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BRIEF REPORT

Tracking *Plasmodium knowlesi* through faecal DNA for monitoring zoonotic transmission in wild macaques across Southeast and South Asia

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We conducted the non-invasive surveillance of *Plasmodium knowlesi* in wild macaques using 4,752 faecal samples collected across nine endemic countries. Parasite DNA was detected in 390 samples (8.2%), with positivity rates ranging from 1.4% to 18.4%. This provides the first field-based evidence that *P. knowlesi* DNA in faeces shed by macaques and present under natural conditions can be detected. These findings validate faecal sampling as a practical and scalable tool for tracking zoonotic-malaria. The results support integration into forest-runoff and rural wastewater surveillance systems, offering new opportunities for early detection of pathogens and environmental monitoring at the human–wildlife interface.

Keywords: *Plasmodium knowlesi,* macaque, faecal surveillance, zoonosis, environmental surveillance

INTRODUCTION

Zoonotic malaria caused by *Plasmodium knowlesi* has emerged as a growing public health threat in Southeast and South Asia, particularly at the human–wildlife interface[1]. Across the Asia-Pacific region, human malaria cases have declined due to effective control measures and targeted interventions[1]. However, human *P. knowlesi* infections have been increasingly reported throughout Asia, with rising incidence and cumulative case numbers, especially among indigenous populations living in forest-fringe communities[1].

Southeast Asia is home to a rich diversity of non-human primates, including 57 monkey species, 18 gibbon species, and three ape species[1]. The predominant natural reservoirs of *P. knowlesi*, long-tailed macaques (*Macaca fascicularis*) and southern pig-tailed macaques (*M. nemestrina*), are highly sympatric and widely distributed throughout the region[1-3]. *P. knowlesi* prevalence in these primates is highly heterogeneous, with reported infection rates ranging from 0% to over 80% in endemic areas[1-3]. The increasing incidence of human *P. knowlesi* cases is strongly associated with deforestation, mining, and other land-use changes, which bring humans into closer contact with vector and reservoir populations[1]. Residence and movement near forest edges are associated with elevated exposure to infected mosquito vectors, raising the risk of sustained transmission.

Traditional surveillance systems rely heavily on human case reporting and invasive sampling of macaques, which are logistically challenging, ethically constrained, and often unfeasible in dense forest or remote rural regions[1, 4-7]. In recent years, environmental surveillance has emerged as a promising proxy for pathogen monitoring, offering earlier detection at lower cost, especially in low- and middle-income countries (LMICs)[8-10]. In forest-adjacent communities, surface water runoff and greywater outflows represent an opportunity to monitor pathogen carriage through environmental DNA within the local non-human primate community for the purpose of estimating zoonotic malaria risk to humans. However, a key limitation in expanding wastewater surveillance for *P. knowlesi* is the lack of understanding regarding the amount of parasite DNA excreted in feces by infected macaques under natural conditions. The amount of *P. knowlesi* DNA present in

faecal material may directly influence the feasibility of using environmental water sources as surveillance substrates. To date, no real-world data exist on the presence and prevalence of *P. knowlesi* DNA in wild macaques' faeces; available information is limited to small-scale, controlled studies in captive or experimentally infected animals[6].

To address this critical gap, we conducted the first large-scale, non-invasive surveillance of *P. knowlesi* in wild macaques across nine countries, using faecal samples collected from diverse ecological zones. Our approach improves on existing monitoring as it achieves parasite detection without animal handling, allowing for broader, more ethical, and repeated surveillance. Importantly, this faecal-based surveillance paradigm provides a scalable model for integrating wildlife pathogen detection into wastewater and runoff-based monitoring systems, supporting proactive surveillance in zoonotic malaria hotspots.

METHODS

Between July and August 2024, we conducted non-invasive faecal surveillance of *Plasmodium knowlesi* in wild macaques across nine endemic countries in Southeast and South Asia (Figure 1a) (Myanmar, Vietnam, India, Indonesia, Cambodia, Philippines, Thailand, Lao PDR, and Malaysia). Sampling was conducted in forested areas and adjacent human–wildlife interface zones, particularly around forest-edge rural communities where macaques frequently interact with human settlements. Fresh faecal droppings were identified by direct observation or by locating deposits on the forest floor, rocks, tree branches, or canopy structures that macaques commonly used for movement or rest. Only visibly fresh faecal material, assumed to have been deposited within the previous 12–24 hours, was sampled. A total of 4,752 faecal samples were collected across all sites (Supplementary Material). All samples were analyzed using a quantitative real-time PCR (qPCR) targeting 18S rRNA gene with *P. knowlesi*-specific probe [7]. All qPCR-positive samples ($Ct < 40$) were subjected to amplification, Sanger sequencing of the 18S rRNA gene to confirm parasite identity, and phylogenetic relationships were inferred using maximum-likelihood analysis (GTR model, 1,000 bootstrap replicates) against reference sequences from GenBank[11, 12]. The study protocol was reviewed by the Institutional Review Board at Yamagata Prefectural Central Hospital (Yamagata, Japan) and deemed exempt from ethical approval, as it involved environmental surveillance and the non-invasive collection of samples from free-ranging environments without direct contact or disturbance. Full protocol details are provided in the Supplementary Methods.

RESULTS

Among 4,752 faecal samples collected across nine countries, 390 samples tested positive for *Plasmodium knowlesi* DNA, resulting in an overall positivity rate of 8.2%. Country-level positive detection rates varied among countries with 17.2% in Lao PDR, 14.5% in Myanmar, 13.4% in Malaysia, 8.5% in Thailand, 8.1% in Cambodia, 6.7% in the Philippines, 3.9% in Indonesia, 2.9%

in Vietnam, and 2.5% in India (Figure 1b). Further analysis revealed varying *P. knowlesi* DNA positivity rates across sampled provinces and states within each country (Figure 1b). In Myanmar, positivity rates ranged from 11.6% in Sagaing Region to 18.4% in Rakhine State. Other observed rates included 12.1% in Kachin, 17.1% in Tanintharyi, and 13.2% in Mon State. In Vietnam, positivity ranged from 1.6% in Khanh Hoa to 4.1% in Phu Yen, with intermediate rates observed in Ninh Thuan (1.7%), Quang Tri (3.6%), and Binh Thuan (3.5%). In India, faecal samples from the Andaman and Nicobar Islands yielded a positivity rate of 2.5%.

In Indonesia, positivity rates across different provinces ranged from 3.1% in Aceh to 5.3% in Jambi, with values of 3.4% in North Sumatra and South Kalimantan, 3.6% in North Sulawesi, and 4.4% in Central Kalimantan. In Cambodia, samples from Battambang and Pailin Provinces showed positivity rates of 8.0% and 8.2%, respectively. In the Philippines, *P. knowlesi* DNA was detected at 8.5 % in Palawan. In Thailand, positivity rates varied across multiple provinces, including Tak (9.1%), Trat (9.3%), Ranong (5.0%), Prachuap Khiri Khan (6.0%), Mukdahan (7.6%), Surin (6.9%), Phatthalung (7.8%), Chumphon (7.8%), Chanthaburi (8.8%), Surat Thani (6.3%), Phang Nga (5.3%), Kanchanaburi (8.0%), Pattani (11.8%), Songkhla (14.3%), and Narathiwat (13.6%). In Laos PDR, rates were 16.7% in Savannakhét and 17.7% in Louangnamtha. In Malaysia, Sarawak and Sabah showed positivity rates of 13.6% and 13.3%, respectively. 18S rRNA gene concentrations were estimated per millilitre of original sample, calculated from Ct values using a standard curve with volume correction. Median 18S rRNA gene concentrations among qPCR-positive samples ranged between 91.9 (Myanmar) and 121.3 (India) copies/mL with other countries in between. A phylogenetic analysis (Figure 1d) demonstrated that the sequences from environmental faecal samples grouped with genome sequences of human and wild macaques across nine endemic countries in Southeast and South Asia. Samples from the same or neighbouring countries (i.e. sequences from geographically proximate regions) particularly those sharing ecological and forested border zones were often grouped closely suggesting a regional connection.

DISCUSSION

This study provides the first real-world evidence that *Plasmodium knowlesi* DNA can be detected in faecal samples from wild macaques across a broad geographic range in Southeast and South Asia. The ability to detect parasite DNA in faeces, without the need for invasive animal handling, represents a critical advance in zoonotic malaria surveillance, particularly in remote, logistically challenging, or ethically sensitive regions. Our results show that *P. knowlesi* is being shed in faecal material at detectable levels under natural conditions, and at positivity rates consistent with known ecological risk zones for human–macaque–mosquito interactions[1, 3, 5, 13-15]. Notably, the majority of our faecal sampling sites were in areas where naturally acquired human *Plasmodium knowlesi* infections have previously been reported [1-3, 13-15]. Many of our sampling locations, such as Kachin and Tanintharyi in Myanmar, Tak and Prachuap Khiri Khan in Thailand, Palawan

in the Philippines, and Sarawak in Malaysian Borneo, have also been associated with human cases of *P. knowlesi* reported in the past[1-3, 13-15]. Samples from these places supports the ecological link between macaque reservoir infection and human zoonotic transmission.

The detection of *P. knowlesi* DNA in environmental faeces has direct implications for wastewater-based and runoff-integrated surveillance systems. In forested regions and rural fringe communities, surface runoff, collected from soils, leaf litter, rocks, tree branches, and other areas frequented by macaques, can carry residual faecal matter into streams, drainage canals, and open greywater systems. This creates an opportunity to passively monitor wildlife-derived pathogen signals in the environment, using techniques adapted from wastewater epidemiology. However, translation from faecal detection to environmental DNA (eDNA) surveillance in runoff or wastewater must account for dilution and degradation . In open environments, parasite DNA concentrations are likely reduced by hydrological dispersion, UV exposure, temperature, and microbial activity. Detection would therefore require larger-volume filtration or molecular concentration methods, potentially supplemented by passive samplers deployed over time to accumulate dilute DNA signals. Furthermore, the proximity of macaques to small rural communities raises the possibility that integrated human–wildlife wastewater streams could serve as a viable matrix for simultaneous surveillance of zoonotic pathogens, including *P. knowlesi* and our findings provide the necessary groundwork for implementing this strategy. The positivity rates observed in wild macaque faeces across nine countries (2.5%–17%) indicate that *P. knowlesi* DNA is shed at detectable quantities under natural field conditions. However, shedding intensity may fluctuate depending on ecological conditions, host infection dynamics, and local transmission intensity [4, 6, 7]. Consequently, whether these concentrations remain detectable following environmental dilution and degradation requires empirical validation in field-based eDNA studies. These data are in line with previous studies in captive macaques under controlled experimental infections, where *P. knowlesi* DNA has been detected in faeces, albeit at lower sample sizes and without environmental complexity [6]. By replicating and extending these findings to wild, free-ranging animals across natural habitats, our study confirms the ecological validity of faeces-based detection.

Importantly, the spatial resolution offered by our dataset, including subnational variation in positivity rates and phylogenetic clustering, further supports the utility of faecal surveillance to identify localized hotspots of parasite circulation. This granularity could help target vector control or diagnostic interventions in areas of heightened risk, especially where human cases are underreported due to poor healthcare access or misdiagnosis.

In summary, this study not only fills a critical gap in our understanding of *P. knowlesi* faecal shedding under natural conditions, but also establishes a scalable, ethical, and field-adaptable platform for monitoring zoonotic malaria at the human–environment–wildlife interface. As environmental DNA tools continue to expand in scope, integrating faecal surveillance into broader wastewater and runoff monitoring systems could play a transformative role in early warning systems for zoonotic spillover in the Asia-Pacific region and beyond.

Conflicts of interest: The authors declare that they have no known potential conflict of interest or competing financial or non-financial interest in relation to the manuscript.

Data availability statement: The authors confirm that the data supporting the findings of this study are available within this article and its additional information. All genome sequences and associated metadata in this study are published in the NCBI GenBank (Accession no: PX532648 - PX533034).

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Ethical Approval: The Institutional Review Board at Yamagata Prefectural Central Hospital (Yamagata, Japan) reviewed the study protocol and determined that ethical approval was not required, as the project qualified as non-invasive environmental surveillance. Ethical clearance or formal waivers were also obtained, as applicable, from relevant institutional or governmental authorities in each participating country.

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Inclusion and diversity: We, the authors of this paper, embrace inclusive, diverse, and equitable conduct of research. Our team comprises individuals who self-identify as underrepresented ethnic minorities, gender minorities, members of the LGBTQIA+ community, and individuals living with disabilities. We actively promote gender balance in our reference list while maintaining scientific relevance.

Author contributions: D.L.W., M.A., C.H., P.H., H.H., P.P., and S.A. conceived the study, acquired funding, and supervised the investigation. D.L.W., M.A., C.H., P.P., and P.H. contributed to data curation, formal analysis, and writing of the original draft. D.L.W., M.A., P.P., C.H., C.M., B.C., K.M., L.C., S.F., A.T.H., S.L., A.C.S., N.K.D.R., T.S.H., K.S., N.N., S.H.N., Y.S., P.O., P.K., R.T., A.K., C.E., L.R., and W.K.C.P.W. were responsible for data collection, technical support, supervision, and manuscript review. Ö.K., T.K., P.G.H., T.A., A.R.Z.R, A.Ki., S.T., P.H., A.Kh., D.S., K.S., S.A., and H.H. provided technical support, resources, and critical review of the manuscript. Ö.K., T.K., P.G.H., T.A., A.Ki., S.T., P.H., A.Kh., D.S., K.S., S.A., X. Y., and H.H. supervised technical work for detection and environmental surveillance, contributing to critical review and editing. Y.W. designed the technical aspects of the drone transport system. T.C., J.Z., H.M., J.J.V.B., Ö.K., T.K., P.G.H., T.A., A.Ki., S.T., P.H., A.Kh., D.S., K.S., S.A., and H.H. contributed to supervision, conceptualization, critical review, and manuscript editing. All authors read and approved the final manuscript.

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FIGURE HEADINGS

Figure 1. Geographical distribution, prevalence, and phylogenetic relationships of Plasmodium knowlesi detected in environmental faecal samples across nine Asian countries. (a) Sampling locations across Myanmar, Vietnam, India, Indonesia, Cambodia, the Philippines, Thailand, Lao PDR, and Malaysia. (b) *P. knowlesi* positivity rates varied by country and province/state (c) Distribution of faecal 18S rRNA gene concentrations (copies/mL) among qPCR-positive samples by country. Boxes represent the interquartile range (IQR), with the central line indicating the median. Whiskers extend to 1.5× the IQR from the first and third quartiles. Points beyond the whiskers are shown as individual outliers, (d) Phylogenetic clustering of *P. knowlesi* sequences from environmental faecal samples alongside human and macaque isolates.