DISCUSSION
5. Discussion

Over the past two decades, the incidence of the fungal infection has increased dramatically. The morbidity and mortality associated with these infections are substantial, and it is clear that fungal diseases have emerged as important public health problems. The array of opportunistic fungal pathogens is almost unlimited (Schell, 1995).

There are number of the new development in fungal infection these included (i) changes in fungal infection epidemiology (ii) there is a new understanding of pathogenesis (iii) there are new methods of diagnosis and (iv) new approaches for treatment (New formulations and the combination therapy).

Given the degree of immunosuppression that may be generated iatrogenically or secondary to HIV infection, any fungus present in the environment may cause localized or invasive infection when introduce into the appropriate host.

There is a striking contrast in the awareness and impact of HIV/AIDS between developed and developing countries. In USA and Europe, about 75 percent of the infected people are aware of their HIV status, whereas about 80-90 percent infected people in developing countries, including in India, who have never been tested for HIV and remain unaware of their infection (Banerjee, 2005; United Nation AIDS program 1998; WHO 1999). The reasons for this are: (i) limited mass access to health care facilities and (ii) lack of basic infrastructure for early diagnosis of primary HIV infection. Early accurate diagnosis of the opportunistic infections is the key for success of effective HIV/AIDS disease management. The infective organisms responsible for opportunistic infections differ in characteristics from that of conventional communicable disease, and are mainly low or non-virulent. These could be nonpathogenic in an
individuals with intact immune system (C. albicans) or known pathogens presenting in different way than usual in immunocompetent individuals (Cryptococcus neoformans) or in the form of increased virulence, recurrence, multidrug resistance (Mycobacterium tuberculosis) or typical presentation (Dermatophytosis) (Banerjee et al, 2001; Banerjee, 2005; Marques et al, 2000).

In the recent years the definition of conventional opportunistic infection is changing and the concept is developing purely on the basis of ‘host-parasite’ interaction (Casaddevall & Pirofski, 2002). The clinical course of HIV disease and pattern of opportunistic infections varies from patient to patient and from country to country. Occurrence of various AIDS associated illness determines disease progression (Kumarasamy et al, 2005). Highly Active Antiretroviral Therapy (HAART) has led to a considerable decline in the HIV disease progression rate and HIV related opportunistic infections, especially in developed countries. Unfortunately, antiretroviral treatment for almost 90% of the HIV infected population is not available because of cost concerns in the developing countries (Saksena & Smit, 2005)

Oropharyngeal candidiasis was at the top of the list of the opportunistic infections in HIV/AIDS patients before the era of HAART. Although the incidences of the opportunistic infections have been reduced around the globe by HAART the situation remains the same in most of the developing countries, including India where patients can barely afford this treatment. Oropharyngeal candidiasis is commonest opportunistic infection seen in Indian HIV/AIDS; however most of the reports are based on clinical observations. There are very few systematic studies on species-specific isolation of Candida, its pattern of antifungal susceptibilities, from Indian HIV positive patients, which is vital for
Discussion

patient management. Moreover, no serious attempt was made so far to detect the source of primary infections and genetic relatedness of the strains. This prospective study was an attempt mainly to analyse the present status of oropharyngeal candidiasis in HIV/AIDS patients. This study affirmed that about 75 percent of the patients studied, had oropharyngeal candidiasis, which is lower than the western figure of about 90% (Fiedl, 2002; Holmstrup et al, 1990; Odds, 1988; Vazquez 2000). One can speculate that probably Candida carriage rate in Indian population is less. It can be assumed the kind of the tradition and practices related to Indian populations is different, same practices are not there among western populations. These practices might be having some role to reduce the carriage of Candida among Indian population. For example diet in India encompasses diversity unknown to most western countries, with many dietary patterns emanating from cultural and religious aching that have existed for thousands of years. Indian food contains different verities of spices, some items used as spices are a medicinal herbs or seeds. Also high percentage of Indian population is vegetarian and factors involved in vegetarianism in India are religious or ethical beliefs. Income considerations or poverty, and because of this reason Indian population are less alcohol consumers and less smoking compared to western populations as alcohol consumption might has a role in the oral Candida carriage and infection. Indian populations also have habit of chewing betel nuts and other several preparations. The chewing habit will increase the salivary flow rate, which considered as one the main factor, which may be reducing the Candida oral carriage. However, these are hypothesis factors and speculations they need further detail objective study to prove them.
There is not much change observed in predominant species of *Candida* reported in the present study versus the report in 1993 from the same institution, where *C. albicans* was also heading the list of all the isolates (Mirdha et al, 1993) in spite of the fact that presently studied patient’s population was much larger and the duration of the study was much longer. A small percentage of other species have also been isolated as *C. parapsilosis* (8%), *C. glabrata* and *C. krusei* (3%) each. Most interesting is that no *C. tropicalis* or *C. dubliniensis* was isolated. This is a surprising observation compared to the reports from developed world, where non-*C. albicans* Candida is a major problem in HIV/AIDS (Hoegal et al, 1998; Jabra-Rizk et al, 2001; Martinez et al, 2002; Meeus et al, 2002; Tumbarello et al, 1999). Antifungal drug prophylaxis is rarely practiced in India and this may be the reason for this observed fact (Lattif et al, 2004). Non-*C. albicans* Candida is not a major problem at this point of time in Indian HIV/AIDS patients in this particular hospital where the study was conducted.

Currently, the most widely used drug for treating candidiasis is fluconazole, a triazole drug that can be administrated orally and is effective for oral and invasive candidiasis (Maenza et al, 1997; Makarova et al, 2003; Perea et al, 2001; Rex et al, 2000; Vazquez, 2003). Fluconazole is also used as prophylaxis for prevention of cryptococcal meningitis in HIV/AIDS patients (Armengou et al, 1996; Viard et al, 1995). Since late 1990’s reports are pouring in regarding fluconazole resistance in *Candida* both in the laboratory strains (Kakeya et al, 2000; Kantarcioğlu & Yucel, 2002; Makarova et al, 2003; Walmsley et al, 2001) and also failure in treatment with it in clinics (Dupont et al, 2000; Kantarcioğlu & Yucel 2002; Vazquez 2003; White et al, 1997) especially in HIV/AIDS patients.
Hence state of susceptibility pattern of these recent prevalent strains was
determined using standard protocol of NCCLS broth microdilution
method (NCCLS, 2002). It is a base line data, as no such test was done in
earlier study in the similar group of patients. Fortunately significant level
of azole resistance was not found among the *Candida* isolates from this
group of patients. In-vitro antifungal susceptibility test is not a routine
diagnostic procedure in laboratory services in India therefore the present
result could not be compared with any published report from this region
except similar study from Bombay where HIV/AIDS is rampant,
Ramakrishna *et al*, (2000) in their study also did not detect fluconazole
resistant in their thirty four isolates tested, though testing susceptibility
was different in their study, was by the well diffusion procedure. It is not
very clear why fluconazole resistance was not seen in *Candida* species
isolated from these patients.
A couple of years back it could have been explained on the basis of the
non-affordability by the patient of the expensive drugs like fluconazole,
which was not in use as a routine prophylactic antifungal drug. However,
over the years the situation has changed, now an increasing numbers of
patients are getting the treatment by fluconazole. A small group of
fluconazole resistant strains (5%) isolated in this study is from patients
who never took fluconazole out of 75 isolates studied. These resistant
isolates can be described as primary or intrinsic resistant strain and is a
very serious concern. Xu *et al*, (2000) reported in their study that the
rate of fluconazole resistant strains of *C. albicans* was 2.5% from patients
never treated with fluconazole among the forty isolates they had studied.
Various mechanisms of azole and polyenes resistance have been reported
in *C. albicans*. These include point mutations and over expression of
*ERG11* gene that encodes for the target enzyme, cytochrome P-450 14α
demethylase (Lamb *et al*, 1997; Sanglard *et al*, 1995).
Over expression of drug efflux pumps namely ABC (CDR1 and CDR2) and MFS (CaMDR1) transporters (Prasad et al, 1995; Sanglard et al, 1995; White et al, 1997, 1998) are a very important cause of drug resistance in *C. albicans*. Thereafter defect in \( \Delta^5,6 \) desaturase enzyme; ERG3 has also been implicated as an important cause of azole resistance (Kelly et al, 1997). Resistance to polyenes like amphotericin B and nystatin has been attributed to enhanced membrane fluidity and alteration in membrane sterols (Kelly et al, 1997). Most of these mechanisms have been well characterized in matched as well as unmatched sets of clinical isolates from HIV patients (Franz et al, 1998a; White et al, 1997; White et al, 2002). In several instances the mechanisms of azole resistance described above have been inadequate in fully explaining the resistance pattern exhibited by *C. albicans* clinical isolates. There has been evidence of existence of other mechanisms of resistance in these isolates that have not been found till date (Franz et al 1998a; Sanglard et al 1995).

The various unknown mechanisms of drug resistance in *C. albicans* can only be deciphered when different groups of samples from diverse patient populations are tested. One such group primarily comprises of the intrinsically resistant clinical isolates. Though intrinsic resistance is very well defined in non-*C. albicans* Candida species (Odds 1993; Rex et al, 1995), it is rarely reported in *C. albicans* (Goff et al 1994; Magaldi et al, 2001). Since intrinsic resistance develops without any previous exposure to fluconazole so the mechanisms of resistance prevalent in these isolates might be altogether different from what we observed in fluconazole adapted strains, since fluconazole prophylaxis is rarely practiced in India (Lattif et al, 2004). However, this is only one time testing, the study should continue with repeated isolates from same patients at different time interval.
Factors that contribute to fluconazole resistance are numerous and include a) the degree of immunosuppression of the patient, b) the contribution from other chemotherapeutic drugs c) the degree of previous exposure to fluconazole and d) intrinsic resistance of Candida species (Harry et al., 2002; White et al., 1997). The MIC data of this study contradicts the common assumption that isolates of C. albicans from persons who were never exposed to fluconazole will be susceptible to fluconazole. Although the frequency of resistance may not be high in the absence of fluconazole, but this finding should alert the clinical laboratory personnel to consider determining the MIC of fluconazole for a patient’s isolates of Candida species before fluconazole is administered to treat candidiasis (Goff et al., 1994; Xu et al., 2000).

Interest in assessing the genetic relatedness of isolates of the same species has grown rapidly in recent years especially for epidemiology. The molecular typing of all the C. albicans isolates reported here revealed them to be unrelated to each other (figure 13). The total unrelated-ness of the isolates indicates the different source of each infection and the commensal status might become pathogen as HIV disease advances. Using the computer-based pattern analysis programs, like DENDRONE used in this study, it was possible to build up databases of DNA fingerprinting patterns and perform identity searches of new patterns in these databases. The DENDRON software packages can perform more sophisticated similarity calculations and cluster analysis of the DNA fingerprinting patterns in the databases.

DNA fingerprinting analysis software has made it possible for researchers to evaluate large numbers of complex DNA fingerprinting pattern in a short time. Computer-based analysis programs are now increasingly being used for epidemiological typing (Blignaut et al., 2002; Gener-Smidt
The development of computer programs for automated analysis of DNA fingerprinting patterns help to perform sophisticated comparisons of large number of complex patterns. However, the programs are not completely automatic but require the user to make critical decision that affect the way in which analysis is done and the final results analysed (Gener-Smidt et al., 1998; Rementeria et al., 2001). 

The similarity analysis study of the DNA fingerprinting patterns has revealed that all the isolates collected from different HIV/AIDS patients were unrelated. The degree of unrelatedness of these isolates, as highlighted by cluster analysis further reaffirms relationship between commensalism and pathogenesis. Since no data and knowledge is available about the commensal status of the outpatients surveyed, it is not possible to speculate about the primary source of infection. The possibilities of a commensal organism becoming a pathogen, however, cannot be totally ruled out.

CD4⁺-T-cells lymphocyte; also known as T helper cells are the coordinators of the body’s immune response e.g. providing help to B cells in production of antibodies, as well as in augmenting cellular immune response to antigens. These CD4⁺-T-cells are the primary target of HIV infection. The loss of these cells in HIV infection, both qualitatively and quantitatively, results in weakening of the immune response and the ability of the host to respond to foreign antigens, thus rendering the host susceptible to infections and leading ultimately to the immune deficiency syndrome (AIDS) (Pattanapanyasat & Thakar, 2005).

Correlation between CD4⁺-T-cell counts and onset of oropharyngeal candidiasis in HIV/AIDS patients has been observed in several western literatures (Brambilla et al., 2001; Varagas & Sophie, 2002; Veazey et al,
2001; Wozniak et al, 2002) however such reports are lacking in Indian hospitals. It is known that macrophages and neutrophils primarily achieve the immune response against Candida species infection. Although a number of studies have shown that HIV-positive patients in early stage of infection maintain neutrophils but their macrophage function is compromised (Fidel, 2002a, b, c; Kirkpatrick, 1984; Poor & cutler, 1981). The progression of HIV infection is largely dependent on the interaction between the viral factors and host factors. HIV primarily infects the CD4+ -T-cell lymphocytes in the body. The infection brings about the destruction of CD4+-T-cells through multiple mechanisms including apoptosis, not only HIV infected cells but also HIV uninfected CD4+-T- cells (Paranjap, 2005). The loss of CD4+-T-cell population ultimately leads to the inability of the infected person to deal with opportunistic organisms like Candida species. The half-life of the most HIV infected T cells in-vivo is 12–36 hours, (Neumann et ai, 1995). It was also shown that the adherence ability of C. albicans increased in relation to the progression of HIV infection and a decrease of CD4+-T-cell count also occurred (Pereiro et ai, 1997). The mechanisms underlying the dramatic impact of HAART on the incidence of oropharyngeal candidiasis and esophageal candidiasis have received close attention (Angel et ai 1998; Autran et al, 1997; Bektic et al, 2001; Cassone et al, 2002) and provide valuable insights into understanding the perturbations of mucosal defense mechanisms against C. albicans in HIV-infection. Several observations indicate that increases in CD4+-T-cell counts in response to HAART confer immunologic reconstitution and a decreased incidence of opportunistic infections. Therefore, the patients with low CD4+-T-cell counts present an increased chance of severe oropharyngeal candidiasis. The present study also supports the same, where patients with low CD4+-T-cell counts
developed extensive oropharyngeal candidiasis lesions. In India, anti retroviral therapy is prescribed depending on CD4+T-cell counts. Testing of CD4+T-cell count is expensive and the facility for this is available only in a few selected Institutions. The present study suggested that severity of oropharyngeal candidiasis detected clinically can provide a rough estimate of CD4+T-cell counts depending on which anti retroviral therapy can be instituted if needed.

The present study is the first ever survey of oropharyngeal candidiasis in HIV/AIDS patients in India where a comprehensive laboratory data has been generated, which will help not only in patient management but also future clinical research.

Patients with diabetes mellitus are at increased risk of vulvovaginal candidiasis. Diabetes mellitus has been considered a predisposing factor for vulvovaginal candidiasis. Diabetes mellitus is increasing by an alarming rate indeed and by the year of 2025 the total number of people with diabetes mellitus is projected to reach 300 million worldwide (Amos et al, 1997). The problem is alarming in developing countries, particularly in India, where the number of people with diabetes figure is expected to rise to more than 80.9 million by the year of 2030 from 31 million, as reported in the year 2000 from India (Bjork et al, 2003; Wild et al, 2004). Vulvovaginal candidiasis is a common complication of diabetic women. Few Indian reports are available regarding specific agents in vulvovaginal candidiasis; however, similar data in diabetic patients is scarce, not only in India but also worldwide. The present study specially surveyed the vulvovaginal candidiasis in diabetic women and non-diabetic women as controls.

In this study a total 350 vaginal swabs were collected from 175 patients. Two swabs have been collected from each patient of the hundred diabetic and seventy-five non-diabetic women.
The results of the present study showed that the percentage rate of the vulvovaginal candidiasis was 45% in diabetic and 25% in non-diabetic women. The difference in the rate of vulvovaginal candidiasis infection between the two groups of patients was found to be statically significant (chi-squared 9.11, \( P=0.0025 \)). State of diabetes mellitus increases the chances of vulvovaginal candidiasis due to certain important factors like: (i) increase in the adherence ability of *Candida* species, particularly *C. albicans* to the vaginal mucosa membrane (de Leon et al, 2002) (ii) mucosal secretions in diabetic patients contain high concentration of glucose, which can serve as nutrient for *Candida* species (Soble, 1997) (iii) hyperglycemia limits neutrophil functions (Fidel, 2002 a, b). Among the diabetic group, women with vulvovaginal candidiasis had significant higher mean HbA 1c when compared to those who had no such infection, 12.8±2.6% HbA 1c of the diabetic with vulvovaginal candidiasis and 9.7±1.7% HbA 1c for diabetic with out vulvovaginal candidiasis. The observation of significantly higher total HbA 1c in patients with diabetes mellitus and vulvovaginal candidiasis lends support to the link between hyperglycemia environment and increased risk of vulvovaginal candidiasis. Higher HbA 1c can be due to the worsening of glycaemic control after vulvovaginal candidiasis. There seems to be a significant link between hyperglycaemia and vulvovaginal candidiasis (Goswami et al, 2000). The oral carriage of *Candida* species was significantly higher in diabetic patients compared with the non-diabetic (Belazi et al, 2005). Due to paucity of reports on the prevalence rate of vulvovaginal candidiasis in diabetic patients, this data could not be compared, with other results barring the report of Goswami et al, (2000). This indicates there is not much variation of the prevalence rate over the years despite inclusion of large number of patients in the present study. Compared to reports of vulvovaginal candidiasis in non-diabetic patients the isolation


rate is more in diabetic patients comparable with other report in non-diabetic.
In woman with diabetes, the monocytes/macrophages are dysfunctional and fail to suppress the spore germination process (Fiedl, 2002b; Sobel, 2004; Fiedl, 2005). In the diabetic-mouse model, serum also permits spore germination, while normal sera are relatively inhibitory (Fidel et al, 1996). Once infection is established, neutrophils play a pivotal role in fighting fungal infections in the normal host. Despite the large size of the hyphal elements and their subsequent inability to be ingested by the inflammatory cells, neutrophils are still able to mediate fungal killing. Neutrophils are chemotactically attracted to the hyphae on which they attach and spread. Using their oxidative cytotoxic system, neutrophils damage and kill the fungal elements without accompanying phagocytosis. In diabetes, each of the four phases of neutrophil activation is impaired (Fiedl, 2002a, c). Chemotaxis, the phagocytic functions (adherence and spreading), and finally the oxidative burst are all inhibited in the ketoacidotic state, essentially inducing functional neutropenia (Fiedl, 2002a, c; Sobel, 2004). Hyperglycemia is the major cause of increased susceptibility of diabetic patients to vulvovaginal candidiasis. Increased glucose levels in genital tissues enhance yeast adhesions and growth. Vaginal epithelium cells bind to \textit{C. albicans} with greater propensity in diabetic patients than in non-diabetic women, regardless of whether the patients are premenopausal, postmenopausal or pregnant (Bohannon, 1998).

The species identification revealed that \textit{C. albicans} tops the list of isolates both in diabetic patients and non-diabetic. The data presented herein are in agreement with study of de-Leon et al, (2002) in reporting \textit{C. albicans} to be the major etiological agents in diabetic patients except that their isolation of \textit{C. albicans} was slightly lower (41%). In the present study the
isolation of *C. albicans* from non-diabetic patients is much lower than the experience of Eckert *et al.*, (1998) and Ribeiro *et al.*, (2001) where they found it in range of 85%–90%. It is difficult to ascertain the reason for this difference; however, higher isolation of *C. glabrata* may be one of the possibilities to reduce the isolation of *C. albicans* in this study. In the present decade it has been found that the non-*C. albicans* *Candida* species are emerging as both colonizers and pathogens in different clinical conditions (Abi Said *et al.*, 1997; Gottlieb *et al.*, 2001; Nguyen *et al.*, 1996; Wingard, 1995).

Several risk factors are typically associated with occurrence of non-*C. albicans* *Candida* species infections (Krcmey & Barnes, 2002). *C. glabrata* was the major non-*C. albicans* *Candida* isolated from both the groups of diabetic and non-diabetic women. There are number of reports about the isolations of non-*Candida albicans Candida*. Some showed high isolation rate of *C. glabrata* (Abu-Elteen, 2001; Geiger *et al.*, 1995; Holland *et al.*, 2003) similar to the present observation, whereas many have reported much lower isolation rate (Chong *et al.*, 2003; Corsello *et al.*, 2003; Otero *et al.*, 1998) though, most of the reports are only from nondiabetic women. Earlier studies revealed almost the same isolation rate of *C. glabrata*, which play a significant role in vulvovaginal candidiasis. Although, a higher incidence of it was expected in the diabetic women, the same was not proved and it could not be concluded as to why both non-diabetic and diabetic have almost similar infection rate by this species of *Candida*.

*C. glabrata* was considered by some investigators as commensals/colonizers and there was doubt about its pathogenic role (Fidel *et al.*, 1999; Sobel, 1998; Tietz *et al.*, 1995). Present study showed a definite role of *C. glabrata* as pathogen as all the isolates are from patients with symptoms of curdy white secretions, same as found earlier.
(Goswami et al, 2000). Not much change in the isolation rate of *C. glabrata* has been noticed in an interval of five years. The reasons of this epidemiological shift for instance, *C. albicans* to *C. glabrata*, observed in vulvovaginal candidiasis have yet to be adequately explained. Nguyen et al, (1996) explained the emerging increase of isolation of *C. glabrata* because it has inherent decreased susceptibility to azole relative to *C. albicans*, which appears to have emerged as a significant cause of the infection since the introduction of azole drug fluconazole in the early 1990s. Which could be the reason for the higher isolation rate?

Interestingly, the isolation rates of other non-*Candida albicans* *Candida* were much less in both the groups of the patients. In contrast, *C. glabrata* grows only as a yeast form in-vivo, secreted hydrolases are minimal, and adhesion is relatively weak (Vermitsky & Edlind, 2004). The potential reason for the *C. glabrata* infections are increasing is their intrinsically low susceptibility to azoles. Further more *C. glabrata* can readily undergo mutation to frank azole resistance either in-vitro or in-vivo (Vermitsky & Edlind, 2004).

However, the mean HbA1 levels between diabetic patients who grew *C. albicans* and *C. glabrata* indicated that degree of glycaemic control is not a major factor in explaining the higher prevalence of the *C. glabrata* in them. Factors other than the glycaemic control might play an important role in determining predilection of patients with diabetes mellitus to *C. glabrata* infection. Excessive epithelial adhesion and colonisation of the fungus is related to the adherence capability of the fungus as well as the poor innate immunity in the compromised host. The poor innate immune response of the compromised host, such as patients with diabetes mellitus, has been attributed to the variability in the expression of the constitutive peptides lactoferrins, lysozyme and defensins, which are responsible for local mucosal defence mechanism (Dale 2003; Feng et al,
2005; Jurevic et al, 2003). Among these β-defensins are cationic peptides that have anti-bacterial, antiviral as well as anti-fungal properties by virtue of their capability to induce pore formation in the cell wall of the organism (Dale 2003; Feng et al, 2005; Jurevic et al, 2003). Interestingly, Jurevic et al, (2003) have recently, reported higher prevalence of single nucleotide polymorphism (SNP) C→G at position—44 in the 5’ untranslated region of the β-defensin gene in patients with diabetes mellitus as compared to the non-diabetic controls. Further the presence of SNP in the β-defensin gene was also associated with higher C. glabrata carriage rate in the oral cavity of the diabetic patients. It has been postulated that candidate SNP in the β-defensin gene might lead to reduced expression of the cationic anti-microbial peptide. There is a possibility that similar pathogenetic mechanism might also lead to higher predisposition of diabetic women to vulvovaginal candidiasis due to C. glabrata.

Fluconazole can effectively treat mucosal candidiasis; however, its use can lead to colonization with less susceptible species and to gain resistance among normally susceptible strains (Sobel, 2003; Sobel et al, 2001).

There are limited data regarding the antifungal susceptibility of yeasts causing vulvovaginal candidiasis, since cultures are rarely performed (Richter et al, 2005). In the present study 22% of the fluconazole resistant Candida strains appear to have caused at least partial failure in clinical cure. All the patients were treated at least once with a supervised dose of fluconazole (150 mg). There was no significant difference between diabetes and non-diabetic women in the Candida species-specific fluconazole response. The percentage of patients who continued to show poor response to fluconazole when C. glabrata was the species isolated. It is difficult to resolve whether higher persistence of Candida growth
following fluconazole therapy in diabetics reflects a mere *Candida* carriage or persistent active infection or re-infection. Molecular typing of the *Candida* isolates might differentiate persistent infection vs. re-infection. Sobel *et al.*, (1992) have reported that initial strain of *C. albicans* isolated in non-diabetic patients with recurrent vulvovaginal candidiasis is unique to each patient (Sobel, 1992; Vazquez *et al*). Further in follow up study of 10 non-diabetic women with recurrent vulvovaginal candidiasis, eight continued to show same strain when followed up for a mean period of 35.3 months (Sobel, 1992). Similar data is not available for patients with diabetes mellitus.

It is often assumed, that resistance to fluconazole occurred independently and the horizontal transfer of resistant isolates among hosts is rare (Klepser *et al*, 1997). The greater significant is there acquired fluconazole resistance in *C. albicans* strains and the potential for an increase in vulvovaginal candidiasis due to non-*C. albicans* *Candida* species with intrinsic fluconazole resistance (Sobel & Chaim, 1997). The present study showed that *C. glabrata* was the second species of *Candida* responsible for the vulvovaginal candidiasis; the isolation percentage is almost equal to *C. albicans*.

A number of studies confirmed that the administration of fluconazole might lead to the acquisition of resistance in the commensal strains (White *et al*, 1998). Factors contributing to fluconazole resistance are numerous as mentioned here. These factors either singly or in combinations may contribute for observed resistance for some of these isolates especially isolated from diabetic women. In-vitro susceptibility test can predict the therapeutic out come and also generate significant data for the trend of resistance development of the prevalent strains in a particular institution, which is vital for the antifungal therapy policy. The definitive diagnosis of the *Candida* species, its susceptibility patterns and
appropriate diabetes control, could lead to better vulvovaginal candidiasis management in diabetic women. However, this is only one time testing, the study should continue with repeated isolates from the same patients at different time interval. Lack of correlation between in vitro fluconazole susceptibility profile and recurrence of vulvovaginal candidiasis have been recently reported by Sobel et al, (2003) in patients with HIV, and they concluded that susceptibility testing for women with complicated vulvovaginal candidiasis would be unjustified. The above facts suggest that host factors might have role in determining the response of fluconazole in patients with vulvovaginal candidiasis similar to those described by Jurveic et al, (2003). Strains of C. glabrata are genetically tolerant to fluconazole. Hence, accurate species/strain identification of the clinical isolates is also necessary and application of molecular techniques may be helpful in these cases.

There are relatively few studies highlighting the problem of vulvovaginal candidiasis in diabetic women in the Indian hospitals and thus it remains a poorly investigated area.

The genetic relationship among the clinical isolates can be studied by DNA fingerprinting. In the present study all the C. albicans isolates were typed from both the diabetic and the non-diabetic women. The widely used C. albicans specific CARE-2 probe was used. The results showed that each isolate was different and genetically unrelated irrespective to their origin. The total unrelatedness of the isolates may indicate different sources of infection. Since no knowledge about the commensal status of the outpatients surveyed in this study is known, it was not possible to speculate about the source of the infection. This is consistent with the present data on oropharyngeal candidiasis in HIV/AIDS patients (Lattif
et al., 2004). The present finding is important for the patient's management in the medical institution from where data was collected. The genetic nature of the predominant non-\textit{C. albicans} Candida isolates, \textit{C. glabrata} isolates were also evaluated by Arbitrarily Primed PCR (AP-PCR).

This technique was employed to assess the genotypes of the vaginal strains of \textit{C. glabrata} isolated from diabetic and non-diabetic women as control. Primarily to make out if there was any variation of strain isolated from diabetic and non-diabetic women, Becker et al., (2001) have shown that the AP50-1 primer can discriminate and classify \textit{C. glabrata} isolates in to three distinct genotypes (A, B, and C) based on the banding pattern. Preliminary trial was done with the primer (AP50-1) before using it, this standardized AP50-1 primer by Becker et al., (2000, 2001) for genotyping of the \textit{C. glabrata}. This primer was used with three clinical isolates of \textit{C. albicans} and four isolates of \textit{C. glabrata} along with a standard \textit{C. glabrata} ATCC 90030 as a control; this was done on coded bases. AP50-1 showed sufficiently discriminatory polymorphism patterns between the \textit{C. albicans} and \textit{C. glabrata}. Then the same primer was employed for genotyping the twenty five strains of \textit{C. glabrata} isolated from both the groups one single type of profile genotype B was observed for the seventeen isolates of \textit{C. glabrata} from diabetic patients and one isolate was belonged to genotype A.

The seven strains of \textit{C. glabrata} from non diabetic women were also of genotype B. Becker et al., (2000, 2001) found the genotypes A and B were wide spread within German and Hungarian isolates of \textit{C. glabrata} which were genotyped using same primer. To draw any meaningful conclusions it is required to continue this investigation with more number of isolates over a long period of time.
Discussion

The present study is the first report from India which where the genotyping of *C. glabrata* has been initiated, and the results showed two different genetic variation of *C. glabrata* strains which have been found to be associated with vulvovaginal candidiasis.

The gravity of the situation concerning vulvovaginal candidiasis in diabetic women in India cannot be overemphasized. Chronological analysis of the published reports (Table 1) from India is sketchy. After three reports in 1960s, there is a long interval till 2000 (except one each in 1970 and 1980) about the isolation of yeast from vulvovaginal candidiasis. Even then the scenario in diabetic women is not clear except a report which appeared in 2000 (Goswami *et al.*, 2000) from All India Institute for Medical Sciences (AIIMS). The present study is the second report from the same tertiary care hospital, which has extensive aims and objectives. Awareness has been generated in this topic by the outcome of this study about the actual problem faced by the clinicians and patients. The present study specifically looks for the basic data regarding specific etiological agents of vulvovaginal candidiasis in diabetic women and it was observed that an alarmingly high population is suffering from the disease. This certainly reflects an enhanced diagnostic and evaluative capacity of interested individuals. The aims of the present study were to estimate the vulvovaginal candidiasis in diabetic women and to generate awareness in the patients and healthcare professionals about the epidemiology as the research has been started on the genotyping of the predominant non-*C. albicans* *Candida* strains were obtained from the diabetic women with vulvovaginal candidiasis.

The clinical picture of infectious diseases in India is quite nebulous; since a great many infections are co-existent. Diabetes mellitus is
increasing by an alarming rate indeed and by the year of 2025 the total number of people with diabetic mellitus is projected to reach 300 million worldwide (Bjork et al, 2003). The problem is particularly alarming in the developing countries, particularly India, where the diabetes mellitus figure is expected to rise to more than 80.9 million by the year of 2030 (Bjork et al, 2003) and consequential increase in vulvovaginal candidiasis is expected. Proper control of glucose levels is therefore paramount in management of vulvovaginal candidiasis. Limited data suggest that the primary causative organism of vulvovaginal candidiasis in diabetic women often differs from non-diabetic and may be more difficult to treat than that usually found in non-diabetic patients. Diabetic mellitus patients themselves are responsible for day-to-day management of their diabetes, which requires considerable time and effort. Therefore, any therapy for vulvovaginal candidiasis in addition to being safe and free of drug interactions that might be compromising glucose control, should fit into the context of everyday life and not further complicate daily routines. Awareness must be spearred and high level of alertness has to be maintained at both clinical and laboratory levels to improve management needs of diabetic mellitus patients. The present study is probably the first ever survey of vulvovaginal candidiasis in diabetic women from India where a comprehensive laboratory data has been generated.

Most of the pathogens including Candida species have developed an effective battery of putative virulence factors and particular strategies to help out in their capacity to colonize host tissues, cause disease, and overcome host defenses [Calderone & Fonzi, 2001]. The glyoxylate cycle plays an important role in the pathogenicity of a number of organisms, some of them performed up to the gene level [Lorenz & Fink 2001; Wang
et al, 2003]. The virulence factors expressed or required by Candida species, and particularly in C. albicans, known to cause infections, may well vary depending on several factors: (i) the type of infection (i.e., mucosal or systemic), (ii) the site and stage of infection, and (iii) the nature of the host response [Calderone & Fonzi, 2001; Kennedy et al, 1992 a, b].

Therefore, one of the main aims of this study was to evaluate the correlation between the recently discovered virulence factors, namely glyoxylate cycle enzymes [Lorenz & Fink 2001] in a set of C. albicans strains. The C. albicans strains were collected from three different groups of patients having candidiasis at three different body locations. All the strains showed different level of the glyoxylate cycle enzyme activities. The level of the enzyme activities of the two key enzymes of glyoxylate cycle namely isocitrate lyase and malate synthase were significantly higher in the C. albicans strains isolated from the diabetic patients suffering from vulvovaginal candidiasis. The reasons for this finding may be because of the physical as well as the chemical conditions in vulvovaginal, which were different from the oropharyngeal cavity of the HIV/AIDS and skin tissue of the burn patients. These conditions include: (i) pH (ii) temperature (iii) adherence capacity expression of C. albicans and (iv) concentrations of the nutritional material which are necessary for the germination.

In addition, in vulvovaginal candidiasis the pathogen is continuously in an environment undergoing periodic fluctuations in the ratio of β-estradiol and progesterone [Karnani et al, 2004] that may have an additional effect on the expression of the virulence factors of the C. albicans. The data from the present research on vulvovaginal candidiasis on diabetic women showed that women with diabetes mellitus are at a
higher risk for vulvovaginal candidiasis; this has been reported earlier from the same hospital [Goswami et al, 2000]. In the present data no significant difference in the level of malate dehydrogenase and citrate synthase was found among the different group of strains of *C. albicans*. These two enzymes namely malate dehydrogenase and citrate synthase may have a role in cell maintenance at the colonized tissues and may have no significant role in the pathogenesis of *C. albicans*.

The data from the present study showed and confirmed the presence of all the glyoxylate cycle enzymes activity in the *C. albicans* isolates from different group of patients. The two key enzymes isocitrate lyase and malate synthase are expressed more in the vulvovaginal isolates of diabetic women. The differences in the glyoxylate cycle enzymes from different body locations may be according to the different metabolic requirements in various stages of *C. albicans* pathogenesis. The ability of intracellular pathogens to cause infection is related to their capacity to survive and grow inside macrophages or in other cell types. *C. albicans* latent virulence is likely to be related to a similar mechanism of avoiding killing by specialized cells and to the resulting ability to grow in such hostile environments [Lorenz & Fink, 2001; Fidel et al, 2003].

The present study is the first report of the presence of the glyoxylate cycle enzyme activities in a group of clinical *C. albicans* strains, isolated from clinically different patients. No significant relationship however was observed between the decreased susceptibility to fluconazole and the levels of enzyme activity in all the three groups of the strains isolated from patients. The two regulatory enzymes of glyoxylate cycle namely, isocitrate lyase and malate synthase were earlier found to be related to the virulence of *C. albicans* [Lorenz & Fink, 2001] and these two enzymes can very well serve as new targets for the antifungal agents used. The
rise in the incidence of fungal infections has exacerbated the need for the next generation of antifungal agents, since many of the currently available drugs have undesirable side effects, are ineffective against new or reemerging fungi, or lead to the rapid development of resistance [White, 1997; White et al, 1998; Mukherjee et al, 2005].

This study confirms the existing of the glyoxylate cycle enzymes in the clinical isolates of *C. albicans* from differentially affected patients. Since the glyoxylate cycle does not exist in mammalian cells, these enzymes therefore can be a new antifungal target with less toxicity and side effect as the target are present in the yeast cells only.

A question, the answer to which was, to find out the usefulness of the molecular diagnostic methods for the diagnosis of the fungi was due to the following reasons, the conventional diagnostic methods have: (i) no major improvements over the past 100 years (ii) are time consuming/labor intensive (iii) toxigenic fungi loose their viability in air very quickly and (iv) fail to detect moulds in air and dust. On the other hand the molecular methods are: (i) fast (ii) very sensitive (iii) detect non-viable moulds: spores are killed by light and (iv) will detect toxigenic and allergenic moulds in dust.

Random amplification and identification of DNA fragments unique to a certain strain or species AP-PCR enables various microorganisms to be differentiated rapidly and precisely from each other (Burgener-Kairuz et al, 1994; Chang et al, 2001). AP-PCR amplification is achieved through the use of short random primer (s) (usually 10 nucleotides long) at relatively low temperature (normally <40°C). Although the exact mechanism behind AP-PCR are not properly understood, it has been hypothesized that by reducing the stringency of the primer annealing
temperature, a random primer that shows no complete homology to a genome may have a perfect match of two-to-three nucleotides from the 3'-end of the primer to the template strand to allow annealing and priming complementary strand synthesis by the DNA polymerase, as putative three-nucleotide sequence can be found in principle once in a 64-nucleotide sequence \( (4^3 \text{ permutations}) \). When two such annealing and priming events occur within a certain distance of each other and in proper orientation, the sequence between the matching sites can be amplified effectively. Subsequent identification of characteristic DNA band patterns in agarose gel electrophoresis enables differentiation of various microorganisms. The application of AP-PCR obviates the necessity to know the detailed sequences of the genes regions concerned as well as need for further manipulation after amplification.

The successful laboratory diagnosis of fungal infection is directly dependent on the proper collection of appropriate clinical specimens and the rapid transport of the specimens to the clinical laboratory (Koneman et al, 1988; WHO guide line, 1999a, b & 2001).

The accurate identification of Candida species has been problematic with increasing the number of immunocompromised patients. The widespread use of certain medical and surgical practices, are favoring the emergence of normally commensal Candida species as life threatening pathogens. Pathogenic yeast conventional identification protocols are usually time consuming and demonstrate ambiguities when they are used to differentiate among the different Candida species. In addition, non-C. albicans Candida are often intrinsically resistant to antifungal especially to azoles (Becker et al, 2001; Richter et al, 2005).

There are number of factors that show the important of the accurate diagnosis of the non-C. albicans Candida these are: (i) the spread of the
Candida species resistant to antifungal, (ii) threat of nosocomial Candida outbreak (iii) the ability to differentiate between closely related species or previously misidentified yeast (Majoros et al, 2003). Antifungal susceptibility testing of clinical isolates can take some time, and rapid species level identification is, therefore necessary for prompt initiation of appropriate therapy (Freydiere et al, 2003). Therefore, the development of a highly sensitive and specific DNA based probe assay for the identification of Candida adds a potentially valuable new tool for the early detection and identification of Candida. There are number of published reports for identification of Candida species by molecular methods, most of these reports are for the diagnosis, or to differentiate one species of Candida (Andrighetto et al, 2000; Becker 2000, 2001; Burgener-Kairuz et al, 1994; Chang et al, 2001; Haynes & Westerneng 1996; Elie et al, 1998; Fujita et al, 2001; King et al, 1995; Tamura et al, 2000; Williams et al, 1995).

The probe designed in this study composed of 10-nucleotide primer showed Candida strain-specific pattern for each Candida species, which are mostly isolated from clinical samples. The data of the present study describes RAPD method used to distinguish the various strains of clinical Candida species, the causative agent of candidiasis at different body locations in humans. In the present research a method has been characterized that produces simple diagnostic fingerprints that are unique to different isolates of Candida species. Non-culture-based methods may aid the diagnosis of invasive fungal infection because the classic diagnostic tests, such as blood culture, show poor sensitivity for the detection of Candida and Aspergillus species (White et al, 2005). RAPD is much more informative than the conventional methods of diagnosis, because they distinguished between all tested species and
Discussion

required less time to compare with the conventional methods of diagnosis. The training of a person to be familiar with conventional protocols includes a number of tests and needs an expert to diagnose the Candida up to the species level. But in the case of employing the RAPD test less time is needed to train the person to identify the Candida up to the species level from the specific bands pattern exhibited by each species.

The results of the RAPD study showed that a promising random primer (ALI-8) can be used as a diagnostic tool for the diagnosis of different Candida species as this probe demonstrated a species-specific band pattern.