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Comparison of a commercial real-time PCR assay, RealCycler® PJIR kit, progenie molecular, to an in-house real-time PCR assay for the diagnosis of *Pneumocystis jirovecii* infections

Thibaud Guillaud-Saumur [a,b,1], Gilles Nevez [a,b], Amélie Bazire [c], Michèle Virmaux [b], Nicolas Papon [d], Solène Le Gal [a,b,*]

[a] Laboratory of Parasitology and Mycology, Brest University Hospital, Brest, France
[b] University of Brest, GEIHP EA, 3142, Brest, France
[c] Medical intensive care unit, Brest University Hospital, Brest, France
[d] University of Angers, GEIHP EA, 3142, Angers, France

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ABSTRACT

We compared the RealCycler® PJIR kit (Progenie Molecular), available in Europe, to an in-house real-time PCR assay for the diagnosis of *Pneumocystis jirovecii* infections. Excellent agreement was found (concordance rate, 97.4%; Cohen's kappa, 0.918 > 0.8) showing that this commercial assay represents an alternative method for the diagnosis of *P. jirovecii* infections.

Biological diagnosis of *Pneumocystis jirovecii* (*P. jirovecii*) infections is essentially based on the detection of the fungus in pulmonary specimens using microscopic examination and polymerase chain reaction (PCR) assays. *P. jirovecii* infections cover a large spectrum of clinical presentations of which *Pneumocystis* pneumonia (PCP) represents only a part, whereas mild infections revealed by pulmonary colonization may be the main part (Morris and Norris 2012; Nevez et al. 1999). *P. jirovecii* colonization is mostly characterized by a low pulmonary fungal burden for which the diagnosis requires the use of highly sensitive techniques such as PCR assays (Morris and Norris 2012; Nevez et al. 1999; Wakefield et al. 1990). In this study, a commercially available kit, the RealCycler PJIR kit® (Progenie Molecular) (pmPCR), was compared to an in-house real-time PCR (ihPCR) assay widely used in France for *P. jirovecii* infection diagnoses (Totet et al. 2003; Le Gal et al. 2016). Both assays target the mitochondrial large subunit ribosomal rRNA gene of *P. jirovecii* and use an hydrolysis probe with an FAM fluorophore. The technical characteristics of the two assays are detailed in Table 1.

Thirty-four patients monitored at Brest University Hospital, Brest, France, between December 2012 and September 2015 and who underwent pulmonary sampling for *Pneumocystis* infection diagnosis were retrospectively enrolled. In the present study, their pulmonary specimens were retrospectively examined. Patients' characteristics are summarized in Table 2.

Fourteen patients were initially diagnosed with PCP, whereas 15 patients were diagnosed with *P. jirovecii* pulmonary colonization. The diagnosis of PCP was based on clinical and radiological signs compatible with PCP (Centers for Diseases Control and Prevention 1992) and a positive result of *P. jirovecii* detection by microscopic examination and/or the ihPCR assay. *P. jirovecii* was detected in 10 out of the 14 PCP patients by microscopic examination using Wright-Giemsa stain and/or indirect immunofluorescence assay (Monofluo™ Kit *P. jirovecii*, Biorad) and the ihPCR assay (Totet et al. 2003). The ihPCR assay had been performed after DNA extraction (NucliSENS® easyMAG® system, bioMérieux) as described elsewhere by Totet et al. (2003). The fungus was detected only by the ihPCR in 4 PCP patients. *P. jirovecii* was detected only by the ihPCR assay in 15 colonized patients. These 15 patients were considered to be colonized since they presented risk factors for being infected with *P.jirovecii* but had alternative diagnoses of PCP, such as bronchial carcinoma (5 patients), bacterial pneumonia (4 patients), COPD exacerbation, pulmonary fibrosis, idiopathic interstitial pneumonia, drug induced pneumonia, invasive pulmonary aspergillosis, bronchiolitis obliterans (one patient each). Five other patients not infected with the fungus and negative for its detection using microscopic examination and the ihPCR assay were also enrolled. They represented a negative

* Corresponding author. Tel.: +33-2-98-14 51 02; fax: +33-2-98-14-51-49.
E-mail addresses: thibaud.guillaud-saumur@hotmail.fr (T. Guillaud-Saumur), solene.legal@univ-brest.fr, solene.legal@chu-brest.fr, (S. Le Gal).
[1] Alternate corresponding author: Tel.: +33 2 98 14 51 02; fax: +33 2 98 14 51 49.

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Table 1
Technical characteristics of the in-house PCR and the RealCycler® PJIR kit (Progenie molecular).

	ihPCR[a]	pmPCR[b]
Target	mtLSUrRNA[c]	mtlSUrRNA[c]
PCR type	Qualitative or Quantitative real-time PCR	Qualitative real-time PCR
Fluorescent reporter	TaqMan® MGB probe (FAM) (Applied Biosystems)	Hydrolysis probe (FAM)
Detection of PCR inhibitors	Yes (TaqMan® exogenous internal positive control reagents, Applied Biosystems)	Yes (Competitive Heterologous Internal Control (CHIC), Progenie Molecular)
Control of cellular quality	Yes (TaqMan® Rnase P Control reagents (VIC), Applied Biosystems)	No
Passive reference dye	Yes (ROX™, Affymetrix)	No
Ready-to-use PCR mix	No	Yes (AmpliMix containing probe, primers, buffer, polymerase and CHIC)

[a] ihPCR = in-house PCR, modified from Totet et al. 2003.
[b] pmPCR = RealCycler® PJIR, Progenie Molecular.
[c] mtLSUrRNA = mitochondrial large subunit ribosomal RNA.

control group. Extracted DNA specimens were stored at −80 °C for possible reexamination.

The 34 archival DNA specimens were reexamined contemporaneously using the aforementioned ihPCR assay (Totet et al. 2003) and the pmPCR assay, on the 7500 Real-Time PCR system (Applied Biosystems). The ihPCR assay was performed as described by Totet et al. (2003). Briefly, 5 μL of DNA specimen were added to a reaction mixture containing 12.5 μL of TaqMan® Gene expression master Mix (Applied Biosystems), 0.1 μM of the probe and 0.2 μM of PCW3 and PCW4 primers for a final volume of 25 μL. The thermocycling program consisted of activation of the uracil-DNA glycosylase at 50 °C for 2 min, activation of the AmpliTaq® Gold DNA polymerase (Applied Biosystems) at 95 °C for 10 min and 45 cycles with denaturation at 95 °C for 15 sec followed by annealing and extension at 60 °C for 1 min. The cellular quality of pulmonary specimens was controlled using amplification of a single copy human gene (TaqMan® RNase P Control Reagents Kit, Applied Biosystems). An internal positive control (TaqMan® Exogenous Internal Positive Control Reagents, Applied Biosystems) was used to detect PCR inhibitors.

The pmPCR assay was performed according to the manufacturer's recommendations (http://www.progenie-molecular.com/PJIR-U-IN.pdf). Briefly, 7.5 μL of DNA specimen were added to 17.5 μL of AmpliMix for a final volume of 25 μL. The thermocycling program consisted of activation of the Taq polymerase at 95 °C for 15 min and 45 cycles with denaturation at 95 °C for 15 sec followed by annealing at 60 °C for 30 sec and extension at 72 °C for 30 sec. Five additional DNA specimens, one negative and four positive for *P. jirovecii* provided by an External Quality Assessment Programme [Quality control for Molecular Diagnostics (QCMD), Glasgow, UK, http://www.qcmd.org], were also examined in the same manner.

The six specimens initially detected as negative, 5 clinical specimens, and one QCMD quality control, were confirmed as negative with both PCR assays. Among the 33 specimens initially detected as positive (29 clinical specimens and four QCMD quality controls), 31 specimens (27 clinical specimens and four QCMD quality controls) were positive with both PCR assays (Fig. 1). The two remaining specimens led to discrepant results. One specimen obtained from a colonized patient harboring a low pulmonary fungal burden and that initially had a quantification cycle (Cq) value ≥40 using the ihPCR assay was negative with both PCR assays. These discrepant results may be explained by the low fungal burden and DNA degradation due to iterative freezing and thawing. The second discrepant result concerned one specimen from another colonized patient previously known to have a Cq value ≥35. It was confirmed to be positive using the ihPCR assay but it was found negative with the pmPCR assay. Two main hypotheses may be considered to explain this result. It may be either related to a false positive result of the ihPCR assay or to a lack of sensitivity of the pmPCR assay. To test these hypotheses, an end-point PCR targeting another *P. jirovecii* gene, i.e. the cytochrome b gene, was performed as described elsewhere (Esteves et al. 2010). The *P. jirovecii* cytochrome b gene was indeed successfully amplified (data not shown). Thus, the hypothesis of a false positive result of the ihPCR assay can be ruled out and consequently, the pmPCR assay appears to be less sensitive.

Table 2
Characteristics of the 34 patients whose pulmonary specimens were retrospectively re-examined with an in-house PCR assay vs. the RealCycler PJIR kit (Progenie molecular).

	Patients with *Pneumocystis* pneumonia	Patients with *Pneumocystis* colonization	Negative control group
No. of patients	14	15	5
Median age [range in years]	59.4 [34–85]	65.0 [49–88]	65.9 [51–74]
Sex ratio M/F	9/5	10/5	2/3
Clinical underlying condition	Hematological malignancy (3), HIV infection (4), Solid tumor (3), Underlying chronic pulmonary disease[a] (2), Transplantation (1), Corticosteroid treatment (1)	Hematological malignancy (3), Solid tumor (3), Underlying chronic pulmonary disease[a] (8), Transplantation (1)	Underlying chronic pulmonary disease[a] (4), Hematological malignancy (1)
Kind of pulmonary samples	BAL[b] (13), Biopsy (1)	BAL[b] (14), Sputum (1)	BAL[b] (5)
Initial techniques of *P. jirovecii* detection	ihPCR[c] assay [14 of which 10 were positive by microscopic examination (Wright-Giemsa and/or IFA[d])]	ihPCR[c] assay [15 of which none were positive by direct examination (Wright-Giemsa and/or IFA[d])]	Absence of *P.jirovecii* detection neither by microscopic examination nor ihPCR [c] assay

[a] Underlying chronic pulmonary diseases: Chronic obstructive pulmonary disease, pulmonary fibrosis, chronic bronchitis, pulmonary infection or emphysema.
[b] BAL, bronchoalveolar lavage.
[c] ihPCR, in-house PCR.
[d] IFA, indirect immunofluorescence assay (Monofluo™ Kit *P. jirovecii*, Biorad).

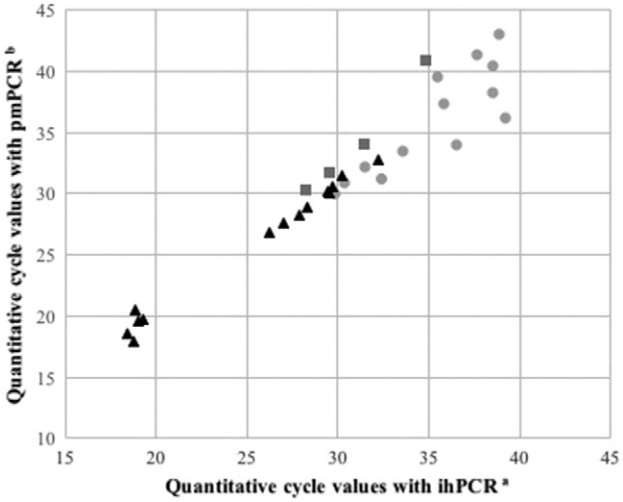

Fig. 1. Distribution of quantitative cycle values obtained with the in-house PCR and the RealCycler PJIR kit (Progenie molecular) for the 31 specimens which were found positive for *Pneumocystis jirovecii* detection with both assays. [a] ihPCR, in-house real-time PCR; [b] pmPCR, RealCycler PJIR kit (Progenie molecular). ■ quality controls; ▲ pulmonary specimens from patients with *Pneumocystis* pneumonia; ● pulmonary specimens from colonized patients.

The concordant rate between the two PCR assays was evaluated at 97.4% (Cohen's kappa = 0.918, IC_{95} [0.76–1]). Considering the ihPCR assay as the gold standard, the sensitivity and specificity of the pmPCR assay were 96.9% and 100% respectively.

Finally, although the RealCycler® PJIR kit (Progenie Molecular) appears to be less sensitive than the in-house real-time PCR assay, excellent agreement between the two PCR assays was observed (concordance rate 97.4%; Cohen's kappa, 0.918 > 0.8). The commercially available kit RealCycler® PJIR kit (Progenie Molecular) may also represent a convenient alternative method for the routine laboratory diagnosis of *P. jirovecii* infections.

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