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Use of Nasopharyngeal Aspirate for Diagnosis of *Pneumocystis* Pneumonia

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Quantitative PCR on nasopharyngeal aspirate (NPA) can achieve high sensitivity and specificity in diagnosing *Pneumocystis* pneumonia (PCP) compared to microscopic examination of bronchoscopic specimens in a population with low HIV prevalence. Since NPA is a minimally invasive procedure, it is ideal as a screening test for PCP.

Currently, microbiological confirmation of *Pneumocystis* pneumonia (PCP) requires the identification of *Pneumocystis* cysts or trophozoites using microscopy. Bronchoscopic specimens are preferred because *Pneumocystis* resides mainly in the alveolar space (1). However, both transbronchoscopic bronchoalveolar lavage and sputum induction pose significant risk to the patient (2–4). Upper respiratory tract specimens are not recommended because of low sensitivity when traditional microscopic examination is used (5). PCR has higher sensitivity than microscopic examination in the detection of *Pneumocystis* (6) and may overcome the problem of low sensitivity associated with microscopic examination of upper respiratory tract specimens. In this study, we sought to determine whether nasopharyngeal aspirate (NPA) specimens could be used for the diagnosis of PCP.

Patients of the Hong Kong West Cluster Hospitals with bronchoscopic specimens (bronchoalveolar lavage fluid [BALF] or bronchial aspirate) submitted to our laboratory for *Pneumocystis* examination from 1 January 2010 to 31 March 2012 were identified using the laboratory information system. In our laboratory, *Pneumocystis* cysts were visualized with methenamine silver stain by using standard procedures (7). With this method, *Pneumocystis* trophozoites or sporozoites could not be detected. Archived NPA specimens from these patients collected during the same hospitalization were retrieved. Only the earliest NPA specimen was tested if multiple specimens were available from the same patient. NPA specimens were collected as described previously (8). Clinical and laboratory data were obtained using the clinical management system. Patients without archived NPA specimens were excluded. A patient was considered to have definite PCP if *Pneumocystis* was identified in the bronchoscopic specimen by microscopic examination (9). This study has been approved by the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster.

DNA was extracted from 200 μl of NPA specimens and from other fungi using the QIAamp DNA kit (Qiagen, Hilden, Germany) and DNeasy plant minikit (Qiagen, Hilden, Germany), respectively, according to the manufacturer's instructions (10). Real-time quantitative PCR (qPCR) targeting a 124-bp fragment of the mitochondrial large subunit rRNA (mt LSU rRNA) gene of *Pneumocystis jirovecii* was performed as described previously (11). Plasmid suspensions were used as standards for quantification and positive controls (Fig. 1).

We verified the positive results from the mitochondrial (mt) large-subunit (LSU) rRNA qPCR (LSU-qPCR) by a conventional PCR targeting the mitochondrial small-subunit rRNA (mt SSU rRNA) gene of *P. jirovecii*. A 220-bp fragment of the mt SSU rRNA of *P. jirovecii* was amplified using 1 μM forward primer (5′-ACC CACGATAAATCTTACCACTTC-3′) and reverse primer (5′-AG CACGTCTGTAGCCCACTT-3′). The PCR mixture (25 μl) contained DNA, PCR buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 3 mM $MgCl_2$), 0.4 mM each deoxynucleoside triphosphates (dNTPs), and 0.625U *Taq* polymerase (Applied Biosystems, Branchburg, NJ). The mixtures were amplified at 95°C for 10 min; 45 cycles of 94°C for 1 min, 63°C for 1 min, and 72°C for 1 min; and a final extension at 72°C for 10 min in an automated thermal cycler (Applied Biosystems, Foster City, CA). PCR products were purified and sequenced as described previously (12).

All statistical analyses were performed using PASW Statistics 18 and VassarStats (http://vassarstats.net/). Sensitivity, specificity, positive predictive value, and negative predictive value were determined using the standard formula (13, 14). A P value of <0.05 was considered to represent statistical significance.

During the study period, a total of 416 bronchoscopic specimens (382 BALF and 34 bronchial aspirate specimens) from 317 patients were submitted to our laboratory for *Pneumocystis* examination, among which *Pneumocystis* was detected in 27 patients by microscopy. Archived NPA specimens were available for 117 patients, and only these patients were included in subsequent analysis (Table 1). NPA specimens were collected from 7 days before to 2 days after the collection of the first bronchoscopic specimen. *Pneumocystis* was detected in bronchoscopic specimens by microscopic examination in 15 patients (14 from BALF, 1 from bronchial aspirate). Among these patients, the median duration from hospital admission to diagnosis was 2 days (range, 1 to 10 days). Eight (53%) out of the 15 patients with *Pneumocystis* detected by microscopic examination of bronchoscopic specimens were non-HIV-immunocompromised patients.

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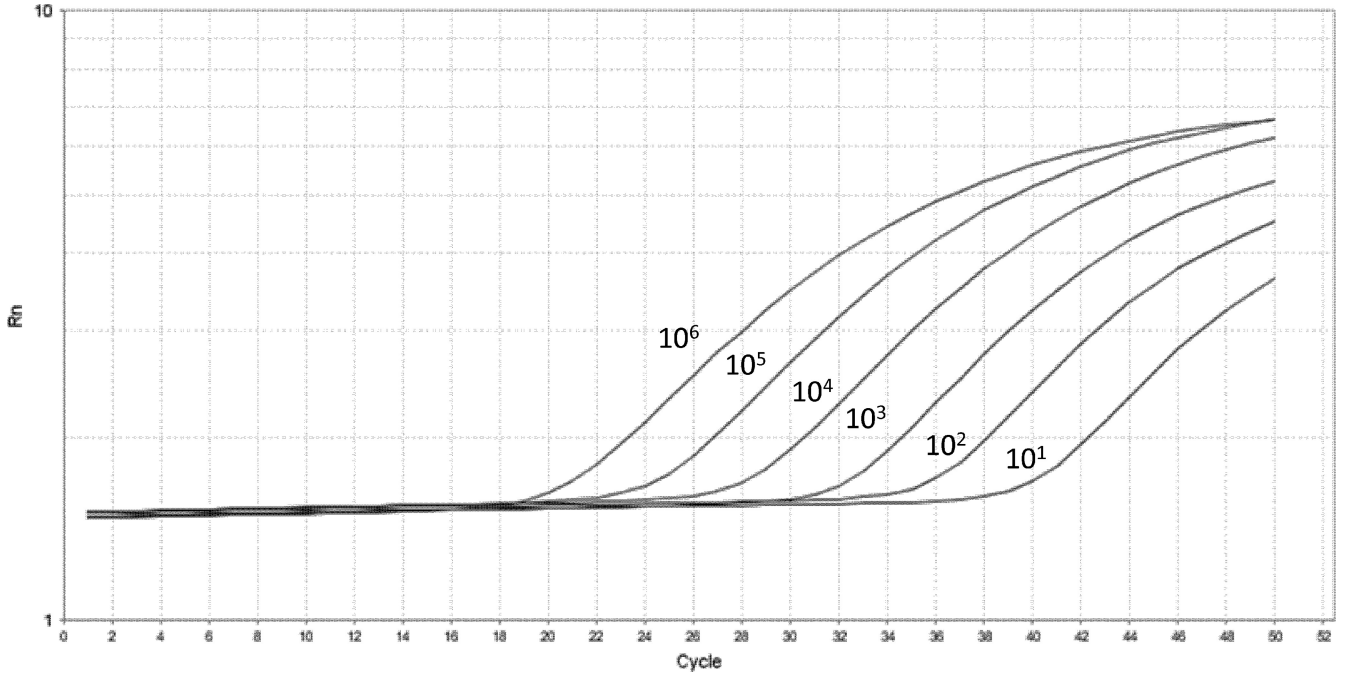

FIG 1 Standard curve for quantitative analysis of *Pneumocystis jirovecii* mt LSU rRNA.

The detection limit for LSU-qPCR was 10 copies per reaction of extracted DNA, corresponding to a cycle threshold value of 41. LSU-qPCR was negative on the DNA extracted from *Candida albicans*, *Cryptococcus neoformans*, *Saccharomyces cerevisiae*, and *Aspergillus fumigatus*. Compared to microscopic examination of the bronchoscopic specimens, LSU-qPCR of NPA specimens had a sensitivity, specificity, positive predictive value, and negative predictive value of 100% (95% confidence interval [CI], 75% to

TABLE 1 Comparison of demographics and clinical characteristics between patients with and without PCP as determined by microscopic examination of bronchoscopic specimens

Characteristic	Value		*P* value[b]
	PCP ($n = 15$)	Non-PCP[a] ($n = 102$)	
Median age (yr) (interquartile range)	46 (41–56)	59 (47–71)	0.012
No. (%) of females	6 (40)	48 (47)	0.783
No. (%) of patients with underlying disease			
HIV	7 (47)	3 (3)	<0.001
Chronic obstructive pulmonary disease	0 (0)	8 (8)	0.594
Bronchiectasis	0 (0)	5 (5)	1.000
Interstitial lung disease	1 (7)	10 (10)	1.000
Lung malignancy	0 (0)	3 (3)	1.000
Active tuberculosis	1 (7)	5 (5)	0.569
Diabetes mellitus	1 (7)	16 (16)	0.694
Solid organ tumor	0 (0)	9 (9)	0.602
Hematological malignancy	0 (0)	22 (22)	0.071
Transplant recipient	7 (47)	26 (26)	0.123
Connective tissue disease	1 (7)	17 (17)	0.461
Immunosuppressive drugs	8 (53)	49 (48)	0.786
Cardiovascular disease	1 (7)	23 (23)	0.301
Liver disease	3 (20)	12 (12)	0.407
Renal disease	5 (33)	16 (16)	0.142
Neurological disease	1 (7)	12 (12)	1.000
Median lymphocyte count, $\times 10^9$ cells/liter (interquartile range)	0.7 (0.4–1.1)	0.6 (0.4–1.1)	0.545

[a] Pneumonia with no causative organism identified (26 patients), bacterial pneumonia (26 patients), viral pneumonia (12 patients), culture-confirmed tuberculosis or empirical antituberculosis treatment (10 patients), culture-confirmed fungal pneumonia or empirical antifungal treatment (6 patients), empirical treatment for PCP (3 patients), bronchiolitis obliterans organizing pneumonia (7 patients), interstitial lung disease (3 patients), organizing pneumonia (3 patients), graft versus host disease (2 patients), gold-induced pneumonitis (1 patient), idiopathic pneumonia syndrome (1 patient), immune reconstitution inflammatory syndrome (1 patient), and flare-up of Churg-Strauss disease (1 patient).

[b] Fisher's exact test was used to compare categorical variables, whereas Mann-Whitney U test was used for continuous variables.

TABLE 2 Performance of LSU-qPCR in NPA specimens for the diagnosis of PCP, with microscopic examination of bronchoscopic specimens as the gold standard

LSU-qPCR in NPA specimens	No. of specimens (microscopic examination of bronchoscopic specimens using methenamine silver staining)		
	Positive	Negative	Total
Positive	15	4	19
Negative	0	98	98
Total	15	102	117

100%), 96.1% (95% CI, 90% to 99%), 78.9% (95% CI, 54% to 93%), and 100% (95% CI, 95% to 100%), respectively (Table 2). All NPA specimens positive for *Pneumocystis* using LSU-qPCR were further confirmed to be positive using mt SSU rRNA conventional PCR (SSU-PCR), while 8.2% (8/98) of NPA specimens negative for LSU-qPCR were positive by SSU-PCR. In true positive cases where bronchoscopic specimens were positive by microscopy and NPA specimens were positive by LSU-qPCR and SSU-PCR, the median *Pneumocystis* load in NPA specimens was lower in non-HIV than in HIV patients, though not reaching statistical significance ($P = 0.203$; Fig. 2).

Four patients were negative for *Pneumocystis* by microscopy of the BALF specimen but positive by LSU-qPCR in the NPA specimens (Table 3). For all four NPA specimens, SSU-PCR was positive, and sequencing of PCR products showed 99 to 100% nucleotide identity with the *P. jirovecii* mt SSU rRNA gene (GenBank accession no. HQ228547.1). Notably, two of these patients were treated with cotrimoxazole due to treatment failure with broad-spectrum antibacterial coverage and a high clinical suspicion of PCP despite negative BALF microscopy result. Both patients improved clinically after empirical treatment with cotrimoxazole. Moreover, these two patients had higher *Pneumocystis* loads than the other two patients who did not receive specific treatment against *Pneumocystis*.

Our study shows that LSU-qPCR in NPA specimens has high sensitivity, specificity, positive predictive value, and negative predictive value for the diagnosis of PCP compared with microscopic examination of bronchoscopic specimens. NPA specimens have several advantages over bronchoscopic specimens. First, NPA is a technically simple procedure and therefore feasible even in busy clinical settings (15, 16). Second, since NPA specimens are often collected upon admission in patients with upper or lower respiratory tract infections, the use of NPA specimens for diagnosis of PCP can potentially minimize the delay compared to when bronchoscopic specimens are used. Third, obtaining NPA specimens is less invasive than obtaining bronchoscopic specimens and therefore is associated with less patient discomfort and a lower risk of complications (17).

In this study, 8.2% of NPA specimens were LSU-qPCR nega-

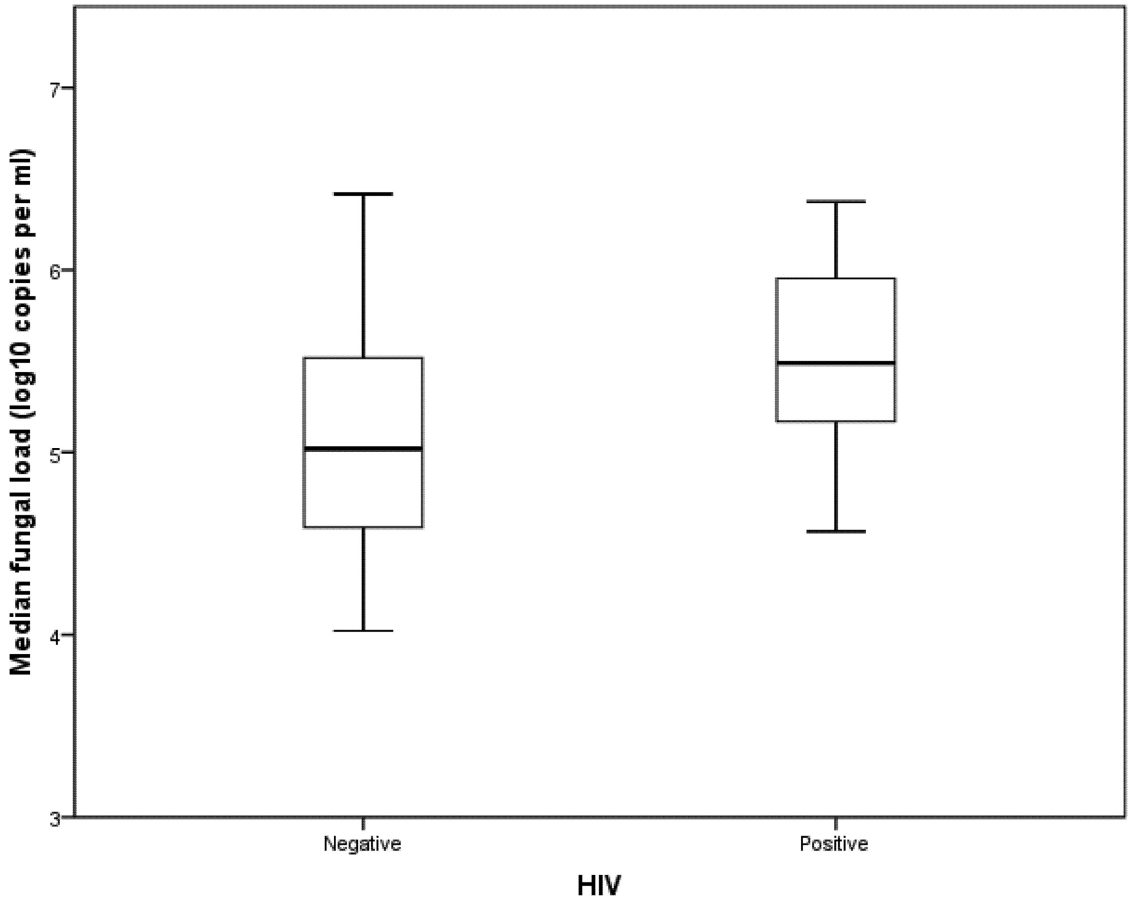

FIG 2 Comparison of *Pneumocystis* load between HIV-positive and -negative patients. Mann-Whitney U test was used for statistical analysis. $P = 0.203$.

TABLE 3 Clinical characteristics of four patients with negative BAL staining and positive NPA PCR for *Pneumocystis*

Patient no.	Sex[h]/age (yr)	Underlying disease	Pulmonary radiological appearance	Lymphocyte count ($\times 10^9$ cells/liter)	Anti-*Pneumocystis* treatment	*Pneumocystis* load (copies/ml)
1	F/52	Systemic lupus erythematosus/scleroderma	Patchy ground glass densities and tiny lung nodules at bilateral upper lobes[a]	0.53	Nil[d]	4.68×10^4
2	M/63	Immunoblastic T cell lymphoma, receiving chemotherapy	Bilateral symmetrical airspace opacities, particularly at the lower zones[b]	0.83	Given cotrimoxazole with clinical improvement[e]	8.88×10^4
3	F/13	Juvenile dermatomyositis	Bilateral symmetrical airspace and reticular opacities, predominantly involving perihilar and lower zones[b]	0.88	Given cotrimoxazole with clinical improvement[f]	1.08×10^5
4	F/54	Lung adenocarcinoma with metastasis to brain, hypertension	Multifocal bilateral patchy consolidation with upper zone predominance[c]	0.60	Nil[g]	3.59×10^4

[a] Positron emission tomography/computed tomography.
[b] Chest radiograph.
[c] Computed tomography.
[d] Clinically improved after receiving empirical tuberculosis treatment for suspected pulmonary tuberculosis.
[e] Received imipenem-cilastatin and ticarcillin-clavulanate before cotrimoxazole therapy.
[f] Received amoxicillin-clavulanate and azithromycin before cotrimoxazole therapy.
[g] Clinically improved after receiving piperacillin-tazobactam and doxycycline for the treatment of community-acquired pneumonia.
[h] F, female; M, male.

tive but SSU-PCR positive, suggesting that the SSU-PCR could be oversensitive in the diagnosis of PCP. Four patients have false-positive NPA LSU-qPCR and SSU-PCR results compared with microscopic examination of BALF specimens. The patients who were given cotrimoxazole due to high clinical suspicion of PCP had higher *Pneumocystis* loads than those without specific treatment for *Pneumocystis*. The result suggests that patients with higher *Pneumocystis* load in NPA specimens have a higher likelihood of true PCP rather than mere colonization.

The use of upper respiratory tract specimens in the diagnosis of *Pneumocystis* infection has been evaluated in previous studies (18–24). Our study differs from these studies in several aspects. First, our study is the first to assess a population consisting of mainly adult non-HIV patients, with less than 10% of HIV-positive patients in the studied population. Second, some of the previous studies used PCR in the BALF as the gold standard for diagnosis, which may be oversensitive due to frequent colonization of *Pneumocystis* in immunocompromised patients.

This is the first study to evaluate the performance of qPCR in NPA specimens for the microbiological confirmation of PCP encompassing mainly non-HIV patients. NPA specimens are superior to bronchoscopic specimens in their ease of collection and low associated risks, making them preferable to bronchoscopic specimens in routine clinical settings. Bronchoscopic procedures should be reserved for patients in whom NPA specimens cannot settle the diagnosis.

Nucleotide sequence accession numbers. The nucleotide sequences obtained in this study have been deposited within the GenBank sequence database (accession numbers JX567346, JX567347, JX567348, and JX567349).

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