

A New Molecular Diagnostic PCR/Microarray Hybridization Prototype Evaluation Compared to Standard PCR/Sequencing for *Pneumocystis jirovecii* Detection

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Abstract

Diagnosis of *Pneumocystis jirovecii* pneumonia (PjP) is challenging. Therefore highly-sensitive and time-efficient new diagnostic tools are needed for early antibiotic treatment start and optimized patient outcome. We evaluated a prototype of a new molecular diagnostic tool using PCR/microarray hybridization for *P. jirovecii* detection in respiratory samples of hospitalized patients suffering from pneumonia. Prototype results were first compared to established routine PCR results and were set in the context of patients' immune status and clinical presentation in an overall assessment (true positive, false negative or false positive). In our study population, *P. jirovecii* was assayed with both detection methods in eleven patients out of 739 evaluable study patients; of these, six cases were detected only with the prototype system, two cases only with site-established PCR and three cases with both methods. From these eleven positive cases, we found that six prototype positive cases were true false positives, three prototype positive cases were confirmed as true positives and two prototype negative cases were true false negatives. Overall prevalence for *P. jirovecii* was low in this study population. Although more positive cases were found using the prototype system, these cases seemed not to be of any clinical significance which might have led to an antibiotic overtreatment of patients under real conditions. However, the correct detection of three positive cases within a few hours might render the prototype a valuable diagnostic tool in the future.

Introduction

Pneumocystis pneumonia (PjP) is a life-threatening opportunistic infection caused by *Pneumocystis jirovecii*, a fungal pathogen which is uncultivable with standard techniques in vitro. PjP is a known serious disease in HIV infected patients, but has a high mortality rate in non-HIV immunocompromised patients as well. Pulmonary co-infection with herpes viruses can worsen clinical outcome [1]. There is an increasing incidence of PjP in immunocompromised patients [2]. Solid organ transplant recipients are of high risk of acquiring PjP within 6 months post-transplantation, associated with a significant mortality rate due to PjP [3]. Pneumocystis carriage was also detected in a significant amount of patients suffering from cystic fibrosis (CF) [4]. PjP-associated mortality in rheumatoid patients using TNF inhibitors agents is higher than in HIV-infected individuals [5]. Careful monitoring, early diagnosis, and proper management are mandatory to secure a good prognosis for these patients [6]. Improved recognition, diagnosis, and prevention of *P. jirovecii* infections are needed to improve outcomes in these patients [7]. PjP diagnosis is based on clinical examination, radiological, clinical chemistry (arterial oxygen saturation, lactate dehydrogenase) and microbiological findings. Radiologically, the most common high-resolution computed tomography (CT) finding of PjP is diffuse ground-glass opacity, followed by nodules, cysts and rare spontaneous pneumothorax [8]. Direct immunofluorescence microscopy is the current gold standard of *P. jirovecii* detection in most microbiology laboratories. PCR is able to detect very low levels of *P. jirovecii* not detectable by routine histochemical staining, especially in patients with previous antibiotic treatment, suggesting a potential replacement of direct immunofluorescence by a real-time PCR assay [9]. The detection of *P. jirovecii* in respiratory samples can be optimized by flow cytometry, using specific monoclonal antibodies [10]. Since *P. jirovecii* is an ubiquitous pathogen and might be detected in small amounts in healthy individuals, findings need clinical interpretations as well as valuable cut off values. Highly-sensitive and time-efficient diagnostic tools are therefore needed for early PjP treatment, especially in patients with impaired immune status [11-14].

Material and Methods

Patient characteristics and specimen handling

We evaluated a new molecular diagnostic prototype using multiplex-PCR combined with microarray hybridization for detection of *P. jirovecii* in respiratory samples (broncho alveolar lavage [BAL], tracheal aspirates [TS], sputum [S]) of hospitalized adult patients suffering from clinical suspected pneumonia (Fig. 1; Curetis AG, Holzgerlingen, Germany). Patients were enrolled from March through September 2012, previous antibiotic treatment was allowed. Specimens were excluded

if the prototype test could not be performed on the same day as the start of microbiological testing, in case of known tuberculosis infection and when sample storage time has exceeded 18 hours after arrival in the laboratory. Samples were pseudonymised and split into three aliquots prior to testing with the prototype; one aliquot was used for routine microbiology, one for testing with the prototype, and the third aliquot was stored frozen (at -20°C or colder) for discrepant result analysis (see below). The prototype test was performed on the same day as the start of standard-of-care testing. Prototype test results were not used for diagnosis, treatment or other patient management decisions. Quality assurance, monitoring, and data management was conducted by Curetis AG.



Figure 1. PCR/Microarray Hybridization Prototype: 1. Universal Analyzer, 2. User Cockpit, 3. Universal Lysator, 4. Cartridge, disposable for clinical applications

Primer	sequence 5' - 3'	position ¹⁾
Pcar-F1	AGT TAC GGC CAT ACC TAC GA	1-16
Pcar-F2	AAG CTA CAG CAC GTC GTA TT	119 - 100

¹⁾reference genome S78185 (SS rDNA): amplicate 124 bp

hybridization probe	sequence 5' - 3'	position ¹⁾
Pcar-S1	FAM-CACCCACTATAGTACTGACGACGCC-TAMRA	89 - 65

¹⁾reference genome S78185 (SS rDNA): amplicate 124 bp

Lambda (Internal Control)

Primer	sequence 5' - 3'	position ¹⁾
Lambda TM-F	5' - GCAGGTCGAAAAATGGGTGGA	2402 - 2422
Lambda R	5' - GCATAAACGAAGCAGTCGAGT	2491 - 2471

¹⁾reference genome J02459 (lambda phage): amplicate 89 bp

hybridization probe	sequence 5' - 3'	position ¹⁾
Lambda S-DY	DY521XLTCA TTG CGT CGC TTT TTG CTG TCC-BHQ2	2467 - 2444

Table 1. Sequence of primers used for *Pneumocystis jirovecii* PCR.

Combined endpoint *P. jirovecii* PCR/Microarray Hybridization

Endpoint *P. jirovecii* PCR was performed with a multiplex PCR in a volume of 30 µl. Primers were directed against the DNA sequence encoding the large subunit of the ribosomal RNA (26S) with a 24 bp forward primer and a 26 bp reverse primer labelled with Atto647N at the 5' site to generate a 332 bp fragment (table 1, 2). Atto647N is a fluorescent label for the red spectral region with strong absorption, high fluorescence quantum yield, high thermal and photo-stability capabilities. Detection was performed by dot blot hybridization [15] with 3 different hybridization probes of 21, 22 and 24 nt complemen-

tary to the central region of the 332bp amplicon. The hybridization probes were immobilized on a nylon substrate. PCR amplification and detection were performed in a combined amplification and detection chamber [16]. PCR was performed in the bottom of the chamber and hybridization buffer was added to the PCR product. By adding hybridization buffer the liquid in the PCR chamber was reaching a nozzle protruding from the center of the combined amplification-detection chamber into the liquid; by applying vacuum the PCR product was pumped via the nozzle through the hybridization membrane.

Reagent	concentration	final concentration	µl per PCR
H ₂ O	0		9.60
FastStart DNA Master PLUS hybprobes	5x	1x	4.00
Pear-F1	20 µM	500 nM	0.50
Pear-F2	20 µM	500 nM	0.50
Pear-S1	20 µM	200 nM	0.20
Lambda TM/LC control mix			1.25
UNG	1 U/µl	0.01 U/µl	0.20
DNA			5.00
Final volume			20.00

Table 2. PCR Mastermix.

Comparison of *P. jirovecii* Detection Methods

In this case series study prototype test results were compared to study site central laboratory specific PCR results for *P. jirovecii* detection. Discrepant results were analysed for clinical relevance of molecular diagnostic tool results. In case of still questionable diagnostic results, sequencing was performed in order to verify differing diagnostic results. For sequencing, DNA from native clinical samples was purified with the QIAGEN DNA blood Mini kit (QIAGEN, Hilden, Germany). Extracted DNA is amplified with endpoint PCR amplification with 35 cycles with the PCR primers described above. Amplicons were purified with the QIAGEN PCR purification kit and were sent to the sequencing laboratory for standard sequencing. For the final result assessment, analyzed cases were assessed as either true positive (prototype result positive, routine PCR/sequencing positive, clinical symptoms/ immunosuppression evident), false negative (negative prototype result, routine PCR/sequencing positive, clinical symptoms/ immunosuppression evident) or false positive (positive prototype result, negative routine PCR/sequencing result, no clinical symptoms/ no immunosuppression).

Study organization

This case series study was performed at five different academic European sites in Belgium (Service de Microbiologie, Hôpital Erasme – Université Libre de Bruxelles, Brussels), Germany (Institute for Laboratory und Transfusion Medicine, Heart and Diabetes Center North Rhine-Westphalia, University Hospital of Ruhr-University Bochum, Bad Oeynhausen; Institute for Medical Microbiology, University Hospital of Friedrich Schiller University, Jena; Institute for Medical Microbiology and Hygiene, University Hospital of Eberhard Karls-University, Tübingen)

and Spain (Department of Pneumology, Hospital Clínic, Barcelona). Developer of the prototype system was Curetis AG, Holzgerlingen, Germany. The protocol of this case series study was reviewed and approved initially by the ethical committee of the Eberhard Karls-University Tübingen, Germany and afterwards by the institutional ethical committees of the other study sites, separately. The case series study was conducted in accordance with the Declaration of Helsinki and ICH-GCP (International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use Good Clinical Practice). With the exception of the Barcelona clinic, 4 of 5 committees waived the need for informed consent due to the low risk of the study.

Results

A total of 739 new patient samples were eligible for study analysis, of which 75 were sputa (S), 421 aspirates (TS), and 234 bronchial lavages (BAL), 9 were unclassified. *P. jirovecii* was detected in eleven patients (1.49 %) out of 739 evaluable patients with suspected pneumonia using either the prototype or conventional PCR as detection method (table 3). Of these, six questionable PjP cases (0,81 %) were detected only with the prototype pneumonia application, two cases only with site-established PCR and three cases with both methods (table 3). An independent academic clinical assessment of these 11 clinical data files was performed in order to correlate patient clinical signs and immune status with *P. jirovecii* findings. During this academic review, six of the prototype -positive cases were classified as false positives as there was no evident immunosuppression or clinical symptoms and negative academic site-established PCR results. The remaining three cases could be confirmed in the academic review as true positives. Clinical patient data (X-ray, CT, symptoms) were compared to Curetis AG study data to additionally cross-check for possibility of PjP. From the eleven patient's detected positive for *P. jirovecii* with either technique, three patients were immunosuppressed, six patients were intubated on intensive care units (ICU), and two patients had a clinical and X-ray signs suggestive for PjP. Therefore, the overall results using the prototype system in the different patient specimens was two false negatives (2xBAL), three true positives (1xS; 2xTS) and six false positives (3xBAL; 2xTS; 1xS; table 3).

Discussion

The comparably low overall detection rate of PjP in the conducted case series could mainly be attributed to the included patient population. Most of the patients were ICU patients, however, only a very small proportion of the case study population represented immunocompromised patients (table 3). Since PjP is almost always associated with immunosuppression, the low incidence of PjP in our study collective was not very surprising. Nevertheless, eleven cases were detected,

No.	Patient characteristics	Spec.	Prototype signal	<i>P. jirovecii</i> PCR	Control-PCR/Sequencing	Final Result Assessment
1	ICU patient, intubated, immunosuppression; clinical symptoms highly suggestive for PjP	BAL	negative	positive	negative	false negative
2	ICU patient, intubated, no immunosuppression, clinically and X-ray confirmed pneumonia, <i>Pseudomonas fluorescens</i> cultured in BAL	BAL	positive	negative	negative	false positive
3	ICU patient, intubated, immunosuppression, clinically and X-ray confirmed PCP, clinical improvement after cotrimoxazole treatment	BAL	negative	positive	positive	false negative
4	ICU patient, intubated, no immunosuppression, clinically and X-ray confirmed pneumonia, <i>Enterobacter cloacae</i> and <i>E. coli</i> cultured in BAL	TS	positive	negative	negative	false positive
5	ICU patient, intubated, no immunosuppression, clinically and X-ray confirmed pneumonia, <i>Pseudomonas aeruginosa</i> cultured in BAL	BAL	positive	negative	negative	false positive
6	ICU patient, intubated, no immunosuppression, ECMO, clinically and X-ray confirmed pneumonia, <i>E. coli</i> cultured in TS	TS	positive	negative	negative	false positive
7	ICU patient, heart transplant patient, intubated, immunosuppression, clinically and X-ray confirmed PCP, <i>P. jirovecii</i> initially confirmed by PCR in TS, died due to PjP, PjP also confirmed by autopsy	TS	positive	positive	positive	true positive
8	ICU patient with liver cirrhosis due to virus hepatitis C (HCV), no immunosuppression, X-ray showed alveolar condensation with pleural effusion, no PjP treatment, full recovery after meropenem plus levofloxacin therapy	BAL	positive	negative	negative	false positive
9	Patient with a history of follicular lymphoma (2006). Clinical presentation with intermittent fever since one week. X-ray showed alveolar condensation suggestive for relapsed lymphoma; but CT was negative. No PjP treatment.	S	positive	no testing	positive	true positive
10	Patient with COPD and Asthma bronchiale, no immunosuppression, <i>S. aureus</i> cultured in sputum, no PjP treatment, full recovery after cephalosporine treatment	S	positive	no testing	negative	false positive
11	Patient with a general state altered in a context of generalized pulmonary adenocarcinoma, corticosteroid therapy, no antimicrobial or PjP treatment, transfer into chronic care facility	TS	positive	no testing	positive	true positive

Table 3. Clinical patient data and *P. jirovecii* case series overall results: Comparison of prototype molecular testing, initial academic on-site PCR and control PCR/sequencing results for final result assessment. BAL = broncho alveolar lavage; TS = tracheal secretion; S=sputum; spec.= specimen. * The *P. jirovecii* status of a patient was regarded as positive if either the initial on-site PCR or the later control PCR was positive

which will be discussed here. In two cases (table 3, No. 1 and 3), the prototype failed to detect *P. jirovecii* in BAL, subsequently, these were classified as false negative. Both cases were related to immunocompromised hosts, presenting a clinical course perfectly matching with PjP. In addition, PjP was detected by on-site *P. jirovecii* PCR in these two patients. The on-site PCR result was also confirmed by control sequencing from the study sample for one case, whereas the other case could not be confirmed. Therefore, one can assume that the amount of *P. jirovecii* DNA present in the samples was below the detection limit of the prototype. Three specimens (2xTS, 1xS) were tested positive for *P. jirovecii* by the prototype (table 3, No. 7, 9, 11). All of them could be confirmed either by on-site PCR and later control sequencing, representing therefore true positive results. One case was related to immunosuppression after heart transplantation (table 3, No.7), presenting with the typical clinical picture of PjP. The other two patients (table 3, No. 9-11) must be classified as asymptomatic carriers for *P. jirovecii*, since they presented with non-PjP related pneumonia, and improved without receiving any PjP active treatment, although a potential immunosuppression could be discussed in these patients (suspicious follicular lymphoma relapse; generalized pulmonary adenocarcinoma; table 3, No. 9, 11). The remaining six cases (table 3, No. 2, 4-6, 8) were classified as false positive, since they were only detected as positive by the prototype and could not be confirmed by on-site PCR or by control-sequencing of remaining study samples. These respective patients were all not immunocompromised and their clinical picture was not consistent with PjP. At the same time, conventional microbiology results revealed *P. aeruginosa*, *E. coli*, *E. cloacae* and *S. aureus* as potentially pneumonia causing pathogens in these patients. Retrospective analysis of these results demonstrated that the false-positive prototype results were probably due to technical reasons. Regarding the turnaround-time (TAT), the average result of system for *P. jirovecii* was available 5.2 hours after specimen arrival in the lab. Results of the on-site PCR testing was ready either the same day or up to four days later. This result demonstrates a significant advantage of the prototype compared to conventional standard of care testing, especially when dealing with critical-ill or immunocompromised patients. The conception of the system as a point-of-care testing (POCT) application allows either a central placement in the core laboratory or a peripheral placement in the ICU, emergency department, or transplantation unit, respectively. The easy-of-use and 24/7 availability renders the system a valuable tool for contemporary and adequate treatment decisions under the guidance of an experienced clinician. Even if there are some drawbacks regarding limit of detection, the availability of results within hours outbalances the disadvantages of the system. The short TAT of this prototype might be able to improve empiric treatment decisions, resulting in optimized outcome in patients suffering from PjP [17,18].

Conclusion

The overall prevalence for *P. jirovecii* was low (11 out of 739) in our case series. Six out of these eleven cases identified by system prototype did not represent clinically and PCR-confirmed PjP cases and were thus classified as false positive, indicating a false positive result rate of 0,81 % (6 out of 739 samples) which might have led to an overtreatment of patients under real conditions. However, the correct detection of three positive PjP cases within a few hours might render the prototype system a valuable diagnostic tool in the future. Since *Pneumocystis* is an ubiquitous pathogen and might be detected in small amounts also in healthy individuals, diagnostic findings need clinical interpretations by an experienced infectious disease specialist or clinical microbiologist, as well as cut off values. A completely automated production process as well as adjustment of cut-off values for the series instruments might increase the robustness of the system in the future.

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Conflict of Interest Statement

Three authors (Anne Thews, Matthias Klein, Gerd Lüdke) of this publication are full-time employees of Curetis AG, the case series sponsor. Three authors (Jan Weile, Ingo Autenrieth, Antoni Torres) served as scientific consultants for the study sponsor. Five authors (Jan Weile, Berit Schulte, Eberhard Straube, Peter M. Keller, Matthias Karrasch) received travel grants to present preliminary data at investigator meetings and scientific conferences.

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