OPPORTUNISTIC FUNGAL PATHOGENS OF LOWER RESPIRATORY TRACT IN HIV SEROPOSITIVE PATIENTS
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HOW TO CITE THIS ARTICLE:

ABSTRACT: Respiratory infections are the major cause of morbidity and mortality in persons with HIV infection. About 70% of HIV/AIDS patients with infection experience a pulmonary opportunistic infection in life time. The nature of pulmonary infection of HIV reflects the level of immunodeficiency. Though increasing number of AIDS cases are being reported from central India, the data on spectrum of opportunistic infections of respiratory tract in HIV seropositive patients from developing countries as well as from the region is scanty. The present study was undertaken to determine the incidence of various fungal pathogens of lower respiratory tract in HIV seropositive patients. A total of 108 HIV seropositive cases presenting with the signs and symptoms of involvement of lower respiratory tract were studied. KOH mount examination revealed fungal elements in 40 samples. Toluidine blue staining and Giemsa staining techniques were used in the present study for the demonstration of Pneumocystis carinii in the sputum. In our series, no specimen revealed forms suggestive of Pneumocystis carinii.

Yeast cells belonging to Candida spp were isolated from 20 cases, 16 isolates belonged to candida albicans & 2 each of candida gullermondii & candida tropicalis, Moulds were recovered from 2 sputum specimens. Both belonged to Aspergillus species, considering morphology on SDA and microscopic morphology in Lactophenol cotton blue (LCB) mount, one species was identified as Asp. flavus and other was Asp. niger.

Although reports of the HIV epidemic emerged from the developed and industrialized countries initially, now focus is shifting fast to South-East Asia in which India contributes the major bulk of cases and at present is in an advanced stage of the epidemic in some states of the country (NACO 2000d). The first case of AIDS in India was detected in 1986, since then HIV infections have been reported in almost all states and union territories (WHO 2003a).

Respiratory infections are the major cause of morbidity and mortality in persons with HIV infection. It is clear that with the progression of HIV infection, the function of pulmonary immunocompetent cells declines. There is severe reduction in concentration of pulmonary CD4 cells and impaired cytolytic activity (Murray and Mills 1990a). About 70% of HIV/AIDS patients with infection experience a pulmonary opportunistic infection in life time (Millar 1996). The nature of pulmonary infection of HIV reflects the level of immunodeficiency (Barlett and Gallant 2004).

Infections with Candida species and Cryptococcus neoformans have been recognized as important complications of HIV infection since the early years of the AIDS epidemic. Shortly thereafter, disseminated fungal infections were included among the indicator diseases diagnostic of AIDS, if they occurred in a patient with laboratory evidence of HIV infection. (Murray and Mills 1990b). Aspergillus species have been isolated from a large number of patients with HIV disease or identified at postmortem examination of patients with AIDS (Niedt and Schinella 1985).
In developed countries, Pneumocystis jiroveci is the most common cause of pulmonary disease in patients infected with HIV. A very high incidence of 60 – 80% of Pneumocystis jiroveci (carinii) (PCP) as a cause of pneumonia in AIDS cases has been reported in United States of America and European countries (Hopewell 1988). PCP has become a less common diagnosis of opportunistic pulmonary infections in AIDS since ART has become widely available (Wolf and Donnell 2001). Very few Indian studies have reported PCP in HIV/AIDS. Though PCP is the most common AIDS defining illness in the developed world, in India very low rates (0.7 - 7%) of PCP have been reported (Kumarasamy et al 2005). In studies conducted in Lucknow and Hyderabad showed 0% of prevalence of PCP in AIDS cases. NACO has reported 4% incidence of PCP and Lanjewar et al (2001) in their autopsy study conducted on AIDS patients noted prevalence of 5%.

Though increasing number of AIDS cases are being reported from central India, the data on spectrum of opportunistic infections of respiratory tract in HIV seropositive patients from developing countries as well as from the region is scanty (Sharma et al 2004).

The present study was undertaken to determine the incidence of various fungal pathogens of lower respiratory tract in HIV seropositive patients.

**MATERIAL & METHODS:** Present study was conducted in the Department of Microbiology, Government Medical College and Hospital, (GMCH), Nagpur from August 2004 to October 2005. A total of 108 patients infected with Human Immunodeficiency Virus (HIV) were included in the study.

**Inclusion criteria:** HIV infected patients presenting with signs and symptoms of lower respiratory tract infection. A detailed history and clinical examination were conducted on each patient as per the proforma. Investigations performed (Total leucocyte counts, CD4 counts) were recorded from the respective case sheet. The cases were classified into I through IV stages on the basis of clinical status of the patients. (Appendix 1).

**Collection of sputum:** Direct or induced sputum was collected in all patients. The patients were advised to collect an early morning fresh sample. In patients having non-productive cough, the sputum was induced with 5% hypertonic saline which was given in the form of an aerosol using nebulizer (Pitchenik et al 1986).

**Processing of sputum sample**

1. **Assessment of adequacy of sputum:** Quality of expectorated sputum was assessed both by macroscopic and microscopic examination. Macroscopically any sample that was thin, watery with no purulent matter was considered inadequate. Microscopically adequacy of sputum was assessed by Barlett's scoring (Koneman et al 1997b; Appendix 2-i).

2. **Each sputum sample was divided into two parts.**

   **Part I**  –  Neat sample
   
   **Part II**  –  Concentrated sample. Concentration of sputum sample was done by Petroff’s method (Cruickshank et al 1975; Appendix 2-ii).

   **Part I - Neat sample was used for**
   
   - Microscopy
     - i) KOH mount (Koneman et al 1997f; Appendix 2-iii)
   - Culture
ii) For fungus

Part II - Concentrated sputum sample was used for

- Microscopy
  i) Giemsa stain (WHO guidelines 2001c; Appendix 2-viii)
  ii) Toluidine blue stain (WHO Guidelines 2001b; Appendix 2-ix)

**Culture of the sputum for the fungus:** Each neat sputum sample was inoculated on two Sabouraud's dextrose agar (SDA) with Chloramphenicol 40 mg/1000 ml (Emmons et al 1977b). One tube was kept at room temperature and the other at 37°C in the incubator. Any significant group of fungal species was identified by as per the standard protocol (Rippon 1988b).

**Identification of fungal isolate**

Fungal growth was identified on the basis of:

a) Rate of growth  
b) Texture, colour of surface, folding of growth.  
c) Pigment on reverse

**A) Candida species**

i) Growth on SDA  
ii) Confirmation of the growth

i) Growth on SDA – The colonies appeared in 3-4 days. They were smooth, creamy, and pasty in consistency.

ii) Confirmation of growth

- Gram stain showed gram positive budding yeast cells (Al-doory 1980b).
- Germ tube test – A yeast colony was transferred and emulsified in a tube containing 0.5 ml of serum and incubated at 37°C for 2½ hours. A drop of emulsion was examined microscopically under 45x for the presence of germ tube (Al-doory 1980b).
- Sugar fermentation test – Different sugars like glucose, lactose, maltose, sucrose at a concentration of 2% with Durham’s tube were used (Al-doory 1980b, Rippon 1988b).
- Cornmeal agar test (CMA). A small amount of the yeast growth was inoculated on CMA with the help of a thick inoculating needle, and was cut through the agar to the bottom of plate. A coverslip was placed on the surface of agar. The plate was incubated at 25°C (room temperature) for 2-4 days. The whole plate was then kept on the stage of the microscope and growth was observed through the coverslip under 10 x and 40 x magnification (Al-doory 1980b)

**B) Cryptococcus neoformans**

i. Growth on SDA – Growth was yeast like, highly mucoid, cream to buff-coloured.

ii. India ink - A wet mount of yeast growth with a drop of India ink was prepared for the presence of capsule (Al - doory 1980c)

iii. Confirmation of the growth

Urease test – Yeast like colonies were inoculated on Christensen’s urea agar, development of pink colour was interpreted as a positive test (Al-doory 1980c).
C) Aspergillus species –
    i. Growth on SDA – Texture, colour of the surface, surface folding and of the reverse of fungal growth were noted till the occurrence of sporulation.
    ii. Confirmation of the growth
        Microscopy – Lacto phenol cotton blue (LCB) mount was prepared and arrangement of conidiophores and phialides were noted (Al-doory 1980d, Rippon 1988c).

RESULTS: Epidemiological characters: A total of 108 HIV seropositive cases presenting with the signs and symptoms of involvement of lower respiratory tract were studied. The maximum number of cases (57) belonged to age group 36 years to 50 years followed by age group of 21 years to 35 years with 40 cases. None of the cases was above 65 years of age. A male preponderance was observed in the present study accounting for 85.18% cases.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Number of cases</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Nil</td>
<td>Nil</td>
</tr>
<tr>
<td>II</td>
<td>13</td>
<td>12.03%</td>
</tr>
<tr>
<td>III</td>
<td>68</td>
<td>62.96%</td>
</tr>
<tr>
<td>IV</td>
<td>27</td>
<td>25%</td>
</tr>
<tr>
<td>Total</td>
<td>108</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 1: Distribution of 108 HIV seropositive cases according to the stage of illness.

Patients presenting with symptoms of involvement of respiratory system predominantly belonged to stage III and IV of the disease. Thirteen patients showing symptoms of respiratory system involvement belonged to stage II. None of the patients with stage I had symptoms of lower respiratory tract involvement.

Estimation of CD4 counts was possible only in 27 cases. The counts observed in these patients are shown in table II.

<table>
<thead>
<tr>
<th>CD4 count</th>
<th>Number of cases</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 50</td>
<td>3</td>
<td>11.11%</td>
</tr>
<tr>
<td>50 - 200</td>
<td>15</td>
<td>55.55%</td>
</tr>
<tr>
<td>200 - 350</td>
<td>9</td>
<td>33.33%</td>
</tr>
<tr>
<td>&gt; 350</td>
<td>Nil</td>
<td>Nil</td>
</tr>
<tr>
<td>Total</td>
<td>27</td>
<td></td>
</tr>
</tbody>
</table>

Table 2: CD4 counts in 27 seropositive cases.
The maximum number of HIV seropositive patients with respiratory infections had CD4 counts between 50 and 200/cumm (55.5%). None of the patients with respiratory symptoms had counts > 350 /cumm.

<table>
<thead>
<tr>
<th>Staining Method</th>
<th>Morphological Features</th>
<th>Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Giemsa stain</td>
<td>Budding yeast cells</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>Cyst or Trophozoite of Pneumocystis carinii.</td>
<td>Nil</td>
</tr>
<tr>
<td>Toluidine blue</td>
<td>Cyst of Pneumocystis carinii</td>
<td>Nil</td>
</tr>
<tr>
<td>KOH mount</td>
<td>Budding yeast cells</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>Budding yeast cells and Pseudo hyphae</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Fungal hyphae-thin, septate with dichotomous, branching</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 3: Morphological features in microscopic examination

In the microscopic examination none of the specimens revealed trophozoites or cystic forms of Pneumocystis carinii in Giemsa or Toluidine blue stained smears. KOH mount examination revealed fungal elements in 40 samples.

Oral mucosal lesions due to Candida (thrush) were present in 46 cases. In 20 cases, a large number of pseudo hyphae were present along with budding yeast cells.

The following criteria were applied to label any candida isolate as aetiological agent of lower respiratory tract infection.

(i) Clinical and or radiological evidence of lower respiratory tract infection.
(ii) Absence of oral thrush
(iii) Presence of pseudo hyphal elements in sputum microscopy along with plenty of polymorphonuclear cell.

Even all above criteria are not sufficient to exclude possible contamination of sputum sample from oral yeast elements; hence the incidence can only be taken as indicative.

<table>
<thead>
<tr>
<th>Candida Species</th>
<th>Number of isolates</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Candida albicans</td>
<td>39</td>
<td>36.11%</td>
</tr>
<tr>
<td>Candida tropicalis</td>
<td>15</td>
<td>13.88%</td>
</tr>
<tr>
<td>Candida guillermondi</td>
<td>6</td>
<td>5.55%</td>
</tr>
<tr>
<td>Candida pseudotropicalis</td>
<td>6</td>
<td>5.55%</td>
</tr>
<tr>
<td>Total</td>
<td>66</td>
<td>61.11%</td>
</tr>
</tbody>
</table>

Table 4: Incidence of various Candida species in sputum samples.

<table>
<thead>
<tr>
<th>Candida Species</th>
<th>Number of isolates</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Candida albicans</td>
<td>16</td>
<td>14.81%</td>
</tr>
<tr>
<td>Candida tropicalis</td>
<td>2</td>
<td>1.85%</td>
</tr>
<tr>
<td>Candida guillermondi</td>
<td>2</td>
<td>1.85%</td>
</tr>
<tr>
<td>Total</td>
<td>20</td>
<td>18.51%</td>
</tr>
</tbody>
</table>

Table 5: Incidence of Candida species causing pulmonary infection
Moulds were recovered from 2 sputum specimens. Both belonged to Aspergillus species. Thin, septate fungal hyphae with typical dichotomous branching were observed in these 2 sputum specimens in direct microscopy of wet mounts. Considering morphology on SDA and microscopic morphology in Lacto phenol cotton blue (LCB) mount, one species was identified as Asp. flavus and other was Asp. niger.

DISCUSSION: The hallmark of infection with HIV is development of progressive immunodeficiency (Wallace JM 1994). The opportunistic infections occur as a result of progressively severe immunodeficiency and are the primary clinical problems associated with HIV infection. About 70% of patients with HIV infection experience a pulmonary opportunistic infection in lifetime and are the cause of death in at least 1/3rd of all AIDS patients (Millar 1996).

In the present series, the maximum number (89.81%) of cases belonged to age group 21-50 years. HIV seropositive survey conducted have shown higher rate of HIV infections among people in 20 to 45 years of age (Folks and Khabbaz 1998). A male predominance was observed in the present study accounting for nearly 85% of cases. In a study conducted by Sharma et al (2004) on 135 patients, male contributes to age between 34 +/- 10 year and female 17%. Similar finding has also been reported in the studies conducted elsewhere in India (Kumaraswamy and Solomon 1995, Ayyagari et al 1999).

HIV disease is a continuous progressive disease; the median time from initial infection to symptomatic disease is being approximately 10 years (Folks and Khabbaz 1998). Several staging systems based on specific clinical and laboratory criteria have been proposed. In the present study, on the basis of clinical findings, the cases were classified into four different categories using WHO guidelines (WHO 2004a). The majority of cases with respiratory findings could be grouped in stage III and IV (87.96%) in the present study. It has been reported that most patients with early HIV disease experience relatively minor, self limited respiratory disease primarily consist of upper respiratory tract infection and acute bronchitis. HIV infected person with moderately advanced immunocompromised state face a continuously increasing risk of bacterial pneumonia and Pneumocystis carinii pneumonia (PCP).

Some laboratory tests have been found to be useful for monitoring the course of HIV infection and predicting progression of the disease. The CD4 count has been most widely used. The absolute number of circulating CD4 lymphocytes has been shown to be a clinically useful indicator of immune function in HIV infected person (Masur et al 1982). It is initially normal and then declines by about 40 - 80 cells/mm$^3$ per year. In the present study estimation of CD4 counts was feasible only in 27 cases as in the initial phase NACO had kept the test chargeable & the patients were not willing to pay the charges of Rs 500/- In as many as 15 of these cases, the counts were between 50-200/mm$^3$. In 3 cases the counts were below 50/mm$^3$ and none of these cases with respiratory symptoms had the counts more than 350/cumm. Opportunistic infections including PCP, have been reported to occur during late symptomatic or advanced stages in patients with low CD4 counts, of usually less than 200 cells/mm$^3$ (Wallace JM 1994).

Toluidine blue staining and Giemsa staining techniques were used in the present study for the demonstration of Pneumocystis carinii in the sputum. In our series, no specimen revealed forms suggestive of Pneumocystis carinii. A very high incidence of 60-80% of Pneumocystis carinii as a
cause of pneumonia in AIDS cases has been reported in western literature (Hopewell 1988). Induced sputum examination has been shown to have sensitivity between 50 and 60% (Pitchenik et al 1986); though patients with PCP rarely produce sputum unless secondary infection is present (Hopewell 1988). Examination of sputum induced by inhalation of aerosolized of hypertonic saline is very useful means of identifying Pneumocystis carinii (Hopewell 1988) however, we did not encounter any positive findings even in the induced specimen in our study. In study conducted in Mumbai on 300 patients, PCP was found in 13%. Median CD4 count of PCP was 96 cells/cumm and bronchoscopy was needed to make a definitive diagnosis in 17 of 38 patients (Udwadia et al 2005). Fishman (1998) has reported incidence of more than 80% of PCP during the course of disease in AIDS patient not receiving prophylactic antibiotics, (Stover et al 1984, Pitchenik et al 1986, Barry and Johnson 2001) PCP was considered as a significant cause of morbidity and mortality in pre anti-retroviral therapy (ART) era. PCP has become a less common diagnosis of opportunistic pulmonary infections in AIDS in the year since ART has become widely available (Wolf and Donnell 2001, Dufour et al 2004). Fishman (1998) believes that the change in the spectrum of opportunistic infections in AIDS reflects factors including use of anti-PCP prophylaxis, prolonged survival of patients on anti-retroviral therapy, improved clinical care and recognition of new or drug resistant organisms. Though PCP infection in HIV/AIDS patients is rampant in Western countries it is rare in equatorial Africa (Clumeck et al 1984). Very few Indian studies have reported PCP in HIV/AIDS. Lanjewar et al (2001) and NACO (2000) have reported incidence of 5% and 4% respectively, in India. Ayyagari et al (1999) studied 46 patients and Shailaja et al (2004) 100 patients and they also did not encounter any case of PCP during their study on pulmonary opportunistic infections in AIDS. The low incidence of PCP in India can attributed to the extensive use of co-trimoxazole for prophylaxis, non-availability of modern sensitive diagnostic techniques like immunofluorescence and predominance of other pulmonary diseases like tuberculosis (Kumarasamy et al 2005).

Fungal elements were revealed in 40 sputum specimen in KOH mount examination; budding yeast cell and pseudolophae were seen in 20 specimens and 2 showed presence of thin septate fungal hyphae with dichotomous branching indicating etiology of mould in this specimen.

CONCLUSION:

- Fungal elements were revealed in two cases in direct microscopy. KOH mount examination revealed presence of pseudo hyphae and yeast cells in 20 (18.51%) cases.
- None of the HIV seropositive cases with symptoms of LRT involvement revealed presence of PCP in Toluidine blue and Giemsa stained sputum smears, indicating very low prevalence of PCP in this region.

REFERENCES:

ORIGINAL ARTICLE

Appendix-1: As per World Health Organization.


Clinical staging of HIV / AIDS infection

A) Clinical Stage – I
1. Asymptomatic
2. Persistent generalized lymphadenopathy (PGL)

B) Clinical Stage - II
3. Weight loss < 10% of body weight.
4. Minor mucocutaneous manifestations (Seborrheic dermatitis, fungal nail infections, recurrent oral ulcerations, angular cheilitis).
5. Herpes zoster within the last 5 years.
6. Recurrent upper respiratory tract infections (i.e. bacterial sinusitis).

C) Clinical Stage – III
7. Weight loss > 10% of body weight.
8. Unexplained chronic diarrhea > 1 month.
9. Unexplained prolonged fever > 1 month.
10. Oral candidiasis (thrush)
12. Pulmonary tuberculosis within past year.
13. Severe bacterial infection (i.e. pneumonia, pyomyositis).

D) Clinical Stage IV
14. HIV wasting syndrome, as defined by CDC.
15. Pneumocystis carinii pneumonia.
17. Cryptosporidiosis with diarrhoea > 1 month.
18. Cryptococcosis, extrapulmonary.
19. Cytomegalovirus (CMV) disease of an organ other than liver, spleen or lymph node.
20. Herpes simplex virus (HSV) infection, mucocutaneous > 1 month or visceral any duration.
22. Any disseminated endemic mycosis (i.e. histoplasmosis, coccidioidomycosis).
23. Candidiasis of the oesophagus, trachea, bronchi or lungs.
25. Non-typhoid salmonella septicemia.
27. Lymphoma.
28. Kaposi’s sarcoma (KS)
29. HIV encephalopathy, as defined by CDC.

**Infectious Agents Commonly Associated with AIDS (Fishman 1998)**

**Viral (with HIV – 1, HIV-2)**
- Cytomegalovirus
- Herpes simplex
- Herpes zoster
- Epstein–Barr virus.
- Parvovirus B19
- HHV-6, HHV-8
- HTLV-1, HTLV – 2.

**Parasite**
- Toxoplasma gondii
- Cryptosporidium
- Isospora belli
- Microsporidium species
- Cyclospora species

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