Research Paper

Detection of *Pneumocystis jirovecii* by quantitative real-time PCR in oral rinses from *Pneumocystis* pneumonia asymptomatic human immunodeficiency virus patients

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A B S T R A C T

Pneumocystis pneumonia (PCP) is a potentially life-threatening fungal infection usually seen in immunocompromised patients. *Pneumocystis jirovecii* can be easily detected from oral rinse samples in HIV patients with suspected PCP. In this study, a quantitative real-time PCR assay was used to establish the frequency of detection of *P. jirovecii* in oral rinses from HIV patients without respiratory symptoms or suspicion of PCP. Two saline oral rinses were collected from 100 ambulant HIV patients and from 60 COPD patients (comparator group). Four HIV patients were positive for *P. jirovecii*. In three patients, the first sample was positive and in one the second one was positive. One of these patients was on PCP prophylaxis and had a CD4$^+$ count of 76 cells/mm^3. The mean CD4$^+$ count for all patients was 527 cells/mm^3. All qRT-PCR test results for the COPD patients were negative. No patient developed PCP at six months follow-up. The qRT-PCR assay can be used to detect *P. jirovecii* DNA in oral rinse samples from HIV patients without evident clinical symptoms, however the oral carriage of this fungus was rare in our cohort of patients. In conclusion, although rare, a positive oral rinse *P. jirovecii* result may reflect colonisation, in particular in patients with HIV. This needs to be kept in mind when using oral rinses and qRT-PCR in the diagnosis of *P. jirovecii* infection.

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1. Introduction

Pneumocystis jirovecii (formerly *Pneumocystis carinii*) is the causative agent of *Pneumocystis* pneumonia (PCP), a potentially life-threatening fungal infection usually seen in immunocompromised or immunosuppressed individuals. If untreated, the mortality rate is as high as 100%, however even with treatment the mortality rate ranges between 10% and 20% [1]. HIV-positive patients with a CD4$^+$ lymphocyte count lower than 200 cells/mm^3 and in particular, lower than 100 cells/mm^3, are the main risk group [2]. In addition, between 10% and 15% of the HIV patients with a CD4$^+$ count higher than 200 cells/mm^3 develop PCP [3]. Although the incidence of PCP in HIV-infected patients has declined after the introduction of antiretroviral (ARV) therapy and the use of PCP prophylaxis, PCP still remains one of the most common opportunistic infections in the HIV population [4]. In HIV-negative immunosupressed patients such as those undergoing intensive anticancer chemotherapy or having an underlying lung disease, the risk for PCP may be even higher than in HIV patients who are on ARV therapy and/or PCP prophylaxis, as reported by several studies [5–8].

Immunocompetent individuals are believed to act as a reservoir for *P. jirovecii* with a potential ability to transmit this organism to susceptible hosts although they rarely show any clinical symptoms of PCP [9]. Carriage of *P. jirovecii* has been reported in immunocompetent patients with pulmonary diseases such as with chronic obstructive pulmonary disease (COPD), lung cancer,

cystic fibrosis or infected with *Mycobacterium tuberculosis* and those having bacterial pneumonia. The prevalence of *P. jirovecii* in those groups of patients ranges from 5% to 55%, depending on the sampling and detection method used, and the stage of the disease [9–12].

Data from the Brittany area of France show that *P. jirovecii* prevalence in patients with cystic fibrosis is lower than those observed in Germany, Spain, Brazil or in other regions of France, illustrating the fact that there are numerous factors that impact on the epidemiology of *Pneumocystis* disease [13].

Although progress has recently been made to culture *P. jirovecii* [14], the gold standard for the diagnosis of PCP is still microscopical examination of respiratory specimens, such as induced sputum or bronchoalveolar lavage (BAL), however lung biopsy has also been used [15–17]. More recently, conventional polymerase chain reaction (PCR) and quantitative real-time PCR (qRT-PCR) are increasingly used as diagnostic tools for PCP, providing important advances compared to microscopy. They target specific loci in *P. jirovecii* DNA and provide faster and more accurate results, requiring only basic molecular laboratory skills [18]. In developing countries with a high burden of HIV, optimal respiratory specimens are not always readily available and the sensitivity of microscopically examined induced sputum is therefore variable. Thus, there is a need for reliable and sensitive tests where specimens obtained using non-invasive methods could be used. Oral rinses have been suggested for diagnosis of PCP as they can be readily obtained avoiding hazardous invasive procedures, especially in severely sick patients [19]. In combination with qRT-PCR they could provide fast diagnosis of PCP.

Since *P. jirovecii* can be easily detected from oral rinse samples in HIV patients with suspected PCP [20–22], we aimed to establish the incidence and level of *P. jirovecii* carriage in HIV patients without any respiratory symptoms or indication of PCP. Moreover, the aim of this study was to investigate the clinical usefulness of oral samples and a molecular detection method (qRT-PCR) in this group of patients. COPD patients with no signs and symptoms of PCP were used as a comparator group. A commercially available CE-marked multiplex qRT-PCR assay (MycAssay *Pneumocystis*; Myconostica, now Novacyt) was used for *P. jirovecii* detection.

2. Material and methods

2.1. Patients

A flow chart (Fig. 1) briefly summarises the patient enrolment and sample processing. A total number of 100 HIV positive patients (Table 1) attending the University Hospital of South Manchester (UHSM, Manchester, United Kingdom) Withington Community Hospital Sexual Health Clinic and 60 COPD patients (comparator group) attending the UHSM Wythenshawe Hospital COPD Clinic
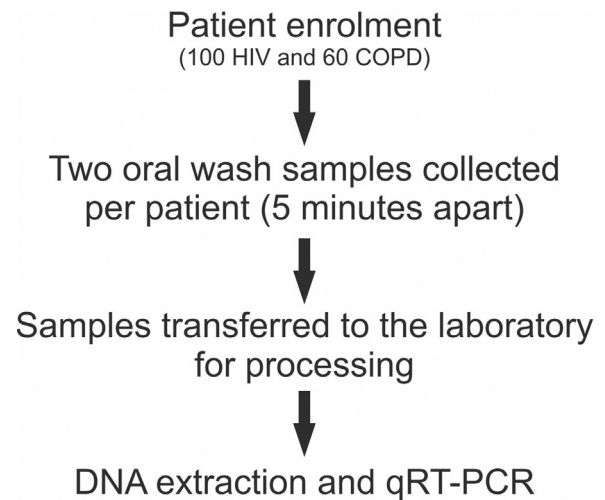

Patient enrolment
(100 HIV and 60 COPD)

↓

Two oral wash samples collected per patient (5 minutes apart)

↓

Samples transferred to the laboratory for processing

↓

DNA extraction and qRT-PCR

Fig. 1. A flow chart showing a brief summary of patient enrolment and sample processing.

were enrolled. The study protocol was approved by the North West 9 Research Ethics Committee Greater Manchester West (Reference: 10/H1014/37). Recruitment was performed in accordance with the International Conference on Harmonisation Good Clinical Practice (ICH-GCP) guidance.

Patients were enrolled based on the following inclusion criteria: (i) confirmed diagnosis of HIV according to the UK National Guidelines [23] and a routine outpatient clinic attendance, (ii) no recent onset or exacerbation of respiratory illness, (iii) no PCP diagnosis for at least the past six months (based on patient records). Patients with an earlier episode of PCP and those on co-trimoxazole or other PCP prophylaxis were also included in the study. Patients less than 18 years old and those in whom the sample collection procedure was unduly risky were excluded from the study. Comparator group patients were enrolled based on the following inclusion criteria:

- confirmed diagnosis of COPD according to the UK National Guidelines [24] and a routine outpatient clinic attendance;
- normal lymphocyte counts;
- no PCP diagnosis for at least the past six months (based on patient records).

2.2. Sample collection and processing

Two oral rinse samples were collected 5 minutes apart from each patient by vigorously washing the oral cavity (but not by gargling the oropharynx to exclude contamination and excretions

Table 1
General HIV patient demographic data and time between CD4+ count and oral sample collection. No demographic data is available for COPD patients.

Patient age	Ethnicity	Gender	CD4+ count (cells/mm^3)	Time between CD4+ count and sample collection
Range: 21–80 Mean: 41	White British: 81 Black British: 2 White Irish: 1 White Other: 3 Black African: 7 Asian not specified: 3 Chinese Other: 1 Not specified: 2	93 males 7 females	Range: 76–1200 Mean: 527	Same month: 48 patients (including 2 PCR positive) 1 month before: 42 patients (including 1 PCR positive) 2 months before: 4 3 months before: 1 4 months before: 3 5 months before: 0 6 months before: 2 patients (including 1 PCR positive)

from the lower respiratory track) with 50 mL of sterile 0.9% saline for 1 minute. Reasoning behind this was that the first sample might contain the higher load of *P. jirovecii* but also PCR inhibitors. Therefore the second sample was collected to investigate if the dilution factor may play a role in detection sensitivity of this organism. Patients were also asked not to eat or drink for at least 2 hours prior to sample collection to minimise possible subsequent qRT-PCR inhibition. No additional examinations (laboratory, radiology) were requested from the patients. Clinical information was collected from medical records and analysed if available.

Following collection, samples were immediately placed on ice and transferred to the laboratory, where they were centrifuged at 7000 x*g* for 10 minutes at 4 °C. The supernatant was decanted and the pellet was resuspended in 1 mL of sterile 1x phosphate buffered saline (PBS). This was subsequently transferred to a 1.5 mL Eppendorf tube and centrifuged at 10,000 x*g* for 2 minutes at 4 °C. All but 50 µL of the supernatant was removed and the pellet was resuspended in the remaining volume. The entire solution was transferred to the bead tube provided in the CE marked MycXtra Fungal DNA Extraction Kit (Myconostica, UK, now Novacyt) and extraction was carried out in accordance with the manufacturer's instructions. Ten microliters of extracted DNA was tested for the presence of *P. jirovecii* DNA using the Multiplex qRT-PCR kit, MycAssay *Pneumocystis*, which targets the *Pneumocystis* ribosomal large sub-unit as per manufacturer's instructions. A Cepheid SmartCycler machine with the version 3 diagnostic software was used. A cycle threshold (Ct) of ≤ 39 was considered positive and Ct>39 was considered negative.

3. Results

The age range of the HIV population was 21 to 80 years, with majority of white British. In the absence of respiratory symptoms, chest radiographs had not been taken for any of the HIV patients within 3 months prior to sampling for this study. None of the HIV or COPD patients developed PCP within 6 months post sampling.

A total of 320 oral rinse samples from 100 HIV and 60 COPD patients were collected and analysed for *P. jirovecii* DNA by a multiplex qRT-PCR assay. Four samples obtained from four HIV patients (4%) were positive for *P. jirovecii.* Of these, the first collected sample was positive from three patients (qRT-PCR Ct values 32.2, 33.2 and 35.8; patient no. 6, 40 and 41, respectively; Table 2) but the second samples were negative. In one patient (no. 50) only the second sample was positive (Ct 32.8). In the case of three qRT-PCR positive patients for whom the first sample was positive, the time between the first sample collection and last meal/drink was 3 h 25 min, 3 h 05 min and 2 h 25 min, respectively. The time between last meal/drink and the first sample collection for the fourth qRT-PCR positive patient was only 50 min. The times for the remaining patients with two negative qRT-PCR results, varied from 23 min to 17 h. Each qRT-PCR sample was monitored for the PCR inhibition against the internal amplification control (IAC) and 7/320 samples failed as the IAC Ct value was not in the acceptable range designed for the kit. These samples were not analysed further. All samples from COPD patients were qRT-PCR negative.

The CD4$^+$ counts for the qRT-PCR positive patients were 76, 391, 448 and 551 cells/mm^3 (Table 2). The cell counts for all patients ranged from 76 to 1200 cells/mm^3 (mean 527 cells/mm^3). The lymphocyte counts for 48 HIV patients were performed on the day of the oral rinse sample collection (of which 2 patients were *P. jirovecii* qRT-PCR positive). Forty-two HIV patients had their CD4$^+$ count analysed within 1 month prior to sample collection (of which 1 patient was qRT-PCR positive), 4 patients within 2 months, 1 patient within 3 months and 3 patients within 4 months. One *P. jirovecii* qRT-PCR positive patient had a CD4$^+$ count analysed 6 months prior to the oral wash sample collection and at that time the lymphocyte count was 551 cells/mm^3. Two of the positive *P. jirovecii* qRT-PCR patients used tobacco but none had any additional immunodeficiency. Of the qRT-PCR positive patients, only one was on ARV therapy at the time of sample collection (tenofovir disoproxil fumarate/emtricitabine (Truvada); patient 41; Table 2).

Six HIV patients were on PCP prophylaxis on the day of oral sample collection, receiving either trimethoprim/sulfamethoxazole (Septrin), diaminodiphenyl sulfone (Dapsone) or atovaquone (Tables 2 and 3). These patients were also on ARV therapy receiving either tenofovir disoproxil fumarate/emtricitabine

Table 2
Positive *P. jirovecii* qRT-PCR HIV patients, their CD4+ count, PCP prophylaxis and ARV therapy at the time of the oral rinse sample collection as well as their previous PCP status. Only one patient in this group was on PCP prophylaxis and ARV therapy.

Patient no.	Gender	Age	Smoker	qRT-PCR result – 1st rinse (Ct)	qRT-PCR result – 2nd rinse (Ct)	CD4$^+$ count; cells/mm3 (months of latest CD4$^+$ count)	PCP prophylaxis	ARV therapy	Previous PCP
6	M	43	No	Positive (33.2)	Negative	391 (−1)	No	No	No
40	M	37	No	Positive (32.2)	Negative	448 (0)	No	No	No
41	F	68	Yes	Positive (35.8)	Negative	76 (0)	Atovaquone	Truvada	No
50	M	49	Yes	Negative	Positive (32.8)	551 (−6)	No	No	No

Table 3
Negative *P. jirovecii* qRT-PCR HIV patients but on PCP prophylaxis and/or on ARV therapy at the time of the oral rinse sample collection. Their CD4 count and previous PCP status is also presented here.

Patient no.	Gender	Age	Smoker	qRT-PCR result – 1st rinse (Ct)	qRT-PCR result – 2nd rinse (Ct)	CD4$^+$ count; cells/mm^3 (months of latest CD4+ count)	PCP prophylaxis	ARV therapy	Previous PCP
14	M	43	No	Negative	Negative	108 (−1)	Dapsone	Truvada	No
32	M	38	No	Negative	Negative	224 (0)	Septrin	Atripla	No
51	M	39	Yes	Negative	Negative	242 (0)	Dapsone	Truvada/kaletra	Yes (in 2008)
72	M	39	No	Negative	Negative	126 (0)	Dapsone	Atripla	No
76	M	44	No	Negative	Negative	122 (−1)	Septrin	Atripla	No
86	M	50	Yes	Negative	Negative	647 (−1)	No	tenofovir/darunavir	Yes (in 1988)

(Truvada), efavirenz/emtricitabine/tenofovir disoproxil fumarate (Atripla), lopinavir/ritonavir (Kaletra), tenofovir or darunavir. One patient (no. 86) was only on AVR therapy but not on PCP prophylaxis. Their CD4$^+$ count ranged from 76 to 647 cells/mm^3 but only one patient (patient no. 41) was *P. jirovecii* qRT-PCR positive (CD4$^+$ count of 76; atovaquone prophylaxis and Truvada therapy). This patient however did not have or developed any symptoms suggestive of PCP during the 6 month follow-up period. Two HIV positive patients had been previously diagnosed with PCP, in 1988 (patient 51) and in 2008 (patient 86) (Table 3). Both were qRT-PCR negative.

4. Discussion

In the current study we aimed to establish the incidence of *P. jirovecii* carriage in HIV patients without any respiratory symptoms or indication of PCP. We also aimed to investigate the clinical usefulness of readily available oral wash samples to detect *P. jirovecii* DNA as this type of specimen may be more suitable for severely ill patients or where bronchoscopy accessibility is limited. We showed that the commercial qRT-PCR assay, MycAssay *Pneumocysis*, can be used to detect *P. jirovecii* DNA from this type of sample in such patients. However, the oral carriage of this organism or its DNA in the group tested was shown to be rare, representing only 4% of the analysed samples in the tested HIV population. Three of the four samples collected from HIV patients had a high qRT-PCR detection signal, indicating a high *P. jirovecii* load. No *P. jirovecii* DNA was detected in the comparator COPD group.

No link was found between the qRT-PCR positivity for *P. jirovecii* and the CD4$^+$ status, as the CD4$^+$ cell count of three out of four positive qRT-PCR patients > 200 cells/mL (Table 2). However, the CD4$^+$ count for one qRT-PCR positive patient was performed 6 months before the oral rinse specimen collection and the CD4$^+$ status of this patient at the time of sample collection was unknown. Therefore it might have changed at the time of this study, resulting in the positive qRT-PCR. The fact that none of the four *P. jirovecii* qRT-PCR positive patients had or developed PCP and all provided only one positive sample suggests that the result could reflect well controlled immune responses against the fungus or good response to PCP prophylaxis as in case of patient no. 41, who was taking atovaquone at the time of oral rinse sample collection. It is also possible that those patients could have acquired *P. jirovecii* in their early life, without developing any symptoms of PCP, while the organism remained latent and was only detected by qRT-PCR in the first of paired samples tested. This could suggest transient colonisation, as the fungal DNA was too low or too diluted to be detected in the second sample examined. However, in patient no. 50 only the second of the paired sample tested was qRT-PCR positive. This may indicate (i) sample variability, (ii) that the first of two mouth rinses was not performed thoroughly, (iii) there was contamination between the samples from the lower respiratory track (e.g. after a cough) or (iv) the first oral rinse sample was contaminated with qRT-PCR inhibitors originating from food/drink. The issue of PCR inhibitors originating from food is well known [25,26] and in case of this patient the time between the sample collection and last meal/drink was only ~50 min (as opposed to other 3 qRT-PCR positive samples for which the time was between 2 h 25 min and 3 h 25 min). This could have resulted in decreased sensitivity or a false-negative result. However, since all samples were monitored for IAC, no inhibition was observed for this sample. In the event of both of the paired samples tested being qRT-PCR positive, fungal load or an ongoing infection should be considered. This also applies to cases with low CT value reflecting high fungal burden. Such results should be further confirmed by

other clinical and laboratory tests. However in this study this was not the case.

None of the patients from the comparator COPD group were positive for *P. jirovecii* DNA by qRT-PCR, suggesting that the *P. jirovecii* colonisation in our COPD cohort is either uncommon, *P. jirovecii* DNA in the samples obtained was too low to be detected by the method used or the sample type is not adequate for this group of patients. However, as none of the patients in this group developed PCP, the level of sensitivity of the sample type appears not to over-diagnose PCP. Although several studies have reported colonisation in COPD patients, with a prevalence of 5 and 55% [11,12], this was not the case in our group of patients using qRT-PCR. A different patient ethnicity in this study compared to previous ones, a different experimental design or that the patients were not colonised by *P. jirovecii* in the first instance could also explain our results.

Our study has some limitations. Firstly, no accompanying sputum samples were collected as patients without any respiratory symptoms cannot produce it. Secondly, no microscopy analyses were performed to confirm the qRT-PCR results. However, as qRT-PCR is more sensitive than microscopy it is highly unlikely that any positive cases were missed. At the same time the proportion of patients with positive results was very low and likely to reflect colonisation. qRT-PCR inhibition originating from food/drink could also have hindered or decrease sensitivity of our results, thus preventing from accurate assessment of incidence of this organism in the tested groups. Although patients were asked not to eat or drink at least 2 h before sample collection, several had had a drink or meal. This should be taken into account if a similar study is performed or results are being clinically assessed. Others have found that qRT-PCR signals can degrade in saliva and oral wash samples, as this is why we placed all the samples in ice for transportation to the laboratory. In addition, no demographic data was available for the comparator group.

In summary, we showed that qRT-PCR assay can be used to detect *P. jirovecii* DNA in oral rinse samples from HIV patients without evident clinical symptoms. However, very few patients were positive. It is likely that the four *Pneumocystis* qRT-PCR positive patients were transiently colonised and did not have ongoing disease as none of these patients developed any symptoms of PCP 6 months after the study.

Author contribution

M.G.F. processed samples, performed experiments and analysed the data; M.G.F., R.R., M.R. and D.W.D. wrote the manuscript; R.R., M.R. and D.W.D. designed the study; S.A. and M.K. selected patients, obtained their consent and collected samples; M.K., S.A., P.B., D.W.D. obtained ethical approval. All authors approved the final version.

Ethical statement

The study protocol was approved by the North West 9 Research Ethics Committee Greater Manchester West (reference: 10/H1014/37).

Disclosure of interest

The authors declare that they have no competing interest.

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