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Detection of *Pneumocystis* DNA in samples from patients suspected of bacterial pneumonia- a case-control study

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Abstract

Background: *Pneumocystis jiroveci* (formerly known as *P. carinii* f.sp. *hominis*) is an opportunistic fungus that causes Pneumocystis pneumonia (PCP) in immunocompromised individuals. *Pneumocystis jiroveci* can be detected by polymerase chain reaction (PCR). To investigate the clinical importance of a positive *Pneumocystis*-PCR among HIV-uninfected patients suspected of bacterial pneumonia, a retrospective matched case-control study was conducted.

Methods: Respiratory samples from 367 patients suspected of bacterial pneumonia were analysed by PCR amplification of *Pneumocystis jiroveci*. To compare clinical factors associated with carriage of *P. jiroveci*, a case-control study was done. For each PCR-positive case, four PCR-negative controls, randomly chosen from the PCR-negative patients, were matched on sex and date of birth.

Results: *Pneumocystis*-DNA was detected in 16 (4.4%) of patients. The median age for PCR-positive patients was higher than PCR-negative patients (74 vs. 62 years, $p = 0.011$). PCR-positive cases had a higher rate of chronic or severe concomitant illness (15 (94%)) than controls (32 (50%)) ($p = 0.004$). Twelve (75%) of the 16 PCR positive patients had received corticosteroids, compared to 8 (13%) of the 64 PCR-negative controls ($p < 0.001$).

Detection of *Pneumocystis*-DNA was associated with a worse prognosis: seven (44%) of patients with positive PCR died within one month compared to nine (14%) of the controls ($p = 0.01$). None of the nine PCR-positive patients who survived had developed PCP at one year of follow-up.

Conclusions: Our data suggest that carriage of *Pneumocystis jiroveci* is associated with old age, concurrent disease and steroid treatment. PCR detection of *P. jiroveci* has low specificity for diagnosing PCP among patients without established immunodeficiency. Whether overt infection is involved in the poorer prognosis or merely reflects sub-clinical carriage is not clear. Further studies of *P. jiroveci* in patients receiving systemic treatment with corticosteroids are warranted.

Background

Pneumocystis pneumonia (PCP) is a common and serious opportunistic infection in immunocompromised patients that is caused by the fungal pathogen *Pneumocystis jiroveci*, formerly known as *P. carinii* f.sp. *hominis* [1]. Because few organisms are found in sputum and the sensitivity of microscopic examination of induced sputum is variable, the diagnostic "gold standard" for PCP continues to be microscopy of bronchoalveolar lavage (BAL) fluid. Although BAL combined with conventional staining has high diagnostic sensitivity (>95%), this procedure is invasive. In order to increase sensitivity in non-invasive specimens, several experimental studies have evaluated PCR detection of *P. jiroveci* DNA as a potential diagnostic test.

We recently described a touch-down PCR method (TD-PCR) with an internal control to detect PCR inhibition [2,3]. On oral wash specimens obtained from HIV-1 infected patients we found positive and negative predictive values of 93 and 91% for the diagnosis of PCP [2]. Further, this method had a 100% sensitivity and specificity among 24 haematological cancer patients [3]. The aim of the current study was to evaluate the clinical significance and prevalence of a PCR positive signal in a broad spectrum of clinical samples from HIV-negative patients with suspected bacterial pneumonia by means of an age and sex matched nested case-control study.

Methods

Clinical specimens

A total of 367 respiratory specimens, including 100 BAL, 126 tracheal aspirates and 141 sputum samples, were obtained from 367 patients suspected of pulmonary infection between November 1997 and August 1999. Of these, 261 specimens were randomly selected from specimens submitted for detection of *Mycoplasma pneumoniae* or *Legionella pneumophila* to Statens Serum Institut (SSI). The remaining 106 respiratory samples represented all specimens submitted for investigation of bacterial pneumonia during a one-week period in August 1999, at the Department of Clinical Microbiology, Herlev University Hospital.

DNA extraction and PCR

DNA was extracted as described [2]. To reduce the risk of a false positive detection of *P. jirovecii* DNA, three distinct genetic loci of *Pneumocystis jiroveci* were amplified by PCR using primer sets that are specific for the human form of *Pneumocystis* infection, *Pneumocystis jiroveci*.

Mitochondrial large subunit rRNA (mtLSU rRNA) was amplified by primers pAZ102 E and pAZ102H [4].

Mitochondrial small subunit rRNA (mtSSU rRNA) was amplified with primers pAZ112-10F and pAZ112-10R [5].

Major Surface Glycoprotein (MSG). Primers JKK14 and JKK17 were used to amplify a highly conserved region of the gene encoding the MSG [6].

The PCR methods amplifying the mtLSU and mtSSU loci were chosen since these methods have been previously validated and are in routine use at SSI [2]. The MSG method was chosen since this method was reported to have increased sensitivity compared to amplification of mtLSU, a finding which have been confirmed in our laboratory (data not shown) [6].

Internal process control for inhibition

To detect PCR inhibition, an internal process control was constructed for each of the three primer sets [2]. When inhibition occurred, a 2-fold dilution of the DNA lysate was retested.

Amplification protocol

PCR was carried out with Platinum Taq (Gibco), with reaction mixtures as reported [2]. Precautions to avoid PCR contamination included barrier methods, multiple negative controls and the use of dUTP. PCR was done blinded to the identity of patients.

The cycling conditions for the mtLSU PCR included a 94°C preactivation of the enzyme for 2 minutes, 10 cycles of touch-down PCR with a 1°C decrement of the annealing temperature per cycle from 72°C to 62°C, followed by 40 cycles with annealing at 62°C/30 seconds with denaturation at 94°C for 15 seconds and extension at 72°C for 15 seconds. Amplification of the mtSSU and MSG genes were done with a touch down step from 65°C to 55°C followed by 40 cycles at 55°C. PCR products were separated on agarose gels with ethidium bromide staining.

Microscopy

Previously frozen PCR positive specimens were evaluated by immunofluorescence staining detecting cyst according to the manufacturers protocol (Pasteur, France) [7].

Data and statistical analysis

Only samples positive by PCR amplification of all three *P. jiroveci* genes were considered true PCR positive. Data on age and sex were available from the laboratory. For each of the 16 PCR-positive cases, we randomly selected four controls matched by age and sex. Clinical data were collected retrospectively by chart review. All 80 patients were followed until October 2000. A clinical diagnosis of pneumonia was made if a newly developed infiltrate was found on the chest X-ray at admission in association with an appropriate clinical history and physical signs for which no other cause was found. Isolation of pathogenic bacteria from respiratory samples or blood culture from the day of admission was considered as definitive microbial diagnosis.

sis. One-month mortality was calculated from day of admission. None of the patients who died underwent autopsy. Two-sided p values were calculated by Fisher's exact test or Mann-Whitney tests with values of < 0.05 considered significant.

Results

A total of 16 patients (4 %) were considered true-PCR positive by amplification of the MSG, mtLSU and mtSSU rRNA genes of *Pneumocystis jiroveci* (table 1). In addition, 5 samples were PCR positive with one or two primer sets (three samples positive in one, two samples in two) but tested negative with the third confirmatory PCR and were considered as PCR negative. Among the 261 patients investigated for *Legionella pneumophila* or *Mycoplasma pneumoniae* pneumonia 11 (4 %) were PCR positive compared to 5 (5 %) of the 106 patients examined for bacterial infection. The median age for PCR-positive patients was higher than for the 346 PCR-negative patients ($p = 0.025$) (table 1). None of the PCR-positive patients were known to be HIV-1 positive at the time of specimen collection or during the follow-up period. To our knowledge none of the PCR-positive cases had been exposed to patients with acute PCP, in particular none were hospitalized at infectious disease departments, which in Denmark are treating the majority of PCP cases. The cases did not receive anti-pneumocystis treatment and were not hospitalised at the same time or at the same hospital.

Table 1: Characteristics of respiratory specimens.

	PCR-negative	PCR-positive
Respiratory specimens		
BAL	96	4 (4%)
Tracheal aspirates	116	7 (6%)
Sputum	134	5 (4%)
Median Age (range), years	62 (1-91)	74* (47-88)
Male / female	195 / 151	6 / 10

* $p = 0.011$, Mann-Whitney.

Two PCR-positive patients were investigated for PCP by microscopy at time of diagnosis, based on fever, diffuse interstitial infiltrates and high LDH occurring after chemotherapy for Hodgkins disease and pneumonia in the setting of immunosuppressive treatment after renal transplantation with delayed response to cefuroxime, respectively. Both patients were negative for *P. jiroveci*. PCP was not suspected or investigated in the remaining 14 PCR-positive patients or in any of the PCR-negative patients. *P. jiroveci* could not be detected by immunofluorescence microscopy in any of the PCR positive specimens.

The findings of the nested case-control study, which compared the 16 patients with a positive *P. jiroveci* PCR to 64 age and sex matched PCR-negative controls, are summarized in table 2. PCR-positive cases had a higher rate of chronic or severe concomitant illness (15 (94%)) compared to controls (32 (50%)) ($p = 0.004$) (Table 2). By comparison of the one-month mortality, seven (44%) cases died compared to nine (14%) of the controls ($p = 0.014$). In all patients death was due to pneumonia and respiratory failure. Among the seven PCR positive patients who died, five had chronic pulmonary disease (COPD), one patient Hodgkin's disease and one patient no prior medical history. *H. influenzae* pneumonia was diagnosed in three patients and *S. pneumoniae* pneumonia in one. At one year of follow-up, none of the 9 surviving patients had developed *Pneumocystis jiroveci* pneumonia.

A higher proportion of PCR-positive patients compared to controls had received treatment with steroids alone (10/16 versus 7/64, $p < 0.001$). In addition, two of the PCR-positive patients were currently immunosuppressed by high-dose chemotherapy or azathioprine, in contrast to controls, of whom none received other immunosuppressive agents at diagnosis. Among patients treated with corticosteroids, the duration of corticosteroid treatment was longer ($p < 0.001$, Mann-Whitney) and the prednisone dosage was higher (median prednisone dosage 37.5 vs. 7.5 mg, $p = 0.026$) in cases compared to controls. However, three of the PCR-positive cases had only received two weeks of corticosteroid treatment. Of the four PCR-positive cases without identified cause of immunosuppression, two patients had verified bacterial pneumonia and two patients had pneumonia of unknown cause. All four patients were old (age 59 to 80 years) with chronic obstructive pulmonary disease (COPD) and/or ischaemic heart disease (IHD).

Discussion

In this study, *P. jiroveci* was detected by PCR in 16 (4 %) of 367 HIV-negative patients suspected of bacterial pneumonia. High age, systemic corticosteroid treatment and a high rate of concurrent illnesses were associated with the finding of a positive *P. jiroveci* PCR. Although the detection of *Pneumocystis* DNA was associated with a poor prognosis, more than half of the patients recovered without treatment of PCP indicating that these patients were colonised or had sub-clinical infection.

In patients without AIDS, treatment with corticosteroids, chemotherapy or other immunosuppressive agents are well established risk factors of clinical PCP [8-10]. PCP is mostly seen following high dose corticosteroid treatment, but no threshold level for immunosuppression exists and even low doses of corticosteroid therapy may predispose to PCP [10]. In clinical practice, however, PCP is rarely

Table 2: Clinical characteristic of 16 *P. jiroveci* PCR-positive patients and 64 age and sex matched PCR-negative controls.

	PCR-positive patients	PCR-negative patients	p-value
N	16	64	
Age, years	73 (53–78)	71 (51–78)	
Male / female	6 / 10	24 / 40	
Dead within one-month	7 (44%)	9 (14%)	p = 0.014
Diagnosis (%)			
Bacterial pneumonia (verified)	3 (19%)	13 (20%)	
Pneumonia, pathogen not identified	6 (39%)	31 (48%)	
Acute bronchitis	5 (31%)	12 (19%)	
Lung malignancy	0	2 (3%)	
Cardiac disease	1 (6%)	4 (6%)	
Other	1 (6%)	3 (5%)	
Concurrent diseases (%)			
Unknown/None	1 (6%)	32 (50%)	
COPD	10 (63%)	13 (20%)	
Malignancy	1 (6%)	4 (6%)	
Lymphoma and bone marrow-transplant	1 (6%)	1 (2%)	
NYHA III/IV	1 (6%)	13 (20%)	
NYHA III/IV and COPD	1 (6%)	1 (2%)	
Chronic renal failure	0	1 (2%)	
Renal transplantation and malignancy	1 (6%)	0	
Total number with concurrent diseases	15 (94%)	32 (50%)	p = 0.004
Immunosuppressive therapy (%)			
None	4 (25%)	56 (87%)	
Steroids alone	10 (63%)	7 (11%)	p < 0.001
Chemotherapy alone	0	1 (2%)	
Chemotherapy and steroids	1 (6%)	0	
Azathioprine and steroids	1 (6%)	0	
Total number receiving steroids	12 (75%)	8 (13%)	
Corticosteroid dose ^a	37,5 (8,8–58,8)	7,5 (5–27,5)	p = 0.026
Duration of corticosteroid therapy, weeks (range)	66 (1–102)	8 (1–42)	p < 0.001

All values are Median (25–75 percentiles). ^a Corticosteroid dose in daily equivalents of prednisone in milligrams at time of diagnosis. COPD: Chronic obstructive pulmonary disease. NYHA: New York Heart Association.

considered in patients presenting with pneumonia, except patients with known predisposing factors such as HIV/HIV, chemotherapy treated malignancy or transplant related immunosuppression. In the current study, PCP was suspected in only two patients with lymphoma and transplantation. Interestingly, three PCR-positive patients had only received corticosteroid treatment for two weeks, suggesting that even short-term immunosuppression may predispose to colonisation.

Previously, several studies have evaluated the use of PCR for diagnosis of PCP [4,7,11]. Most studies have found that PCR is more sensitive than conventional staining methods with the ability to detect infection in oral wash, sputum and tracheal aspirates, in which conventional staining has inadequate sensitivity. These samples are considerably easier to obtain than BAL. The use of nested PCR have been shown to increase sensitivity in comparison with single-round PCR, however the additional ma-

nipulations increase the risk of false positive results [11,12]. Consequently, an optimised single round PCR protocol (TD-PCR) has been chosen for routine use at Statens Serum Institut [2].

The exact mode of acquisition and transmission of *P. jiroveci* has been debated but is still not completely understood. For many years PCP was believed to be caused by reactivation of latent infection based on a high prevalence of persistent antibodies to *P. jiroveci* from an early age [13,14]. This view was changed when it was observed that animal hosts [15,16] and immunocompetent individuals [17] do not seem to carry *Pneumocystis* after primary infection. Hence, infection is now primarily believed to be caused by new infection, rather than reactivation of latent organisms. But if infection is cleared, what constitutes the source of new infections? Although the natural reservoir for *P. jiroveci* remains undefined, recent experimental animal studies and studies in humans have suggested that

asymptomatic immunocompetent carriers may be one source of infection. Immunocompetent mice can be transiently colonised with *P. carinii* and are able to transmit the infection to mice with severe combined immunodeficiency [18]. In humans subclinical colonisation have been described in both immunosuppressed and immunocompetent individuals by PCR. However, the extent of colonisation has been debated with different rates of *P. jiroveci* carriage reported. Nevez *et al.* reported that 20 % of 169 HIV-negative patients with moderate or severe immunosuppression were positive by nested PCR; none of the patients developed PCP [19]. In agreement with this study studies by Olsson *et al.* and Takahashi *et al.* reported a PCR positive rates of 28% and 20%, respectively among HIV-negative immunosuppressed patients without definite or probable PCP [20,21]. Sing *et al.* found by nested PCR that 17–20% of immunocompetent patients with primary pulmonary disorders were nested PCR positive without developing PCP [11,22]. These observations suggest that colonization with *P. jiroveci* may be more common than previously thought, even in immunocompetent individuals. However, in contrast to Sing *et al.*, we observed a much lower rate of PCR positivity (4%) among our patients with community-acquired pneumonia.

Whether the relatively low rate of *P. jiroveci* detection is a result of real population based differences in *P. jiroveci* carrier rates or stems from differences in PCR methods is unknown. PCR is extremely sensitive and will detect both living and dead organisms. A positive PCR in an asymptomatic patient could reflect PCR contamination, colonization or transient carriage of a very low number of *P. jiroveci* organisms. In our study, the risk of false positive reactions was reduced by only considering samples true PCR positive if three different *P. jiroveci* gene targets could be amplified, whereas the other studies [11,19,22]. only amplified a single gene with nested PCR, which may increase the risk of false positive signals. In 5 patients we detected *P. jiroveci* DNA with one or two of the three PCR primer amplifications but failed to generate a PCR signal in the third confirmatory setup. These samples possibly represent cases in which the *P. jiroveci* DNA was at the threshold of detection. Interestingly, the frequency of positive specimens were comparable in BAL, tracheal aspirates and sputum (4–6%) and were equivalent in patients suspected of bacterial and atypical bacterial pneumonia (3–4%), suggesting that the rate of positive specimens was not related to respiratory sample type. Further, nosocomial transmission was unlikely, since the patients were not admitted to the same ward. The low rate of PCR positivity we observe is comparable to reports by Visconti *et al.* [23], where only 2.5% of 78 immunocompetent patients were positive by nested PCR; Oz *et al.* [24], in which no *P. carinii* DNA by single PCR were detected in any of 258 upper respiratory tract specimens from 86 immunocompetent indi-

viduals in the absence of microscopy verified PCP, and a Danish study[25] where 4.7% of 1762 consecutive lung biopsies at autopsy were microscopically positive for *P. carinii* [25].

Because of the retrospective design and limited sample size, our study has limitations. The clinical significance of a positive *P. jiroveci* PCR is unclear. In terms of demographics and comorbidities, a wide spectrum of patients was included with an overall low pre-test probability of PCP, which were only clinically suspected in a few patients. A higher mortality (54%) was observed in PCR-positive cases compared to controls (18%), a rate that is similar to the 18% mortality rate reported for elderly patients with community-acquired pneumonia [26]. However, since autopsies were not done, the causes of deaths are unknown. Whether detection of *P. jiroveci* is an independent predictor of death or merely a marker of severe illness is therefore not known. We were unable to detect *P. jiroveci* by microscopy in any of the 16 PCR-positive samples, indicating that the number of organisms was below the detection limit of conventional staining. However, 10 of the samples were sputum or tracheal aspirates, in which microscopy has limited diagnostic sensitivity. Further, 9 of the PCR positive patients recovered without specific anti-pneumocystis treatment and another pulmonary infection were found in 4 patients. Nevertheless, these findings do not rule out that *P. jiroveci* may have contributed to the severity of clinical disease since concomitant pulmonary infections may be detected in 35–58% of AIDS-associated PCP [8,10].

Conclusions

Our results show, that *P. jiroveci* may more easily colonise individuals with severe or concomitant disease, in particular in connection with steroid treatment. These individuals may represent a possible reservoir for the circulation of Pneumocystis organisms in the community. Because *P. jiroveci* DNA was also detected in immunocompetent individuals recovering without specific anti pneumocystis therapy, our results support that subclinical infection or colonisation may happen in adult immunocompetent individuals, although rarely. Our findings affirm that the interpretation of a positive PCR must be done with great caution, in particular in patients without known risk factors for PCP. In such patients, confirmation of PCP should preferably be sought by conventional staining methods. Possibly, a improved method of PCR diagnosis may be quantitative PCR, since recent research suggest that application of a cut-off value to quantitative PCR could have a role in distinguishing between colonisation and clinical disease [27]. Additional studies of colonisation and the possible pathogenic role of *P. jiroveci* in patients without HIV, in particular COPD patients are warranted.

Competing interests

None declared.

Authors' contributions

JHL, JSJ and BL were responsible for conception and design of the study. JHL collected all data, performed statistical analysis and wrote first draft of the manuscript. BD and JSJ were responsible for laboratory PCR analysis. BL supervised the study and together with TLB and JSJ participated in design and critical review of the manuscript. All authors have read and approved the manuscript.

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