Molecular Diagnosis of Malaria Infection: A Survey in a Hospital in Central Italy

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Abstract

Malaria is a dramatic disease caused by the protozoan parasites *Plasmodium*. The diagnosis is mainly based on microscopy and rapid diagnostic tests (RDT). Molecular approaches based on PCR techniques may be an alternative tool particularly favourable in regions with declining prevalence. This work aimed to assess pros and cons of molecular diagnosis of malaria in a district of Central Italy were several tens of imported malaria cases are diagnosed every year. Thirty-three blood samples were analysed by microscopy, RDT and molecular techniques to monitor the relative efficiency in malaria diagnosis. Molecular analysis and microscopy diagnosed 32 out of 33 samples as positive for malaria, while RDT only 29. More differences concerned the diagnosis of mixed infections. Our findings remark the importance of the molecular approach in supporting and improving malaria diagnosis. In the cases here presented, the molecular analysis was particularly useful to unveil parasites presence in infections not detectable by blood smear analysis and to additionally solve real and/or presumed mixed infections.

Keywords: Malaria, *Plasmodium*, PCR, Clinical diagnosis

Abbreviations: Rapid diagnostic Test (RDT)

Introduction

Malaria is a dramatic disease caused by the protozoan parasites of *Plasmodium* genus, with *Plasmodium falciparum* being the deadliest. Up to date 212 million malaria cases estimated worldwide in 2015 [1] and 429 thousand deaths. The diagnosis based on only the clinical symptoms is often not sufficient since this disease shows a wide range of non-specific symptomsun distinguishable by other diseases [2]. For this reason, in non-endemic areas, malaria continues to pose challenges in diagnosis and management [3]. Common malaria symptoms are headache and myalgia. Other symptoms like fever for up to two days, fatigue, loss of appetite and body pains can be observed. Other symptoms may include nausea, vomiting, dry cough, icterus, confusion and respiratory distress [3].

The frequency of febrile episodes depends on the parasite species: a) tertian for *P. vivax* and *P. ovale* occurring every 48 hours, c) quartan for *P. malariae* every 72 hours and d) quotidian for *P. falciparum* every 24 hours [3]. In non-immune patients, malaria usually starts suddenly with a severe feeling of sickness and fever, often reaching 39 °C and higher [2]. Malaria cannot be confirmed clinically. The standard method for malaria diagnosis is based on the examination of Giemsa-stained thin and thick blood films at light microscopy. This approach is rapid, easy to perform and sensitive, but requires a high level of expertise [4].

New rapid diagnostic tests (RDTs) for antigen detection are increasingly used, permitting a reliable detection of malaria infections particularly in remote areas with limited access to good quality microscopy services. RDTs are immuno chromatographic tests targeting antigens of one or more *Plasmodium* species [5]. Nevertheless, these methods have limitations because have limited sensitivity and specificity [6]. False positives are often observed, particularly after treatment, as the detected parasite antigens can remain in the circulation after parasite clearance.

Alongside, several *Plasmodium* genus- or species-specific PCR assays have also been developed for malaria diagnosis [7-11]. PCR-based assays present various advantages over microscopy and RDT: they are highly specific and highly sensitive

[12-14]. Moreover, as few as five parasites per microliter of blood can be detected [15]. Thus, they are ideal for revealing the presence of mixed infections overlooked by conventional methods [13,10,16]. In non-endemic countries PCR can be of extreme help to unveil false negative and mixed infections. Here, a cohort of malaria patients of the S. Lucia Hospital of Macerata in Marche region (central Italy) were recruited to confirm, and in some cases to clarify, the clinical diagnosis using PCR-based methods.

Material and Methods

Patients

Thirty-three blood samples were collected from patients with clinical symptoms referable to malaria (Table 1). The clinical diagnosis was performed by Giemsa-stained thin and thick blood films and/or RDTs tests. Patients were informed about the molecular diagnosis of malaria.

Table 1: Diagnostic data of malaria patients. The table showed information about patients and the results of three different diagnostic methods.

Sample	Source	Sex	Age	Microscopically Diagnosis	Antigen (RDTs)	Molecular Diagnosis
1	Ghana	M	34	*P. falciparum*	F	*P. falciparum*
2	Nigeria	M	55	neg	neg	*P. falciparum*
3	Nigeria	M	26	*P. falciparum*	F + O	*P. falciparum*
4	Senegal	M	36	*P. falciparum*	F + O	*P. falciparum*
5	Pakistan	F	24	*P. vivax*	O	*P. vivax*
6	Nigeria	F	25	*P. ovale/P. malariae*	O	*P. malariae*
7	Nigeria	M	40	*P. falciparum*	F	*P. falciparum*
8	Pakistan	M	41	*P. vivax*	O	*P. vivax*
9	Cameroon	M	65	*P. falciparum*	F	*P. falciparum*
10	Nigeria	F	45	*P. falciparum/P. malariae*	F + O	*P. falciparum*
11	Guinea	F	16	*P. falciparum*	F	*P. falciparum*
12	Pakistan	M	42	*P. vivax*	O	*P. vivax*
13	Nigeria	M	47	neg	F	*P. falciparum*
14	Nigeria	M	2	*P. falciparum*	F + O	*P. falciparum*
15	Nigeria	F	27	*P. falciparum*	F + O	*P. falciparum*
16	Nigeria	F	5	*P. falciparum*	F + O	*P. falciparum*
17	Somalia	M	33	*P. ovale*	O	*P. ovale*
18	Nigeria	M	36	*P. falciparum*	F + O	*P. falciparum*
19	Sierra Leone	M	29	*P. falciparum*	F	*P. falciparum*
20	Pakistan	M	33	*P. vivax*	O	*P. vivax*
21	Benin	M	32	neg	neg	*P. malariae*
22	Nigeria	M	12	*P. malariae (uncertain diagnosis)*	neg	*P. malariae*
23	Nigeria	M	8	neg	neg	neg
24	Nigeria	M	33	*P. falciparum*	F	*P. falciparum*
25	Nigeria	M	31	*P. falciparum*	F + O	*P. falciparum*
26	Nigeria	M	18	*P. falciparum*	F + O	*P. falciparum*
27	Pakistan	M	26	*P. vivax*	O	*P. vivax*
28	Senegal	M	35	*P. falciparum*	F + O	*P. falciparum*
29	Nigeria	F	8	*P. falciparum*	F + O	*P. falciparum/P. vivax*
30	Nigeria	M	31	*P. falciparum*	F + O	*P. falciparum*
31	Nigeria	F	37	*P. falciparum*	F + O	*P. falciparum*
32	Nigeria	M	7	*P. falciparum*	F	*P. falciparum*
33	Pakistan	M	44	*P. vivax*	O	*P. falciparum/P. vivax/ P. malariae/P. ovale*

F: *P. falciparum*; O: other *Plasmodium* species; neg: negative

DNA extraction and PCR assays

Genomic DNA was extracted from blood samples using a JetFlex Genomic DNA Purification kit (Genomed, Lohne, Germany) according to the manufacturer's instructions. Molecular identification of the *Plasmodium* species was performed following PCR amplification protocols described in Snounou et al. [11]. Each experiment included four positive controls (consisting of *P. falciparum*, *P. vivax, P. malariae and P. ovale*, genomic DNA, respectively, from positive blood samples) and one negative control (consisting of sterile double-distilled water). PCR was performed using the Dream Taq DNA polymerase kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA) according to the manufacturer's protocol. The PCR products were verified through electrophoresis run on agarose gel 1% with ethidium bromide and displayed using the UV light.

Results and Discussion

The molecular analysis confirmed that approximately 97% of analysed samples (32/33) resulted malaria positives (Table 1). Only a single sample resulted negative. This refers to an African patient with repeated episodes of fever, but both RDT and the thick blood analyses were also negative. Three more samples were diagnosed negative by the means of RDT one of whose malaria positive (although uncertain) at microscopy analysis. The PCR based diagnosis diagnosed two *P. malariae* and one *P. falciparum* infections.

Eight samples were microscopically confirmed while RDTs indicated a generalist "non-*P. falciparum*". In all these cases the PCR-based approach could precisely diagnose the infective malaria species. Among the eight cases, two were peculiar:

A. In the first case the morphologic diagnose was uncertain between *P. malariae* and *P. ovale*, while the PCR clarifies the presence of *P. malariae* only.

B. In the second case, a simultaneous co-infection of four *Plasmodium* species (*P. falciparum, P. ovale, P. vivax* and *P. malariae*) species were molecularly detected in a Pakistan patient, where the thick blood film's observation showed the presence of *P. vivax* and the RDTs indicated "non-*P. falciparum*". This result is not surprising since such kinds of multi-species malaria co-infections have been reported [17,18].

Interestingly, 13 samples were diagnosed by RDTs, based on the detection of a double band, as mix infections. The molecular diagnosis, instead, has ascertained one mixed infection (*P. falciparum/P. vivax*) in a Nigerian patient, where rare cases of co-infections were already described [19]. In all the other cases, PCR-based assays identified *P. falciparum* parasites. This may be explained by false positive signals due to high parasite densities of *P. falciparum* revealed in RDTs tests [5]. Eight more cases were independently diagnosed as *P. falciparum* by all the diagnostic methods. These results highlighted that RDTs offer a real-time detection of malaria infections, but the diagnosis can eventually result incomplete or wrong without the support of a solid microscopical and/or molecular analysis.

Conclusion

Our findings remark the importance of molecular approaches in supporting and improving malaria diagnosis. In the cases here presented, the molecular analysis was particularly useful to reveal the parasite in infections not detectable by blood smear analysis and to solve real and/or presumed mixed infections.

Acknowledgement

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