COMPARATIVE YIELD OF DIFFERENT RESPIRATORY SAMPLES FOR DIAGNOSIS OF *PNEUMOCYSTIS CARINII* INFECTIONS IN HIV-SEROPOSITIVE AND SERONEGATIVE INDIVIDUALS IN INDIA

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Abstract. Respiratory specimens were prospectively examined for *Pneumocystis carinii* from 53 patients. The majority of specimens were comprised of expectorated sputum, induced sputum, broncho-alveolar lavage (BAL), and tracheal aspirates. In only four patients *Pneumocystis carinii* (*P. carinii*) was detected. All the samples were produced by broncho-alveolar lavage. *Candida* spp and *Aspergillus* spp were also identified in a small number of patients. Acid-fast-bacilli were not detected in any of the cases under study. There were no sex-related differences in distribution. The present prospective study was undertaken in order to determine *P. carinii* infections in human immunodeficiency virus (HIV) seropositive and seronegative individuals. Expectorated sputum samples were probably the major limiting factor in low positivity for detection of *P. carinii* and study of BAL specimens would be more useful for better results.

INTRODUCTION

Infections due to *Pneumocystis carinii* (*P. carinii*) were originally observed during epidemics of interstitial plasma cell pneumonia in institutions caring for newborn infants (Gajdusek, 1957). Since 1960 most reports of pneumonia due to *Pneumocystis* were seen in primary immunodeficient patients or secondary immunodeficiencies caused by immunosuppressive treatment or malignant diseases or organ transplants (Hughes *et al*, 1973; Hamburger *et al*, 1965). In the mid-1980s it was reported that *P. carinii* pneumonia (PCP) is an important cause of morbidity and mortality in HIV infected individuals.

Clinical diagnosis of *P. carinii* pneumonia (PCP) is often difficult. Pathophysiological symptoms, chest roentogenograms and unresponsiveness to treatment with conventional antibiotics usually suggest PCP. Direct demonstration of the organism (*P. carinii*) is often difficult or not possible because the trophozoites or cyst of the organism are either not present in abundant numbers or not uniformly spread out in the alveoli of infected lungs HIV infected patients are more susceptible to infection with *P. carinii* than HIV-negative individual and have a greater risk of progression to active disease. Therefore, the incidence of *P. carinii* in a population of HIV infected patients might as well serve as an indicator of transmission in general. Number of different techniques have been used by several investigators for the diagnosis of *P. carinii.*

The first reported cases of HIV infection diagnosed in India were among commercial sex workers in erstwhile Madras in May 1986. Since 1988, a significant increase in HIV seroprevalence has been detected in high risk populations and by the end of 1992, HIV infection had been reported from all states in India (Bollinger *et al*, 1995). So far, very limited information is available about the frequency of overt or latent infection with *P. carinii* in India. In this prospective report a systematic effort has been made to detect *P. carinii* in respiratory specimens obtained from suspected patients and data is presented on the assessment/distribution of pneumocystosis in HIV serpositive and HIV seronegative individuals. The definitive diagnosis of *P. carinii* can only be made by morphological or histopathological demonstration of organism. In the present study we performed staining techniques [Gomorimethenamine silver (GMS), Giemsa and immunofluorescence] to detect *P. carinii* from different samples obtained from patients. In doing so, we found a critical pitfall in using staining as the sole diagnostic criteria in diagnosis of *P. carinii* and felt that the further use of cytological study, as well as molecular techniques would be of much importance to ascertain the prevalence of *P. carinii* infection. Polymerase chain reaction (PCR), if done properly has been found to be more sensitive and specific and has been recommended as a gold standard for diagnosis.

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MATERIALS AND METHODS

The study population consisted of patients who were diagnosed having *P. carinii* pneumonia (PCP) episode at our hospital during August 1993 - December 1998. Some patients were also from other hospitals in and around Delhi. Patients with cardinal features of pneumocystosis such as shortness of breath, fever and a dry non-productive cough whether presented individually or in varying combination of symptoms were included in the study. All patients, whose samples were examined for *P. carinii* were also prospectively followed up with respect to their symptoms, clinical signs and laboratory parameters. During the study period, respiratory samples sent for investigation by clinicians were obtained from 53 patients. During hospitalization (except a few patients), fever, prolonged cough, shortness of breath, weight loss and pulmonary infiltrates were the prominent presenting features. Samples obtained from all patients had a strong clinical suspicion suggestive of diffuse interstitial pneumonia or had underlying diseases that might have predisposition to *P. carinii* infection. These patients were divided into two groups according to HIV status. Group-I included 40 HIV seropositive patients with overt clinical signs (dyspnea, fever and diffuse interstitial pneumonia). Group II comprised of 13 HIV seronegative patients with clinical signs of a prolonged serious respiratory infection and had also shown unresponsiveness to conventional antibiotic treatment.

Respiratory specimens, *viz* expectorated sputum, induced sputum, broncho-alveolar lavage (BAL), tracheal aspirates were sent for investigations from these patients to our laboratory. Transbronchial biopsy specimens were not received from any of the patients. Thick viscid samples were treated with sputolysin (alpha-dithierythretol). The washings and other specimens aliquotes were pooled and centrifuged for 5 minutes at low speed and the precipitates/deposits obtained were studied for the presence of *P. carinii*. Of the precipitates/deposits obtained, a minimum of four smears from the representative sample of each study individual were prepared on glass slides and air dried. Smears were stained with Giemsa, Gomori's methenamine silver (GMS) and fluorescein-conjugated monoclonal antibodies for direct immunofluorescence (MeRIFLUOR™ pneumocystis, Meridian Diagnostic, INC, Ohio). One smear was stored at -20ºC as a back-up slide. Both negative and positive cases were rescreened for conclusive results. Immunofluorescence staining was performed according to the instructions of the manufacturer.

Assessment were also made of blood gas analysis, serum lacatate dehydrogenase and CD_4^+ lymphocytes (in HIV patients only) at the time of diagnosis (data not shown). Patients were asked about the development and duration of any newly developed symptoms.

RESULTS

Among the 40 patients studied in HIV seropositive group 3 had AIDS, 21 had primary interstitial pneumonia, 11 had chronic lung disease and 5 had pulmonary infiltrates. Two transplants (heart and renal, one each), two Hodgkin's lymphoma, one with severe combined immunodeficiency disorder (SCID) and 5 immunosuppressed individuals were the major study population in HIV seronegative individuals. Table 1 summarizes the clinical diagnosis along with results. The average age of the patients were 35 years (range 3-77 years): 40 were men and 13 women. The risks of HIV acquisition were through heterosexuality in 30, intravenous drug users in 5, 5 children had acquired HIV through perinatal transmission. Patients were from different parts of the country with different socio-economic background 95% of the HIV seropositive patients with respiratory symptoms studied had a CD_4 lymphocyte count of <200 µl. The most likely cause of clinical respiratory disease in HIV seropositive patients were due to disease progression and others were due to prolonged immunosuppressive therapy and underlying disease.

In 3 (6%) of HIV infected patients and one (1.5%) HIV seronegative patient with severe combined immune deficiency (SCID), *P. carinii* was detected. In all four, the specimens were BAL. Patients from whom *P. carinii* were detected in BAL samples were ultimately diagnosed with *P. carinii* pneumonia.

No classical trophozoites (octanucleate) could be detected, however with GMS, *Pneumocystic carinii* in the form of irregularly shaped purple or lightly bluish stained cysts were seen. These were also demonstrated subsequently by immunoflourescence staining, which showed fluorescent staining with characteristics morphology. One of the BAL samples obtained from a patient with interstitial pneumonitis in which *P. carinii* could not be demonstrated by any of the staining techniques, was considered

Table 1
Clinical diagnosis of patients.

Clinical diagnosis	No.	*P. carinii* (+ve)	*P. carinii* (-ve)
Acquired immune deficiency syndrome (AIDS	3	1	2
HIV seropositive	37		
Interstitial pneumonitis	21	2	19
[a]Chronic lung disease	11	-	11
Pulmonary infiltrates	5	-	5
HIV seronegative	13		
Severe combined immune deficiency syndrome (SCID)	1	1	0
Chronic myeloid leukemia	1	-	1
Acute myeloid leukemia	1	-	1
Hodgkin's disease	2	-	2
Wegner's granulomatosis	1	-	1
Transplant (renal and cardiac	2	-	2
Immunosuppressive therapy	5	-	5
Total	53	4	49

[a]Persistent chest infection.

Table 2
Sampling methods with relative success.

Methods	No. of sample	Positivity (%)
Expectorated sputum	32	0
Induced sputum	13	0
Broncho-alveolar lavage	5	7.5
Tracheal aspirate	3	0
Total	53	7.5

to be a true negative based upon evaluation of the clinical course. Other samples of interest such as induced sputum (n=13) did not show any positivity. In expectorated sputum and tracheal aspirates (n=35), though considered inappropriate for *P. carinii* investigation, the validity of negative results could not be determined nor explained. Different samples with relative success in identification of the organism are shown in Table 2.

Patients with HIV seropositivity who had clinical indications of pneumonia due to *Pneumocystis* had significant radiological changes on chest-roentogenogram as compared to HIV seronegative cases. This is because of incapacitating immunological system in HIV positive patients. Yields of *P. carinii* have been lowest in non-HIV patients, as the low parasite burden makes demonstration of *P. carinii* more difficult than in HIV infected individuals. Acid-fast bacilli were not detected in any of the cases that were studied. This finding is at variance, as HIV and tuberculosis go more hand in hand in countries like India (Singh, 1993). Few species of *Candida* and *Aspergillus* were also detected in few patients.

DISCUSSION

Besides many life-threatening opportunistic infectious agents, pulmonary infiltrates affecting immunocompromised patients could be due to drug toxicity, and respiratory diseases of unknown origin. For HIV related/induced pneumonitis, examination of respiratory samples plays an additional role in the management of infected individuals. The fact that the vast majority of AIDS patient develop PCP during their illness has been a great stimulus for research into PCP and for detection of *Pneumocystis carinii*. It is also true that PCP is one of the major opportunistic infections in immunocompromised patients in whom the pathology may be difficult. Consequently awareness of PCP by clinicians and the role of laboratory tests needs further careful assessment.

Diagnostic success of expectorated sputum has been poor. Estimated yield of correct diagnosis varies from 6-24% with a diagnostic sensitivity of 57% (Walzer *et al*, 1974; Elvin *et al*, 1988). Sputum induction is non-invasive but requires careful supervision and has its inherent adverse effect. It was reported that induced sputum samples were, frequently diagnostic and less expensive, however, in different studies the prevalence of PCP in patients with sputum induction is <50%, questioning its cost-effectiveness and reliability (Chouaid *et al*, 1993; Wehner *et al*, 1994). However, the yield of sputum induction has been improved upon the use of direct fluorescent antibody (DFA) (Halford *et al*, 1994) test or by using molecular techniques (Lipschik *et al*, 1992).

The high sensitivity of broncho-alveolar lavage, with its relatively low level of complication probably makes this technique more useful in detection of *P. carinii* (Golden *et al*, 1986). In our experience, interpretation of BAL samples are far superior to the results obtained by using only sputum (expectorated/induced) samples. The argument in this regard may appear difficult to settle as there may be a certain degree of bias both in personal and institutional basis. In investigation of *P. carinii* infection, several factors plays a role, starting from the degree of immunocompromise to expertise in obtaining the specimen and laboratory investigations. Unfortunately, many clinics do not provide BAL as primary samples specially in HIV patients due to technical difficulties, a major factor in underdiagnosis in developing countries including India. The hypothesis (Kaur *et al*, 1992) that infection prevalent in developed countries might not be seen in the same proportions in less-developed or developing countries depending on various factors is therefore not entirely correct. Failure in detection of *P. carinii* infection as expected in the present study could be either due to under-diagnosis or under-reporting as well as due to the paucity of diagnostic infrastructure in developing countries.

The results of this study however show a high degree of agreement between GMS and fluorescent staining (direct). Our experience has shown that with the help of clinical course as indicator, examination of BAL specimens by using GMS and immunofluorescence staining will achieve a good and reliable sensitivity. A distinct advantage of the fluorescent staining over the other staining techniques or Giemsa was the ease and rapidity with which specimens can be examined. It would also be very useful in samples with a relatively low volume or with few organisms in it. For all practical purposes, BAL specimen can be used as a gold-standard for detecting *P. carinii* in all respiratory specimens.

Systematic studies on pneumocystosis are scarce in Indian sub-continent. Most of the published data are in the form of case reports (Arora *et al*, 1996, 1998; Cherian *et al*, 1997; Bijur *et al*, 1996; Date *et al*, 1995; Singh *et al*, 1993; Sherka *et al*, 1992). Our prospective study indicates that clinical pneumocystosis infections are not quite uncommon, rather they remain clinically inapparent. The direct demonstration of organisms made only in few patients in the present study could be because of inappropriate samples were provided or probably due to the fact that some of our patients were on trimethoprim-sulphamethoxazole prophylaxis, where cessation of the multiplication of organisms can occur (Hughes *et al*, 1975). In addition, trophozoites are more fragile than thick walled cysts which could be an additional factor for not detecting trophozoites (Chatterton, 1990). Yields have also been low in non-HIV patients as the low cysts burden makes demonstration of *P. carinii* more difficult than in AIDS/HIV patients.

Recently, evidence has accumulated that use of polymerase chain reaction (PCR) is sensitive enough to enable a diagnosis to be made from different samples using less invasive techniques (Evans *et al*, 1995; Wagner *et al*, 1997; Robodonirina *et al*, 1999). However, the PCR positive results need to be correlated with relevant clinical symptoms suggesting active infection, as positive PCR for *P. carinii* can also be seen in asymptomatic individuals.

The factors leading to variable success in detection of *P. carinii* in different samples are not well understood. Therefore, each institution may need to examine its own results with different samples as well as the prevalence of *P. carinii* infection in their patient populations. However, further study is warranted using sensitive techniques such as DNA amplification which might increase the sensitivity in both invasive and non invasive samples (*viz* expectorated sputum oropharyngeal washings and blood) in developing countries as well. To the best of our knowledge, this appears to be the first report from this sub-continent dealing exclusively for detection of *P. carinii* in both HIV serpositive and suspected HIV-seronegative individuals.

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