Full Length Research Paper

Pathogenic fungal isolates in sputum of HIV positive patients

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Fungal pathogens were isolated and identified from sputa of 100 HIV positive patients admitted at ART centre, K.G.H, Visakhapatnam during the period of 2 months from June 2010 to July 2010. The sputum samples from HIV positive individuals were collected in a sterile container and processed by standard methods. Two consecutive samples at the interval of three days were collected. If two samples yielded the same growth, then only they were considered as positive for fungal pathogens. Patients age ranged from 6 to 55 years in both sexes. Samples were collected from 67 males and 33 females. Out of 100 samples, single fungal isolates were obtained in 54 samples, mixed fungal species in 20 samples and no fungal isolates in 26 samples. Candida species predominate (42 samples) in single isolates followed by Cryptococcus neoformans (4 samples), Penicillium spp., Aspergillus fumigatus and Aspergillus niger (2 each), Scedosporium apiospermum, Cunninghamella bertholletiae, Sporothrix schenckii and Geotrichum candidicum were also isolated. The Penicillium marneffei isolates (2 samples) were from cases with CD4 counts below 100.

Key words: Opportunistic fungal infections, immunocompromised individuals, CD4 counts, penicillium marneffei.

INTRODUCTION

A predominant source of morbidity and mortality among HIV positive individuals in late stages of HIV infection and low CD4 count below 500/cumm, is opportunistic infection caused by agents that rarely infect immuno competent individuals (Jawetz et al., 2007). The occurrence of opportunistic fungal infections has risen progressively in recent years. Invasive fungal infections has been reported in recent years in 26% of chronically and intensively immunosuppressed patients (Topley and Wilson’s, 2005). Infections with Candida albicans appear when CD4 count is between 500-200/cumm and may be the first indication of immunodeficiency. Cryptococcal infection occurs when CD4 count has fallen below 150/cumm (NACO Specialist's Training and Reference Module, 2000). Penicilliosis is observed in patients with CD4 count of less than100/cumm (Guidelines for prevention and treatment of opportunistic infections in HIV infected adults and adolescents, 2009). The phagocytic cells and lymphocytes (T&B both) are believed to function together in protecting the host against fungal pathogens but the exact degree to which each is involved is not yet fully known. It has been shown that vegetative hyphal structures of Aspergillus and candida are ingested and killed by neutrophils (Jagdish, 2009). Skin and mucosal surfaces play an important role in primary defense against pathogens. The mucociliary action of mucus membrane is the prime clearance mechanism active against inhaled fungal spores. As the HIV positive individuals are prone to get recurrent respiratory infections, the mucosal barrier may be damaged and they are more vulnerable to develop fungal respiratory infections (Topley and Wilson’s, 2005).

The fungal infections depend on, exposure to sufficient inoculum size of organism and general resistance of the host. Immune deficiency predispose to progression of infections by established pathogens that is Penicillium marneffei and Histoplasma capsulatum. Aspergillus spp., Rhizomucor, Absidia spp., Cunninghamella spp., Apophysomyces spp. are mould species commonly
isolated in HIV positive individuals (Harrison’s, 2008). In Thailand *P. marneffei* infection is the third most frequent infection in HIV infected persons after tuberculosis and cryptococcosis (Anantanarayan and Paniker’s, 2009). Now CDC recognized Manipur State, India, as a new endemic area of *P. marneffei* (Guidelines for prevention and treatment of opportunistic infections in HIV infected adults and adolescents, 2009). With introduction of antifungal agents, the cause of candida infections shifted from *C. albicans* to *C. glabrata* and other non albicans species, as *C. glabrata* and *C. krusei* develop resistance to fluconazole (Topley and Wilson’s, 2005). Fungal infections may disseminate and cause fungaemia, which is a grave condition in immunosuppressed individuals. Thus, prompt diagnosis by standard microbiological methods and treatment are crucial.

**MATERIALS AND METHODS**

We got approval from Ethics Committee, Andhra Medical College, Visakhapatnam to conduct the study. The copy of the Ethics Committee Approval was enclosed (Annexure 1). Two consecutive sputum samples at an interval of 3 days were collected from HIV the patients and their CD4 counts were presented in Table 1. All patients complained of cough and fever for more than one week. Sputa samples were collected in a sterile wide mouthed container. Patients were asked to wash their oral cavity with distilled water before collecting sputum in order to avoid contamination of sputum with commensal flora from oral cavity (Koneman color Atlas and Text book of Diagnostic Microbiology, 1997). Specimens were processed by doing Gram’s staining for direct smears and KOH mount. Gram’s stained smears were examined under oil immersion objective of microscope for the presence of inflammatory cells (pus) cells and fungal elements. Quality of the sputum was assessed by examining Grams staining smears. The specimen was considered as acceptable when number of squamous epithelial cells are less than 10/ LPF (Bailey and Scott’s, 2002; Koneman color Atlas and Text book of Diagnostic Microbiology, 1997). The samples that showed fungal elements and pus cells in direct Gram’s staining were processed further (Koneman color Atlas and Text book of Diagnostic Microbiology, 1997; Topley and Wilson’s, 2005).

Sputum was inoculated on two sets of Sabouraud’s dextrose agar (SDA with antibiotic gentamic in alone and SDA with gentamicin and cycloheximide) and incubated at 25°C in BOD for 4 weeks. SDA bottles were examined for growth once in two days during 1st week and twice a week thereafter up to 4 weeks. SDA medium with growth was processed by standard methods (Mackie and McCartney, 2008). Mucoid, yeast like growth was processed by doing Gram staining, capsular staining, germ tube test, urease test and inoculation onto corn meal agar. Gram’s staining was done to all the isolates with mucoid and yeast like growth and observed for gram positive budding yeast cells.

Capsular staining with 10% Nigrosin: All the isolates with mucoid and yeast like growth were tested for capsulated budding yeast cells for identification of *Cryptococcus neoformans*.

Germ tube test: All candida isolates were tested for germ tube formation. A colony was inoculated in human serum and incubated at 37°C. After 2 to 4 h wet mount was prepared and observed for germ tubes.

Chlamydospore formation: All candida isolates were tested for the production of chlamydospores in corn meal agar. After inoculation and incubation at 25°C, the plates were examined under microscope for the presence of chlamydospores.

Urease test: Christensens’ Urease test was done for *Cryptococcus*. *Cryptococcus neoformans* was urease positive. Lactophenol cotton blue (LPCB) mount was done for filamentous growth to see arrangement of conidia.

Slide cultures: Slide culture in Mycology is used to study undisturbed morphological details of fungi, particularly relationship between reproductive structures like conidia, conidiophore and hyphae (Jagdish, 2009). When two samples yielded the same fungal isolate, then only the fungal isolate was considered as causative agent.

Identification of *P. marneffei*: *P. marneffei* is a dimorphic fungus. At 25°C on SDA it grows as a mycelial fungus producing rapidly growing greenish yellow sporulating colony with a red centre and dark green edges with diffusible brick red pigment. At 37°C on SDA it produces smooth glabrous off white yeast like growth with little pigment. Microscopically the fruiting heads sometimes have terminal conidia larger than the ones beneath them called Corda’s phenomenon, characteristic of *P. marneffei* (Figure 4).

**RESULTS**

Out of 100 sputum samples, 54 samples yielded single fungal isolates. Mixed fungal isolates were observed in 20 samples and rest of 26 samples were negative for
fungal growth. Out of 54 single isolates, Candida spp. were predominant isolates. Those yeasts were identified in 42 samples. C. albicans in 18, candida non albicans in 24. Other fungi were isolated mainly Cryptococcus neoformans (4 samples), Penicillium spp. and Aspergillus fumigatus (each one in 2 samples) Scedosporium apiospermum (Figure 1), Cunninghamella bertholletiae (Figure 2), Geotrichum candidicum and Sporothrix schenckii (Figure 3) (each in one sample) as shown in Table 2. Among mixed isolates Candida spp. were isolated in 13 cases and in 7 cases several moulds were isolated as shown in Table 2. P. marneffei was isolated in samples in combination with C. albicans in one sample and with A. fumigatus in another sample. The CD4 counts of P. marneffei was both below 100.

Out of total 96 isolates Candida spp. was the major group comprising 55 in number (57%). C. albicans in 26 samples (27%) and non albicans spp. in 29 (30%) Next common isolate was Aspergillus spp.in 13 samples that is 13.5% (with A. fumigatus and A. niger each one in 6 samples, A. flavus in one sample) followed by Penicillium spp. in 6 (6.25%) including two P. marneffei (Figure 4) isolates. C. neoformans and S.schenckii (Figure 3) each one in 5 samples (5.2%), Geotrichum candidicum in 4 (4.16%), Wangiella dermatitidis (Figure 5) and Scedosporium apiospermum each one in 2 samples, Absidia corymbifera (Figure 6), Cunninghamella berthollatiae, Nigrospora spp (Figure 7) and Prototheca spp. each one in one sample were isolated.

DISCUSSION

The number of opportunistic fungal infections in general have increased in HIV positive patients and therefore the incidence of fungal infections has also been on the rise. Recent pandemic of AIDS caused by HIV virus has been the precipitating cause for the increased incidence of two common fungal diseases i.e., isosophageal candidiasis and cryptococcosis. Candida is part of normal flora of upper respiratory tract, when immunity is low, it acts as an opportunistic pathogen. Usually HIV positive individuals also have other predisposing factors like neutropenia, lymphopenia, selective T cell defects, altered monocyte macrophage function, frequent use of antibiotics for prophylaxis and treatment of various bacterial infections, lowered immunity due to low CD4 count (Ananthnarayan, 2009). All these factors contribute to the development of opportunistic fungal infections like pulmonary candidiasis, cryptococcosis, zygomycosis, aspergillosis.

Invasive fungal infections like Candidiasis, Aspergillosis, Mucormycosis, Cryptococcosis, Histoplasmosis and Coccidiodomycosis are known infections whereas infections with S. schenckii (Figure 3) and P. marneffei are emerging ones in HIV positive patients (Carol, 1998). Other fungi that cause pulmonary infections in HIV/AIDS patients are Scedosporium apiospermum, (Figure 1),
Table 2. Fungal pathogens in mixed infection.

<table>
<thead>
<tr>
<th>No.</th>
<th>Names of the fungal pathogens in mixed infection</th>
<th>No. of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Non albicans Candida spp. + Aspergillus fumigatus + Aspergillus niger</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>Candida albicans + Penicillium spp. + Wangiella dermatitidis</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>Candida albicans + Penicillium marneffei</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>Candida albicans + Absidia corymbifera</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>Candida albicans + Sporothrix schenckii</td>
<td>2</td>
</tr>
<tr>
<td>6</td>
<td>Non albicans Candida spp. + Aspergillus niger</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>Candida albicans + Aspergillus niger</td>
<td>1</td>
</tr>
<tr>
<td>8</td>
<td>Non albicans Candida spp. + Geotrichum candidicum</td>
<td>1</td>
</tr>
<tr>
<td>9</td>
<td>Candida albicans + Cryptococcus neoformans</td>
<td>1</td>
</tr>
<tr>
<td>10</td>
<td>Candida albicans + Nigrospora spp</td>
<td>1</td>
</tr>
<tr>
<td>11</td>
<td>Non albicans Candida spp. + Wangiella dermatitidis</td>
<td>1</td>
</tr>
<tr>
<td>12</td>
<td>Scedosporium apiospermum + Geotrichum candidicum</td>
<td>1</td>
</tr>
<tr>
<td>13</td>
<td>Aspergillus fumigatus + Penicillium marneffei</td>
<td>1</td>
</tr>
<tr>
<td>14</td>
<td>Sporothrix schenckii + Penicillium spp.</td>
<td>1</td>
</tr>
<tr>
<td>15</td>
<td>Aspergillus fumigatus + Aspergillus niger</td>
<td>1</td>
</tr>
<tr>
<td>16</td>
<td>Aspergillus fumigatus + Prototheca spp.</td>
<td>1</td>
</tr>
<tr>
<td>17</td>
<td>Aspergillus niger + Sporothrix schenckii</td>
<td>1</td>
</tr>
<tr>
<td>18</td>
<td>Aspergillus niger + Aspergillus flavus</td>
<td>1</td>
</tr>
</tbody>
</table>

Figure 4. Photomicrograph of *P. marneffei* showing Corda’s phenomenon.

Figure 5. Photomicrograph of *W. dermatitidis*.

Figure 6. Photomicrograph of *A. corymbifera*.

Figure 7. Photomicrograph of *Nigrospora* spp.
Candida corymbifera (Figure 6), Wangiella dermatitidis (Figure 5) (Topley and Wilson’s, 2005).

Today’s concern about Candidiasis is emergence of fluconazole resistant C. albicans in AIDS patients with recurrent attacks of oral thrush and less susceptibility of C. krusei and C. glabrata to fluconazole. C. albicans is generally considered as major pathogen among Candida spp (Topley and Wilson’s, 2005).

In the present study 74 sputum samples yielded 96 isolates. Out of 96 isolates Candida spp. were 55 (C. albicans in 26 samples, non albicans in 29 samples) showed the change in the trend of candidial infections towards non albicans spp. As non albicans like C. krusei and C. glabrata develop resistance to fluconazole, commonly used antifungal drug for prophylaxis, isolation of more number of non Albicans spp. is gaining importance. Sailaja et al. (2004) in Hyderabad, India also isolated more number of non albicans spp. in 18 cases than C. albicans in 6 cases (Sailaja et al., 2004). Aruna Agarwal et al. (2005) Punjab isolated C. albicans in 20 (62.5%) out of 32 isolates (Usha et al., 2005). Jha et al. (2006) from Madurai isolated 20 C. albicans and 10 non albicans from 462 samples of lower respiratory tract infections (Jha et al., 2006). Prasobh et al. (2009) Tamilnadu, performed resistotyping of 350 C. albicans isolated from sputum samples. All the above studies were geographically from the same region. C.albicans was isolated from sputum samples in all these studies. In the study of opportunistic fungal infections in HIV/AIDS patients by Rakhamanova (1998), the culture positivity for C. albicans and C. neoformans in pulmonary infections was 4% each (Rakhamanova et al., 1998). Yongabi et al. (2009) isolated 12 strains of C. albicans from 98 sputum samples albicans spp. predominate in the present study and study by Sailaja et al. (2004) where as C. albicans was predominate species in Jha study. Only C. albicans was mentioned in other studies. But the importance of C. albicans as causative agent in pulmonary infection was observed in all these studies.

Aspergillus spp. were the next commonest fungi isolated in the present study in 13 samples (13.5%). A. fumigatus and A. niger each from 6 samples and A. flavus from one sample were isolated where as Sailaja et al. isolated A. niger in one case. Nash et al. (1997) identified 17 cases of AIDS related pulmonary aspergillosis at autopsy (Nash et al., 1997). Mylonakis et al. (1998) after reviewing 342 AIDS cases concluded that invasive pulmonary aspergillosis usually occur among patients with less than 50 CD4 cells /cummm (Mylonakis et al., 1998).

C. neoformans was isolated from 5 samples (5.2%) in the present study, 2 samples from a study by Sailaja et al. (2004). Isolation was 4% by Rakhamanova (1998) and 2.04% by Yongabi et al. (2009). A retrospective study by Mirvium L Cameroon between 1981 and 1989 revealed 12 cases of cryptococcal pneumonia with culture positivity in 10 cases (Mirvium et al., 1990). C. neoformans as causative agent of pulmonary fungal infection was established in all these studies. Other organisms isolated were Penicillium spp. (including two P. marneffei) in six Geotrichum candidicum in four, Wangiella dermatitidis and Scedosporium apiospermum each one in two, Cunnighamella bertholletiae, A. corymbifera, Nigrospora spp. and Prototheca spp. each one in one sample. The above fungi were isolated for the first time in our geographical area. The emerging fungus P. marneffei was isolated from sputum in two samples in this study. The same was reported from four autochthonous cases by Singh et al. (1999) from Manipur India (Singh et al., 1999). Ranjana et al. (2002) reported disseminated P. marneffei infection in 50 cases from the same hospital (Ranjana et al., 2002). CDC recognized Manipur, India, as new endemic area of this fungus and observed that penicillosis was seen in HIV positive patients who have CD4 counts less than 100/cummm (Guidelines for prevention and treatment of opportunistic infections in HIV infected adults and adolescents, 2009).

Gupta et al. (2007) from New Delhi isolated P. marneffei from lymph node and blood cultures of HIV positive patient with multiple lymphadenopathy and massive hepatosplenomegal. Bhagyabathi et al. (2009), Imphal, India isolated P. marneffei from HIV positive individuals whose CD4 counts were less than 100 (21.4%) (Bhagyabathi et al., 2009). Bharathi et al. (2010) Thanjavur, India isolated the same in one case of HIV positive patient.

Conclusions

It is concluded that Candida species (C. albicans in 26 samples and non albicans in 29 samples) were the most common species isolated in the present study. Next common species were Aspergillus species and Cryptococcus neoformans. Geotrichum candidicum, Wangiella dermatitidis, Scedosporium apiospermum, Cunnighamella bertholletiae, Absidia corymbifera, Nigrospora spp. and Prototheca spp. were also isolated though in less number of cases, stressing the need for more number of studies for fungal pathogens in HIV positive patients. Isolation of P. marneffei an emerging fungal pathogen in two cases with CD4 counts less than 100 in the present study. We made an attempt for the first time in Andhra Medical College, Visakhapatnam to isolate fungal pathogens from lower respiratory tract. Still it needs further and extensive study. It is necessary to screen all HIV positive cases with cough for fungal pathogens in addition to bacterial pathogens. By that early diagnosis and early treatment reduces further complications.

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REFERENCES


ANNEXURE 1

VISAKHAPATNAM

LETTER OF CONSENT

This letter of consent is hereby accorded to ..................

Dr. N. Bharathi, Asst. Prof. Medicine for conducting
the research work titled: Study of fungal pathogens...

in ...epidemic of HIV positive individuals...

................... after the

necessary scientific evaluation and ethical review of the

above cited research protocol, by the ETHICS COMMITTEE.

ANDHRA MEDICAL COLLEGE, VISAKHAPATNAM.

SECRETARY

CHAIR PERSON