Molecular Evidence of Malaria and Zoonotic Diseases among Rapid Diagnostic Test–Negative Febrile Patients in Low-Transmission Season, Mali

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Abstract. From November to December 2012 in Sélingué-Mali, blood samples from 88 febrile patients who tested negative by malaria Paracheck® rapid diagnostic tests (RDTs) were used to assess the presence of sub-RDT Plasmodium falciparum as well as Borrelia, Coxiella burnetii, and Babesia applying molecular tools. Plasmodium sp. was present among 57 (60.2%) of the 88 malaria RDT–negative patients, whereas the prevalence of Borrelia, C. burnetii, and Babesia were 3.4% (N = 3), 1.1% (N = 1), and 0.0%, respectively. The additional diagnostic use of polymerase chain reaction (PCR) identified a high proportion of Plasmodium sp.-positive samples and although this may be a concern for malaria control, the respective PCR-identified malaria infections were less likely responsible for the observed fevers given the low parasite density. Also, the low infection levels of Borrelia and C. burnetii and lack of Babesia among the febrile patients call for further studies to assess the causes of fever among malaria RDT–negative patients in Sélingué.

Worldwide, an estimated 438,000 people died of malaria in 2014.1 Approximately 82% of malaria cases and 90% of malaria deaths occurred in sub-Saharan Africa (SSA), with children under 5 years of age and pregnant women the most severely affected.1 Yet, strong decreasing trends of malaria-related morbidity and mortality have been observed in SSA in recent years,1 most likely as a result of intensified investments in control measures such as long-lasting insecticide-treated nets, the use of malaria rapid diagnostic tests (RDTs), and efficacious treatment (artemisinin-based combination therapies). In Mali, malaria is endemic and strongly seasonal in many parts of the country, as most of the cases (60–70%) are observed between August and November.2

The diagnosis of malaria relies on deployment of RDTs with considerable challenges during the low-transmission season when parasite loads may be low resulting in reduced sensitivity of RDT diagnosis.3 Furthermore, management of RDT-negative fever cases are difficult in remote rural settings as differential diagnosis on malaria-like symptoms is limited due to low index of suspicion among health staff and inadequate diagnostic capacity. Hence, diseases that can be mistaken as malaria in rural tropical settings are often underestimated and left untreated. This includes several zoonotic diseases such as rickettsiosis, Q fever, babesiosis, and borreliosis.4 Notably, relapsing fever (borreliosis) has long been recognized as a major cause of disease and death in several regions of Africa, yet is considered vastly underreported.5,6

Q fever is caused by the Gram-negative bacterium Coxiella burnetii (Coxiellaceae family).7 It is found worldwide in wild rodents and birds. The main hosts are domestic animals such as sheep, goats, and cattle.8,9 Borreliosis is a genus of the spirochete phylum causing relapsing fever and transmitted primarily by ticks/lice, depending on the species. In west Africa, small mammals and birds are the main hosts of Borreliosis leading to tick-borne relapsing fever in humans.9 Babesiosis is a zoonosis caused by tick-transmitted intraerythrocytic protozoa of the phylum Apicomplexa.9 The main pathological event is lysis of erythrocytes resulting in hemolytic anemia.9

Only few studies have investigated the possible contribution of Q fever, borreliosis, and babesiosis to the disease burden in SSA. In Mali, a previous serological study has reported a seroprevalence of Q fever of 28% in Bamako and 51% in Mopti among fever patients seeking treatment in outpatient clinics.10 With regard to Borrelia, previous studies report 10% active spirochete infections among humans,11 and a seroprevalence among cattle of 7% and 38% of Babesia bovis and Babesia bigemina, respectively.12

A recent study performed in Sélingué showed an unexpected weak correlation between fever and malaria infections among children < 10 years of age as diagnosed by RDT during the low-transmission season.13 Thus, the present study aimed to explore this observation further by initially examining the prevalence of sub-RDT malaria infections using highly sensitive nested polymerase chain reaction (PCR), and thereafter, exploring other causes of fever including the potential role of zoonotic infections by retrospectively examining the malaria RDT–negative fever cases for Borrelia, C. burnetii, and Babesia infection using PCR-based methodologies.

Sélingué is located in the Guinean Sudan Savanna areas of Mali about 130 km southwest of Bamako. Malaria transmission is seasonal with more than 60% of malaria incidence observed during the wet season (June–November). The population is composed mainly of farmers and fishermen, where the majority of families keep livestock (cattle, goats, and sheep), increasing the risk for zoonotic diseases.

The study was conducted from November 15 to December 31, 2012. Participants were recruited at the district health center, and all participants were tested with the Malaria RDT Paracheck® Pf (Orchid Biomedical Systems, Goa, India), (a histidine-rich protein [HRP]-2 test for Plasmodium falciparum malaria). Patients that were RDT positive were treated with artemether–lumefantrine according to national guidelines and excluded from further study. For the present study, the inclusion criteria were subjects presenting with a malaria RDT–negative test, age ≥ 5 years, axillary temperature ≥ 37.5°C, and acceptance to sign the informed consent. Into BD vacutainer® (Becton, Dickinson and Company, Franklin Lakes, NJ)
containing ethylenediaminetetraacetic acid. 1.5 mL blood samples were collected, and then separated into two tubes of red blood cells (RBC) and plasma and stored frozen at −20°C. Samples were thawed and depending on the volume of the plasma samples, between 10 and 130 μL of plasma was added back to the corresponding RBC. DNA was then extracted from the remixed full blood samples using Nucleospin® Blood QuickPure kit (Macherey-Nagel, Düren, Germany).

Q FEVER PCR

A conventional PCR assay based on Frangoulidis and others14 was used to confirm C. burnetii infections. For Babesia and Borrelia PCR, nested PCR developed by Persing and others15 and Nordstrand and others,16 respectively, were used. For these three diagnostic PCRs, a set of positive and negative controls were used, including samples containing DNA extracted from healthy Danish blood donors, from a patient with P. falciparum parasitemia, as well as DNA isolated from Babesia-, Borrelia-, and C. burnetii-positive patients, which were kindly provided by Bjørn Kantso, Statens Serum Institute in Copenhagen, Denmark.

MALARIA PCR

A nested PCR assay was used to identify samples containing Plasmodium malaria not identified by RDTs. Two primers were used as outer: rPLu6 (TTAAAATTGTTGACGTAAAACG) and rPLu5 (CTGTGTTGTCCTTTAAACTC), originally described in Snounou and others.17 Primers for the nested PCR were Plasm-all-n1fw (CCCTCAGTACCTTATGAGAAATC) and Plasm-all-n2fw (TCGTCGATCTCTTGTTCTT).

All samples that were identified as Plasmodium positive were selected and run in separate nested PCRs to identify the Plasmodium species of infection as described in Snounou and others.17 Positive controls were DNA extracts from patients with confirmed P. falciparum, Plasmodium malariae, and Plasmodium vivax infections, whereas negative controls were DNA extracted from healthy Danish blood donors.

In total, 88 blood samples from malaria RDT–negative febrile patients were examined by subsequent PCR analyses. Of these, 56 (63.6%) were between 5 and 14 years, 27 (30.7%) between 15 and 44 years, and 5 (5.7%) above 44 years of age. Clinical diagnosis classified 39 (44.3%) as typhoid fever, 26 (29.5%) as acute respiratory infection, and 23 (26.1%) as digestive disorder. Of the 88 malaria RDT–negative patients, a total of 57 (64.8%) were found positive by nested PCR (Table 1). Plasmodium falciparum was the main species found in 35 (63.1%) of samples. However, coinfections were also observed: three (5.3%) P. falciparum + P. malariae and two (3.5%) P. falciparum + P. malariae + P. ovale. Three patients were infected with Borrelia (two males 15 and 17 years of age and one female 21 years of age), whereas one patient, (11-year-old male) was tested positive for C. burnetii. None of the patients were PCR positive for Babesia. (Table 1).

In the present study, 64.8% of the 88 febrile patients with a negative Paracheck Pf RDT were found positive for malaria by nested Plasmodium PCR. Numerous reports have shown greater specificity and sensitivity of using PCR as opposed to conventional diagnosis using either microscopy or RDTs, and most likely, the many RDT negatives that were PCR positive were due to low parasite density infections. Alterna-

### Table 1

<table>
<thead>
<tr>
<th>Age groups (n years)</th>
<th>Malaria infection</th>
<th>Borrelia infection</th>
<th>Q fever infection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
<td>n (%)</td>
</tr>
<tr>
<td>5–14</td>
<td>56 (75.0)</td>
<td>1 (1.8)</td>
<td>1 (1.8)</td>
</tr>
<tr>
<td>15–44</td>
<td>27 (44.4)</td>
<td>2 (7.4)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>≥45</td>
<td>5 (60.0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Total</td>
<td>88 (64.8)</td>
<td>3 (3.4)</td>
<td>1 (1.1)</td>
</tr>
</tbody>
</table>

PCR = polymerase chain reaction.

CONCLUSIONS

The additional diagnostic use of PCR identified a high proportion of Plasmodium spp.-positive samples and although this is a concern for malaria control, the respective PCR-identified malaria infections were less likely responsible for the observed fevers since they are of low density, below the threshold for RDT detection. The diagnosed cases of Q fever and borreliosis were few but acute. RDTs improved considerably the quality of malaria case management. However, molecular-based assays could be important in the context of malaria elimination efforts.21 Further studies are needed on the epidemiology of zoonotic diseases (as well as other febrile diseases) in the study area.

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