Development and evaluation of a real-time PCR assay for detection of *Pneumocystis jirovecii* DNA in bronchoalveolar lavage fluid of HIV-infected patients

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Short title: Real-time PCR for detection of *Pneumocystis jirovecii* DNA

Key words: PCP, real-time PCR, bronchoalveolar lavage, diagnosis

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Abstract

Background: *Pneumocystis* pneumonia (PCP) is conventionally diagnosed by identifying *Pneumocystis jirovecii* in lower respiratory tract samples using cytochemical stains. Molecular diagnosis of PCP is potentially more sensitive.

Objectives and methods: To use an extensively optimized real-time polymerase chain reaction (PCR) using primers designed to hybridise with the *P. jirovecii* heat-shock protein 70 (HSP70) gene to quantify *P. jirovecii* DNA in bronchoalveolar lavage (BAL) fluid from HIV-infected patients with and without PCP and to compare this assay with conventional PCR targeting the *P. jirovecii* mitochondrial large subunit rRNA gene sequence (mt LSU rRNA).

Results: Sixty-one patients had 62 episodes of PCP (defined by detection of *P. jirovecii* in BAL fluid by cytochemical stains and typical clinical presentation). Quantifiable HSP70 DNA was detected in 61/62; range ~13-18608 [median ~332] copies/reaction and detectable, below the limit of quantification (~5 copies/reaction, <LQ) in 1/62. Seventy-one other patients had 74 episodes with alternative diagnoses. Quantifiable HSP70 DNA was detectable in 6/74(8%) episodes; range ~6-590 [median ~14] copies/reaction and detectable but <LQ in 34/74(46%). Receiver-Operator Curve analysis (cut-off >10 copies/reaction) showed clinical sensitivity =98% (95% Confidence Interval (CI) =91-100%) and specificity =96% (95%CI =87-99%), for diagnosis of PCP. By contrast, clinical sensitivity and specificity of mt LSU rRNA PCR was 97% (95%CI =89-99%) and 68% (95 CI =56-78%), respectively.

Interpretation: The HSP70 real-time PCR assay detects *P. jirovecii* DNA in BAL fluid and may have a diagnostic application. Quantification of *P. jirovecii* DNA by real-time PCR may also discriminate between colonization with *P. jirovecii* and infection.

The fungal pathogen *Pneumocystis jirovecii* is the cause of *Pneumocystis* pneumonia (PCP) in humans. [1] Diagnosis of *P. jirovecii* infection is hampered by the lack of a sustainable in *vitro* culture method. Diagnosis of PCP is typically made by direct examination of respiratory samples (bronchoalveolar lavage [BAL] fluid or induced sputum) after staining in order to detect the cyst form of *Pneumocystis*. [2] Some laboratories also use immunofluorescence stains to enhance sensitivity. Use of cytochemical stains for diagnosis is time consuming and it may be difficult to maintain laboratory diagnostic expertise, because of the lower incidence of PCP since the introduction of highly active antiretroviral therapy.

Molecular diagnostic techniques, using the polymerase chain reaction (PCR), are more clinically sensitive when compared with staining for detection of *P. jirovecii* in BAL fluid and induced sputum. [3-6] Molecular techniques however, may identify *P. jirovecii* DNA in respiratory samples from patients without clinically apparent PCP. [7-10] suggesting asymptomatic carriage, or ‘colonization.’ Several studies have used real-time PCR for detection of *Pneumocystis carinii*, [11] *Pneumocystis murina* [12] and *P. jirovecii* in respiratory specimens. [13-21] Real-time PCR allows accurate quantification of DNA and the potential to discriminate between asymptomatic carriage of *P. jirovecii* and clinical disease based on pathogen load. The objective of this study was to compare real-time PCR using primers designed to hybridise to the heat shock protein 70 (HSP70) gene of *P. jirovecii* [22,23] with conventional PCR using primers designed to the large subunit of mitochondrial rRNA (mt LSU rRNA) [3,4,10] for detection of *P. jirovecii* DNA in bronchoalveolar lavage (BAL) fluid from patients undergoing diagnostic bronchoscopy. Patients had either proven PCP or confirmed alternative diagnoses.
Methods

Selection of a new molecular target

The target region of HSP70 was obtained for *P. jirovecii* by designing generic primers to the flanking regions of *P. carinii*. Nucleotide sequences for all *Pneumocystis* types, available from public data repositories were searched using BlastX [24] against the non-redundant protein database. Expressed sequence tag data were assembled into contigs using CAP3 prior to searching, in order to remove redundancy and improve sequence accuracy. Sequences with high quality matches (bit score >60) to proteins from a broad spectrum of eukaryotic species were considered further (minimally: *Caenorhabditis elegans*, *Drosophila melanogaster*, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and *Neurospora crassa*).

The corresponding region was amplified using *P. jirovecii* as template and sequenced. Real-time PCR for *P. jirovecii* HSP70 (*PjHSP70a*) was designed to amplify a 106 base pair region of the *P. jirovecii* sequence and not the corresponding sequence of other *Pneumocystis* species, or a wide range of potential fungal, bacterial or mycobacterial pathogens (Table 1).

Table 1. Fungal, bacterial and mycobacterial organisms used for cross-reactivity assessment of the specificity of the *PjHSP70a* PCR assay.

<table>
<thead>
<tr>
<th>Organism</th>
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<tbody>
<tr>
<td><strong>Fungi</strong></td>
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<tr>
<td><em>Aspergillus flavus</em></td>
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<tr>
<td><em>Aspergillus fumigatus</em></td>
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<tr>
<td><em>Aspergillus niger</em></td>
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<tr>
<td><em>Aspergillus terreus</em></td>
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<tr>
<td><em>Candida glabrata</em></td>
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<td><em>Candida krusei</em></td>
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<tr>
<td><em>Candida parapsilosis</em></td>
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<tr>
<td><em>Candida tropicalis</em></td>
</tr>
<tr>
<td><em>Cryptococcus</em> sp.</td>
</tr>
<tr>
<td><em>Pneumocystis carinii</em> &amp;</td>
</tr>
<tr>
<td><em>Pneumocystis wakefieldiae</em></td>
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<tr>
<td><em>Pneumocystis jirovecii</em></td>
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<tr>
<td><em>Pneumocystis murina</em></td>
</tr>
<tr>
<td><em>Saccharomyces cerevisiae</em></td>
</tr>
<tr>
<td><em>Trichosporon</em> sp.</td>
</tr>
<tr>
<td><strong>Bacteria</strong></td>
</tr>
<tr>
<td><em>Burkholderia cepacia</em></td>
</tr>
<tr>
<td><em>Haemophilus influenzae</em></td>
</tr>
<tr>
<td><em>Propionibacterium</em> sp.</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
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<tr>
<td><em>Streptococcus agalactiae</em></td>
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<tr>
<td><em>Streptococcus mitis</em></td>
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<tr>
<td><em>Streptococcus oralis</em></td>
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<tr>
<td><em>Streptococcus pneumoniae</em></td>
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<tr>
<td><strong>Mycobacteria</strong></td>
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<tr>
<td><em>Mycobacterium avium</em></td>
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<tr>
<td><em>Mycobacterium tuberculosis</em></td>
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<tr>
<td><strong>Mammalia</strong></td>
</tr>
<tr>
<td><em>Human DNA</em></td>
</tr>
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</table>
DNA was extracted from these isolates using the DNeasy tissue kit (QIAGEN, Crawley, UK) following manufacturer’s instructions and was used as a template for the *Pj*HSP70a.

**Patients & samples**

One hundred and thirty-six BAL samples were obtained from 132 adult HIV-infected patients (112 men) undergoing diagnostic bronchoscopy. Sixty-one consecutive patients with PCP had 62 episodes of PCP; one patient had two episodes of PCP (interval =9 weeks). All had typical clinical and radiographic presentations, identification of *P. jirovecii* cysts in BAL fluid by Grocott-Gomori methenamine silver staining, [2] and response to specific anti-PCP therapy. [25,26] A further seventy-one consecutive patients (74 episodes) investigated contemporaneously did not have PCP, clinically or radiographically, had negative results from Grocott-Gomori staining, and did not receive specific anti-*Pneumocystis* treatment. All had alternative diagnoses, as described previously, [10,26-28] comprising pulmonary Kaposi sarcoma =21 (one patient had two BAL; interval =27 weeks), bacterial bronchitis =12, lymphocytic interstitial pneumonitis =5 (one had two BAL; interval =24 weeks), *Cryptococcus neoformans* pneumonia =3, pulmonary tuberculosis =3, *Aspergillus fumigatus* bronchitis =3 (one patient had two BAL; interval =3 weeks, the diagnosis then was bacterial bronchitis), *S. pneumoniae* pneumonia =2, bronchiectasis =2, self-limiting fever and dyspnoea =2 patients. One patient each had cytomegalovirus pneumonitis, pulmonary *Mycobacterium avium* infection, *Salmonella typhi* septicaemia, tracheitis, adenocarcinoma of bronchus, primary effusion lymphoma, pulmonary Castleman disease and HIV ‘constitutional’ disease. No patient with an alternative diagnosis developed PCP during 6 months follow up. We recorded outcome (survival at one month) in all patients. Additionally, in those with PCP, we recorded receipt of specific anti-*Pneumocystis* prophylaxis, disease severity (PaO₂, breathing room air) and number of days of specific anti-*Pneumocystis* therapy before BAL. All bronchoscopies were performed by RFM; BAL was performed in a standardized way, as previously described. [25,26] All BAL samples were coded and analyses by PCR were performed ‘blind’ to patient’s clinical and laboratory details. All patients undergoing bronchoscopy gave informed written consent and the study was performed with Local Research Ethics Committee approval.

**DNA extraction**

DNA extraction from BAL fluid was first done using the DNeasy tissue kit and subsequently using the QIAamp UltraSens Virus Kit (both QIAGEN, Crawley, UK), following manufacturer’s instructions. Total DNA was extracted from 200 µL (DNeasy) or 750 µL (QIAamp) of BAL fluid. RNA quench was also included in the latter extract, as suggested by manufacturer, and the extracted sample was eluted in 60 µL of elution buffer by contrast with the DNeasy extraction (100 µL of elution buffer). QIAamp UltraSens Virus Kit was chosen as it was specifically designed to extract DNA from liquid samples, enabled a greater volume to be extracted from BAL fluid and facilitated generation of a higher concentration of extracted nucleic acids. Consequently, the final QIAamp eluate achieved a 12.5-fold concentration, compared with a 2-fold increase with the DNeasy kit. When the two extraction techniques were both applied to BAL samples a greater amount of DNA was extracted with the QIAamp method compared with the DNeasy method (Figure 1).

**Molecular assessment of *P. jirovecii* DNA in BAL fluid**

Attempts were made to use the mt LSU rRNA assay by real-time PCR, but efficient amplification was not possible and so conventional PCR was used for comparison with the *Pj*HSP70a assay.

**PjHSP70a**

The *Pj*HSP70a assay consisted of a 12.5 µl reaction using 1U of Hot Taq (BioGene, Kimbolton, UK) with supplied buffer, 3 mM MgCl₂, 200 µM each dNTP, 250 µg/µL tRNA (Sigma, UK) and 300 nM of the forward (5’-CGTCTTGTAACCACTTCATTGC-3’) and reverse (5’-
AGTCCGTTTAGCAGCTAC-3') primers and 75 nM of the probe (Fam 5'-AAGAAAGATCTTCCAGGG-3' BHQ1; underlined bases are 'locked' nucleic acids). The PjHSP70a real-time PCR assay was performed using the Rotorgene 3000 (Corbett Research, Sydney, Australia) with an initial denaturation of 95°C for 8 minutes followed by 45 cycles of 10 seconds: 95°C, 20 seconds: 72°C and 30 seconds: 72°C. Results were determined by comparing unknowns with a 10-fold dilution series from ~10^6 - ~1 copy/reaction. Dilution series were made from preparations of pGEM-T easy (Promega, Southampton, UK) plasmid containing the amplicon of interest. The plasmids were linearised prior to re-purification using a PCR cleanup kit (Qiagen) and quantified using Pico green reagent (Molecular probes, Invitrogen, Paisley, UK). Reactions were setup using the CAS liquid-handling robot (Corbett Research) placed in a laminar-flow hood to reduce contamination. All clinical samples and standards were run as duplicate PCR reactions. Negative controls (ultra-pure RNAse-free water, Sigma) were distributed to provide maximum information as to the potential source of any contamination. Three negative controls were aliquoted prior to dilution series, three further controls were established before aliquoting clinical samples and an additional four controls were prepared at the end. In summary, ten negative controls were included in each PCR reaction and in total 110 negative control PjHSP70a reactions were performed in the assessments.

Initially reactions were also assessed by agarose gel electrophoresis (3% agarose gel with TBE and Ethidium Bromide) and products were sized using the HYPER ladder IV and V (Bioline, London, UK). Amounts of extracted DNA used for the PjHSP70a assay were 0.5% of extract obtained by DNAeasy and 2% of extract obtained by QIAamp UltraSens. As real-time PCR results are quantitative a receiver-operator curve (ROC) was generated from the data. No universal reference material was available from which to compare findings, so ROC analysis was used to establish the optimum cut-off, which enabled discrimination between PCP and alternative diagnoses.

**mt LSU rRNA**

The mt LSU rRNA assay was a 25 µL reaction using 1 U of Hot Taq (BioGene) with supplied buffer, 3 mM MgCl₂, 200 µM each dNTP, 250 µg/µl tRNA (Sigma) and 200 nM of primers pAZ102-H and pAZ102-E (5). The mt LSU rRNA PCR assay was performed using the Palm-Cycler (Corbett Research) with an initial denaturation of 95°C for 8 minutes followed by 40 cycles of 30 seconds: 95°C, 30 Seconds: 62°C and 60 seconds: 72°C. Reactions were assessed by agarose gel electrophoresis (1% Agarose gel with TBE and Ethidium Bromide) and products sized using the 3 µl [8 ng/µl] HYPER ladder IV (Bioline).

Samples with PCR product showing band density >24 ng/400 base pairs HYPER ladder IV band were scored as positive; samples with no product or product showing ≤density were scored as negative. Real-time PjHSP70a and conventional mt LSU rRNA PCR data were compared.

**Statistical analysis**

For patients with PCP duration of anti-*Pneumocystis* therapy before BAL, PaO₂ levels and quantity of *P. jirovecii* DNA were log₁₀ transformed to fit a normal distribution. Univariate analyses were performed using Spearman’s rank test for continuous variables and the Mann-Whitney test for binary variables to assess correlations between variables and the amount of detectable *P. jirovecii* DNA in BAL samples. Stata version 9.0 was used for statistical analysis; p <0.05 was regarded as statistically significant.

**Results**

**Molecular assay detection sensitivities**

The PjHSP70a assay reproducibly detected as few as ~5 copies/reaction, (Table 2)

Table 2. Characteristics of PjHSP70a assay.
and had a dynamic range across 9 orders of magnitude (Figure 2a). When the same dilution experiment was performed with the mt LSU rRNA assay, detection sensitivity was \( \sim 10^5 \) copies/reaction, despite extensive optimisation (Figure 2b).

**Patients**

Among those with PCP one month mortality was (9/62) 14.5%; all patients with alternative diagnoses survived. Among patients with PCP \( \text{PaO}_2 \) ranged from 5.1-13.2 kPa (median =9.6). Ten patients were receiving prophylaxis. Seven patients had pulmonary co-pathology; pulmonary KS =three, *C. neoformans* pneumonia =two, pulmonary *Strongyloides stercoralis* infection =one and pulmonary tuberculosis =one patient.

**Detection of *P. jirovecii* DNA by real-time PCR using the PjHSP70a assay**

In the QIAamp-extracted samples quantifiable *P. jirovecii* DNA was detectable in 61/62 episodes of PCP (Figure 3), ranging from \( \sim 13 \) to 18608 copies/reaction [median \( \sim 332 \)]. ROC analysis demonstrated a clinical sensitivity of 98% (95% Confidence Interval (CI) =91-100%) and specificity of 96% (95% CI =87-99%) when a quantitative threshold of >10 copies/reaction was used to define a diagnosis of PCP (Figure 4), (sensitivity and specificity were derived from analysis of patients and not total episodes).

One patient with PCP had detectable *P. jirovecii* DNA, but below limits of quantification (<5 copies/reaction). This patient had first episode PCP, a CD4 count of 0 cells/µl, had not received anti-*Pneumocystis* prophylaxis and had received five days of high-dose co-trimoxazole before BAL was performed. Necropsy confirmed PCP.

Among 71 patients (74 episodes) with an alternative diagnosis, quantifiable (>5 copies/reaction) *P. jirovecii* DNA was detectable in 6 (8%) episodes ~6 to 590 copies/reaction [median =14] (Table 3),

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>CD4 count (cells/µL)</th>
<th>Receiving <em>Pneumocystis</em> prophylaxis (drug)</th>
<th><em>P. jirovecii</em> DNA (~copies/reaction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspergillus bronchitis</td>
<td>30</td>
<td>Yes (neb pent)</td>
<td>590</td>
</tr>
<tr>
<td>Bacterial bronchitis</td>
<td>20</td>
<td>No</td>
<td>49</td>
</tr>
<tr>
<td>Pulmonary KS</td>
<td>190</td>
<td>Yes (co-trimoxazole)</td>
<td>18</td>
</tr>
<tr>
<td>LIP</td>
<td>140</td>
<td>Yes (co-trimoxazole)</td>
<td>10</td>
</tr>
<tr>
<td>Bacterial bronchitis</td>
<td>200</td>
<td>No</td>
<td>6</td>
</tr>
<tr>
<td>Bacterial bronchitis</td>
<td>240</td>
<td>No</td>
<td>6</td>
</tr>
</tbody>
</table>

Key: neb pent = nebulised pentamidine; KS = Kaposi sarcoma; LIP = lymphocytic interstitial pneumonitis.

detectable but below the limits of quantification in a further 34 (46%) and was undetectable in 35 (46%). Levels of detectable, quantifiable, *P. jirovecii* DNA in those with PCP were higher than in those with alternative diagnoses; \( p =0.0001 \) (Figure 3).
Among patients with PCP there was no correlation between the quantity of detectable \textit{P. jirovecii} DNA and patient’s prior receipt of prophylaxis, duration of anti-\textit{Pneumocystis} therapy prior to diagnosis, \( \text{PaO}_2 \), or presence of pulmonary co-pathology. Of 110 negative controls only one had detectable DNA (at <5copies/reaction). None of 9 other negative controls in that reaction produced detectable signal.

\textit{Detection of \textit{P. jirovecii} DNA using PCR at the mt LSU rRNA} 
\textit{P. jirovecii} DNA was detected using the mt LSU rRNA assay in BAL fluid from 61 of 62 (96\%) episodes of PCP and from 24 of 74 (32\%) episodes with an alternative diagnosis (Figure 4). Using the defined cut-off of >24 ng/reaction gel gave a clinical sensitivity and specificity of 97\% (95\% CI =89-99\%) and 68\% (95\% CI =56-78\%), respectively for diagnosis of PCP.

\textbf{Discussion}

This study describes the design, optimisation and performance of a real-time PCR assay intended for detection of \textit{P. jirovecii}. Numerous reports describe molecular assays using conventional PCR to diagnose PCP, the most common being variations on the method targeting mt LSU rRNA. \cite{2,3,10} More recently real-time PCR has been used for detection of \textit{P. jirovecii} DNA in respiratory samples and for diagnosis of PCP. \cite{13-21,29} The present study differs from previous reports by the strategy used for optimising DNA extraction and by the gene target used for analysis. We chose an \textit{in silico} strategy to find an optimal molecular target in the HSP70 gene and compared this method with a well-described assay targeting mt LSU rRNA. \cite{2,3,10}

There are many reasons for selecting this new target: the HSP70 gene is conserved across eukaryotic organisms, \cite{22,30} yet the sequence identity is not. Phylogenetic analysis suggests this sequence is unlikely to be lost, reducing the likelihood of sensitivity being compromised. As this sequence is diverse, the potential for a molecular method detecting other organisms that may be present within the sample, either as a co-pathogen or as a commensal, are greatly reduced. This PCR assay is specific for \textit{P. jirovecii} as it does not detect other species of \textit{Pneumocystis}, including \textit{P. carinii}, \textit{P. murina} or \textit{P. wakefieldiae}, or a wide range of fungal, bacterial or mycobacterial potential pathogens. Furthermore, by designing the HSP70 assay to detect part of the coding sequence, polymorphisms affecting the resultant heat shock protein are also less likely than if the assay amplified a less conserved non-coding region or a highly variable protein such as the major surface glycoprotein.

When the \textit{Pj}HSP70a assay is used to amplify DNA from BAL fluid the amounts of \textit{P. jirovecii} HSP70 DNA detected from patients with PCP and those with alternative diagnoses were significantly different. Using a quantitative cut-off of >10 copies/reaction ROC analysis shows clinical sensitivity =98\% and specificity =96\%. Further, in those with PCP the amount of \textit{P. jirovecii} DNA in BAL fluid was not associated with disease severity and outcome, suggesting that other factors are implicated in the pathogenesis of PCP.

Interpretation of the significance of finding \textit{P. jirovecii} DNA in a respiratory sample is hampered by the observation that the organism may be asymptptomatically present in the lung, either transiently or latently. \cite{7-10,31} Furthermore, a ‘negative’ result from attempts at DNA detection, in the context of asymptomatic carriage becomes more difficult to interpret when using conventional PCR. A faint band following gel electrophoresis might be due to low starting copy number, which may or may not be attributable to ‘colonization’; however inhibition and/or low assay efficiency may also contribute to such a finding. There are further difficulties if a mitochondrial sequence, such as mt LSU rRNA is used as little is known about the physiology of \textit{P. jirovecii} and how mitochondrial DNA copy number (a factor of mitochondrial numeracy and genome replication) may vary according to different states of disease, ‘colonization’, or in response to treatment. We found that the mt LSU rRNA PCR assay had an analytical sensitivity for detection of \textit{P. jirovecii} DNA of \( \sim 10^5 \) copies/reaction. This result is surprising and may in part be explained by the
secondary structure found in both primers pAZ102-H and pAZ102-E, when analysed using mfold [32] and by the 5°C difference in melting temperature (Tm) between these primers, identified using the nearest neighbour prediction method. [33] Both of these factors could affect PCR efficiency at this locus. This finding suggests that mitochondrial sequences can be in great abundance, at least 1000 copies to every one of HSP70, when *P. jirovecii* is present in BAL fluid as analytical sensitivity was comparable to the single copy *Pj*HSP70a assay. The mt LSU rRNA PCR assay can be nested to improve sensitivity, with a reported detection sensitivity of a single-copy target [34]. Our data suggest that nested PCR at the mt LSU rRNA locus is unnecessary as, with an optimized extraction procedure, single round PCR at this locus has a sensitivity of 97%. Whichever conventional PCR strategy is chosen, improved analytic sensitivity will be achieved at the cost of reduced specificity, as increasing numbers of patients with low burden asymptomatic ‘colonization’ are detected. Therefore a quantitative method is essential.

Studies which attempt to quantify *P. jirovecii* DNA from BAL fluid and which aim to correlate genome copy numbers with clinical situations (colonization or PCP) may potentially be confounded by the influence of variable dilution of BAL fluid caused by variations in bronchoscopic technique. In this study we attempted to control for this as one person performed all BAL, using a standard technique. Future studies which control for heterogeneity caused by dilution, either by inclusion of internal amplification controls [35] or by normalising to genomic DNA, [36] may enable better clinical correlations to be made.

Several real-time PCR assays, using a variety of gene targets, have been described for detection of *P. jirovecii* in respiratory samples; [12,16,19] a high inter-laboratory agreement among real-time PCR assays has been described. [29] Alvarez-Martinez et al compared nested PCR with real-time PCR using the dihydropteroate synthase gene target applied to BAL fluid samples. [19] Nested PCR had an analytical sensitivity of 94% and a specificity of 81% for detection of *P. jirovecii*. Real-time PCR detected *P. jirovecii* DNA from 67/70 patients with microscopically confirmed PCP, range =1-10⁶ copies/µl of sample [mean =1.8 x 10⁴] and from 3/70 with negative microscopy for PCP, range =1-10 copies/µl of sample [mean =4], giving a clinical sensitivity of 94% and a specificity of 96%. [19] Larsen et al described development of a quantitative touch-down real-time PCR using the multi-copy major surface glycoprotein (MSG) gene for diagnosis of PCP. [12] Lower respiratory tract samples (BAL fluid and induced sputum) from patients with PCP contained 2.4-1 040 000 copies of MSG gene/reaction (median =417) and from those without PCP contained 0.3-248 copies/reaction (median =2.6). Discrimination between infection and colonization was possible by using an arbitrary cut-off of 10 copies/reaction. However, applying this cut-off meant that the false positive detection rate fell, at the cost of an increase in false negative results. [12] In a follow up study the authors applied the MSG real-time PCR assay to oral wash samples for diagnosis of PCP and demonstrated a clinical sensitivity of 88% and specificity of 85%. [16] A marked treatment effect was observed in those with PCP. Samples obtained ≤1 day after start of treatment contained a median of 417 MSG gene copies/reaction (range 0-21 290) and in samples from those who had received >1 day of treatment median was 7 copies/reaction (range 0-3,673). Clinical specificity was increased to 100%, but sensitivity was reduced to 75%, by applying a post hoc cut-off of 50 copies/reaction. [16] This observation contrasts with the findings of our study, as we found that in those with confirmed PCP there was no correlation between duration of therapy prior to obtaining the BAL fluid sample, and HSP70 copy number. In the present study, if all patients with detectable *P. jirovecii* DNA using the *Pj*HSP70a assay are considered positive, then clinical sensitivity would be 100% and specificity would be 37%. In order to discriminate between those who did and did not have PCP we used a cut-off of ~10 copies/reaction, based on ROC analysis.

Assessments of new molecular diagnostic methods are often reported on a “yes the pathogen is present” versus “no it is not present” basis, dependent on whether or not a nucleic acid target is detected in a clinical sample. From such analysis positive and negative predictive values are derived.
from what is often a small and highly polarised group of individuals. Diagnostic assays are often not fully characterised, making a negative result difficult to interpret as it is uncertain whether this is a true negative result, or is due to sampling procedure, extraction technique or the PCR assay itself. In this study we have demonstrated the importance of DNA extraction techniques, as we compared two procedures and show improved detection of *P. jirovecii* DNA using QIAamp UltraSens. We used a large number of negative controls were used to exclude potential contamination. Only one of 110 negative controls had detectable signal; this result would have been considered negative using ROC analysis. We interpret this as representing sporadic contamination (~1 % of assays).

In summary, molecular detection tests require considerable optimisation in order to be diagnostically useful. In particular, variations in DNA extraction methods may influence the amount of detectable *P. jirovecii* DNA in BAL fluid. Real-time PCR using the HSP70 genomic target enables discrimination of PCP from other infections that form part of the differential diagnosis among immunosuppressed patients. Real-time PCR targeting HSP70 has a potential diagnostic application. A larger prospective translational study is required to define the suitability of this diagnostic approach in a routine clinical setting. The finding of *P. jirovecii* DNA in BAL fluid in some patients without confirmed PCP is consistent with the concept of ‘colonization’ and underscores the need for a quantitative approach to detection.

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**Conflict of Interest**

Professor RF Miller is Co-Editor of *Sexually Transmitted Infections*, part of the BMJ Publishing Group.

**GeneBank**

Accession number = DQ987621

**References**


**Figure legends**

Figure 1. *P. jirovecii* DNA detected in BAL fluid from patients with PCP extracted with DNAeasy (left) and QIAamp UltraSens (right). Data are shown as scatter and bar and whisker plots (median, 25th & 75th percentiles and range).

Figure 2. Agarose gel showing analytical sensitivity and dynamic range of PCR assays

a) *PjHSP70*. Product = 106 base pairs.

b) mt LSU rRNA. Product = 348 base pairs.

Figure 3. Real-time *PjHSP70a* PCR results obtained from QIAamp UltraSens extracted samples from 61 patients with 62 episodes of PCP (left) and from 71 patients with 74 episodes with alternative diagnoses (right). Data are shown as scatter and bar and whisker plots (median, 25th & 75th percentiles and range).
Figure 4. Receiver-operator curve (red line) analysis of real-time *Pj*HSP70a PCR and mt LSU rRNA (△) PCR assays. The black circle depicts >10 copies cut-off, used to generate clinical sensitivity and specificity of the *Pj*HSP70a assay.
Figure 1

HSP70 ~copies/ reaction

DNAeasy

QIAamp
Figure 2

Part a: 
- *PjHSP70a 106 bp*

Part b: 
- *mt LSU rRNA 348 bp*
Figure 3
Development and evaluation of a real-time PCR assay for detection of Pneumocystis jirovecii DNA in bronchoalveolar lavage fluid of HIV-infected patients

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model does apply to occupational causation overall. However, as they readily acknowledge, an attempt to stratify this relationship according to whether the causative agent is of high molecular weight (HMW) or low molecular weight (LMW) is limited by the small size of the study population. An epidemiological approach offers a means of complementing the observations from this type of clinical study in order to better characterise how the frequency of rhinitis varies with type of exposure.

National reporting schemes for occupational diseases such as The Health and Occupation Reporting (THOR) network in the UK provide data on the causative agents for a large number of cases of OR and OA reported by networks of respiratory and occupational physicians. In a preliminary study we have determined the number of cases of rhinitis (with or without asthma) as a proportion of the total number of cases of rhinitis and asthma for the 15 respiratory sensitisers most frequently reported to THOR during the decade 1997–2006.

The data suggest significant differences in the rhinitis:asthma ratio between the various causative agents. Few, if any, of the cases of OR reported to THOR would have been confirmed by acoustic rhinometry, and their diagnosis is probably based on a history of nasal symptoms, sometimes accompanied by rhinoscopy, which can have poor specificity. This, together with potential reporting biases, limits conclusions that can be drawn from these provisional THOR data. They do, however, raise the possibility that respiratory sensitisers differ in the extent to which they cause rhinitis compared with asthma. This is particularly evident when the rhinitis:asthma ratio is compared for the two most frequently reported respiratory sensitisers—laboratory animals and isocyanates—with a much higher proportion of rhinitis associated with the former.

A number of toxicokinetic factors, such as particle size or solubility, could determine the relative distribution of a sensiser in the upper and lower airways. Toxicodynamic factors might relate to molecular weight, with the suggestion from the data that higher molecular weight agents might be preferentially associated with rhinitis. The THOR data are consistent with the hypothesis that OR (when in conjunction with OA) is more likely to be caused by sensitisers that cause disease through IgE-mediated mechanisms. On the other hand, LMW agents such as isocyanates, which may cause OA by non-IgE mechanisms, do not associate strongly with OR. Morphine, which had the highest rhinitis:asthma ratio in our study, may act through direct mast cell degranulation. Further clinical and epidemiological research could help to substantiate such mechanistic hypotheses. Castano and colleagues have shown that the united airways disease can apply to occupational causation, but this might not be consistent across the diverse range of respiratory sensitisers.

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CORRECTION
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J F Huggett, M S Taylor, G Kocjan, et al. Development and evaluation of a real-time PCR assay for detection of Pneumocystis jirovecii DNA in bronchoalveolar lavage fluid of HIV-infected patients. Thorax 2008;63:154–9. There is a typographical error in one of the PCR primers, which will result in the assay not working if this is copied. The forward primer that currently reads 5′-AGTCCGTTTAGCAGCTAC-3′ should read 5′-AGTCGGTTTAGACGCAGCTAC-3′.