spp. + *Strongyles* + *Entamoeba* spp.	1	4.35	0.67-20.50
Eimeria spp. + *Ascaris* spp. + *Entamoeba* spp.	1	4.35	0.67-20.50
Strongyles + *Entamoeba* spp. + *Strongyloides* spp.	1	4.35	0.67-20.50
Strongyles + *Ascaris* spp. + *Entamoeba* spp.	1	4.35	0.67-20.50
Eimeria spp. + *Strongyles* + *Ascaris* spp.	2	8.70	2.55-27.18
Quadruple Infection	5	21.74	9.83-42.18
Strongyles + *Ascaris* spp. + *Entamoeba* spp. + *Metastrongylus* spp.	1	4.35	0.67-20.50
Eimeria spp. + *Strongyles* + *Ascaris* spp. + *Strongyloides spp.*	1	4.35	0.67-20.50
Eimeria spp. + *Strongyles* + *Entamoeba* spp. + *Strongyloides spp.*	1	4.35	0.67-20.50
Eimeria spp. + *Strongyles* + *Ascaris* spp. + *Entamoeba* spp.	2	8.70	2.55-27.18
Protozoan + Protozoan	1	4.35	0.67-20.50
Double infection	1	4.35	0.67-20.50
Eimeria spp. + *Entamoeba* spp.	1	4.35	0.67-20.50

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Conflict of interest statement

The authors declare no conflict of interest

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