Progress in diagnosis of opportunistic infections in HIV/AIDS

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The impact of HIV/AIDS is different in developed and developing countries including India. Limited access to health care facilities, lack of infrastructure for diagnostic set up and cost of anti retroviral therapy are some of the reasons. Early accurate diagnosis of opportunistic infections (OIs), the common presenting symptom of the patients, is the key for success of effective management. This review attempts an overview of few of the important OIs with which majority of Indian patients present in the clinics. Emphasis has been on conventional method of diagnostic approach, which is possible in most of the diagnostic laboratory set up in India. Awareness of the disease and maintenance of high index of clinical suspicion are required. An integrated approach to patient management with active interaction between clinicians and microbiologists would be highly beneficial. Introduction of routine in vitro antimicrobial testing system especially for Candida sp, Cryptococcus sp and Mycobacterium sp, is also important, in order to obtain a baseline data on the susceptibility pattern, which not only have therapeutic relevance, but also can predict in advance, any shift in these patterns in the Indian population. Administrative support for the skill development of personnel, facility for data preservation and telemedicine can extend the diagnostic expertise to the remote areas, without affecting the patients mobility.

Key words Antifungal susceptibility test - Candidosis - Cryptococcosis - opportunistic infections - Penicillosis marnefii - Pneumocystis carinii - tuberculosis

Definition of the problem: There is a striking contrast in the awareness and impact of HIV /AIDS between developed and developing countries. In USA and Europe, about 75 per cent of infected people are aware of their HIV serostatus, where as about 80-90 per cent of infected people in developing countries, including in India, have never been tested for HIV and remain unaware of their infection1-5. The reason for this is multifactorial including: (i) limited mass access to health care facilities and (ii) lack of basic infrastructure for early diagnosis of primary HIV infection (PHI) which includes expertise, equipment, reagent/s, and insufficient funds to support awareness/preventive campaigns and also specific treatment like highly active antiretroviral therapy (HAART). Thus the morbidity and mortality from this infection continue to rise. Early accurate diagnosis of opportunistic infections (OIs), the common presenting symptoms of this group of patients, may help in effective disease management. With careful investigations on the present status of the prevailing agent causing OI may also predict the individual’s HIV sero-status6, 7.

The infective organisms responsible for OIs differ in characteristics from that of conventional communicable disease, and are mainly low or non virulent. Hence, these could be, nonpathogenic in an
individuals with intact immune system (*Candida albicans*) or known pathogens presenting in a different way than usual in immunocompetent individuals (*Cryptococcus neoformans*) or in the form of increased virulence, recurrence, multi-drug resistance (*Mycobacterium tuberculosis*) or atypical presentation (dermatophytosis)\(^7\)\(^-\)\(^9\).

The clinical manifestations of HIV infection in India (like other developing countries) are diverse. Spectrum of OIs with which most of the patients present in the clinics, reflects a wide variety of other endemic diseases prevalent within each region. In contrast to the developed world, a small number of opportunistic pathogens cause a majority of the clinical infections in India. *Pneumocystis carinii* (syn. *P. jeroveci*)\(^10\) are rarely documented in India\(^11\)\(^-\)\(^13\) . Few reports are available for *Toxoplasma gondii* infection\(^14\)\(^,\)\(^15\). *Penicillium marneffii*, one of the emerging AIDS defining infections, reported only from North Eastern State (Manipur)\(^16\). While maximum number of reports are available on important mucocutaneous lesions like oropharyngeal candidosis (OPC)\(^9\)\(^,\)\(^13\)\(^,\)\(^17\)\(^-\)\(^27\), status of valvovaginal candidosis in HIV-positive women is not known in India. The reasons could be few environmental exposures, difference in host susceptibility, earlier death owing to exposure to more virulent organisms and diagnostic difficulties are some of the factors. Other contributory factors may be the prevalence of particular pathogens in an environment, ecological factors that result in exposure to these pathogens, and paucity of experienced and trained personnel to look for these infections\(^5\)\(^,\)\(^7\)\(^,\)\(^8\).

In recent years the definition of conventional OI is changing and the concept is developing purely on basis of ‘host- parasite’ interaction\(^28\). In this review we intend to focus mainly on the fungal OIs, as it is one of the early manifestations with which Indian HIV positive patients present in clinics\(^9\), diagnostic clinical mycology set up and expertise available only in few institutions\(^7\)\(^,\)\(^8\). Experiences at the All India Institute of Medical Sciences (AIIMS), New Delhi, one of the premier tertiary care centres in India, will be highlighted.

Determining the spectrum of OIs and the changing pattern over the years, in a given region requires adequate surveillance and good diagnostic services that are not available in many parts of the country. OI that can be diagnosed with reasonable accuracy by physical examination (oral candidosis) or by inexpensive laboratory techniques (India ink), may be documented more frequently than OI requiring more cumbersome procedures of collection of specimen, *P. carinii* (syn. *P. jeroveci*), or that requires more expensive diagnostic technology (viruses). Besides biases in diagnosing and reporting, OI may be important among socially disadvantaged groups with limited access to diagnostic and health care services (women, elderly, and children). Further, difference in clinical definitions may make comparison between published reports difficult. Thus much less is known about frequency of different OIs in India in the backdrop of HIV/AIDS. In addition, a greater exposure to a wide variety of relatively virulent pathogens (*M. tuberculosis*) or lack of access to medical care and treatment, rather than inherent difference in the rate of decline of immunological function\(^1\)\(^-\)\(^5\) may also be responsible of present state of knowledge in India.

In India, quite often the diagnosis of OI is made only on clinical signs and symptoms or when illness is quite advanced, and by then it may be polymicrobial in nature\(^29\)\(^,\)\(^30\) . There is paucity of reports about nature of etiological agents causing various clinical manifestations in HIV disease\(^9\)\(^,\)\(^25\). Hence, integrated investigative procedures are vital, specially in early stages of HIV positivity.

**Candidosis and Cryptococcosis as co-infection in HIV**

Mucocutaneous candidosis is probably one of the commonest manifestations of HIV-positive status worldwide with oropharyngeal candidosis (OPC) being most widely reported. In India, its incidence has been reported from 50 to 100 per cent\(^9\)\(^,\)\(^13\)\(^,\)\(^17\)\(^-\)\(^27\) . Type of lesions may vary and some of the patients may lack classical picture of oral thrush especially when CD 4 count is quite high. In our recent study on OPC in 125 HIV-positive patients\(^27\), we found that patients can present with gingivitis, glossitis, while the unusual manifestations like generalized cutaneous, non healing ulcers, perineal cutaneous lesions or
disseminated candidosis were first reported in our initial studies with agent isolated only C. albicans17-19. Several studies since1992, with increased number of patients with OPC, still show the prevalent isolate as C. albicans, though emergence of non albicans Candida species, in concordance with experience of other Indian investigators, has also been reported20-27. Surprisingly, C. dubliensis, one important species of Candida reported in OPC mainly from abroad31 is still not seen in Indian HIV-positive population with infection; barring only one report from a carrier32. The reason for this discrepancy is difficult to explain, however, the type of oral hygiene in different groups of patients along with their flora before active infection may have some role27. This study also revealed good correlation of extent of lesion along with CD4 count. It suggested that initiation of HAART treatment may be indicated on physical assessment of OPC in HIV-positive patient which indirectly can reduce the cost of investigation for CD4 count27. Candida oesophagitis, one of the AIDS defining infections, scarcely reported in Indian HIV-positive patients, is also seen in a non immune suppressed patient in southern India33. Same also applies on Candida vulvovaginitis33. Probably it may be due to the cumbersome procedure of collection of sample and lack of enthusiastic investigator specifically looking for this type of lesion. Gender bias for seeking medical help, common in India, cannot be ruled out.

We have found different species of Candida (like C. glabrata) causing majority of infection in vulvovaginitis in Indian diabetic women34. Species identification has therapeutic impact also, as C. glabrata has inherent resistance to commonly used fluconazole27,34.

Inexpensive conventional diagnostic test can differentiate majority of common pathogenic species of Candida35,36 (Fig. 1).

Cryptococcosis, one of the AIDS defining infections, once considered as “sleeping disease”, became an “awakening giant” within a couple of years, and has now been predicted as the “Mycosis of the future” with a predilection that for every million patients with AIDS, 50,000-100,000 will contract cryptococcosis37,38. Chronic meningitis is a main manifestation of this infection. Reports of it, from various states of India have increased more in the AIDS era. However, a review in 20017, on the status of cryptococcosis in India strangely reveals more cases from the northern part, where the HIV prevalence rate is low compared to high HIV prevalent states in the southern or western India (Fig. 2). Since then the overall scenario has not changed much. This discrepancy probably is due to under reporting and misdiagnosis of cases.
Over the years, three successive studies expanding a period of 12 yr (1992-2004), in our laboratory, revealed that parallel to increase in number of HIV-positive patients at AIIMS, HIV co-infection with *Cryptococcus*, has also increased from 20 per cent in 1992-1996 to 37 per cent in 1996-2000 to 49 per cent in 2000-2004 (Fig. 3). This is probably the first study which observes the successive increase of cryptococcosis in this group of patients. Interestingly, the recent study (2000-2004) (Banerjee and Jain-unpublished data), we also found that 23 per cent of HIV positive patients presented with non-meningitis manifestations especially with pyrexia of unknown origin (Fig. 4).

Paediatric cryptococcosis which was rare in earlier days, is now being increasingly reported from India, and in HIV-positive children manifestation may be both of chronic meningitis and PUO (cryptococcaemia). In case of cryptococcal meningitis therapeutic management may improve the condition. However, our experience reveals that once cryptococcaemia establishes, patients hardly survives even after proper dosage of specific antifungals. Hence, early detection is critical.

Biological amplification or positive culture is gold standard for documented infection. In contrast to the colony morphology of different *Candida* species, which is quite homogenous, we have seen different colonial variety in case of *Cryptococcus*. There is no correlation between capsule size, (direct microscopy) serotype / variety status and colony morphology, in isolates from HIV-positive patients. Both smooth and mucoid forms have been observed in these isolates in addition to few dry irregular, sectored colonies, first time recorded in Indian patients.

Pathogenic role of the clinical isolates of *Cryptococcus* needs to be tested for production of melanin. Melanin negative albino isolate is considered as non-pathogenic. Surprisingly we have isolated and identified melanin negative albino strains in two patients and in one exceptional case of HIV-positive patients with chronic meningitis there was a dual infection by both melanin positive and negative strains of *C. neoformans* (var grubii) . Whether, these isolates are with some genetic variation of the same strain or two entirely different strains causing infection is presently under investigation by molecular characterization in our laboratory (Banerjee and Mandal-unpublished data).
Identification of serotype/variety status of isolates of Cryptococcus has significant relevance on the therapeutic outcome. Majority of the isolates from HIV-positive patients in India, are serotype A, var grubii, consistent with the fact that this is the most common serotype worldwide. However, in contrast to the conventional belief that var gatti (serotype B) does not infect HIV-positive patients, there are few reports, both from north and south India, about the isolation of it in HIV infected patients. This indicates the widespread reservoir of this agent in India, which is evidenced by a significant epidemiological study from the north. Identification of this variety of the isolate is particularly important as it is relatively refractory to the treatment and prolonged course and/or increased dosage of antifungals may be necessary. Besides, sequale of cryptococcosis is much more in gatti variety.

Serology, detection of antibody and/or antigen and/or metabolites, is one of the important adjuncts for supportive evidence of any infection. For instance, in case of cryptococcosis, polysaccharide antigen detection is one of the important supportive evidences of infection. Of the various available test systems, latex agglutination (LA) is a popular test. This test, though expensive is presently used in many diagnostic laboratories in India. However its utility in diagnosis of case of cryptococcosis in HIV-positive patients is superfluous, as in this group of patients with symptoms of meningitis even a few cells in CSF can be demonstrated by rapid and cheap method like India
ink/Nigrosin, antigen detection by expensive commercial kit may not be always necessary. Once antigen is detected in the system, it takes long time to get it cleared even after, microbiological and clinical cure. Besides, performance of different commercial kits varies. In an in vitro study using the isolate from HIV positive patient it was observed that antigen can be detected in the suspending extracellular fluid even when Cryptococcus was not detected by direct microscopy (by Gram stain/ India ink) or grown on culture. Detection of only antigen does not always indicate active infection. Direct demonstration of capsulated budding yeast cells followed by subsequent culture may be enough to start specific antifungal therapy, which is vital for this serious often fatal infection.

Follow up and monitoring is necessary in this group of patient, as relapse rate is quite high, even after initial successful therapeutic outcome. It is believed now, that like tuberculosis, relapse in cryptococcosis, may also be due to re-activation.

In vitro antimicrobial test is one of the integral part of a diagnostic set up in clinical microbiology. In contrast to wide application of the in vitro antibacterial susceptibility test, development and adoption of in vitro antifungal test is still in infancy in India. This test system is essential for therapeutic guidance in fungal OI in HIV disease, particularly in case of fluconazole, widely used triazole, which is being used for prophylaxis of OPC and life long maintenance therapy in patients who have been treated for chronic meningitis with amphotericine B.

Few institutions have adopted the guidelines laid down by the National Committee of Clinical Laboratory Standards while others are using indigenously developed test. We have optimized NCCLS macro- and micro-broth dilution and conventional agar dilution techniques especially for Candida species and Cryptococcus species. We observed that in contrast to the worldwide report of appearance of growing population of fluconazole resistant C. albicans, only 6 per cent of our isolates from OPC were resistant against fluconazole. On the other hand, isolates of Cryptococcus, though not overly resistant, 16 per cent showed higher minimal

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**Fig. 5.** Morphological variations in colony of C. neoformans

**Fig. 6.** Simultaneous isolation of the melanin positive and negative strain in L-Dopa medium, by glass bead method. White arrow showing brown melanin positive strain; black arrow indicates melanin negative strain.
inhibitory concentration (MIC) against fluconazole. Amphotericin B resistant *C. neoformans* infection has been experienced in Tuberculosis Research Centre, Chennai (TRC) in one of their HIV-positive patient (Soumya Swaminathan-personal communication). Situation though is not alarming at this point, surveillance programmes need to continue, to forecast the advent of a resistant population.

We feel, NCCLS M27A, test is quite cumbersome, labor intensive, expensive and where reproducibility is variable. It is recommended for the use only in referral laboratory, where specific skill and expertise are available. Probably, NCCLS M44 and M44A disk diffusion technique will be more useful in routine diagnostic service. This is currently being investigated in our laboratory. Strains showing resistance by NCCLS (M27A), must also be checked by conventional agar dilution method, simultaneously, to ascertain its true resistance nature, as also *in vivo* clinical correlation with the patient on therapy. A team of clinician and clinical microbiologist, should take care of HIV patients/samples. Intra-laboratory network for the exchange of strains can generate documented and reproducible data for Indian isolates.

**HIV co-infection with other fungi**

*Pneumocystis carinii* (syn. *P. jeroveci*) which was one of the important OI with which patient first reported and diagnosed as HIV disease, rarely documented in Indian patients, except for few occasional reports which varies from 6-10 Per cent. Why *P. carinii* is uncommon in Indian HIV-positive patient is not known, however, collection of appropriate sample before specific treatment is a major hurdle. In an earlier prospective study in our laboratory, sputum samples from 53 HIV-positive patients, clinically diagnosed as interstitial pneumonia, were screened conventionally for the presence of *P. carinii* (syn. *P. jeroveci*). The organism could be detected only in 4 patients (7.54%) in an ongoing prospective study on more than 100 bronchoalveolar lavage (BAL) fluid samples of known HIV-positive patients, using calcoflour white and silver methanamine staining as a tool for direct demonstration, *P. carinii* could be demonstrated in only one sample (Fig. 7). This confirms the rarity of the infection in India.

*Penicilliosis marnefii,* is a new emerging fungal disease in HIV-positive patients. It is surprising that earlier reports mainly confined to the patients from North Eastern region of India (Manipur), where reservoir of this infection had been identified as bamboo rat.

Mere isolation of *Penicillium* with red pigment does not necessarily indicate the species *P. marnefii.* Direct demonstration of the yeast with characteristic septa in clinical specimen followed by successful culture is a definitive diagnostic criterion. However, once isolated, its thermal dimorphism needs to be proved by growing the isolates at two different temperatures and by suitable animal pathogenicity.
Tuberculosis as a co-infection with HIV

Tuberculosis is an important invasive OI, seen as HIV co-infection. Amongst the large number of reports available pulmonary tuberculosis is being reported more than any other clinical forms. Detailed information about HIV/TB co-infection is available elsewhere.

All forms of tuberculosis, in HIV-positive patient, except when cavitation occurs, are paucibacillary in nature. Hence collection of appropriate sample and extensive search of evidence for presence of bacilli, on repeated specimens or any other supportive evidence (like PCR) is essential to be sure of definitive diagnosis. Microscopy of sputum is of great value in detection of open or infectious cases of tuberculosis. In developing countries including India, the establishment of a good sputum microscopic service is of prime importance for detection and treatment of open cases.

Smears are stained by Ziehl-Neelsen (ZN) or by one of its various modifications. Grading of the positive smears gives a broad indication of the severity of disease and the response to therapy during treatment of the patient. Direct fluorescence has been used in some laboratory for rapid detection of the agent however, it needs fluorescent microscope and specialized skilled personnel. Culture should be attempted in all suspected cases. Modified Petroff’s method followed in most of the laboratories. At AIIMS, New Delhi in addition, CDC, recommended NALC-NaOH (N-acetyl L-cysteine) concentration technique is being followed for better chance of isolation of bacilli.

There are very few reports of non-tuberculous mycobacteria (NTM), M. avium complex (MAC) before the AIDS era, but the scenario has changed during the present AIDS pandemic in the developed world, but hardly reported in Indian patients. In a prospective study at AIIMS, New Delhi, 97 clinical samples investigated microbiologically (sputum 84, CSF 11, pleural fluid 1, biopsy 1) collected from 94 HIV seropositive patients, revealed that the presence of NTM (57.2%) was slightly higher than M. tuberculosis (42.8%) in the study group. The test was based on conventional techniques of smear/or culture. NTM were detected on smear as well as isolated on culture repeatedly, without any other known respiratory pathogens including M. tuberculosis. These were isolated from 2-3 consecutive sputum samples, confirming the etiology. All patients in this study had advanced stage of HIV disease with CD4 count < 200/µl. (Singh and Khatter – unpublished observation). Of the 17 positive cultures of Mycobacteria, 14 could be identified up to species level depending upon the conventional biochemical reactions. This study also revealed the distribution of species amongst 8 NTM as MAC 3, M. fortuitum 2, M. phlei 2 and M. vaccae 1.

Multi drug resistance (MDR) in M tuberculosis infection is a major problem. In India, the prevalence of MDR TB has increased from zero in 1982 to 10.7 per cent in 1995. However, its relation to isolates from HIV-positive patient is hardly reported.

Parasitic infection as co-infection with HIV

Parasitic diseases like cryptosporidiosis, microsporidiosis, isosporiosis and cyclosporiosis, either singly or in different combinations, have been well documented in Indian patients, pre- and post- mortem. These are diagnosed by direct demonstration of the agent in concentrated stool samples, by modified acid fast stain or safranin methylene blue stain, modified trichrome stain / Gram chromotrop stain / modified acid fast stain. In a recent case report from our laboratory, simultaneous co-infection with three coccidian parasites, Cyclosporidium, Isospora and Cyclospora has been demonstrated in a HIV positive child.

Toxoplasmosis, a zoonotic disease caused by ubiquitous intracellular protozoan Toxoplasma gondii, with worldwide distribution. It has emerged as an important OI in HIV disease however; there is paucity of reports from Indian patients.

Other bacterial co-infection with HIV

Though conventionally infected by bacteria with low virulence, at the same time HIV positive patients...
are not spared by classical virulent ones either. Important OIs like *Rodococcus equi* or *Nocardia* though reported from abroad, are hardly seen from Indian patients. Spectrum of bacterial infection is changing, towards the tilt of infection caused by conventional pathogen.

**Other viral co-infection with HIV**

Any virus can infect HIV infected patients. Important OIs are herpes simple virus (HSV), varicella–zoster virus (VZV) and cytomegalovirus (CMV). Herpes simplex virus type 1 (HSV1) and type 2 (HSV2) cause primary and recurrent oral, genital and rectal ulceration and occasionally disseminated visceral, CNS disease and VZV lesions. In HIV infected persons, re-activation of VZV causes prolonged and severe manifestation of herpes zoster. CMV is responsible for greater proportion of OI in HIV-infected persons with advanced immune deficiency. Retinities is most frequent clinical manifestation of CMV though other manifestations like gastrointestinal disease, encephalitis and pneumonia may occur.

The other viruses associated with HIV infected persons include Epstein Barr virus (EBV) Kaposi’s sarcoma-associated herpes virus (HHV8), human papilloma viruses (HPV), molluscum contagiosum virus, hepatitis B virus (HBV) and Hepatitis C virus (HCV). Detailed information of HIV HBB/HCV co-infection is available elsewhere.

**Concluding remarks**

Recent advances have improved our prophylactic, diagnostic and therapeutic capabilities for a variety of infections / diseases. Diagnostic capabilities of various OI, specifically those commonly seen in HIV disease, can be done with the tools now available in India. Early effective diagnosis depends upon high index of clinical suspicion followed by good laboratory practice. Absence of pathognomonic signs and symptoms suggestive of classical manifestations of particular infection in this group of patients often makes it difficult to recognize it, especially in its early stage when it is relatively easy to manage the patient. Taken as one instance is that in India majority of cases with PUO, with which a large number of HIV infected patients present in the clinics quite often diagnosed clinically as ‘tuberculosis’ and anti tubercular treatment (ATT), in various combination is often instituted empirically. Only in absence of clinical response (the trial period varies according to choice of physician) are the other possibilities considered. This time lag is critical especially in immunocompromised patients in particular in HIV infection.

Diagnosis is still by and large based on conventional method of direct demonstration, histopathology, as well as culture, and on rare occasions special confirmatory test such as animal pathogenicity are used. For evidence of invasive disease organ-wise, classical biopsy is preferred specially in early stage of infection, as it can detect small number of agent in the tissue as well as surrounding tissue reaction, though some investigators prefer fine needle aspiration cytology (FNAC). Conventional diagnostic procedure may not always offer the expected discriminatory power. It mainly depends upon the stage of the disease, suitable collection of appropriate specimen in adequate amount and proper processing in the laboratory. For example, to differentiate from colonization to the actual pathogenic role of a particular microbe, multiple samples, specifically from the site that is normally not sterile, is advisable. For definitive diagnosis of OI, repeated demonstration and / or isolation of the same microbe from the same site of lesion or same microbe from multiple sites (depending on clinical presentation) is essential. Stringent adherence of protocol for evidence of ‘documented’, ‘possible’, and ‘probable’ infections are needed when an unusual agent with low virulence is seen in a clinical specimen. Quality control needs to be maintained at each step, starting from collection to the processing of samples in the laboratory till the final identification. Knowledge about proper collection and transportation should be disseminated amongst medical and paramedical staff.

A separate investigative procedure specifically for OI may not always be possible in many institutions. Only a few of the premier institutions have taken a lead in this respect, for instance, at AIIMS, New Delhi special laboratory facility for the investigations for
infections in immunocompromised patient (ICP lab) has been established in the department of Microbiology. Telemedicine adapted in such institutions, can extend the diagnostic expertise to the remote area, without affecting the patients mobility.

Scenario of present status of OIs is expected to change in highly active antiretroviral therapy (HAART) era. Improvement, upgradation and networking of an effective data preservation system specially that of the laboratory documented cases of unusual agents causing OIs, is essential of the Indian institutions.

A high level of alertness is needed at both clinical and laboratory level and routine surveillance studies need to be undertaken. Institutions in India and other developing countries need to be equipped to face the emerging challenge, in the form of updating the present knowledge, by way of education and training of the personnel, acquisition of skills of improved procedures, and their implementation in appropriate settings with adequate administrative support.

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