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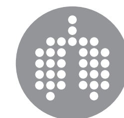

Quantitative PCR to diagnose *Pneumocystis* pneumonia in immunocompromised non-HIV patients

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ABSTRACT: The utility of quantitative *Pneumocystis jirovecii* PCR in clinical routine for diagnosing *Pneumocystis* pneumonia (PCP) in immunocompromised non-HIV patients is unknown.

We analysed bronchoalveolar lavage fluid with real-time quantitative *P. jirovecii* PCR in 71 cases with definitive PCP defined by positive immunofluorescence (IF) tests and in 171 randomly selected patients with acute lung disease. In those patients, possible PCP cases were identified by using a novel standardised PCP probability algorithm and chart review. PCR performance was compared with IF testing, clinical judgment and the PCP probability algorithm.

Quantitative *P. jirovecii* PCR values $>$1,450 pathogens·mL^{-1} had a positive predictive value of 98.0% (95% CI 89.6–100.0%) for diagnosing definitive PCP. PCR values of between 1 and 1,450 pathogens·mL^{-1} were associated with both colonisation and infection; thus, a cut-off between the two conditions could not be identified and diagnosis of PCP in this setting relied on IF and clinical assessment. Clinical PCP could be ruled out in 99.3% of 153 patients with negative PCR results.

Quantitative PCR is useful for diagnosing PCP and is complementary to IF. PCR values of $>$1,450 pathogens·mL^{-1} allow reliable diagnosis, whereas negative PCR results virtually exclude PCP. Intermediate values require additional clinical assessment and IF testing. On the basis of our data and for economic and logistical limitations, we propose a clinical algorithm in which IF remains the preferred first test in most cases, followed by PCR in those patients with a negative IF and strong clinical suspicion for PCP.

KEYWORDS: HIV negative, immunocompromised, molecular diagnosis, *Pneumocystis jirovecii*, *Pneumocystis* pneumonia, quantitative PCR

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The organism *Pneumocystis jirovecii*, previously known as *Pneumocystis carinii*, is the causative agent of *Pneumocystis* pneumonia (PCP). In immunocompromised HIV-negative patients, PCP is a feared disease and particularly challenging to diagnose for several reasons. First, the clinical presentation is unspecific and any acute febrile respiratory disease with radiological infiltrates may be due to PCP [1, 2]. Secondly, PCP has been reported in nearly all drug-induced immunocompromised conditions (*e.g.* haematological and solid organ transplant, and rheumatological and haemato-oncological disease) [3–7]. Thirdly, low disease incidence may lead to delayed diagnosis and increased mortality [8]. Lastly, HIV-negative patients with PCP may rapidly develop respiratory insufficiency and death within a few days [1, 9].

A PCP diagnosis requires pathogen identification in respiratory samples. Several immunohistochemical and immunofluorescent (IF) antibody staining techniques are available [10]. Direct IF antibody stains with *P. jirovecii* monoclonal antibodies display higher sensitivity and specificity compared with conventional staining methods [1, 11, 12]. Lower pathogen density in HIV-negative patients has been reported to reduce test sensitivity [13, 14], with inflammatory responses against *P. jirovecii* being hypothesised to play a more important role than the pathogen itself in these patients [15]. Several PCR methods

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amplifying different *P. jirovecii* genes detect *P. jirovecii* in respiratory samples, with sensitivities as high as 96% and specificities >90% [11, 16–20] when compared with gold standard staining techniques. The true performance, however, is difficult to extrapolate from published data in HIV-positive patients to HIV-negative patients because of the lower pathogen burden and potentially higher false-negative staining in the latter population. Furthermore, airways colonisation of 15.5%–58.8% [21–24] lowers specificity. In these patients, clinical data should therefore be considered in addition to classical staining tests in interpreting PCR results. A major concern with qualitative PCR is its inability to differentiate colonisation from infection, a drawback that might be overcome by quantitative PCR. The utility of this approach has been previously reported in HIV-positive patients [16] and only suggested in HIV-negative patients [25, 26], but insufficient data are available to support its introduction in routine clinical practice. A recent report suggested that, from a small sample of seven HIV-negative patients, differentiation between ''clinical high- or low-probability pneumonia'' is feasible by quantitative PCR [27]. Larger studies integrating clinical judgment are therefore required to assess the value of quantitative PCR in discriminating simple colonisation from PCP in HIV-negative patients. Furthermore, the utility of quantitative PCR *versus* classical direct staining should be defined and the results integrated in a clinical algorithm.

We aimed to test the performance of quantitative PCR in immunocompromised HIV-negative patients, using IF as the gold standard to diagnose definitive PCP and clinical and radiological patient characteristics to diagnose possible PCP. Moreover, we hypothesised that: 1) a negative PCR could exclude PCP; 2) a cut-off value existed to discriminate between IF-positive and IF-negative patients; and 3) a cut-off would enable discrimination between colonisation and infection when IF was negative. Data were incorporated into an algorithm with complementary roles for IF testing and PCR that might be adapted to local resources.

MATERIAL AND METHODS

Study population and data collection

The study was conducted in Bern University Hospital (Bern, Switzerland) and included hospitalised patients during a 10-yr period (1999–2009). Patients aged >16 yrs were retrospectively identified in an electronic database at the Institute for Infectious Diseases of the University of Bern. The following patient groups were defined: group 1: definitive PCP diagnosed by positive IF testing in bronchoalveolar lavage fluid (BALF); group 2: random selection of 171 HIV-negative IF-negative adults evaluated for acute lung disease with BALF during the same period. The following data were retrospectively analysed: demographics; clinical symptoms (respiratory symptoms: cough, dyspnoea and fever); need for additional oxygen and/or mechanical ventilation; radiological studies; laboratory values; microbiological results; medical history (including chronic lung disease and reason for and type of immunosuppressive therapy); antibiotic therapy; and discharge diagnosis. The local ethics committee approved this study (Kantonale Ethikkommission Bern, decision Nr. 28-08-09).

Definitions: PCP diagnosis

Definitive PCP was diagnosed in the presence of a compatible clinical presentation of PCP and a positive IF test in BALF. For negative IF, no single internationally accepted definition exists for the diagnosis of PCP. We established a clinical algorithm to classify acute lung disease according to the likelihood of PCP (''PCP probability algorithm''). The algorithm is based on the assumption that PCP can be excluded in immunocompromised patients with acute lung disease compatible with PCP and a good outcome who are not receiving specific *P. jirovecii* therapy. Possible PCP was diagnosed in deceased patients and/or patients having received an active drug against *P. jirovecii* for >5 days, if the following additional four elements were present: 1) compatibility of clinical signs (at least two symptoms: fever; dyspnoea; cough); 2) presence of hypoxia (arterial oxygen saturation <93% and/or need for supplementary oxygen of >2 $L\cdot min^{-1}$ and/or mechanical ventilation); 3) compatible radiological findings (interstitial pattern and/or ground glass opacities on computed tomography); and 4) immunosuppressive state. In all other patients, the diagnosis was PCP excluded.

Two physicians (infectious disease and lung specialists), both with long-standing experience in infectious lung disease in immunocompromised hosts and blinded to the PCR results, independently reviewed patient records.

Bronchoalveolar lavage procedure, microbiological analysis and P. jirovecii testing

The bronchoalveolar lavage (BAL) procedure was performed following a standardised protocol. In cases of suspected infection in immunocompromised patients, BALF was routinely examined as follows: bacterial, fungal and mycobacterial culture; herpes simplex virus 1/2; cytomegalovirus (culture and PCR); Epstein–Barr virus (PCR); and HSV1, HSV2 and VZV (antigen testing, culture and PCR). For the *Pneumocystis* diagnostic tests, 10-mL BALF aliquots were centrifuged. 25 μL of the resuspended pellet was used for direct IF testing (Monofluo *P. jirovecii* IFA Test Kit; Bio-Rad, Redmond, WA, USA) and the remaining pellet was stored at -80°C for retrospective *P. jirovecii* real-time PCR. See online supplementary material for details of: the BAL procedure; IF testing, including semi-quantitative cyst/trophozoite determination; PCR technique; and *P. jirovecii* quantification expressed in $pathogens\cdot mL^{-1}$.

Statistical methods

Data were analysed with Stata software version 10.1 (StataCorp LP, College Station, TX, USA). Quantitative PCR values (log_{10} transformed) were compared with a nonparametric Mann–Whitney U-test because data were not normally distributed. Categorical variables were compared with Fisher exact tests. Statistical significance was defined as a p-value <0.05 (two-tailed). Graphics were generated with GraphPad Prism 5.02 (GraphPad Software, Inc., San Diego, CA, USA).

RESULTS

Utility of qualitative real-time P. jirovecii PCR in diagnosing definitive PCP

During the study period, 81 cases of definitive PCP among 1,158 BALFs performed in HIV-negative immunocompromised patients were diagnosed by positive IF testing, currently

considered the diagnostic gold standard [10]. BALF material from 71 out of 81 was available for retrospective PCR testing. Patient clinical characteristics are summarised in table 1 (group 1). A retrospective review of clinical and radiological data by two independent physicians, blinded for *P. jirovecii* PCR results, confirmed the diagnosis of PCP in all patients. PCR in BALF detected *P. jirovecii* in all 71 patients, resulting in a sensitivity of 100.0% for diagnosing definitive PCP.

Utility of quantitative real-time P. jirovecii PCR in diagnosing definitive PCP

We used a three-step approach to determine a cut-off value for the diagnosis of definitive PCP when applying quantitative real-time PCR testing. First, we confirmed the positive correlation between semi-quantitative determination (+ to +++) of cysts/trophozoites in IF staining and quantitative PCR values expressed as pathogens·mL^{-1} (fig. 1a). Secondly, we performed serial dilution of a strongly positive PCP case, yielding a detection limit for positive IF at 1,522 pathogens·mL^{-1} (not shown). Thirdly, we randomly selected 171 IF-negative BALF samples from HIV-negative patients evaluated for acute lung disease (group 2). In this group, PCR was positive in BALF from 18 patients (10.5%). The two patient groups were merged to determine cut-off *P. jirovecii* PCR values for positive IF (definitive PCP) in 242 BALF samples. Performance of the standard recommended diagnostic approach using IF as the first test is summarised in table 2. PCR values in definitive *versus* nondefinitive PCP are depicted in figure 1c. The quantitative PCR had a sensitivity of 100% (95% CI 94.9–100%), a specificity of 89.5% (95% CI 83.9–93.9%) and a negative predictive value of 100% (95% CI 97.6–100%) when compared with positive IF testing. The receiver operating characteristic (ROC) curve for positive PCR values showed an area of 0.992 (95% CI 0.983–1.000; fig. 1b), suggesting that quantitative *P. jirovecii* PCR is a reliable method for diagnosing definitive PCP. With this approach, two clinically useful cut-off

TABLE 1 Patient characteristics

Characteristics	Group 1: IF positive	Group 2: IF negative
Subjects	71	171
Age yrs	58.92 (17–83) ± 15.89	56.68 (15–82) ± 13.55
Male/female	45/26 (63.4)	109/62 (63.7)
Immunocompromised	71 (100)	136 (79.5)
Autoimmune disease#	18 (25.3)	53 (31.0)
Rheumatoid arthritis	4	17
Giant cell arteritis	6	0
ANCA-positive vasculitis	4	2
Systemic sclerosis	0	9
Granulomatosis with polyangiitis	0	6
Other	6	20
Solid organ transplantation#	28 (39.4)	37 (21.6)
Liver	1	6
Heart	10	15
Kidney	16	14
Lung	0	5
>1 organ	1	1
Active haemato-oncological disease#	23 (32.4)	41 (24.0)
Solid tumour	3	6
Acute myeloid leukaemia	1	9
Acute lymphoblastic leukaemia	1	2
Chronic lymphatic leukaemia	3	4
Hodgkin's lymphoma	3	2
Non-Hodgkin's lymphoma	12	14
Multiple myeloma	2	9
Other haematological disease	0	9
Other cause of immunosuppression#	2 (2.8)	5 (2.9)
Death	18 (25.4)	16 (9.34)
***Pneumocystis* diagnosis**		
Definitive PCP (positive IF testing)	71 (100)	0 (0)
PCR *Pneumocystis jirovecii* results		
Positive qualitative PCR	71 (100)	18 (10.5)
Quantitative PCR (log_{10}) in case of positivity median (range)	3.8 (1.94–6.17)	2.18 (1.15–4.72)

Data are presented as n, median (range) ± SD, n (%), unless otherwise stated. IF: immunofluorescence; ANCA: antineutrophil cytoplasmic antibody; PCP: *Pneumocystitis* pneumonia. #: the most relevant immunosuppression is chosen. For the specified diagnoses, more than one diagnosis per patient is possible.

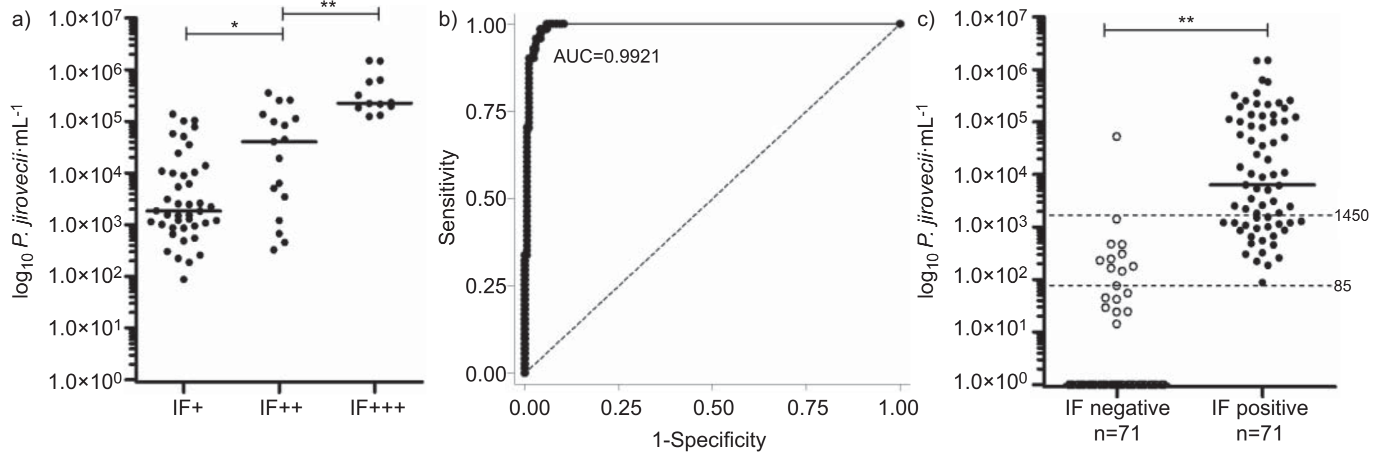


FIGURE 1. *Pneumocystis jirovecii* PCR values compared with direct immunofluorescence (IF) testing in bronchoalveolar lavage fluid. a) Correlation between semiquantitative estimation of *P. jirovecii* density in direct IF staining and PCR values. Semiquantitative microscopy results are provided (number of cysts/trophozoites per field of vision, magnitude 200 ×) with the following scores: IF+: few (<1); IF++: many (1–10); and IF+++: abundant (> 10). b) Receiver-operating characteristic curve showing positive PCR value *versus* definitive *Pneumocystis* pneumonia (PCP) as defined by positive IF testing. c) *P. jirovecii* PCR values in cases of positive IF testing (definitive PCP) and negative IF testing. Two cut-offs are shown. At >1,450 pathogens·mL^{-1}, the positive predictive value for positive IF testing (and definitive PCP) is 98.0%. At 1–85 pathogens·mL^{-1}, the negative predictive value is 100% for positive IF testing. At 85–1,450 pathogens·mL^{-1}, PCR is not able to predict IF test results. In this case, additional IF testing is helpful in diagnosing definitive PCP. AUC: area under the curve. *: $p<0.05$, **: $p<0.001$ (nonparametric Mann–Whitney U-test).

values can be defined (fig. 1c), with performances summarised in table 3 and integrated in an algorithm (see ''PCR first'', fig. 2). At <85 pathogens·mL^{-1}, no definitive PCP case was diagnosed. Therefore, a PCR below this threshold has a negative predictive value of 100% (95% CI 97.7%–100%). At a value >1,450 pathogens·mL^{-1}, all patients but one were diagnosed with definitive PCP. Therefore, values above this cut-off have a positive predictive value of 98.0% (95% CI 89.6–100%). At 85–1,450 pathogens·mL^{-1}, a range of uncertainty exists; quantitative PCR is unable to predict IF results and consequently is not helpful in diagnosing definitive PCP.

Utility of quantitative real-time P. jirovecii *PCR in differentiating colonisation from possible infection*

No standard definition or diagnostic test for PCP in the absence of pathogen identification currently exists. Although the diagnosis is commonly based on clinical suspicion, the clinical presentation in HIV-negative patients is highly non-specific. We therefore reviewed all cases using two different approaches: a standardised ''PCP probability algorithm'' and a retrospective case review by two independent physicians. The PCP probability algorithm classified episodes as ''possible PCP'' or ''PCP excluded'' (see Methods section). By analysing acute respiratory disease in 171 IF-negative patients with this algorithm, we identified 14 cases of possible PCP and 157 of PCP excluded. Real-time PCR performed on BALF was positive in 18 (10.5%) of these 171 episodes. Clinical characteristics from those patients are summarised in online supplemenatary table 1. Of note, possible PCP was diagnosed by the algorithm in only three (16.7%) out of 18 patients with positive PCR but negative IF testing. The remaining 15 (83.3%) patients were classified as PCP excluded and considered colonised. In their review of patient data, two independent physicians agreed with the PCP probability algorithm in all cases. No

TABLE 2 Results of the diagnostic procedure according to the performance of the currently recommended standard diagnostic approach with immunofluorescence (IF) as first test

First test	Second test	Results			
IF	PCR	Total subjects	Definitive PCP (IF test positive)	Possible PCP	PCP excluded
Positive	Positive	71 (100)	71 (100)	0	0
	Negative	0	0		
Negative	Positive	18 (10.5)	0	3 (16.6)	15 (83.3)
	Negative	153 (89.5)	0	1 (0.65)#	152 (99.3)

Data are presented as n (%). PCP: *Pneumocystitis* pneumonia. #: PCP could not be formally ruled out in only one patient with rheumatoid arthritis and lung involvement. The treating physicians prescribed 3-week therapy against *Pneumocystis jirovecii*, but judged the acute lung disease as ''more likely to be caused by autoimmune disease exacerbation rather than PCP'' in the discharge letter. Retrospective reviewers unaware of the PCR result agreed with this interpretation.

TABLE 3 Results of the diagnostic procedure according to a theoretical approach based primarily on quantitative PCR *Pneumocystis jirovecii* as the first test#

First test	Second test	Results			
PCR *P. jirovecii* copies·mL^{-1}	IF	Total subjects	Definitive PCP (IF test positive)	Possible PCP	PCP excluded
>1450	Positive	50 (98.0)	50 (100)	0	0
	Negative	1 (2.0)	0	0	1 (100)
85–1450	Positive	21 (70)	21 (100)	0	0
	Negative	9 (30)	0	2 (22.2)	7 (77.7)
1–85	Positive	0	0		
	Negative	8 (100)	0	1 (12.5)	7 (87.5)
Negative	Positive	0	0		
	Negative	153 (100)	0	1 (0.65)¶	152 (99.3)

Data are presented as n (%). IF: immunofluorescence; PCP: *Pneumocystitis* pneumonia. #: definitive conclusions are possible only with high copy numbers (>1,450 copies·mL^{-1}) and negative results. In the range 1–1,450 copies·mL^{-1}, quantitative PCR is not able to differentiate between ''possible PCP'' and ''PCP excluded'' and clinical suspicion remains essential. ¶: PCP could not be formally ruled out in only one patient with rheumatoid arthritis and lung involvement. The treating physicians prescribed 3-week therapy against *Pneumocystis jirovecii*, but judged the acute lung disease as ''more likely to be caused by autoimmune disease exacerbation rather than PCP'' in the discharge letter. Retrospective reviewers unaware of the PCR result agreed with this interpretation.

statistical differences in PCR values were noted between PCP excluded (colonisation) and possible PCP (fig. 3). Despite the small number of cases, these results suggest that differentiation between PCP excluded (colonisation) and possible PCP infection in cases of negative IF is impossible from PCR values alone.

Use of quantitative real-time P. jirovecii *PCR to exclude PCP*

PCR testing is highly sensitive for *P. jirovecii* detection. We tested the ability of a negative PCR result to exclude PCP, as recently reported [28]. First, all 153 patients with negative PCR results had negative IF tests, which excluded definitive PCP by definition. Secondly, a clinical diagnosis of PCP (clinically compatible, but IF negative) was excluded by applying the PCP probability algorithm combined with an independent review by two physicians in 152 (99.3%) out of 153 patients with negative *P. jirovecii* PCR. In 153 PCR-negative patients with acute lung disease, the PCP probability algorithm detected 11 cases of possible PCP. To minimise missed PCP diagnoses, we designed the algorithm to maximise sensitivity at the expense of lower specificity. Additional analysis of those 11 patients by two physicians revealed an alternative diagnosis in 10: acute exacerbation of interstitial lung disease (ILD; n=1); alveolar proteinosis (n=1); septic shock with acute respiratory distress syndrome (n=3); bacterial/fungal pneumonia in aplasia (n=2); definitive invasive pulmonary aspergillosis (n=2); and nosocomial bacterial pneumonia (n=1). Retrospectively, PCP could not be excluded with certainty in only one patient, who had seronegative polyarthritis-associated ILD and had received 3 weeks of therapeutic trimethoprim-sulfamethoxazol with adjunctive corticosteroids. The clinical manifestation was compatible with PCP, but the treating physician and two reviewers judged acute ILD exacerbation to be more likely. In summary, our data strongly suggest that a negative PCR safely rules out PCP.

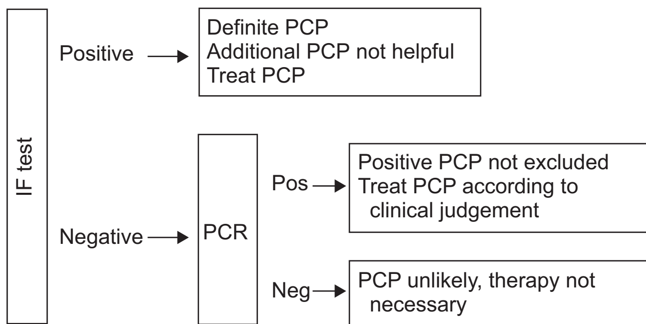
Decision-making algorithms to use quantitative PCR in the diagnosis and therapy for PCP in HIV-negative patients

Performance of IF and PCR in our populations according to the test sequence is summarised in tables 2 and 3. The data from our study were first integrated in the current diagnostic approach with IF as the initial diagnostic test (algorithm 1, ''IF first'') to guide clinical management of immunocompromised HIV-negative patients with possible PCP (fig. 2). Our data suggest that quantitative PCR could in theory be used as the first test and replace IF in most cases (alternative algorithm 2, ''PCR first'').

DISCUSSION

The gold standard for PCP diagnosis is microscopic visualisation of *P. jirovecii* [1, 2]. Several PCR techniques amplifying different *P. jirovecii* genomic regions have been described, but to date, none has been introduced into clinical practice. Initial reports using qualitative PCR identified a significant number of asymptomatic *P. jirovecii* carriers [29]. Discriminating between colonisation and infection is central in defining the role of PCR testing. Quantitative PCR represents an attractive approach, as first suggested by LARSEN *et al.* [30]. BALF from HIV-positive patients with PCP has a significantly higher pathogen density than does BALF from colonised patients [16]. This result cannot, however, be automatically extrapolated to HIV-negative patients, as a lower pathogen burden may cause PCP [14].

In our study, the quantitative PCR method showed good correlation with pathogen density estimated by IF staining. A cut-off of >1,450 pathogens·mL^{-1} predicted in 98% of cases a positive IF test, providing a definitive PCP diagnosis. This finding is in accordance with a recent report in a mixed HIV-positive and HIV-negative patient population, in which the authors proposed a slightly higher cut-off (1,900 pathogens·mL^{-1}) using quantitative PCR of a different gene (mitochondrial



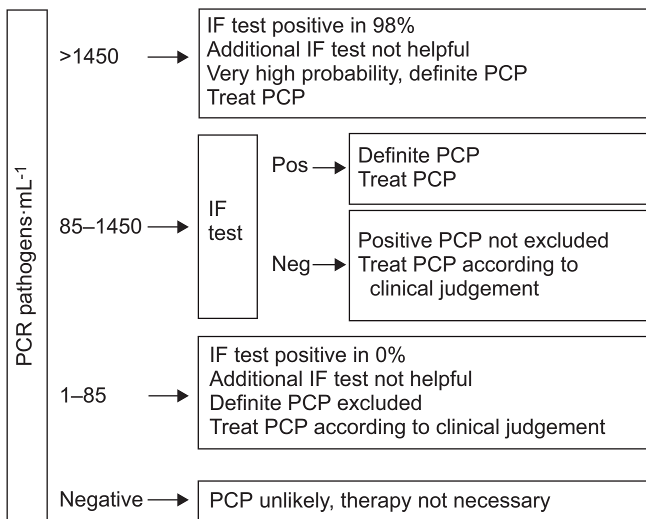


FIGURE 2. Decision-making algorithms in the diagnosis and therapy of *Pneumocystis* pneumonia (PCP) in HIV-negative patients with clinically suspected PCP. On the basis of our data, the ''immunofluorescence (IF) first'' algorithm should be preferred for logistical and economic reasons. IF allows rapid diagnosis of PCP, and in cases of IF negativity, PCR should be performed to exclude PCP. However, the use of PCR as first test is theoretically possible and presented as an alternative. Note that quantitative PCR is not able to differentiate between colonisation and infection: therefore, the PCR test should be performed only in cases of PCP suspicion. Pos: positive; Neg: negative.

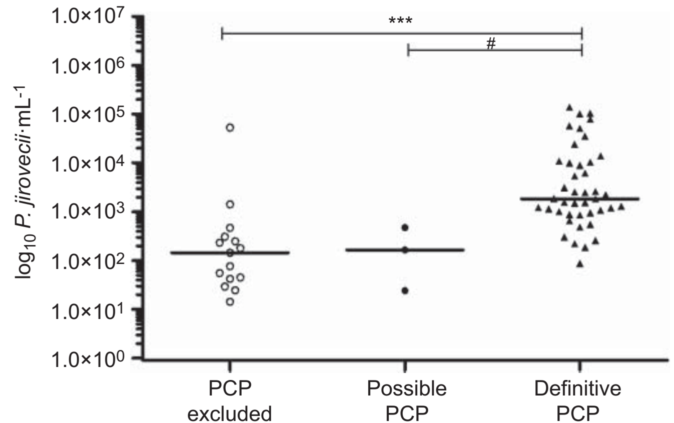


FIGURE 3. Definitive clinical judgment according to PCR value with positive *Pneumocystis jirovecii* PCR in bronchoalveolar lavage fluid (BALF). Definitive *Pneumocystis* pneumonia (PCP) was defined as positive immunofluorescence (IF) testing (patient group 1; table 1). In group 2, of 171 IF-negative patients, a positive PCR result was detected in 18. With extensive clinical review, these cases were diagnosed either as possible PCP or PCP excluded. No statistical significance was detected for *P. jirovecii* pathogen density in BALF; therefore, PCR testing in cases of negative IF testing does not allow discrimination between possible PCP and *P. jirovecii* colonisation (PCP excluded). #: $p=0.01$; ***: $p<0.001$ (nonparametric Mann–Whitney U-test).

large subunit ribosomal RNA) [27]. Therefore, quantitative PCR >1,450–1,900 pathogens·mL^{-1} might replace IF in diagnosing definitive PCP.

Immunocompromised HIV-negative patients may have rapidly progressive PCP in the presence of low pathogen density [13, 14], and the decision to treat is merely clinical, as staining procedures, including IF testing, are insufficiently sensitive to exclude PCP. PCR testing has a higher sensitivity, but its use is limited by *P. jirovecii* colonisation causing lower specificity. The distinction between colonisation and infection is relevant in immunocompromised HIV-negative patients, as colonisation rates increase with immunosuppression; rates up to 58% are reported [21–24]. From our data, we conclude that positive PCR values <1,450 pathogens·mL^{-1} may occur with both colonisation and infection and that PCR is not helpful in distinguishing between the two clinical conditions, as a discriminating cut-off could not be defined. In the range of 85–1,450 pathogens·mL^{-1}, PCR is unable to predict results of IF testing, and additional staining is advisable. PCP is then diagnosed from a positive IF result, whereas a negative IF test cannot exclude PCP, and patients should be treated according to clinical suspicion. Importantly, as patients with a low pathogen density of 1–85 pathogens·mL^{-1} may still have PCP and additional IF testing is not helpful (negative predictive value 100%), the decision to treat should be based purely on clinical grounds.

ALANIO *et al.* [27] concluded that a lower cut-off value exists for diagnosing colonisation excluding infection (<120 pathogens·mL^{-1}). However, we believe that the results of that study and ours do not support the existence of lower cut-off values to reliably exclude PCP. First, we identified one patient (P11; online supplementary table 1) with 24 pathogens·mL^{-1} and a clinical picture of PCP. Secondly, ALANIO *et al.* [27] described a collective of only seven HIV-negative patients with PCP, and reported no case of HIV-negative patients with clinical PCP and negative IF testing. PCP in HIV-negative patients with negative IF is rare but constitutes a diagnostic challenge [8, 13, 14]. Analysing 171 random BALF samples, we detected three patients with possible PCP and negative IF tests (P2, P3 and P11; online supplementary table 1) with PCR values of 164, 474 and 24 pathogens·mL^{-1}. Considering the low patient numbers in both reports, definitive conclusions cannot be drawn in the absence of larger studies. Clinicians should be aware that PCP with very low pathogen density is rare but possible and that a positive PCR result never allows *a priori* exclusion of PCP. Our data suggest that future attempts to define a cut-off value to distinguish colonisation from possible infection will probably fail. This contrasts with previous results in HIV-positive patients, where such a cut-off exists [16].

A further salient finding of our study is the utility of a negative PCR in BALF for excluding PCP in HIV-negative patients. Our result validates this recently published concept in a large population of 242 HIV-negative patients [28]. We identified only one patient with negative *P. jirovecii* PCR in whom PCP could not be formally ruled out. Therefore, a negative PCR in BALF allows reliable exclusion of PCP and safe discontinuation of therapy.

Our results were used to establish an algorithm (algorithm 1) that integrated PCR in the current routine diagnostic approach based on IF testing. Algorithm 1 proposes maintaining IF as the first diagnostic procedure (''IF first''), allowing rapid and economic diagnosis of definitive PCP. With negative IF results and high clinical suspicion, however, PCR is mandatory for excluding PCP. Our data suggest that quantitative PCR could theoretically replace IF as first test, not taking into account economic and logistic issues. Algorithm 2 summarises these results and proposes a possible alternative strategy (''PCR first''). Quantitative PCR used as a first step allows diagnosis of definitive PCP (>1,450 pathogens·mL^{-1}) and PCP exclusion with therapy discontinuation (negative *P. jirovecii* PCR) in one single investigation. For values of 1–1,450 pathogens·mL^{-1}, clinicians should know the major limitations of the test, remembering that PCP cannot, unfortunately, be ruled out and that the decision is primarily a clinical one.

Our results do not support routine replacement of IF by PCR testing as the initial test in cases of PCP suspicion. The IF first approach remains preferable for several reasons: PCR testing is more time-consuming (technician and sample processing time is 4.5 h *versus* 2 h), is logistically more demanding and generates higher costs than IF without offering major advantages. Importantly, PCR testing does not allow discrimination between colonisation and infection, a major issue for the clinician. The option of PCR first could, however, be considered in centres with a high volume of analyses in which PCR may be economically advantageous, as many analyses are performed in parallel.

Our study has several limitations. First, cases were analysed retrospectively. To reduce biases and review cases objectively, we defined the stringent PCP probability algorithm. Moreover, two experts independently reviewed patient documents blinded to PCR results. Secondly, formally, patients with ''probable PCP'' have a negative ''gold standard'' test for PCP, *i.e.* direct pathogen visualisation. However, as discussed previously and commonly reported [8, 13, 14], PCP can occur with pathogen densities below the detection limit of microscopic visualisation methods. Lacking a better gold standard test, we proposed a ''case definition for possible PCP'' requiring clinical criteria usually applied by clinicians to ''diagnose and treat PCP'' even in cases of a negative IF test in the presence of high clinical suspicion. Thirdly, the number of IF-negative and PCR-positive patients with possible PCP is small. However, given the rarity of the disease, much larger studies would be required that are unlikely to be performed.

In summary, although PCR testing is able to predict IF positivity with a positive predictive value of 98%, its main advantage lies with its capacity to formally exclude PCP when IF is negative and PCP is clinically strongly suspected. As a positive PCR result in IF-negative BALF may be associated both with colonisation and infection, it is essential to perform a quantitative PCR only in patients with a high index of clinical suspicion for PCP. Therefore, routine BALF testing with PCR should be strongly discouraged in the absence of a PCP-compatible clinical presentation. The main reason for this recommendation is that clinicians might be tempted to treat colonised (non-infected, non-PCP) patients with unnecessary exposure to potentially toxic anti-*P. jirovecii* treatments. Though a relevant percentage of immunocompromised patients are *P. jirovecii* colonised, the clinical consequences of *P. jirovecii* detection in asymptomatic immunocompromised patients are currently unknown, *e.g.* whether colonisation could progress to PCP [29, 31]. To treat or not to treat colonisation, and if yes, how, remains a relevant dilemma that should be prospectively analysed in longitudinal studies by taking into account potential advantages of quantitative PCR testing.

We conclude that an optimal resource-sparing approach to diagnose PCP in HIV-negative immunocompromised is initial IF testing, complemented by PCR when IF is negative despite clinically suspected PCP. Though a ''PCR first'' approach could theoretically be applied, we suggest that clinicians should continue to perform IF as an initial test for economic and logistical reasons. The major limitation of PCR testing is *P. jirovecii* biology, as very low *P. jirovecii* density may be associated with PCP. Negative *P. jirovecii* PCR testing rules out PCP, but the biological limitation precludes defining a specific cut-off value that can be utilised to exclude PCP, underscoring the importance of thorough clinical judgment.

STATEMENT OF INTEREST

None declared.

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