Indigenous *Plasmodium ovale* Malaria in Bangladesh

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Abstract.   In spite of the high prevalence of malaria in Southeastern Bangladesh, there remains a significant shortage of information regarding the presence of three of five human malaria parasites: *Plasmodium ovale*, *P. malariae*, and *P. knowlesi*. The presence of *P. ovale* and *P. knowlesi* has previously never been reported from Bangladesh. We used a genus- and species-specific nested polymerase chain reaction, targeting highly conserved regions of the small subunit ribosomal RNA (SSU rRNA) gene, to investigate the presence of malaria parasites in a total number of 379 patient samples in a survey of patients with febrile illnesses in the Chittagong Hill Tracts in Southeastern Bangladesh. We identified the first cases of *P. ovale* in Bangladesh. They were confirmed by sequence analysis; 189 of 379 samples (49.9%; 95% confidence interval = 44.9–54.9%) were positive for *Plasmodium* sp. by PCR. *P. falciparum* monoinfections accounted for 68.3% (61.3–74.5%), followed by *P. vivax* (15.3%; 10.9–21.2%), *P. malariae* (1.6%; 0.5–4.6%), *P. ovale* (1.6%; 0.5–4.6%), and mixed infections (13.2%; 9.1–18.8%). We found no evidence of *P. knowlesi* in this region.

INTRODUCTION

Almost 1 million people die of malaria every year, and recent reports of emerging artemisinin resistance in Southeast Asia will add another challenge to malaria control efforts.[1–3] High standards in the diagnosis of the malaria-causing *Plasmodium* species are essential to control and adequately treat malaria. Despite its known limitations, microscopy remains the gold standard of malaria diagnosis in the field and frequently, not even microscopy is available in resource-limited environments. This may lead to a significant underestimation of the true malaria burden, especially of less prevalent and less documented species such as *P. ovale*, *P. malariae*, and *P. knowlesi* in Asia.

The Chittagong Hill Tracts in Southeastern Bangladesh are known to be highly endemic for *P. falciparum* with reported resistance to chloroquine and sulfadoxine/pyrimethamine.[4] Virtually all malaria infections were previously attributed either to *P. falciparum* or *P. vivax*.[5–8] Recent surveillance studies based on the diagnosis with rapid diagnostic tests (RDTs) indicate *P. falciparum* as the dominant species in malaria-endemic districts of Bangladesh with a country-wide prevalence of 3.58% (compared with only 0.21% for *P. vivax*).[9] The existence of infections with *P. malariae* with a prevalence of 1% in Bangladesh was first reported from a study conducted in the Chittagong Hill Tracts.[10] Both *P. ovale* and *P. malariae* are typically found at very low prevalence in Southeast Asia.[11] *P. ovale* is known to be endemic in subSaharan Africa, the Middle East, Irian Jaya. and Papua New Guinea, but using the availability of polymerase chain reaction (PCR)-based techniques for the diagnosis of malaria, this parasite has recently also been reported from a number of countries in South and Southeast Asia.[12–16] So far, infections with *P. ovale* have not been reported from Bangladesh.

In recent years, human cases of *P. knowlesi* infections have been reported from Southeast Asia, especially by the Kuching group in Malaysia, and although originally classified as a sim-

ian malaria parasite, *P. knowlesi* is now generally recognized as the fifth human malaria species.[17] PCR-confirmed cases have been documented in Malaysia, Thailand, Myanmar, Singapore, and the Philippines.[18–24] *P. knowlesi* has a daily (quotidian) asexual cycle, potentially resulting in life-threatening hyperparasitemia and hepatorenal dysfunction. A number of fatal cases have been reported from Malaysia.[19] Identification of *P. knowlesi* solely based on microscopy remains difficult.[18,25] Several Macaque species, including the traditional hosts of *P. knowlesi*, have their habitats in Bangladesh, and populations of critically endangered *Macaca fascicularis* are known to be endemic in the very southeastern parts of the country.[26] The limited distribution of mosquitoes of the *Anopheles leucosphyrus* group, the only known vector of *P. knowlesi*, restricts the current distribution of *P. knowlesi* to a limited area in Southeast and parts of South Asia, including the Chittagong Hill Tracts in Bangladesh.[27,28]

The primary aim of this study was to establish the prevalence of all five malaria species among febrile patients in the Chittagong Hill Tracts in Bangladesh with special emphasis on the three rare malaria species.

MATERIALS AND METHODS

Study population. Diagnostic samples were collected from febrile patients in the course of field surveys in Bandarban District in the Chittagong Hill Tracts in 2007/2008 and a hospital-based survey at the Malaria Research Initiative Bandarban (MARIB) field site in 2008/2009. Male and female volunteers of any age with acute fever or a history of fever within the past 72 hours were included in this study. Venous blood was only drawn from patients 8 years or older. Written informed consent was obtained from all study participants or their legal representatives before blood samples were collected. The study protocol was reviewed and approved by the Ethical Review Committee of the International Center for Diarrhoeal Disease Research, Bangladesh.

Malaria diagnosis. Thick and thin blood smears were prepared and examined in duplicate by two expert microscopists blinded to each other's results after staining with Giemsa (Merck KGaA, Darmstadt, Germany). In thick films,

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200 oil-immersion fields were screened before declaring a slide negative. If the parasite count on the thick film was too numerous to count, the number of parasites per 2,000 red blood cells was counted on the thin film. RDTs (FalciVax; Zephyr Biomedicals, Goa, India) based on the detection of *P. falciparum*-specific histidine-rich protein 2 (HRP2) and *P. vivax*-specific lactate dehydrogenase (pLDH) were used in 357 patients.

Blood sample collection for PCR. From all patients over the age of 8 years, 100 µL of venous whole blood and from children below 8 years, two drops of finger-prick blood were transferred onto filter paper (903; Schleicher & Schuell BioScience GmbH, Dassel, Germany). A total of 379 filter papers were prepared in duplicate, air dried at room temperature, and stored in airtight containers at 4°C until further processing by PCR.

DNA isolation and purification. A modified chelex-based DNA extraction method using the InstaGene Whole Blood Kit (Bio-Rad Laboratories, Hercules, CA) was used for the extraction and purification of *Plasmodium* DNA from the blood spots on filter paper.

To ensure comparable quantities of blood, samples of exactly 4 mm in diameter were punched out of the blood spots. Blood spots were soaked overnight in 100 µL phosphate-buffered saline (PBS) at 4°C, and the DNA extraction was performed on the following day as previously described.[29,30] The resulting supernatant was purified with InstaGene Matrix two times.

Parasite detection by nested PCR. Nested PCR assays were performed as described previously.[31,32] The specific primers bind in highly conserved regions of the small subunit ribosomal RNA (SSU rRNA) gene that was used for the detection of genus- and/or species-specific *Plasmodium* DNA.

In the nested PCR, we used the genus-specific primers rPLU1 and rPLU5 for the first PCR and rPLU3 and rPLU4 for the second PCR. Whenever this genus-specific nested PCR gave positive results, species-specific Nest 2 PCRs were performed for species determination using the following internal primer pairs: rFAL1 and rFAL2 for *P. falciparum*, rVIV1 and rVIV2 for *P. vivax*, rMAL1 and rMAL2 for *P. malariae*, rOVA1 and rPLU2 for *P. ovale*, and Pmk8 and Pmkr9 for *P. knowlesi*.[18,31,32] The oligonucleotides were obtained from Microsynth (Microsynth AG, Balgach, Switzerland).

We used a template of 5 µL DNA in a 50 µL reaction (GoTaq PCR Core System; Promega, Madison, USA) for the first amplification and 5 µL Nest 1 product in 50 µL reactions for the amplification in Nest 2 (GoTaq PCR Core System). In all experiments, a negative control of water and known positive controls were run with the samples. The PCR products of Nest 2 amplifications were analyzed by gel electrophoresis with 2% agarose and ethidium bromide staining. The individual interpreting the PCR results was blinded to the results of microscopy and RDT.

Sequence analysis. The amplicons of the *P. ovale*-positive samples from the nested PCR were further analyzed by sequencing for species confirmation. PCR products were purified using the Xact DNA Gel Extraction Kit (GenXpress, Wiener Neudorf, Austria) and sequenced using the BigDye Sequencing Kit and an automatic 310 ABI PRISM sequencer (PE Applied Biosystems, Weiterstadt, Germany).

RESULTS

A total number of 189 of 379 [49.9%; 95% confidence interval (CI) = 44.9–54.9%] filter papers from patients with febrile

illnesses gave positive results for *Plasmodium* spp. with genus-specific primers by nested PCR. All 159 samples classified as positive by microscopic examination were confirmed as being positive. In addition, 30 samples that were diagnosed as negative by microscopy were positive in the genus-specific nested PCR. Species-specific PCR showed that of 189 *Plasmodium* spp. positive samples, 154 (81.5%; 95% CI = 75.3–86.4%) were positive for *P. falciparum*, 50 (26.5%; 95% CI = 20.7–33.2%) were positive for *P. vivax*, 7 (3.7%; 95% CI = 1.8–7.4%) were positive for *P. malariae*, and 3 (1.6%; 95% CI = 0.5–4.6%) were positive for *P. ovale*. All samples tested negative for *P. knowlesi*.

We found 164 (86.8%; 95% CI = 81.2–91.1%) monoinfections and 25 (13.2%; 95% CI = 9.1–18.8%) mixed infections; 129 (68.3%; 95% CI = 61.3–74.5%) patients presented with *P. falciparum*, 29 (15.3%; 95% CI = 10.9–21.2%) with *P. vivax*, 3 (1.6%; 95% CI = 0.5–4.6%) with *P. malariae*, and 3 (1.6%; 95% CI = 0.5–4.6%) with *P. ovale* monoinfections. In addition, 21 (11.1%; 95% CI = 7.4–16.4%) patient samples contained DNA of both *P. falciparum* and *P. vivax*, 2 (1.1%; 95% CI = 0.3–3.8%) of *P. falciparum* and *P. malariae*, and 2 (1.1%; 95% CI = 0.3–3.8%) triple infections with *P. falciparum*, *P. vivax,* and *P. malariae* (Table 1). One patient presented with *P. ovale* two times in the course of this study. Based on the PCR results, 30 (7.9%; 95% CI = 5.5–10.8%) microscopy slides were classified as false negative, and none were false positive. Compared with the 25 PCR-confirmed samples with mixed malaria infections, only 9 (36%) were read as positive for mixed infections in microscopy, and only 6 (24%) were diagnosed as mixed infections by RDT.

All three samples positive for *P. ovale* in PCR were proven by DNA sequencing (GenBank Accession numbers: HM196277, HM196278) and after unblinding, were also found positive for *P. ovale* on microscopical reexamination.

DISCUSSION

For 2006, the World Health Organization estimated almost 3 million malaria cases and 15,000 deaths in Bangladesh in mostly unconfirmed cases. Among microscopy-confirmed malaria infections, *P. falciparum* was the dominant species, causing more than 70% of all malaria cases.[1] A recent report based on microscopic diagnosis indicated that 70.3% of all malaria cases in the Chittagong Hill Tracts were caused by *P. falciparum*, 29.6% were caused by *P. vivax*, and only 0.01% were read as mixed infections.[7] A study using microscopic and molecular methods for diagnosis conducted between 2000 and 2002 revealed that of those slides considered positive for *Plasmodium* spp., 84% were *P. falciparum* monoinfections or mixed infections, 15% were *P. vivax*, and 1% were *P. malariae*.[10] Our study shows comparable results for *P. falciparum*

TABLE 1

Comparison of malaria diagnosis by nested PCR, microscopy, and FalciVax-RDT

	Neg	Pf	Pv	Pm	Po	Pk	Pf + Pv	Pf + Pm	Pf + Pv + Pm
PCR*	190	129	29	3	3	0	21	2	2
Microscopy*	220	122	29	3	0	0	6	0	0
RDT†	228	108	15	–	–	–	6	–	–

neg = negative; Pf = *P. falciparum*; Pv = *P. vivax*; Pm = *P. malariae*; Po = *P. ovale*; Pk = *P. knowlesi*; Pf + Pv = *P. falciparum + P. vivax*; Pf + Pm = *P. falciparum + P. malariae*; Pf + Pv + Pm = *P. falciparum + P. vivax + P. malariae*.
* $n_{PCR} = n_{microscopy} = 379$.
† $n_{RDT} = 357$; diagnosis of Pm, Po, and Pk are not possible with FalciVax-RDTs.

with 81.5% (of which 68.3% were monoinfections) and *P. vivax* with 26.5% (of which 15.3% were monoinfections). However, none of the earlier studies ever reported any cases of *P. ovale*.

We report the first three cases of *P. ovale* in Bangladesh. All of them were monoinfections and originated from the Chittagong Hill Tracts. Two of the positive samples found in this survey were seen in the same patient who originally tested negative in the RDT but was diagnosed with *P. malariae* based on microscopy (assuming that *P. ovale* was not an option, because it had never been seen in Bangladesh before). Two months after being treated for his suspected *P. malariae* infection, the patient returned with signs and symptoms consistent with malaria. This sample was confirmed to be *P. ovale* by molecular techniques, suggesting a relapse with *P. ovale*.

The nested PCR assay used in this study is highly sensitive with a documented limit of detection of 6 parasites/µL.[31] *P. ovale* was found in 1.6% of the *Plasmodium*-positive samples. *P. ovale* had previously been reported from nearby Rakhine State in Myanmar. It has also been reported from other parts of Myanmar and a surprisingly high prevalence of up to 6.1% in Tanintharyi Division in 1996.[11,33] In the same study, *P. malariae* was reported from 15.2% of all cases. Other PCR-based studies in Southeast Asia report up to 4% of *P. ovale* malaria in Northeastern Cambodia and 1.03% in Thailand.[16,22,34]

The impact of newly emerging pathogens in public health is increasing. Although our data do not confirm the presence of *P. knowlesi* in Bangladesh, our knowledge of the reservoir, vectors, and ecology of this malaria parasite indicates that Southeastern Bangladesh may be a an environment in which *P. knowlesi* is likely to be found. Human infections are easily mistaken for *P. falciparum* infections when the parasites are in the stage of young trophozoites.[27] In studies based on microscopic diagnosis, it may also easily be mistaken for the morphologically similar parasite *P. malariae*.[25] This parasite should, therefore, always be considered whenever patients are from or report a travel history to remote areas of Southeast Asia and are diagnosed with *P. malariae* malaria based on microscopic examination.[35,36]

Relative to microscopy, molecular methods not only tend to improve the sensitivity and specificity of diagnostic studies, but they also result in higher estimates of malaria prevalence, particularly for the rare species *P. malariae* and *P. ovale*, and higher rates of mixed infections.[37] Studies conducted in Lao People's Democratic Republic (PDR) (23.1%) and Northwestern Thailand (23–24%) in which molecular techniques were used all suggested the presence of high numbers of mixed infections.[22,34] In our survey, we found 25 cases (13.2% of all positive samples) of mixed infections, of which the combination of *P. falciparum* and *P. vivax* was most frequently seen. Triple infections with *P. falciparum*, *P. vivax*, and *P. malariae* are rare and were found in only two patients coming from the same small village (Nathogri, Rowanchari Subdistrict).

Accurate diagnosis of *Plasmodium* spp. is essential for optimizing malaria-treatment guidelines. Further studies assessing the prevalence of the rare species *P. ovale*, *P. malariae*, and *P. knowlesi* in South Asia are, therefore, urgently needed to better understand the species distribution and to allow for adapting treatment strategies.

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