Original Article

Frequency of *Pneumocystis jirovecii* in sputum from HIV and TB patients in Namibia

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Abstract

Introduction: The opportunistic fungus *Pneumocystis jirovecii* causes *Pneumocystis* pneumonia (PcP), which is a life-threatening infection in HIV/AIDS patients. The seemingly low prevalence of *P. jirovecii* pneumonia in sub-Saharan Africa has been a matter of great debate because many HIV/AIDS patients reside in this region. The lack of suitable diagnostic practices in this resource limited-region has been added to the uncertainty of PcP prevalence. Only a few studies have evaluated the utility of easily obtainable samples such as expectorated sputum for diagnosis of PcP. Thus, the aim of the current study was to evaluate the effectiveness of expectorated sputum for the routine diagnosis of PcP in a resource-limited sub-Saharan African setting.

Methodology: Randomly collected sputum samples were analysed by microscopy after Grocott's methenamine silver (GMS) stain staining and by qPCR to determine the minimum frequency of detectable *P. jirovecii.*

Results: A total of 475 samples were analysed. Twenty five (5.3%) samples were positive for *P. jirovecii*, i.e., 17 (3.6%) using both qPCR and GMS staining and eight (1.7%) using qPCR only. *P. jirovecii* was present in 8/150 (5.3%) HIV-positive and tuberculosis (TB) smear-negative patients, and in 12/227 (5.3%) TB smear-negative patients with an unknown HIV status. The minimum frequency of PcP was 3.6% in Namibian HIV and TB patients, while the actual frequency is likely to be 5.3%.

Conclusion: This study demonstrated that expectorated sputum can be used routinely for the diagnosis of PcP by GMS, although qPCR is more sensitive, and it requires less time and skill.

Key words: fungal diseases; HIV; opportunistic infections; Pneumocystis jirovecii; tuberculosis; sputum; Namibia

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Introduction

Pneumocystis pneumonia (PcP) is caused by the opportunistic fungus *Pneumocystis jirovecii* and is a life-threatening respiratory disease that became clinically relevant when it led to the discovery of HIV/AIDS in homosexual males in the United States of America [1]. Aggressive HIV/AIDS management strategies that combine highly active antiretroviral therapy (HAART) for HIV and PcP chemoprophylaxis with trimethoprim sulfamethoxazole have led to a significant decline in the overall incidence of PcP in the developed world [2]. However, PcP remains a potentially fatal disease in many immunocompromised patients worldwide [3].

In the era of HAART therapy, PcP is still associated with high mortality rates in AIDS patients, especially in developing countries and medically underserved communities in the developed world [4,5]. HIV patients who are at risk of contracting PcP include those who are unaware of their HIV status, patients with drug-resistant HIV, and those who are non-compliant or incompatible with antiretroviral therapy or prophylaxis [3,6].

The successful diagnosis of PcP requires an evaluation of the clinical signs in at risk patients and radiographic evidence; however, the clinical and radiographic findings are non-specific and they may overlap with those of other lower respiratory tract infections [7]. Thus, a definitive diagnosis requires laboratory identification of the organism in respiratory specimens. As *P. jirovecii* cannot be cultured, a definitive diagnosis has relied heavily on the detection of the organism's cysts by microscopy. Effective

sampling procedures are important for detecting the presence of the organism [8-10].

A variety of respiratory specimens have been used for diagnosis, including lung biopsy, bronchoalveolar lavage (BAL), induced and expectorated sputum, nasopharyngeal aspirates and, more recently, oral washings. The high morbidity associated with biopsy specimens has limited their clinical utility so BAL has largely been the sample of choice [8]. Bronchoscopy is unpleasant and requires special expertise, and thus so the use of induced sputum is preferred [9]. The induction of sputum using nebulised saline has generally been preferred over spontaneously expectorated sputum because it is believed to yield higher quality clinical material. However, sputum induction may be unsafe or impossible in some patients, particularly in infants and weaker AIDS patients, because of the risk of haemoptysis in patients with tuberculosis or chronic pulmonary aspergillosis and because the healthcare worker is potentially exposed to *Mycobacterium tuberculosis* (including multidrug-resistant (MDR) and extensively drug resistant (XDR) TB [11,12]. The labour intensiveness of sputum induction and the requirement for special patient preparation also means it is not feasible in health care settings that handle a large volume of patients [13].

Material derived from expectorated sputum is used routinely in clinical microbiology laboratories to determine the aetiology of pulmonary infections; however, few studies have studied the usefulness of expectorated sputum for the diagnosis of PcP. This is mainly due to the assumed marginal benefit of using induced sputum material because expectorates are generally contaminated with saliva [14]; however, one study reported a 55% detection sensitivity for *P. jirovecii* in expectorated sputum [15], which was similar to the sensitivity with induced sputum [16,17]. A retrospective review of PcP cases by the same investigators also found no significant difference when induced or expectorated sputum were submitted for investigation at their institution [18]. Moreover, comparative evaluations of the general specimen quality of induced and expectorated sputum concluded that sputum induction did not improve the specimen quality substantially [14,19].

The molecular detection of *P. jirovecii* might facilitate the diagnosis and management of PcP. A variety of polymerase chain reaction (PCR) techniques based on several unique regions in the *Pneumocystis* genome have been evaluated as potential diagnostic markers [20-22]. PCR protocols that amplify these target genes have all demonstrated higher sensitivity than conventional diagnostic techniques. Targeting the mitochondrial large subunit rRNA (*mtLSU rRNA)* also facilitates the detection of *P. jirovecii* in samples derived from the oropharynx [13,23], which may be attractive for the rapid screening and diagnosis of PcP.

Despite its strong association with AIDS patients in the Western world, PcP always had a low prevalence in sub-Saharan African AIDS patients [24-28], which has caused it to be deprioritised as a relevant respiratory opportunistic pathogen in African health care settings. The main respiratory opportunistic infection associated with HIV/AIDS in these regions is pulmonary tuberculosis (TB) [28]. In the past decade, however, much debate has surrounded the status of PcP in African populations because it has been shown that its prevalence may be higher in Southern Africa, particularly in children infected with HIV [29-31]. The widespread use of cotrimoxazole prophylaxis for pneumococcal infections has provided some sense of medical reassurance with respect to PcP, although without any direct supporting data.

A lack of resources and capacity means that routine *Pneumocystis* testing in Africa requires simple and accurate methods using easily obtainable samples, which do not demand special staff training or equipment. Indeed, this lack of capacity explains why routine PcP testing is not performed in Namibia, which forces clinicians to treat patients based on risk factor analysis and empirical clinical and chest radiography evidence. The HIV prevalence in Namibia is among the highest in the world and the capital city, Windhoek, has a prevalence of 14.6% [32].

In Namibia, there is no access to PcP diagnostic testing. HIV-positive patients with a CD4 count of < 350 cells/μL are given PcP prophylaxis according to the World Health Organization (WHO) guidelines [33]. Patients with respiratory symptoms are tested for TB and bacterial pneumonia. HIV testing is conducted on a voluntary basis. However, TB smear-negative patients who choose not to be tested for HIV forfeit the opportunity of PcP prophylaxis because CD4 testing is only carried out for HIV-positive patients. The current study evaluated the effectiveness of expectorated sputum for the routine diagnosis of PcP in a resource-limited setting in Windhoek, Namibia by using samples submitted to a central reference laboratory for TB microscopy.

Methodology

Study environment and design

This prospective laboratory-based study was conducted at the Namibia Institute of Pathology in the Central Reference Laboratory in Windhoek, Namibia. The study used 475 expectorated sputum samples, which were sent to the Namibia Institute of Pathology between June 2011 and August 2011 for TB investigation using Auramine O staining [34]. Residual samples measuring at least 2 ml were stored at 4°C and used for fungal DNA extraction, quantitative PCR (qPCR) and cytospin centrifugation before subsequent GMS staining. Sputum samples from subjects aged ≤ 16 years were excluded as according to the law in Namibia study participants must be aged 16 or over. The clinical details of the patients were obtained from the hospital records, including recent drug therapy.

Sputum processing

Sputum samples were examined visually and their appearance was recorded. The sample was transferred to a 50 ml Falcon tube and homogenised and decontaminated with dithiothreitol (DTT) using a commercially available Sputasol (Oxoid, Basingstoke, UK), according to the manufacturer's instructions. The sputum digest was aliquoted into two equal volumes, one for DNA extraction and another for cytospin slide preparation. All samples were handled in a Class II biosafety cabinet, decontaminated before use to prevent cross-contamination. Moreover, the air within the cabinet was monitored using an air-sampler to control for any contaminants.

DNA extraction

DNA was extracted using a commercially available MycXtra kit (Myconostica Ltd, Manchester, UK) according to the manufacturer's instructions. After extraction, the DNA was stored at –80°C.

Microscope slide preparation

The sputum digest was diluted with 5 ml of PBS (pH 7.4), as described previously [35]. Additional PBS was added to more mucoid samples. A slide was prepared with 0.5 ml samples by centrifugation at 1200 rpm for 10 minutes using a Shandon Cytospin 3 Cytocentrifuge (Thermo Scientific, Waltham, USA). The slides were spray-fixed immediately using a commercial aerosol cytological fixative (Fencott, Cape Town, South Africa).

Sample transport

DNA samples and microscope slide preparations were shipped to the United Kingdom for analysis. The DNA extracts were shipped on dry ice.

Pneumocystis qPCR

qPCR was performed using the MycAssay *Pneumocystis* Assay (Myconostica Ltd, Manchester, UK), which detects the amplified *mtLSU rRNA* region of the *P. jirovecii* genome by molecular beacon PCR technology [7]. The assay contains an internal amplification control DNA sequence, which is not present in *Pneumocystis* and other fungal, bacterial or human genomes, to exclude the presence of any PCR inhibitory substances, thereby confirming the validity of the test.

Grocott's methenamine silver staining and microscopical analysis

The fixed sputum smears were stained with GMS, as described previously [36]. A positive *P. jirovecii* control slide was stained using the same solution alongside each set of slides. A positive identification of *P. jirovecii* was made if characteristic dark-stained cysts were observed with a crushed ping-pong ball appearance.

Statistical analysis

Data were collected daily using data collection forms. After checking for accuracy, the data were entered in a bespoke Excel database before preliminary analysis. The data were then exported to the StatsDirect statistical program (StatsDirect, Altrincham, UK Ltd. 2008) for statistical analysis. The Wilcoxon-Mann-Whitney test was used to test associations between the numerical data.

Ethics statement

Approval for this study was obtained from the Research Ethics Committees at The University of Manchester and the Namibian Ministry of Health and Social Services in April 2011.

Results

Patient demographics

Three hundred and ninety-nine (84.0%) of the 475 specimens were received from local TB clinics in the city of Windhoek, Namibia. Sixty-two (13.0%) of the specimens were from hospitalised patients in the Windhoek Central and Katutura Hospitals. Fourteen (3.0%) of the specimens were provided by the Windhoek Central Intensive Care Unit (ICU). Of the

subjects, 226 (47.6%) were females and 249 (52.4%) were males. The mean and median ages of the patients were 38 and 36 (range = 17–88) years, respectively (Table 1). One hundred and seventy-five (36.8%) subjects were known to be HIV-positive and 39 (8.2%) were HIV-negative. The HIV status of 261 (54.9%) patients was unknown.

HIV and TB status

Of the 175 samples from HIV-positive patients, 80 (45.7%) were obtained from males. Twenty-five (14.3%) of the HIV-positive patients were acid-fast bacillus (AFB) smear positive for TB. The median CD4 count for HIV-positive patients was 282 cells/µl (range = 5–1031) (Table 1). CD4 counts of < 200 cells/µl were observed in 53 (30.3%) of the HIV-positive patients. The median CD4 count for HIV-positive, TB smear-positive patients was 124 cells/µl, which was significantly lower than the median count of 261 cells/µl for HIV-positive, TB smear-negative patients ($p = 0.025$).

Other infections

Other respiratory pathogens were isolated from the sputum of 44 (9.3%) patients by routine laboratory examination. Of these, 27 (5.7%) contained *Candida albicans*, four (0.8%) contained 'yeast other than *C. albicans*' (*C. glabrata*, *C. parapsilopsis* and *C. tropicalis*, based on morphological and biochemical tests), four (0.8%) contained *Staphylococcus aureus*, three (0.6%) contained *Klebsiella pneumoniae*, two (0.4%) contained *Haemophilus influenzae*, two (0.4%) contained *Pseudomonas* spp. and two (0.4%) contained other Gram-negative bacilli. Multiple organisms were isolated from six patients. PcP was detected in three of the patients with other respiratory pathogens, two were infected with *C. albicans* and the other had a 'yeast other than *C. albicans*'.

PcP detection

P. jirovecii was detected in 25/475 (5.3%) patients. Seventeen patients (3.6%) tested positive for *P. jirovecii* by qPCR and direct microscopy (Table 2). Eight patients (1.7%) were positive only by qPCR. None of the samples tested were positive by direct microscopy alone.

Table 1. Demographics of the study subjects (n = 475)

	All	HIV-positive	HIV-negative	HIV-unknown
n (%)	475 (100)	175 (36.8)	39 (8.2)	261 (55.0)
Male (%)	249 (52.4)	80 (45.7)	19 (48.7)	150 (60.2)
Female (%)	226 (47.6)	95(54.3)	20 (51.3)	111 (49.1)
Age mean; median (range)	38; 36 (17–88)	39; 37 (18–72)	35; 33 (18–64)	38; 34 (17–88)
Males	39; 37 (19–88)	42; 39 (24–59)	33; 31 (19–46)	39; 35 (19–88)
Females	37; 36 (17–79)	37; 37 (18–72)	38; 33 (18–64)	37; 34 (17–79)
TB-positive (%)	64 (13.5)	25 (14.3)	5 (12.8)	34 (13.0)
TB-negative (%)	411 (86.5)	150 (85.7)	34 (87.2)	227 (87.0)
*CD4 median (range)	–	282 (5–1031)	–	–
TB-positive	–	124 (5–664)	–	–
TB-negative	–	261 (10–1031)	–	–
CD4 >200 cells/µl	–	98	–	–
CD4 100–200 cells/µl	–	28	–	–
CD4 <100 cells/µl	–	25	–	–

* Only CD4 counts determined within one month of the study were considered. The CD4 counts for 19 HIV-positive patients were either unknown or disregarded based on this criterion.

Table 2. Concordance of the qPCR assay with direct GMS microscopy

		GMS direct microscopy results		
		Positive	Negative	
PCR results	Positive	17	8	PPV 17/(17+5) = 68%
	Negative	0	450	NPV 450/(450+0) = 100%
		Sensitivity 17/(17+0) = 100%	Specificity 450/(450+8) = 100%	

Table 3. Study subjects that tested positive for PcP (n = 25)

	All	HIV-positive	HIV-negative	HIV-unknown
n (%)	25 (5.3)	10 (5.7)	3 (7.7)	12 (4.6)
Male (%)	12 (4.8)	5 (6.3)	2 (10.5)	5 (3.3)
Female (%)	13 (5.8)	5 (5.3)	1 (5.0)	7 (6.3)
Age mean; median (range)	42; 39 (18-73)	43; 42 (32-59)	41; 29 (20-73)	41; 35 (18-70)
Males	44; 35 (27-73)	47; 50 (33-59)	51; 51 (29-73)	37; 30 (27-70)
Females	40; 44 (18-64)	39; 39 (32-45)	20; 20 (20-20)	44; 48 (18-64)
TB positive (%)	3 (4.7)	2 (8.0)	1 (20)	0 (0)
TB negative (%)	22 (5.4)	8 (5.3)	2 (5.9)	12 (5.3)
*CD4 median (range)	–	129 (5-783)	–	–
TB positive	–	335(5-664)	–	–
TB negative	–	129 (20-783)	–	–
CD4 >200 cells/ul	–	4	–	–
CD4 100-200 cells/ul	–	3	–	–
CD4 <100 cells/ul	–	3	–	–

* Only CD4 counts that were determined within one month of the study were considered.

Table 4. Characteristics of samples that tested positive for PcP

Demographics			Sputum characteristics		HIV		TB	*Pneumocystis* detection		
Patient number	Age	Sex	Specimen volume (ml)	≠Specimen appearance	HIV status	*CD4 count (cells/ul)	AFB smear	qPCR		GMS Direct Microscopy
								Result	Ct	
1	32	F	3.5	mucoid	P	783	N	P	35.5	N
2	36	F	2.0	muco-salivary	P	664	Scant	P	37.6	P
3	45	F	2.0	muco-salivary	P	462	N	P	35.8	P
4	44	F	2.0	muco-salivary	P	325	N	P	37.7	P
5	59	M	2.0	muco-salivary	P	130	N	P	38.8	P
6	50	M	2.0	muco-salivary	P	128	N	P	36.4	P
7	36	M	2.0	muco-salivary	P	127	N	P	32.1	N
8	57	M	7.5	mucoid	P	60	N	P	33.9	P
9	33	M	2.0	muco-salivary	P	20	N	P	36.6	P
10	39	F	6.5	muco-salivary	P	5	+++	P	37.8	P
11	29	M	2.0	mucoid	N	U	++	P	36.8	N
12	73	M	7.0	muco-salivary	N	U	N	P	36.4	N
13	20	F	2.0	muco-salivary	N	U	N	P	35.3	P
14	31	M	2.0	mucoid	U	U	N	P	36.9	P
15	18	F	4.0	muco-salivary	U	U	N	P	35.1	N
16	28	M	2.0	muco-salivary	U	U	N	P	34.3	P
17	70	M	5.0	muco-salivary	U	U	N	P	37.2	P
18	49	F	2.0	muco-salivary	U	U	N	P	38.6	N
19	35	F	2.0	mucoid	U	U	N	P	36.9	N
20	46	F	2.0	muco-salivary	U	U	N	P	36.3	P
21	48	F	4.5	mucoid	U	U	N	P	37.7	P
22	27	M	4.0	muco-salivary	U	U	N	P	37.5	N
23	64	F	3.5	mucoid	U	U	N	P	37.2	P
24	30	M	2.0	muco-salivary	U	U	N	P	35.5	P
25	48	F	2.0	mucoid	U	U	N	P	37.4	P

P = positive; N = negative; U = unknown; *CD4 lymphocyte count median = 129 cells/µl.; ≠ 17 (68.0%) mucosalivary specimens; 8 (32.0%) mucoid specimens

The sensitivity, specificity, positive predictive value, negative predictive value and diagnostic odds ratio for the qPCR were 100%, 98.3%, 68.0%, 100% and ∞, respectively, when compared with GMS as the gold standard.

Of the 25 patients that tested positive for *P. jirovecii* by qPCR, 10 were HIV-positive and three were HIV-negative (Table 3). The HIV status of the remaining 12 patients was undetermined.

The median CD4 count for HIV-positive patients that tested positive for *P. jirovecii* was 129 cells/µl (range = 5–783 cells/µl). This was significantly lower than the median CD4 count of 284 cells/µl (range = 6–1031 cells/µl) for HIV-positive patients where *P. jirovecii* was not detected (p = 0.028).

PcP and TB status

Only three (4.7%) of the patients with *P. jirovecii* had positive AFB smears (Table 3). Of these, two were HIV-positive and one was HIV-negative. *P. jirovecii* was detected in 22 (5.4%) patients with negative AFB smears. Eight (5.3%) of the TB smear-negative patients with *P. jirovecii* were HIV-positive. An example of silver staining from a sputum sample of one of the HIV-positive, PcP positive patient (Table 4, patient 8) is shown in Figure 1 with a large number of *P. jirovecii* cysts.

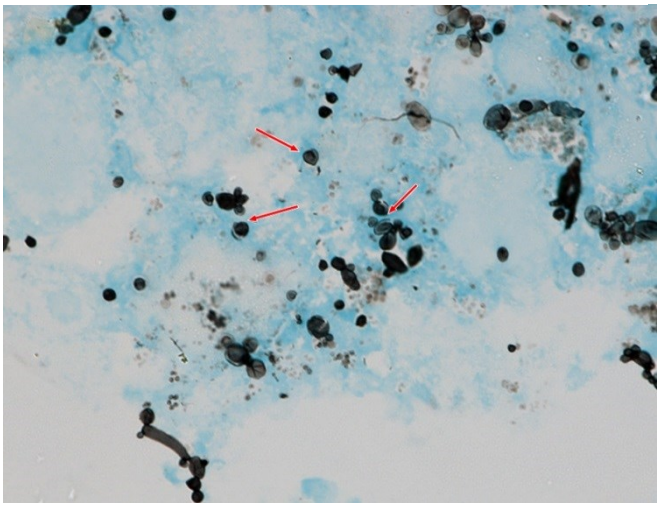
P. jirovecii DNA quantification: HIV and TB status

The assay limit of the blank was the cycle threshold (Ct) and all values < 39 were considered positive. The median *P. jirovecii* Ct value for HIV-positive, TB-negative patients was 36.1, which was not significantly different from the median of 37.7 obtained for the HIV-positive, TB-positive group (p = 0.196). Similarly, no significant differences were observed between the median *P. jirovecii* Ct value for HIV-positive patients (Ct = 36.5) compared to patients with unknown HIV status (Ct = 37.1) (p = 0.490). The median Ct value for patients where *P. jirovecii* was identified by GMS was 36.9, which was not significantly different from the median of 36.6 in GMS-negative patients (p = 0.520).

P. jirovecii DNA quantification and CD4 lymphocyte count

No significant differences were observed between the median *P. jirovecii* Ct value for patients with a CD4 lymphocyte count of <200 cells/µl (Ct = 36.5; n = 6), compared to the patients with a CD4 lymphocyte count of >200 cells/µl (Ct = 36.7; n = 4) (p = 0.500).

Figure 1. GMS-stained sputum smear for HIV- and PcP-positive patient 8 (see Table 4). The silver-stained crushed ping-pong ball-shaped cysts of *P. jirovecii* are indicated by arrows



Specimen volume and quality

Of the 475 sputum specimens, 297 (62.5%) were mucosalivary, 87 (18.3%) were mucoid, 52 (11.0%) were mucopurulent, 16 (3.4%) were mucoid and bloody, 13 (2.7%) were mucopurulent and bloody and 10 (2.1%) were mucosalivary and bloody. Seventeen (68.0%) of the specimens where *P. jirovecii* DNA was detected were mucosalivary in appearance and eight (32.0%) were mucoid (Table 4). Nine (75%) of the specimens in which PcP cysts were observed after GMS staining were mucosalivary and three (25%) were mucoid. No *P. jirovecii* DNA or cysts were found in mucopurulent or bloody sputum samples. *P. jirovecii* was not found in mucopurulent or bloody sputum samples. The minimum specimen volume of all samples analysed was 2.0 ml. The median specimen volume of *P. jirovecii*-positive samples was 2.0 ml (range = 2.0–7.5).

Discussion

PcP remains a fatal respiratory disease in AIDS patients, even in the era of HAART therapy. Previously, the prevalence of PcP was not well established in sub-Saharan African AIDS patients. Recent reports from different regions of Africa showed that it may be an emerging opportunistic infection in African AIDS patients, which may have been underreported initially. Over the past decade, studies have indicated that PcP may be more frequent in TB smear-negative patients [33,37]. The blurred picture of PcP prevalence in Africa may be due to

empirical therapy and prophylaxis using cotrimoxazole in HIV/AIDS patients.

The current study showed that PcP was present in Namibian HIV and TB patients, although it was not very frequent; however, it was an important differential diagnosis in patients who present with pulmonary symptoms, especially those with negative AFB smears. *P. jirovecii* was detected in only 5.3% of 475 expectorated sputum samples analysed by PCR and direct GMS microscopy. BAL analysis is considered to be the ideal sample for the effective detection of *P. jirovecii*, but its collection procedure is invasive and it requires trained respiratory specialists and expensive equipment. This makes it impractical in resource-limited regions of sub-Saharan Africa. Induced sputum may have a diagnostic yield that is comparable to BAL specimens and it is more cost-effective; however, it is also of limited use because it requires staff training, patient preparation and dedicated rooms (unless performed outside, which is a challenge during the rainy season). A few studies have compared the value of induced sputum with expectorated sputum and they concluded that the difference in yield was negligible [15,18]. The present study demonstrated that expectorated sputum can be used for the reliable detection of *P. jirovecii*, especially when used in conjunction with sensitive PCR technology.

P. jirovecii was detected in 25 patients by qPCR and in 17 patients by GMS microscopy. The lower GMS detection sensitivity may have been due to the lower fungal burden in certain patients or poor specimen quality, and it was demonstrated previously that GMS microscopy was about 25% less sensitive compared with a combination of methods that did not include PCR [38]. If this approach had been applied to our patients with positive PCR results, GMS should have diagnosed 19 cases rather than 17. A positive GMS stain generally indicated PcP, so this may be considered the minimum frequency of infection (3.6%).

TB smear-negative patients were found to have a significantly higher median CD4 cell count than TB smear-positive patients ($p = 0.025$). *P. jirovecii* was detected more frequently in TB smear-negative patients than TB smear-positive patients. Simultaneous co-infection with *M. tuberculosis* and *P. jirovecii* is not thought to be common, which may explain why the prevalence of *P. jirovecii* appeared to be low in regions with high *M. tuberculosis* burdens. PcP was detected in three of the patients with other respiratory pathogens, two of whom were infected with *C. albicans* and one with yeast other than *C. albicans*. The presence of PcP may indicate colonisation of these patients; however, it was more likely that *Candida* was a co-existing oral pathogen in patients with PcP, or at least in those who were HIV-positive, because oral candidiasis is common in these patients.

In HIV-positive patients, CD4 lymphocyte quantification is useful for assessing the risk of PcP development. Patients with CD4 counts of < 200 cells/µl generally had an increased risk [39], and 90% of patients with PcP had a CD4 count of <200 cells/µl; however, other risk factors may also contribute, such as malnutrition and corticosteroids [40]. In this study, the median CD4 count in HIV patients with *P. jirovecii* was 129 cells/µl. This group of patients had a definite risk of developing PcP. There was no correlation between the CD4 count and the quantitative *P. jirovecii* DNA load but this may have been due to the small number of PcP cases detected. Prior prophylactic therapy also lowered the frequency of infection (by 10% to 47% with a CD4 count of < 100 cells/µl) [41]. In this population, all of the patients with a CD4 count of ≤ 350 cells/µl received PcP prophylaxis in the form of cotrimoxazole, according to the WHO guidelines [42]. A recent meta-analysis of nine studies showed that cotrimoxazole significantly reduced the mortality of HIV patients who received antiretroviral treatment [43]. Prophylaxis against PcP is an expedient measure because of the lack of adequate diagnostic services in this setting; however, at least 10% of patients could not take cotrimoxazole because of an intolerance or allergy, which left them unprotected, and this was also the case for those whose HIV status was unknown. Our data also suggest the remote possibility of cotrimoxazole resistance by *P. jirovecii* in Namibia, which demands further research.

The current study detected *P. jirovecii* in expectorated sputum volumes as low as 2 ml after TB smearing. Previous studies detected *P. jirovecii* in expectorated sputum volumes of 2.5 ml using PCR and direct microscopy [44]. It is not known whether a smaller volume of sputum would be adequate, and this needs to be studied. It is not possible to conclude that 2 ml of expectorated sputum was always an adequate sample for PcP diagnosis using qPCR because we did not prospectively exclude PcP in negative patients. This is a difficult problem to resolve in this type of resource-limited setting, especially because HIV status of many patients is often unknown or disapproved. It can be concluded that the minimum frequency of PcP in unselected patients from Namibia who had been tested for TB was 5.3% in HIV-positive, TB-negative

patients and TB-negative patients with an unknown HIV status.

Thus, for a Namibian adult with HIV who undergoes sputum examination to investigate pulmonary TB, there is an approximately 1:20 to 1:25 likelihood of PcP (5.3% and 3.6% by PCR and GMS detection, respectively). This frequency is sufficiently high to indicate that laboratory testing for PcP is required to confirm a definitive diagnosis, especially in those with an unknown HIV status. This frequency also supports a strategy of widespread cotrimoxazole prophylaxis as primary prevention in HIV-positive patients with CD4 cell counts of < 200 cells/µl.

Conclusion

The current study demonstrated that expectorated sputum can be used in resource-limited settings in sub-Saharan Africa for the routine diagnosis of PcP. The laboratory results should be interpreted in conjunction with the clinical and chest X-ray findings. Further investigations should be conducted to evaluate the efficacy of using oral wash specimens for diagnosis, especially in patients who are unable to produce sputum spontaneously, which is often the case with PcP patients.

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