PCR-BASED METHODS TO THE DIAGNOSIS OF IMPORTED MALARIA

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Summary:

Rapid and precise diagnosis of malaria is needed to take care febrile patient returning from endemic areas. Since the first description of the diagnosis of *Plasmodium* infection by polymerase-chain-reaction (PCR), the role of this kind of molecular method in the laboratory diagnosis of imported malaria is still a topical question. PCR-based assays were found to be more sensitive and more specific than all conventional methods. The highest contribution of the molecular diagnosis is that a PCR negative result would ascertain the lack of any malaria infection, thus quickly orienting the investigations toward other aetiology. This technique should be now considered as the gold standard for the diagnosis of imported malaria.

KEY WORDS : imported malaria, molecular diagnosis, PCR.

Since the first description, in 1990, of the diagnosis of *Plasmodium falciparum* infection by means of polymerase-chain-reaction (PCR) (Jaureguiberry *et al.*, 1990), the role of this kind of molecular method in the laboratory diagnosis of imported malaria is still a topical question. Albeit considered the gold standard as regards sensitivity and specificity, the use of PCR-based assays for routine detection of malaria infection is debatable for many people, who consider the practical requirements (expensive apparatus, need for qualified technicians, usage costs), together with the risk of contamination and the problems of emergency diagnosis, to be out of proportion given the known benefits. However, permanent technological improvement has made PCR faster and more and more user-friendly.

A SENSITIVE ASSAY

For the laboratory diagnosis of malaria, PCR-based assays were found to be more sensitive than all conventional methods, whatever the type of PCR-based test used (see above) or the nature of primers that were used. At its best, the power of detection of thick blood smear examination ranged from 10 to 50 parasites/µL (Guerin *et al.*, 2002; Moody, 2002). Quantitative Buffy Coat (QBC®) tests were not more efficient (Wongsrichanalai *et al.*, 1991) and dipstick assays exhibited a poor sensitivity when the parasitemia value was under 100 parasites/µL (Moody, 2002). Conversely, in 20 studies (Table I), PCR's sensitivity for the detection of *P. falciparum* ranged from 0.004 to 30 parasites/µL. However, some of these results should be carefully considered, since they were experimental and dilution ranges were done in very different manners, using either blood from infected subjects, or from *P. falciparum* cultivation (often with no indication concerning synchronization, so the presence of multinucleated schizonts might be suspected), or from extracted DNA (with different methods of extraction). For species other than *P. falciparum*, comparing sensitivity results were impossible due to the lack of any means of cultivation and also to the variation of the proportion of schizonts between different patients.

Concerning the diagnosis of imported malaria, only papers that compared PCR and conventional microscopy during a limited period of time and in all patients suspected to be infected were analyzed. In 15 studies including 2,416 subjects, 1,108 patients were found to be malaria positive by conventional microscopy, and 1,204 (+ 10 %) by PCR (range: from 0 to + 13 %). All the patients positive by microscopy were also positive by PCR, whatever the *Plasmodium* species. When compared to the set of the requests, the gain of sensitivity was 4.4 % (range: from 0 to + 11 %) (Rubio *et al.*, 1999; Filisetti *et al.*, 2002; Morassin *et al.*, 2002; Richardson *et al.*, 2002; de Monbrison *et al.*, 2003; Padley *et al.*, 2003; Patsoula *et al.*, 2003; Perandin *et al.*, 2003; Calderaro *et al.*, 2004; Fabre *et al.*, 2004; Perandin *et al.*, 2004; Rougemont *et al.*, 2004; Whiley *et al.*, 2004; Machouart *et al.*, 2006; Vo *et al.*, 2007). This improvement in sensitivity affected mainly falciparum malaria. *P. falciparum* is the most frequently detected agent of imported malaria, especially in the European Union where the closest endemic zone is sub-Saharan Africa (Jelinek *et al.*, 2002). Moreover, even chemoprophy-

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Authors/Year	Apparatus	PCR type	Revelation	Detected species[1]	Gene's target	Sensitivity for *Pf* (parasite/µl)
Arai *et al.,* 1994	Classical	Nested	Ethidium bromide (gel)	*Pf*	DHFR	1.3
Ciceron *et al.,* 1999	Classical	Simplex	Probe (southern blot)	Genus *Pf, Pv*/	SSUrRNA	3.0
Tham *et al.*, 1999	Classical	Simplex	Probe (gel)	Genus *Pf, Po, Pv*/	Cox 1/Plastid	0.01
Fabre *et al.,* 2002	Real-time	Multiplex & simplex[2]	Syber green®	Genus/*Pm*	Cox 1/Plastid/ SSUrRNA	0.035
Filisetti *et al.,* 2002	Classical	Simplex	Ethidium bromide (gel)	*Pf*	STEVOR	0.01
Myjak *et al.*, 2002	Classical	Nested	Ethidium bromide (gel)	4	SSUrRNA	0.38
Rubio *et al.*, 2002	Classical	Nested & multiplex	Ethidium bromide (gel)	4	SSUrRNA	0.01
De Monbrison *et al.,* 2003	Real-time	Simplex	Syber green®	Genus & 4	SSUrRNA	30
Kho *et al.*, 2003	Classical	Multiplex	Ethidium bromide (gel)	*Pf, Pv*	SSUrRNA	0.1
Patsoula *et al.,* 2003	Classical	Multiplex	Ethidium bromide (gel)	*Pf, Pv*	SSUrRNA	1.0
Calderaro *et al.,* 2004	Classical	Nested	Probe (enzyme-linked)	4	SSUrRNA	0.07
McNamara *et al.*, 2004	Classical	Simplex	Probe (gel)	4	SSUrRNA	0.1
Montenegro *et al.*, 2004	Classical	Nested	Ethidium bromide (gel)	Genus	SSUrRNA	0.07
Perandin *et al.,* 2004	Real-time	Simplex	Probe	*Pf, Po, Pv*	SSUrRNA	1.0
Whiley *et al.,* 2004	Classical	Simplex	Probe (enzyme-linked)	4	SSUrRNA	1.4
Mangold *et al.,* 2005	Real -time	Simplex	Syber green®	4	SSUrRNA	1.0
Elsayed *et al.,* 2006	Real-time	Simplex	Probe (molecular braecon)	*Pf*, Genus	Cox 1/Plastid/ SSUrRNA	0.004
Machouart *et al.,* 2006	Classical	Simplex	Probe (enzyme-linked)	4	SSUrRNA	10
Vo *et al.,* 2007	Real Time	Simplex	Syber green·	4	AQP/ECPR, Pos25/CS	15
Gama *et al.,* 2007	Real time	Simplex	Probe (TaqMan®)	*Pf*	SSUrRNA	0.5
	Classical	Simplex	Ethidium bromide (gel)	*Pf*	SSUrRNA	0.5

[1]*Pf, Pm, Po, Pv: Plasmodium falciparaum, P. malariae, P. ovale, P. vivax.*
[2]For *Pm, Po, Pv.*

Table I. – PCR's sensitivity for the detection of *P. falciparum* ranged from 0.004 to 30 parasites/µL in 20 studies.

laxis that is not correctly followed out remains rather active on species other than *P. falciparum*, apart from the chloroquine-resistant *P. vivax* strains, which are still rarely encountered in Europe.

A SPECIFIC METHOD

The nature of the target for primers or probes, as determined by the studies of the *Plasmodium* genome whose results are available in GenBank®, guarantees the specificity of PCR results. A further precaution has often been the sequencing of PCR products.

Concerning *Plasmodium* species identification, literature analysis reports frequent and sometimes substantial discrepancies between the results from microscopy and those from PCR. From the last 12 published studies that have compared the performance of thin blood smear examination and PCR, 17.1 % (range: 1.5-39) of 923 species identifications by microscopy were corrected after PCR checking (Rubio *et al.*, 1999; Morassin *et al.*, 2002; de Monbrison *et al.*, 2003; Perandin *et al.*, 2003; Calderaro *et al.*, 2004; Di Santi *et al.*, 2004; Ndao *et al.*, 2004; Perandin *et al.*, 2004; Rougemont *et al.*, 2004; Whiley *et al.*, 2004; Machouart *et al.*, 2006; Vo *et al.*, 2007). The rate of misdiagnosis varied from 20 % to 50 % for *P. malariae, P. ovale* or *P. vivax* (Morassin *et al.*, 2002; de Monbrison *et al.*, 2003; Perandin *et al.*, 2003; Calderaro *et al.*, 2004; Di Santi *et al.*, 2004; Ndao *et al.*, 2004; Perandin *et al.*, 2004; Rougemont *et al.*, 2004). Concerning mixed infections, practically 100 % of the microscopy results were false, either by excess (diagnostic of a mixed infection when there was a single species) or by omission (reverse situation). *Plasmodium falciparum* identification appeared to be more reliable, with no misdiagnoses found in eight studies (Morassin *et al.*, 2002; de Monbrison *et al.*, 2003; Calderaro *et al.*, 2004; Ndao *et al.*, 2004; Perandin *et al.*, 2004; Whiley *et al.*, 2004; Machouart *et al.*, 2006; Vo *et al.*, 2007), a 1.7 to 2.8 % misdiagnosis rate in three studies (Rougemont *et al.*, 2004; Rubio *et al.*, 1999; Perandin *et al.*, 2003) and 8.4 % of misdiagnoses in one study (Di Santi *et al.*, 2004).

However, the high specificity of PCR methods demonstrated the other side of the coin, namely the intrinsic risk of these tests being unable to detect new molecular variants. This phenomenon was reported concerning *P. malariae* in China (Liu *et al.*, 1998; Kawamoto *et al.*, 2002), *P. ovale* in the Thai – Myanmar border region (Kawamoto *et al.*, 1996) or in Africa (Calderaro *et al.*, 2007) and also *P. vivax* in Papua-New Guinea, where the so-called *P. vivax*-like species was described (Qari *et al.*, 1993). Moreover, newly emergent infec-

tions by zoonotic primate *Plasmodium* can go undiagnosed by species-PCR, which occurred with the Borneo focus of *P. knowlesi* (Singh *et al.*, 2004).
When primers or probes are chosen then designed, these two points should be kept in mind and correlated with future uses of the PCR test, *e.g.* routine diagnosis in a westernized country or, conversely, epidemiology surveys from specimens collected in the field. The concomitant use of an assay detecting the *Plasmodium* genus, whatever the origin – Man, subhuman primate or even rodent – of the species involved, appears to be an excellent safeguard against rare but possible false negative results.

PCR AND THE DIAGNOSIS OF IMPORTED MALARIA

Even now, PCR assay may be considered the gold standard for the diagnosis of malaria, therefore replacing thick smear examination. This situation is due to both high sensitivity and specificity, as detailed above. While PCR is used more and more in laboratories of Parasitology, its exact place in the full range of malaria diagnostic tests has yet to be explicitly established.
Obviously, the emergency diagnosis of malaria will for a long time remain the prerogative of microscopy (thin smear or fast thick smear examination (Thellier *et al.*, 2002), QBC®) associated with immunochromatographic methods. Such a test combination can detect all infections displaying a moderate to high parasitemia, which ensures within less than half an hour the diagnosis of severe malaria. PCR should therefore be considered a second-line method.
The first question about the routine use of PCR for the diagnosis of imported malaria concerns the schedule of use, daily, weekly or bimonthly, and also the type of specimens concerned, all or only those posing a problem. Based on a 8-year experience, we subsequently believe PCR should be done on a daily basis. In this case, low parasitemia infections due to, *e.g.* incorrect chemoprophylaxis or an immune status, would be promptly identified, despite having escaped the first-line methods. Anyone who deals with tropical medicine has had a personal example of a patient displaying chronic fever along with a recent history of travel in a malaria-endemic area and who is finally diagnosed as malaria-infected after many days of hospitalization and multiple conventional investigations. A request for PCR would quickly resolve this diagnostic quandary (Puente *et al.*, 2000; Antinori *et al.*, 2001; Speers *et al.*, 2003). Moreover, it should be underlined that, according to our experience, the predictive negative value of a PCR-based result is 100 %. A negative result would ascertain the lack of any malaria infection, thus quickly orienting the investigations toward other hypotheses. With the expected reduction of hospitalization time, substantial money saving could be achieved, as – in France – daily costs are approximately 800 € in a Department of Internal Medicine or Infectious/Tropical Diseases.
Parasitemia level can be quantified by real-time PCR. However, this can be more easily achieved by the combined use of blood count results and thin smear examination.
Since real-time PCR can detect and quantify very low parasitic loads, it has been hypothesized that this assay could be a valuable tool for the monitoring of falciparum malaria therapy. Two studies indicated that the persistence of positive PCR results after a 5-day treatment was consistent with treatment failure (Kain *et al.*, 1994; Ciceron *et al.*, 1999). However, such a result can also be due to the presence of gametocytes (Smalley & Sinden, 1977), which removes any purpose from this kind of molecular follow-up.
From a financial point of view, PCR is said to be a costly method. It is clear that technical requirements, such as room layout in a laboratory, together with the purchase of a thermocycler and the required ancillaries, are not inexpensive. However, one should consider that this price is on a constantly decreasing slope and these investments are also indispensable for other diagnoses in Parasitology, such as that of congenital toxoplasmosis. Many hospitals have also chosen the solution of a technical platform pooled with different laboratories. *In fine*, it appears obvious that PCR use improves the diagnosis of imported malaria, but also that this method is restricted to health centers, such as University Hospitals, for whom malaria identification is an important and routine problem. For other structures, the combination of conventional microscopy with immunochromatographic methods is certainly the best choice.

CONCLUSION

Currently, PCR assay may be considered the "gold standard" for malaria diagnosis, given both its high sensitivity and specificity, along with the robust predictive value of a negative result. PCR-based methods should therefore be included in the panel of diagnostic tools for imported malaria infection for any center of reference. This is based on both scientific reasons and also interesting from a "service" point of view, whose importance is constantly increasing, to help meet the duty of best efforts.

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