



Evaluation of a new commercial real-time PCR assay for diagnosis of *Pneumocystis jirovecii* pneumonia and identification of dihydropteroate synthase (DHPS) mutations

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ABSTRACT

The PneumoGenius® real-time PCR assay is a new commercial multiplex real-time PCR method, which detects the *Pneumocystis* mitochondrial ribosomal large subunit (mtLSU) and two dihydropteroate synthase (DHPS) point mutations. To evaluate the clinical performance of this new real-time PCR assay we tested 120 extracted DNA samples from bronchoalveolar lavage specimens. These set of extracted DNA samples had already tested positive for *Pneumocystis* and patients had been classified in probable and unlikely PCP in a previous study. To evaluate de accuracy of the DHPS mutant's identification, an "in house" PCR and sequencing was performed. The sensitivity and specificity of PneumoGenius® PCR in discriminating between probable and unlikely *Pneumocystis* pneumonia (PCP) were 70% and 82% respectively. PneumoGenius® PCR was able to genotype more samples than "in house" DHPS PCR and sequencing. The same DHPS mutations were observed by both methods in four patients: two patients with a single mutation in position 171 (Pro57Ser) and two patients with a double mutation in position 165 (Thr55Ala) and in position 171 (Pro57Ser). A low rate of *P. jirovecii* (4.5%) harboring DHPS mutations was found, comparable to rates observed in other European countries. The PneumoGenius® real-time PCR is a suitable real-time PCR for PCP diagnosis and detection of DHPS mutants. The added value of DHPS mutation identification can assist in understanding the role of these mutations in prophylaxis failure or treatment outcome.

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1. Introduction

Pneumocystis jirovecii is an opportunistic fungal pathogen that remains an important cause of pneumonia in immunocompromised patients. The diagnosis of *Pneumocystis* pneumonia (PCP) is based on clinical and radiological manifestations in combination with microbiological evidence. The complexity of PCP diagnosis has already been extensively described, especially for non-HIV immunocompromised patients because of unspecific signs and symptoms and sensitivity limitations of staining techniques (Tasaka and Tokuda, 2012; Walzer et al., 2008). Polymerase chain reaction (PCR) assays have improved diagnostic accuracy and can be especially helpful in those cases of false negative direct examinations (Alanio et al., 2011; Botterel et al., 2012; Flori et al., 2004; Montesinos et al., 2015; Robert-Gangneux et al., 2014).

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Trimethoprim-sulfamethoxazole (TMP-SMX) is the recommended first line treatment and prophylaxis regimen for PCP. This combination inhibits 2 enzymes in the folate metabolism: dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS). Long-term exposure to low levels of TMP-SMX has been identified as a risk factor for developing an infection with *P. jirovecii* DHPS mutants or for selecting DHPS mutants (Kazanjian et al., 1998; Ma et al., 1999; Mei et al., 1998). Nevertheless, the association between DHPS *P. jirovecii* mutants and TMP-SMX prophylaxis or treatment failure in PCP remains unclear (Alvarez-Martinez et al., 2008; Kazanjian et al., 1998; Ma et al., 1999; Mei et al., 1998; Valerio et al., 2007; Yoon et al., 2013).

In this study, we evaluated the new commercial PneumoGenius® real-time PCR (PathoNostics, Maastricht, The Netherlands), that combines *P. jirovecii* amplification with the detection of DHPS mutations. At the same time, we estimated the rate of *P. jirovecii* harboring DHPS mutations in our hospital.

2. Patients and methods

2.1. Patients and clinical samples

We carried out this study on 120 DNA extractions from bronchoalveolar lavage specimens (BALs) which had been tested previously in order to

compare an “in house” PCR (TaqMan-based real-time PCR selecting the beta-tubulin gene like target) to Bio-Evolution *Pneumocystis* quantitative real-time PCR (Bio-Evolution qPCR) (Bry-sur-Marne, France). Briefly, BALs were collected in the context of undetermined pneumonia and were tested positives for *Pneumocystis* by PCR. An infectious disease physician classified the episodes in probable ($n = 34$) or in unlikely PCP ($n = 86$). Probable PCP was diagnosed in patients receiving anti-pneumocystis treatment if all following additional characteristics were present: compatibility of clinical signs among patients with underlying immunodeficiency, presence of hypoxia, compatible radiological findings and response to anti-pneumocystis treatment with absence of alternative diagnosis. Patients not compatible with all criteria described above or patients with respiratory symptoms not typical for PCP or no typical radiological infiltrations and patients with treatment response to other antimicrobial agents or other definite diagnosis were defined as unlikely PCP. One hundred thirteen patients were non-HIV immunocompromised patients and 7 were HIV patients (Montesinos et al., 2015). Patients' past history of *P. jirovecii* prophylaxis or treatment were acquired retrospectively by medical chart review.

2.2. PathoNostics PneumoGenius® real-time PCR

The PathoNostics real-time PneumoGenius® PCR assay is a new commercial multiplex real-time PCR, which detects the *Pneumocystis* mtLSU and DHPS *fas* gene mutations. The point mutations in the DHPS *fas* gene related to resistance can be identified by using melting curve analyses to discriminate between wild type and mutant strains. Stored DNA samples were tested with this real-time PCR according to the manufacturers' instructions using an LC480 real-time system. The cycle threshold value (Ct value) was registered for mtLSU (*P. jirovecii*) positive samples.

2.3. Detection of DHPS mutations: “in-house” PCR and sequencing

The same set of extracted DNA samples were tested for DHPS analysis as described by Dini et al. (2010). Briefly, nested PCR was performed to amplify a 278-bp region encompassing polymorphic nucleotide positions ¹⁶⁵A/G and ¹⁷¹C/T. The nested PCR products were sequenced using BigDye Terminator chemistry and the DNA sequences were analyzed with Bionumerics 6.5 (Applied Maths, Belgium).

DHPS genotypes were classified as: Wild type: no mutation observed; DHPS mut55: single mutation in position 165 (Thr55Ala); DHPS mut57: single mutation in position 171 (Pro57Ser); DHPS mut55–57: double mutation (165 + 171).

2.4. Statistical analysis

Statistical assessment of differences in the means of *P. jirovecii* DNA concentration (Ct value) from probable PCP and unlikely PCP patients was performed using the Student's *t* test. The Mann-Whitney *U* test was used to assess the difference between the medians of Ct values obtained by “in house” PCR and PneumoGenius® PCR. Correlation between three real-time PCR results was analyzed by Spearman's coefficient of rank correlation test. A receiver operator characteristic (ROC) curve was constructed and used to define optimal cut-off values in order to discriminate the probable PCP from the unlikely PCP groups. The comparison of the area under the curve (AUC) of the three ROC curves and kappa statistics were also analyzed. $P < 0.05$ was considered statistically significant. Statistical analysis was performed with MedCalc software (Mariakerke, Belgium).

3. Results

3.1. PneumoGenius® PCR results

3.1.1. *Pneumocystis* DNA amplification

Pneumocystis DNA amplification by the PneumoGenius® PCR was observed in 118 samples. Two samples with the “in house” PCR (both

Ct 40) failed in the amplification. The median Ct value obtained with the PneumoGenius® PCR was statistically lower than the in house real-time PCR median Ct value ($P < 0.001$) (Fig. 1).

In the box and whisker plots, the boxes contain 50% of sample data, with the median Ct value indicated by horizontal bar. Mean Ct values are indicated by an orange marker. The whiskers contain 1.5× the interquartile range. Ct values were significantly lower in the probable PCP group when compared with the unlikely PCP group ($P < 0.001$).

The mean Ct value in the probable PCP patients group (Ct = 30) was significantly lower than the mean Ct value of unlikely PCP patients (Ct = 35) ($P < 0.001$) (Fig. 2).

In the box and whisker plots, the boxes contain 50% of sample data, with the median Ct value indicated by horizontal bar. Mean Ct values are indicated by an orange marker. The whiskers contain 1.5× the interquartile range. Ct values were significantly lower in the probable PCP group when compared with the unlikely PCP group of patients ($P < 0.001$).

An estimated cut-off value (Ct = 32) was calculated by ROC curve to evaluate the clinical performances of PneumoGenius® PCR: the sensitivity and specificity of PneumoGenius® PCR for discriminating probable from unlikely PCP were 70% and 82% respectively; positive and negative predictive values were 60% and 87% respectively; positive and negative likelihood ratios were 3.95 and 0.37, respectively.

We observed a good correlation between PneumoGenius® PCR and “in house” PCR results ($r = 0.80$; 95% 0.70–0.80; $P < 0.001$) and Bio-Evolution qPCR results ($r = -0.90$; 95% CI $-0.93/-0.86$; $P < 0.001$) (Figs. 3 and 4). Comparison of the areas under de curve (AUCs) showed that the three real-time PCR methods had comparable diagnostic performances for the discrimination of probable and unlikely PCP (Difference between Bio-Evolution qPCR and PneumoGenius® PCR areas = 0.00849; 95% CI = -0.02 to 0.03; $P = 0.5$. Difference between “in house” PCR and PneumoGenius® PCR areas = 0.066; 95% CI = -0.003 to 0.1; $P = 0.062$) (Fig. 5).

3.1.2. Detection of DHPS mutations by PneumoGenius® PCR

Amplification of DHPS gene was observed in 118 samples but melting peaks were only observed in 89 samples. The results and clinical description of patients harboring DHPS mutants are included in Table 1. Based on the PneumoGenius® results, the rate of *P. jirovecii* harboring DHPS mutations in our hospital population was 4.5% (4 out of 89). During the study period, only 12 patients were on TMP-SMX prophylaxis at the moment of PCP diagnosis.

3.2. Detection of DHPS mutations by “in-house” PCR and sequencing

Amplification and sequencing of the DHPS *fas* gene was successfully performed in 72 extracted DNA samples. A 100% agreement was

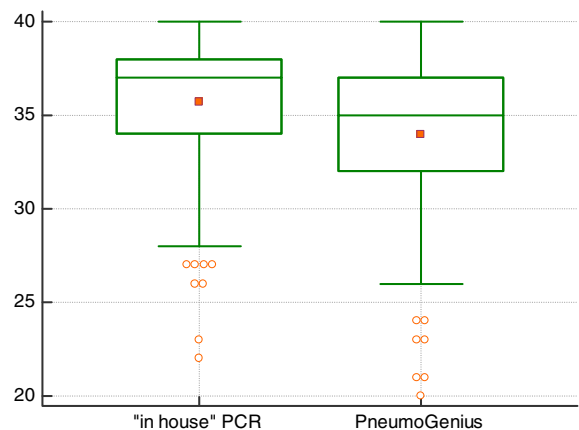


Fig. 1. Cycle threshold (ct) values obtained by the “in house” and PneumoGenius® real-time PCR.

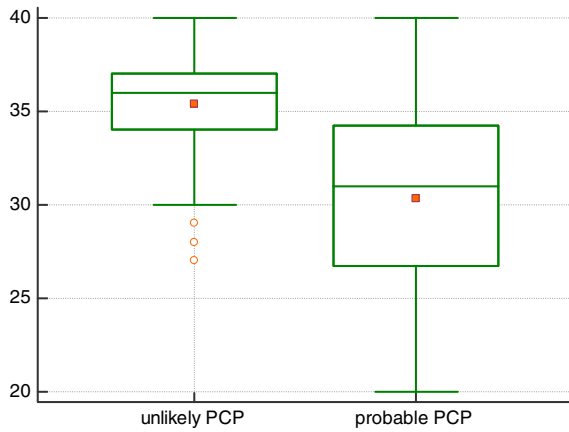


Fig. 2. Cycle threshold (Ct) values obtained by PneumoGenius real-time PCR in the group of patients with unlikely PCP and probable PCP.

obtained between PneumoGenius® PCR and the “in house” PCR and sequencing method for DHPS mutation identification (Table 1).

4. Discussion

In this study, we evaluated the PathoNostics PneumoGenius® real-time PCR, a new commercial PCR that combines *Pneumocystis jirovecii* detection and DHPS mutation identification.

In the first part of this work, we evaluated the clinical performance of the PneumoGenius® real-time PCR for the diagnosis of PCP, testing stored DNAs from previously analyzed BALs in order to compare an “in house” PCR and the Bio-Evolution qPCR, another commercial real-time PCR. The high percentage of non-HIV immunocompromised patients included in this study (93%) could explain the lower clinical performances obtained in comparison with those observed by others (Alanio et al., 2011; Botterel et al., 2012; Robert-Gangneux et al., 2014). Low-burden *Pneumocystis* infections and overlapped zones of PCR results have been extensively observed in the literature for this group of patients (Botterel et al., 2012; Montesinos et al., 2015; Robert-Gangneux et al., 2014). As expected, we observed lower Ct values with the PneumoGenius® PCR in comparison to the Ct values obtained by our “in house” PCR assay. PCR assays targeting multi-copy genes like PneumoGenius and Bio-Evolution qPCR (mtLSU) were found to have higher sensitivity than those who target a single copy gene like the ‘in house’ PCR does (*β-tubuline* gene) (Alanio et al., 2011; Botterel et al., 2012; Robert-Gangneux et al., 2014). The clinical performance of PneumoGenius® PCR in discriminating between probable and unlikely PCP was similar to the clinical performance observed

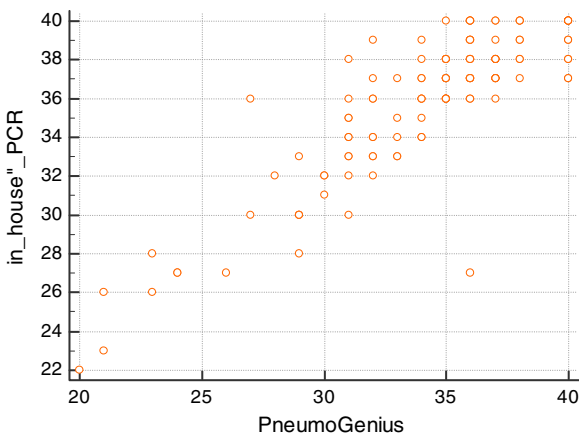


Fig. 3. Scatter diagram showing correlation between CT values by PneumoGenius and “in house” real-time PCR.

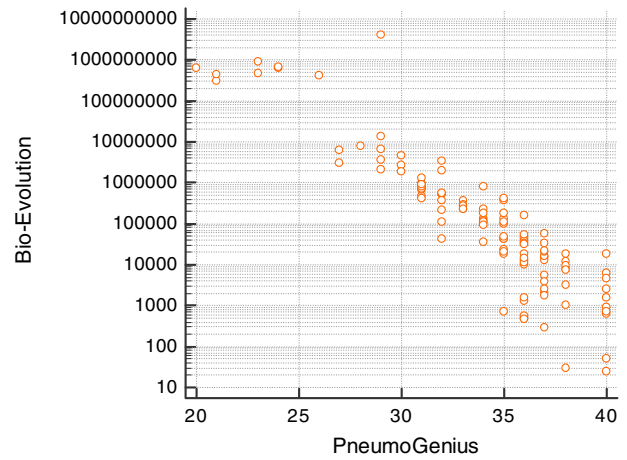


Fig. 4. Scatter diagram showing negative correlation between ct values by PneumoGenius real-time PCR and copies/mL by Bio-evolution qPCR.

previously by Bio-evolution qPCR and an “in house” PCR on the same set of samples (Montesinos et al., 2015). Two samples were not detected by the PneumoGenius® PCR probably because of low fungal burden and/or supplementary freezing–thawing cycles of DNA samples.

The detection of *Pneumocystis* in persons without signs or symptoms of PCP is defined as colonization. Children, HIV and non-HIV immunosuppressed patients, chronic lung diseases patients, inter alia, may be colonized by *Pneumocystis*. Although the clinical significance is still unknown, colonization would maintain the presence and transmission of *Pneumocystis* (Morris and Norris, 2012; Morris et al., 2008). In that context, the higher sensitivity of PCR methods brings a new difficulty in diagnosis and patient management: the discrimination between colonization and active PCP. This is especially difficult in non-HIV immunocompromised patients who are susceptible to develop low-burden *Pneumocystis* infections.

In the second part of the study, we evaluated the detection of DHPS mutations by the PneumoGenius® PCR in comparison with an “in house” DHPS PCR and sequencing and we determined the rate of *Pneumocystis* DHPS mutants in our hospital. The PneumoGenius® PCR was able to genotype 17 more samples than the “in house” method. Nevertheless, 25% of the samples remained untypeable. In these

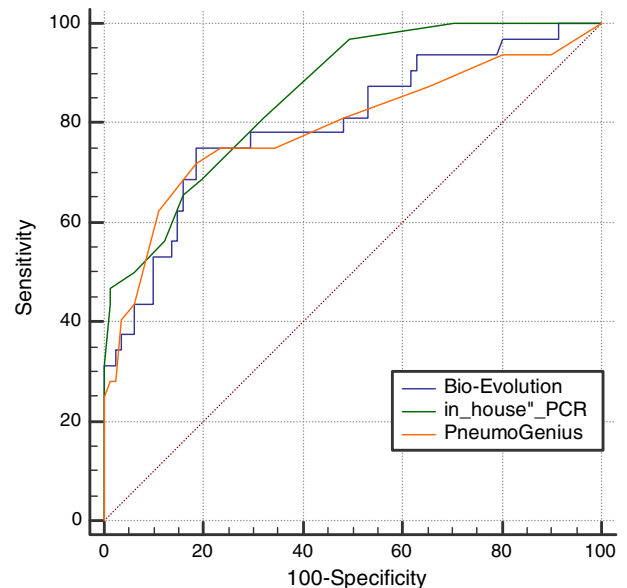


Fig. 5. Comparison of AUC curves of CT values obtained by “in house” and real-time PneumoGenius real-time PCR and copies/mL by Bio-Evolution qPCR.

Table 1

Pneumocystis jirovecii DHPS genotypes detected by “in house” method and PneumoGenius® and description of patients harboring these DHPS mutants admitted at Hôpital Erasme in Brussels between January 2010 and September 2014.

DHPS genotype	Nucleotide at position (amino acid at position)		“In-house” method No. of patients	Pneumo Genius® No. of patients	Underlying disease	Prophylaxis	Treatment	Outcome
	165 (55)	171 (57)						
Wild type (wt) (W165/W171)	A (Thr)	C (Pro)	68	85				
DHPS mut55 (M165/W171) ^a	G (Ala)	C (Pro)	0	0				
DHPS mut57 (W165/M171) ^b	A (Thr)	T (Ser)	1	1	Renal transplantation	none	TMP-SMX	Deceased
DHPS mut55–57 (M165/M171) ^c	G (Ala)	T (Ser)	1	1	Severe Rheumatoid Arthritis ^f	none	none	Good
Mixed infection (mut55 + wt)			1	1	HIV patient	TMP-SMX	Pentamidine	Good
Not genotyped			48 ^d	31 ^e	Sjögren's syndrome	none	Pentamidine ^g	Good

^a Mutation at nucleotide position 165.

^b Mutation at nucleotide position 171.

^c Double mutation at nucleotide position 165 and 171.

^d No amplification of DHPS gene.

^e No amplification in 2 samples and no melting peaks observed in 29 samples.

^f Patient considered to be colonized by *P. jirovecii*.

^g Patient was switched successfully to pentamidine after poor clinical response despite 10 days of TMP-SMX treatment.

samples a very low fungal load was observed (PneumoGenius® mean CT value = 38). DHPS *fas* gene is a single copy gene, which explains this lower sensitivity in limited fungal burden samples.

To our knowledge, this is the first evaluation of *P. jirovecii* DHPS mutation rates in Belgium. We observed a low rate of *P. jirovecii* harboring DHPS mutations (4.5%) in our hospital population. Others European countries have also reported low prevalence of DHPS mutations, for example: Italy: 8% (Valerio et al., 2007), Portugal: 7% (Esteves et al., 2008), Spain: 3.7% (Alvarez-Martinez et al., 2008) and Sweden: 0% (Baser et al., 2012). Nevertheless, large variations in prevalence between cities in the same country have been observed. For example, in France the prevalence ranges from 0% in Brest to 40% in Paris (Le Gal et al., 2012; Magne et al., 2011). Differences in geographical prevalence of *P. jirovecii* DHPS mutants may be due to intrinsic epidemiological factors and to differing use of sulphonamides for PCP prophylaxis (Alvarez-Martínez et al., 2010; Magne et al., 2011). Only 12 patients (10%) were in TMP-SMX prophylaxis at the moment of *Pneumocystis* detection by PCR which could explain the low rate of DHPS mutants.

An increased risk of DHPS mutants has been shown among patients reporting sulfa-prophylaxis or treatment (Stein et al., 2004). In contrast, the relation of DHPS mutations with mortality and poor outcome is unclear. Recent studies have demonstrated a successful outcome in most patients with PCP containing DHPS mutants treated with therapeutic doses of TMP-SMX, suggesting that mortality is related primarily to the underlying severity of illness and the initial severity of PCP (Alvarez-Martinez et al., 2008, 2010; Baser et al., 2012; Dini et al., 2010; Esteves et al., 2008; Le Gal et al., 2012; Magne et al., 2011; Morris and Norris, 2012; Morris et al., 2008; Yoon et al., 2013). In this study, it was difficult to evaluate prophylactic and/or treatment failures since only a low rate of *P. jirovecii* harboring DHPS mutations was observed. However, among the few patients harboring mutants, 1 patient failed to respond to TMP-SMX treatment and 1 patient failed to respond to TMP-SMX prophylaxis suggesting a possible correlation. Yoon et al. suggest further studies on different DHPS genotypes taking into account well defined clinical variables and outcomes to better understand the clinical impact of these mutations (Yoon et al., 2013).

5. Conclusion

The PneumoGenius® PCR showed comparable clinical performance to other PCR assays for detection of *P. jirovecii* but has the added value of simultaneous identification of DHPS mutations. However, to

discriminate PCP and colonization, the use of multiple diagnostic methods remains essential. A low rate of *P. jirovecii* harboring DHPS mutations was found in our institution and is comparable to rates observed in other European countries. In our opinion, the additional information about DHPS mutation in the same test could assist in understanding the role of these mutations in prophylaxis failure or treatment outcome. It is necessary to continue studying on *P. jirovecii* mutations related to sulfa-drug resistance, as they could lead to higher levels of resistance in the future.

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Transparency declarations

None to declare.

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