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▶ **To cite this version:**

Hélène Guegan, Florence Robert-Gangneux. Molecular diagnosis of Pneumocystis pneumonia in immunocompromised patients. Current Opinion in Infectious Diseases, 2019, 32 (4), pp.314-321. 10.1097/QCO.0000000000000559 . hal-02153616

HAL Id: hal-02153616

https://univ-rennes.hal.science/hal-02153616

Submitted on 12 Sep 2019

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Molecular diagnosis of *Pneumocystis* pneumonia in immunocompromised patients

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This is a non-final version of an article published in final form in Current Opinion in Infectious Diseases: August 2019 - Volume 32 - Issue 4 - p 314–321

ABSTRACT

***Purpose of review**: Pneumocystis* pneumonia (PCP) is a frequent opportunistic infection associated with a high mortality rate. PCP is of increasing importance in non-HIV immunocompromised (IC) patients, who present with severe respiratory distress with low fungal loads. Molecular detection of *Pneumocystis* in broncho-alveolar lavage (BAL) has become an important diagnostic tool, but quantitative PCR (qPCR) needs standardization.

***Recent findings**:* Despite a high negative predictive value, the positive predictive value of qPCR is moderate, as it also detects colonized patients. Attempts are made to set a cut-off value of qPCR to discriminate between PCP and colonization, or to use non-invasive samples or combined strategies to increase specificity.

***Summary**:* It is easy to set a qPCR cut-off for HIV-infected patients. In non-HIV IC patients, a gain in specificity could be obtained by combining strategies, i.e. qPCR on BAL and a non-invasive sample, or qPCR and serum beta-1,3-D-glucane dosage.

Keywords: Pneumocystis jirovecii, Pneumocystis pneumonia, immunocompromised patients, molecular diagnosis, PCR

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INTRODUCTION

Pneumocystis jirovecii (Pj) is responsible for *Pneumocystis* pneumonia (PCP), a life-threatening fungal infection in immunocompromised patients, ranking first among opportunistic pathogens revealing the HIV-positive status (1,2) and in HIV-infected patients who do not comply with antiretroviral therapy or anti-*Pneumocystis* prophylaxis (3). Over the last decade, PCP has been also recognized as a major opportunistic infection in non-HIV immunocompromised patients, such as transplant patients, patients with hematological malignancies or solid cancers, and patients receiving corticosteroid therapy or other immunosuppressive drugs (4). The growing number of immunosuppressive procedures led to an increasing frequency of PCP diagnoses in non-HIV compared to HIV-infected patients (3,5,6). The heterogeneity of immune background in this non-HIV population can influence the clinical and radiological signs, which can be misleading, since they are often less typical than in HIV patients (2,7).

Mortality in the non-HIV-infected immunosuppressed population is generally higher than that in HIV infection, reaching 50% in some studies (8,9). Whether this poor outcome is due to the patient immune background itself, or to gaps in diagnosis or management of patients at risk, is unclear. However, it is now widely recognized that evolution of the disease is more acute than in HIV-infected patients, with rapid clinical deterioration even with low fungal loads (2). Therefore, rapid and reliable diagnosis is of critical importance.

As Pj cannot be cultured, diagnosis relies on its detection in broncho-alveolar lavage (BAL) or other lung specimens, by microscopic examination and PCR. While microscopy is helpful to diagnose PCP in HIV-infected patients who usually harbor high fungal loads, molecular diagnosis is critical to detect low Pj counts in non-HIV patients (6), and PCR has been included in the guidelines for the diagnosis of PCP in patients with hematological malignancies (10). Pj PCR is assumed to have a 100% negative predictive value (NPV). Meanwhile, these sensitive methods have highlighted the possible colonization of hospitalized patients with Pj, thus limiting the positive predictive value (PPV) of PCR result. Therefore, continuous efforts are ongoing to improve the interpretation of molecular results or to develop new strategies of amplification that would be more specific of acute infection.

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In this article, we provide a review of the literature on the molecular diagnosis of PCP, with special emphasis on the interpretation of results and possible complementary diagnostic tools.

MOLECULAR DIAGNOSIS OF PCP: TECHNICAL ASPECTS AND DIAGNOSTIC IMPACT

Gene targets and PCR methods: what's new?

Many gene targets have been described for the molecular diagnosis of PCP and have been assessed in clinical series (Table 1) (11–33). The mitochondrial large sub-unit of ribosomal RNA (mtLSU) has been the first target for PCP diagnosis (11) and it is still the most widely used and reported in the literature. Among the multiple gene targets described, mtLSU, mtSSU and the major surface glycoprotein (MSG) are the only gene targets to be repeated in *Pneumocystis* genome, and thus are associated to a very high sensitivity of detection, compared to single-copy targets. Actually, single copy loci such as 5.8SrRNA, DHFR, DHPS, TS and beta-tubulin have been also investigated for PCP diagnosis, but they are more often used for Pj genotyping in epidemiological studies (34). The use of conventional amplification techniques has been supplanted by real-time PCR, which offers improved specificity and allows to quantify fungal loads in fluid samples. However, no clear correlation has been made to date between parasite concentration and severity of infection, probably, at least partially, because of a variable number of copies of the targeted genes (32)**.

A reverse-transcriptase PCR assay targeting the mRNA from a gene encoding a *Pneumocystis* HSP70 was also investigated with the aim to detect only viable organisms (27,35), to monitor treatment. It was evaluated on a HIV-infected cohort, but despite good performances on BAL compared to microscopy (100% concordance), the detection rate strongly decreased in induced sputum (IS) and oral wash (OW) samples (65% and 35%, respectively), thus hampering the use of less invasive specimens to monitor treatment efficacy after initial diagnosis.

Over the last decade, several commercial quantitative PCR (qPCR) assays have been developed, all targeting mtLSU or MSG gene (Table 2) (36–40). MycAssay *Pneumocystis* (36–40), BioEvolution PCR (39,41) and Amplisens (39,42) have been the most evaluated. Sasso *et*

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al. compared these 3 assays with an in-house RT PCR targeting MSG and found excellent performances of all assays excepted BioEvolution kit, to detect very low fungal burdens (39). Over the past 2 years, a growing number of assays have been commercialized and evaluated in clinical series, yielding heterogenous performances with sensitivity and specificity ranging from 60 to 100% and from 82 to 100%, respectively (42–46).

To date, only 2 real-time assays have been adapted on fully automated platforms combining DNA extraction and multiplex real-time PCR, allowing on-demand molecular testing (47–49*). The BD MAX system was prospectively evaluated and yielded similar sensitivity as a reference qPCR assay (47). Moreover, samples from patients with colonization had significantly higher median amplification cycle threshold (Ct) values than samples from patients with PCP (32.0 vs 25.7; p = 0.002). The Genecube platform was evaluated on BAL and IS samples and yielded similar sensitivity compared with "in-house" qPCR assay (92.3% *versus* 94.9%) (49)*.

As an alternative method to PCR, the loop-mediated isothermal amplification (LAMP) technique has been described to amplify *Pneumocystis* DNA (22). Nakashima *et al*. retrospectively assessed LAMP performances in BAL and IS from 78 non-HIV immunocompromised patients and demonstrated LAMP to have sensitivity and positive predictive value of 95.4% and 91.3%, respectively, higher than that of nested PCR (72.7%, 84.2%, respectively) (50). However, despite these interesting performances, clinical evaluations on larger cohorts are still needed and the absence of fungal load quantification does not help interpretation of Pj presence as colonizing or pathogenic agent.

Sample types: which are best suited?

BAL is considered as the "gold standard" procedure to diagnose PCP, since i) it provides the most appropriate sample to perform microscopic staining to detect trophic forms and cysts of Pj, and ii) it allows to retrieve Pj from the lower respiratory tract (LRT), thus most likely responsible for infection. Therefore, a fair amount of literature evaluated molecular diagnosis in BAL samples.

However, if the patient cannot stand BAL which is an invasive procedure associated to potential complications, tracheal aspirates, induced sputum (IS), or oral washes (OW) can be

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used, as well as nasopharyngal aspirates (NPA) for neonates (51–56). However, these samples from the upper respiratory tract are not suitable for microscopic examination, thus molecular diagnosis is essential, but must be carefully evaluated in clinical practice. Few studies have addressed prospectively a comparative evaluation of molecular diagnosis on paired samples of BAL and other sample types (Table 3). Alanio *et al*. found no significant difference in fungal DNA loads between IS and BAL fluid samples from immunocompromised patients (mixed HIV status)(57). Teh *et al*. in cancer patients obtained higher levels of Pj DNA detection in IS than in BAL, probably because it is less diluted, but found no link with the severity of infection (58).

When comparing PCR on OW and BAL paired samples, the sensitivity was shown to be significantly reduced in the former, but the PCR specificity increased (Table 3). However, it is awkward that Juliano *et al*. found 11 false-negative results of PCR on OW out 61 paired BAL with positive microscopy, suggesting that this sample might be inadequate for the diagnosis of PCP in HIV-negative patients, who present frequently with PCP with negative microscopy (51).

On the other hand, Samuel *et al*. (55) reported NPA to be of interest for PCP confirmation in young children. On a series of 147 NPA, they reported roughly similar DNA detection rates in NPA, compared to paired IS or BAL (49.6% *versus* 55.1%).

Apart from respiratory specimens, the detection of circulating Pj DNA in peripheral blood has been considered in a few reports, based on the Pj transient passage from lung alveoli towards the blood compartment, that would occur during PCP. Whether Pj PCR in blood sample is a valuable tool to diagnose PCP remains controversial. Wang *et al*. (59)* recently demonstrated that despite a moderate sensitivity compared to BAL or sputum (68.6% *versus* 91.4-97.1%), PCR on serum had a higher PPV (96% versus 86.5-87.2%). Therefore, when BAL is not available, they emphasized the relevance of combining PCR on sputum and serum to reach similar performances.

PERFORMANCE OF MOLECULAR DIAGNOSIS

How to evaluate clinical performance?

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The evaluation of the clinical performance of molecular diagnosis of PCP is a difficult task and should be made in each individual institution. Indeed, data from the literature are barely comparable, because i) lack of assays standardization (gene target, PCR method, type of sample and processing before DNA extraction), and ii) lack of standardized score to classify patients for the diagnosis of PCP (definite PCP, probable PCP or colonization), both influence the evaluation of PCR performances. Some authors use radiological and clinical findings associated to a positive microscopic and/or molecular detection of Pj to classify the diagnosis as PCP, while others also consider the clinical improvement after cotrimoxazole therapy. The interpretation of a positive PCR when direct examination is negative is particularly challenging when there is another possible cause for respiratory symptoms, and authors use variable criteria to define PCP in this setting. Additionally, the proportion of patients with probable PCP or colonization may vary according to the population study, i.e. the patients' immune background. Whether Pj detection represents an aggravating factor leading to shortened survival, or only the reflection of colonization, may be difficult to appreciate, and can lead to underestimate the PPV of a molecular assay. Therefore it is important to keep in mind these limitations, as they might greatly impact the estimation of PCR specificity in clinical studies. In any case, the use of microscopic detection as gold standard, should now be definitely avoided.

Sensitivity of PCR on BAL samples is usually considered to be as high as 100%, although unpredictable mutation at the site of probe hybridization can be an anecdotic source of false-negative result (60). Consequently, the 100% NPV allows to use qPCR as an exclusion test, but positive qPCR results need careful interpretation when microscopy is negative. Therefore, attempts have been made to set up a threshold to distinguish between probable PCP and colonization, which is feasible on BAL samples only, provided that lavage volumes instilled are somewhat standardized. Given the absence of Pj international standard material, these cut-offs are assay-specific and are expressed in various units (Ct of amplification or number of copies). As stated above, due to the variability in the number of copies per genome and the unknown proportion of cysts and trophic forms in a given sample, the significance of quantification is still debatable.

Over the past decade, several studies have proposed to define cut-off values in relation to clinical diagnosis. Alanio *et al* (57) estimated the sensitivity and specificity of qPCR, using different qPCR cut-off values to discriminate between PCP and colonization: a >1900 trophic

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form equivalent (TFEq) /ml value giving 100% PPV, and a <120 TFEq/mL value yielding a 100% NPV. A limited number of samples quantified within the grey zone were considered to be undetermined. Other authors proposed cut-off values expressed as cycle threshold (Ct) of amplification or number of gene copies per mL. As shown in Table 4, these thresholds are highly variable, depending on the gene target, the sample input for DNA extraction, the HIV status and the bronchial lavage volume according to local procedures of pneumologists (6,41,61–65). These findings underline the need to standardize analytical procedures to make comparison possible between hospital centers. Overall, the use of a threshold value is easier to handle for HIV-infected patients, but is awkward for non-HIV patients (6,41,62).

Recently, Valero *et al*. (32)** quantified 4 different mitochondrial (mt) genes, and compared their copy numbers to two nuclear single-copy genes in 84 BAL samples from patients with various background. Interestingly, they observed a high variation in the copy numbers of mt genes and found no correlation with the immunofluorescence (IF) detection of Pj, nor the PCP diagnosis. For the six genes tested, there was an overlap in the Ct between IF-positive and IF-negative samples, ranging from 34 to 54%, and a variation of the number of copies according to the fungal load in 5 out of 6. Taken together, mt small sub-unit (mtSSU) rRNA displayed the highest copy number and seemed to be the most stable, thus could be a more sensitive target to detect Pj and probably more robust gene to quantify fungal loads.

In conclusion, as no qPCR assay currently allows to safely distinguish a colonized patient from an infected patient, the absence of Pj DNA detection can be used to rule out PCP, but the presence of Pj DNA in pulmonary samples must be interpreted according to the severity of clinical signs and the immune background of the patient.

Use of qPCR result as a prognosis marker

Although attributable mortality is difficult to evaluate, the co-infection or colonization with Pj could be an aggravating factor, possibly worsening prognosis or reducing survival of patients with cancer, and should be taken into account to improve survival (6). Additionally, several studies indicated that colonization with Pj could be the first step before the development of acute PCP in the following weeks or months, particularly in HIV-negative immunocompromised patients.

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On the other hand, Choi *et al*. reported that negative conversion of PCR result after specific therapy was associated with lower mortality rates (HR 0.433 [0.203-0.928], p<0.05) in HIV-negative patients with severe PCP (66). They suggested that qPCR could be used to monitor treatment efficacy and to guide a switch of treatment, if first-line treatment does not result in negative PCR. Overall, they observed that patient outcome was poorer when time-to PCR negativation was longer. However, we presently lack other studies to validate these findings and to recommend patient monitoring by qPCR at a larger scale.

FUTURE PERSPECTIVE: COMBINED DIAGNOSTIC STRATEGIES?

Beta 1,3-D glucan (BDG) is a polysaccharide of the walls of most fungi, including *Pneumocystis* cysts. It can be detected in serum specimens from patients with PCP, thus has triggered recent interest as a noninvasive diagnostic test for PCP. Several studies have reported the performance characteristics of commercial BDG assays, with a sensitivity ranging from 90 to 100% and a specificity of 78 to 96% (67), depending on the BDG kit and the patient population.

As BDG is not specific for Pj, it should be associated to Pj qPCR to improve its specificity. Morjaria *et al*. (68)** recently reported a retrospective study on 438 cancer patients with suspicion of PCP, and found that in patients with a positive Pj PCR, BDG had a sensitivity, specificity, and PPV of 88%, 85%, and 96%, respectively. Therefore, combining qPCR and BDG would allow to benefit from the 100% NPV of qPCR, and to increase the PPV when qPCR and BDG are both positive. If such strategy is confirmed in other patient groups, it would greatly improve care management.

Finally, new commercial real-time multiplex assays combine the detection of *Pneumocystis* together with dihydropteroate synthase (DHPS) point mutations responsible for resistance to sulfonamides drugs used for prophylactic and curative PCP therapy. PneumoGenius® assay (PathoNostics) has been recently shown to outpace “in-house” DHPS sequencing in terms of *DHPS* successful amplification (89 *versus* 72 BAL), and gave 100% concordant results (46). However, its sensitivity for diagnosing PCP was only moderate (70%).

In conclusion, despite a significant body of literature on molecular diagnosis of PCP, there is still a need for innovative strategies to improve the PPV. Further studies would be welcome

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to precise the interest of combining qPCR on pulmonary samples with serum BDG, and their respective cut-off to allow a reliable discrimination between colonization and active disease. Meanwhile, the search for new specific markers on non-invasive samples would be highly welcome.

Key points:

- Negative qPCR in BAL can rule out PCP
- qPCR cut-off values are unreliable for patients with hematological malignancies
- Negative qPCR result from non-invasive samples cannot rule out PCP whatever the patient background
- Positive qPCR in BAL and BDG detection in serum is a promising strategy to target specific therapy

Conflicts of interest: none

Sources of funding: none

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KEY POINTS

- Negative qPCR in BAL can rule out PCP.
- qPCR cut-off values are unreliable for patients with hematological malignancies.
- Negative qPCR result from noninvasive samples cannot rule out PCP whatever the patient background.
- Positive qPCR in BAL and BDG detection in serum is a promising strategy to target specific therapy.

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Target	Number of repetitions	Main use	No of clinical studies	Year of first publication	Ref
mtLSU rRNA	Multiple	Conv, RT	100	1990 (conv) 2003 (RT)	[11,12]
MSG	Multiple	Conv, RT	33	1999 (conv) 2002 (RT)	[13,14]
ITS	Single	Conv, RT	14	1994 (conv) 2009 (RT)	[15,16]
5S rRNA	Single	Conv, RT	12	1991 (conv) 2001 (RT)	[17,18]
DHPS	Single	Conv, RT	9	2003 (conv) 2006 (RT)	[19,20]
18S rRNA	Single	Conv, LAMP	7	1994 (conv), 2008 (LAMP)	[21,22]
cdc2	Single	RT	7	2006	[23]
DHFR	Single	Conv, RT	6	1991 (conv) 2007 (RT)	[24,25]
Beta-tubulin	Single	RT	4	2005	[26]
HSP70	Single	mRNA viability assay, RT	4	2001 (mRNA), 2007 (RT)	[27,28]
TS	Single	Conv	3	1993	[29]
28S rRNA	Single	Conv	2	1999	[30]
mtSSU rRNA	Multiple	Conv, RT	2	1996 (conv), 2016 (RT)	[31,32▪▪]
Kex-1	Single	RT	2	2009	[33]
5.8S rRNA	Single	RT	1	2007	[25]

28S rRNA, T1-T2 region of the large subunit ribosomal RNA gene; cdc2, cyclin-dependent kinase; conv, conventional PCR; DHFR, dihydrofolate reductase; DHPS, dihydropteroase synthase; HSP70, heat shock protein (*phsb1* gene); ITS, Internal Transcribed Spacer of ribosomal DNA; kex-1, serine endopeptidase; MSG, major surface glycoprotein; mtLSU rRNA, mitochondrial Large sub-unit (23S) of ribosomal RNA; RT, real-time PCR; TS, thymidylate synthase.

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Table 1. Gene targets used in molecular diagnosis of Pneumocystis pneumonia (excluding typing studies)

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Assay	Gene target	Sample type (no)	Criteria of evaluation	Sensitivity (%)	Specificity (%)	Reference
MycAssay™ *Pneumocystis* (MycoNostica Ltd., UK)	mtLSU	BAL (110)	Mixed[a]	93	91	[36]
		BAL (21)	Mixed[a]	88.9	63.4	[37]
		BAL (44)	Mixed[a]	100	94.4	[38]
		BAL, sputum, biopsy (113)	Mixed[a]	91.5	92.6	[39]
		BAL (105)	''In-house'qPCR	'0	86	[40]
BioEvolution *Pneumocystis* (Bioevolution, France)	mtLSU	BAL, sputum, biopsy (113)	mixed[a]	79.7	100	[39]
		BAL (120)	Mixed[a]	73	82	[41]
Amplisens (InterLab Service Ltd., Russia)	mtLSU	BAL, sputum, biopsy (113)	Mixed[a]	94	83.3	[39]
		BAL, sputum, TA (159)	Mixed[a]	56.5	87.4	[42]
RealCycler PJIR kit (Progenie Molecular, Spain)	mtLSU	BAL (32), sputum, biopsy (2)	''In-house' qPCR	'.9	100	[43]
LightMix kit *P. jirovecii* (Tib Molbiol, Germany)	MSG	BAL, sputum, TA (150)	Mixed[a]	78	86	[44]
FTD *P. jirovecii* (Fast Track Diagnostics, Luxembourg)	mtLSU	BAL, IS (150)	Mixed[a]	92.8–100[b]	100–91.6[b]	[45]
Real-Star *Pneumocystis jirovecii* PCR kit (Altona Diagnostics, Germany)	mtLSU	BAL, sputum, TA (159)	Mixed[a]	60.9	95.1	[42]
PneumoGenius (PathoNostics, The Netherlands)	mtLSU	BAL (120)	Mixed[a]	70	82	[46]
BD MAX™ system (BD Diagnostics, USA)	MSG	BAL, sputum, TA (278)	''In-house'qPCR	'0	95.4	[47]
		BAL, sputum (137)	Clinical	92.6	94.5	[48]
GENECUBE system (Toyobo, Japan)	MSG	BAL, sputum (221)	Mixed[a]	92.3	85.7	[49▪]

BAL, bronchoalveolar lavage; IS, induced sputum; TA, Tracheal aspirate.
[a]Clinical features with microscopy results.
[b]Depending on cut-off Ct values.

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Table 2. Performances of commercial quantitative PCR assays for Pneumocystis jirovecii diagnosis

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Sample type (no)	Population/ HIV status	Method/ target	Gold standard	Sensitivity (%)	Specificity (%)	Reference
OW (77)	Adult/HIV+	RT/DHPS, MSG	BAL/IS microscopy	82;85	68;88	[51]
OW (175)	Adult/mixed	Conv/MSG Conv/mtLSU	BAL/IS microscopy	90.6 75.0	96.5 98.6	[52]
OW (31)	Adult/HIV+	RT/mtLSU	sputum PCR	47	100	[53]
OW (48)	Adult/HIV+	Conv/mtLSU	BAL PCR	40	77	[54]
NPA (147)	Children/mixed	RT/MSG	BAL/IS PCR	86	95	[55]
NPA (117)	Adult/mixed (90% HIV-)	RT/mtLSU	BAL/bronchial aspirate microscopy	100	75	[56]
Serum (71)	Adult/HIV-	RT/HSP70	BAL/IS microscopy BAL/IS PCR	77.8 68.6	79.3 97.2	[59▪]
Plasma (41)	Adult/HIV+	RT/mtLSU	sputum PCR	50	100	[53]

BAL, bronchoalveolar fluid; IS, induced sputum; NPA, nasopharyngeal aspirate; OW, oral wash; Conv, conventional nested PCR; RT, real-time PCR.

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Table 3. Clinical evaluations of PCR performances on oral washes, nasopharyngeal aspirates and blood, compared with bronchoalveolar fluid or induced sputum samples

Reference	No of patients	qPCR target	Cut-off value for colonization	Cut-off value for PCP diagnosis	Grey zone No of patients (%)	Sensitivity (%)	Specificity (%)	Sample volume analyzed (ml)
[61]	225	mtLSU	Ct > 35	Ct < 31	38 (17)	59	80	2
[62]	64 HIV+ 166 HIV-	mtLSU	<15 000 copies/ml < 3400 copies/ml	> 15 000 copies/ml > 28 700 copies/ml	0 30 (18)	100 81 or 57[a]	67 or 100[a] 100 or 88[a]	0.5
[6]	162	mtLSU	Ct > 35	Ct < 32	23 (15)	65	89	1
[41]	120	beta-tubulin	Ct > 34	Ct < 34	0	65	74	nd
[63]	35	MSG	<3160 copies/ml	>31 600 copies/ml	9 (25)	80	100	0.8
[64]	128	DHPS	<340 copies/ml	>1300 copies/ml	nd	74	73	nd
[65]	54	MSG	Nd	>54 copies/ml	nd	100	98	2

na, not available; nd, not determined; PCP, *Pneumocystis* pneumonia; qPCR, quantitative PCR.
[a]When merging probable and definite PCP cases.

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Table 4. Clinical studies defining cut-off values for quantitative PCR interpretation and corresponding performances of the assays

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