Review of Literature
REVIEW OF LITERATURE

2.1 INCIDENCE

2.1.1 Historical review

Hippocrates in his "Epidemics" described aphthae or thrush (white patches) in debilitated patients, and the presence of this clinical condition has been recognized for centuries. The important association of vaginal candidiasis and thrush in the newborn was made by Haussmann in 1875. He demonstrated the transmission of the fungus to the mouths of babies from lesions in the vagina. He also produced vaginitis in a healthy gravid female by inoculating Candida into her vagina.

The relationship of dermato candidiasis to thrush was recognized as early as 1771, by Rosen von Rosenstein. Systemic disease by hematogenous spread was described by Zenker in 1861.

His patient was debilitated and had oral thrush, an association noted many times previously. The patient succumbed to a brain infection, however and Candida was demonstrated in the lesion, indicating that the fungus could spread to other organs. Intestinal disease was described by Parrot in 1870 and in 1877, he also noted the first pulmonary infection. Disseminated infection
involving many organ systems was recorded by Schmorl in 1890. The first descriptions of the other forms of *Candida* infections were onychomycosis by Dubandorfer in 1904 and cutaneous disease by Jacob in 1907. Chronic mucocutaneous disease was probably first described by Forbes in 1909, the distinction of it as a separate entity of familial inheritance was made by Schulz in 1925, and its association with endocrine dysfunctions was pointed out by Sutphin in 1943. *Candida* cysts was first recorded by Rafin in 1920, osteomyelitis by Conner in 1928, endocarditis as a separate entity by Joachim and Polyes in 1940, and endogenous dissemination resulting in endo ophthalmitis in 1943 by Maile. By the early 1940’s it thus became evident that Candidiasis was the most common protean fungus infection. Statistically it was a common infecting agent of the skin, mucosa and vagina but was rarely a cause of serious systemic disease.

A revival of interest in systemic candidiasis and *Candidal* endocarditis took place after 1940. The occurrence of candidiasis as a sequel to the use of antibacterial antibiotics particularly broad-spectrum antibiotics, evoked a great surge of research. The results have demonstrated the delicate ecosystem of which *Candida* is a member. Many fatal cases of candidiasis occurred following abrogation of this balance. In 1940 Joachim and Polyes described candidal endocarditis as a hazard of heroin injection. About the same time, the association of candidiasis and steroid therapy, immunosuppressive drugs, cytotoxic agents and immune defects became apparent. Presently *Candida* is recognized as one of the most frequently encountered fungal opportunists and is now regarded as the commonest cause of serious fungal disease.
2.1.2 ABO blood groups and *Candida* carriage rate

Adherence of *Candida* species to mucosal epithelial cells is thought to be the primary event necessary in order to establish colonization. *Candida* species adhere more readily to buccal and vaginal epithelial cells from some donors than others suggesting phenotypic differences in susceptibility to colonization (Sobel *et al.*, 1981). Correlation between ABO blood groups and infectious diseases including mycotic infections are well documented (Deresinki *et al.*, 1979; De Restrepo *et al.* 1983; Clemens 1989) and blood group determinants have been shown to function as microbial receptors. The ABO blood groups are not confined to erythrocytes but are present on many cells including mucosal epithelial cells (Burford-Mason *et al.* 1988).

2.1.3 Role of *Candida* in pathogenesis of antibiotic associated diarrhoea in infants

Infectious diarrhoea is a cause of illness throughout the world, leading to high mortality with loss of time from work and school in Western countries and high mortality in developing countries (Gorbach 1987). In Western countries the average person has one or two episodes of diarrhoea a year, the recorded attack rates being highest in young children. In developing countries the incidence figures are twice as high and diarrhoea has an even greater impact on children (Gorbach 1987).

Bacterial pathogens account for the severest forms of infectious diarrhoea, but they are not necessarily the most common causes. In terms of
occurrence viral agents such as rotavirus and Norwalk viruses are more frequent (Gorbach 1987).

The cause of diarrhoea associated with antibiotic use remains elusive in many cases. Apart from *Clostridium difficile* no other intestinal pathogen has been consistently associated with nosocomial diarrhoea (Yannelli *et al.*, 1988).

The cause and pathogenesis of antibiotic associated diarrhoea remains poorly defined. The demonstration of pseudo membranes on colonoscopy is significant. Some investigators consider the isolation of *C. difficile* from such patients to be diagnostic, especially in out breaks (Danna *et al.*, 1991).

Antibiotics may predispose to diarrhoea by suppressing intestinal bacteria and disturbing the local microbial ecology. The emergence of *C. difficile* as one cause of antibiotic associated diarrhoea is consistent with this hypothesis. Thus it is reasonable to search for other causes among microorganisms such as yeasts that receive a competitive advantage in the presence of antibiotics. It has been known that *C. albicans* may cause inflammatory lesions of the alimentary tract. Despite well documented fatal cases of candidal enteritis reported from pre-antibiotic days to the present, the disease is rarely suspected and evenless commonly diagnosed.

Furthermore, oral antibiotic treatment disrupts the ecology of the indigenous intestinal microflora, allowing *Candida* and other fungi to overpopulate the gastrointestinal tract (GI) and subsequently pass through the intestinal mucosa to initiate systemic infections (Kennedy 1981; Kennedy and
Volz. 1983; Giuliano et al. 1987). Dissemination of fungi from the GI tract is considered to be an important portal of entry into the host and an important cause of systemic infection, particularly in patients compromised immunologically or patients undergoing prolonged antibiotic therapy (Krause et al., 1969, Stone et al., 1974).

Lederer and Todd (1949) listed thrush esophagitis or enteritis as the cause of death in 26 infants under one year of age, on whom autopsies were performed in the course of one year.

However, the saprophytic occurrence of Candida in the alimentary canal of 10 to 15% of healthy individuals handicaps assessment of its etiologic significance in a given case of enteritis. Kozinn and Taschajian (1962) studies showed an incidence of 24% in 250 consecutive pediatric cases of diarrhoea. These figures indicate that C.albicans may exist in the intestines of patients with a frequency similar to that reported for healthy persons but permit no conclusion regarding its etiologic significance (Kaplan et al. 1991).

The difficulty of differentiation between Candida, the intestinal saprophyte and the enteropathogen may explain why this organism was not mentioned in review of the etiology of infantile diarrhoea (Kane et al. 1976; Gorbach 1987).

Since stool cultures are obviously inconclusive, additional diagnostic criteria are required to establish the diagnosis of candidal enteritis.
It has been demonstrated repeatedly that the invasion of mucous membranes by *C. albicans* was accompanied by the formation of mycelium. The eruption of clinical oral thrush lesions in newborn infants coincides with the first appearance of mycelia (M) in oral smears. Prior to the onset of clinical manifestations direct oral smears are negative for candidal elements or show the yeast forms only (Y). In the same way all reports of fatal enteric candidiasis stress massive mycelial invasion of the oesophageal or intestinal wall in typical autopsy findings (Wagner *et al.*, 1958).

Rogers (1957) was the first to interpret the presence of mycelia in fecal smears as a sign of overt intestinal candidiasis and proposed this phenomenon as a diagnostic criterion.

An investigation of the diagnostic reliability of mycelial and yeast forms of *C. albicans* in direct fecal smears was of great importance. Enteric candidal lesions are inaccessible to direct visualization unless they are located in the lower intestine within the reach of sigmoidoscope (Kozinn *et al.*, 1962). Autopsy reports indicates, however that in infants and children the oesophagus and the small intestine are the predominant sites of infection.

Direct microscopic examination of fecal smear permits differentiation between saprophytic and pathogenic phase of intestinal candidiasis (Kozinn and Taschdjian 1962). The pathogenic phase was characterized by the presence of mycelia (M) in direct smears. When *C. albicans* was present saprophytically, direct fecal smears were negative or showed yeast (Y) forms only. Furthermore, Kozinn *et al.*, 1962 found that MY (Mycelia and Yeast) positive fecal smears
were found with nine times more frequency in abnormal than in normal appearing stools of infants with oral thrush who yielded *C. albicans* in stool cultures. Candidal enteritis was diagnosed on the basis of MY positive fecal smears in six patients who yielded the organism in cultures only. The validity of the prior diagnosis was tested by the comparative response of the MY positive and MY-negative patients to specific antifungal therapy. Kozinn *et al.*, 1962 demonstrated, that 88% of the patients responded clinically and mycologically as predicted on the basis of direct mycological stool examinations. Of the MY positive patients, 80% responded to nystatin within an average of 3 days therapy. From the above observation they concluded that the presence of mycelial forms in direct smear in patients with diarrhoea furnishes a clinical clue to candidal etiology of the enteritis.

β-lactamases are enzymes that inactivate the β-lactam antibiotics by breaking their β-lactam ring structure. These enzymes are nearly ubiquitous among bacterial species and only small number of pathogens do not produce them (Acar *et al.*, 1989). The role of these enzymes in resistance to β-lactam antibiotics is widely recognized (Lindh *et al.* 1990).

Despite the increasing importance of β-lactamases in recent years, the appearance of these enzymes is not a new phenomenon (Abraham *et al.* 1940). Resistance as a result of the production of β-lactamases is a world wide problem.

Although β-lactamases are widely distributed throughout the microbial kingdom (Richmond and Skyes 1973), there are few reports of β-lactamases
production in yeasts (Mehta and Nash 1978). \(\beta\)-lactamases activity is also observed with whole cells and cell free extracts in three yeast cultures including \textit{C. albicans} by Mehta and Nash in 1978. Little is known about the \(\beta\)-lactamase production of \textit{Candida} species and quantitative determination of \(\beta\)-lactamase production in AAD cases (Antibiotic associated diarrhoea) has not been reported.

2.1.4 \textit{Candida} Infection in Burn Patients

The incidence of burn wound sepsis is very high in the Indian population as the socio-economic conditions and the standard of personal hygiene are poor and the hot moist climatic conditions encourage microbial growth. These three factors are rarely present in the temperate zone countries of Europe and North America (Thangam Menon 1984).

Disseminated fungal infections have represented an increasingly frequent cause of septic death in many areas of medicine. The incidence of yeast infection in burned patients has steadily increased (Spebar et al. 1981; Pensler et al. 1986). Multiple factors have contributed to the development of this problem (Gauto et al. 1977). Widespread use of topical and systemic antibiotics while leading to better control of bacterial infection predisposes to the development of fungal infection. The use of techniques such as hyperalimentation which are of importance in burn management therefore adds another portal of entry for systemic infection. The majority of fungi encountered have been yeasts of the \textit{Candida} species (Law et al., 1972). The other mycosis like aspergillosis and mucormycosis are rare in burned patients.
Immunodeficient patients are commonly a target of opportunistic fungal infection (Klein et al., 1978, Young et al., 1979).

*Candida* species are a common cause of opportunistic infection in burned patients (Meunier et al., 1983). Early diagnosis is difficult and therapy of established infection is often unsuccessful. Suppression of *Candida* has been attempted with varying results. The number of sites colonized and degree of colonization have been positively correlated with the risk of invasive infection.

Disseminated candidiasis has long been recognized as a problem among patients whose resistance to infection is impaired. A steady and progressive increase in such infections at the Institute of surgical research of Brooke Army Medical Centre had been noted by Nash et al. 1971. A similar increase in such infections mainly due to *Candida* organisms occurred at the Shriners Burns Institute from 1965 (Law et al. 1972). In this study the incidence of wound colonization by yeasts varied from 33% in 1965 to 82% in 1969. In 1969, 47% of all patients yielded *C. albicans* and 35% other species of *Candida* when surface swabs of burn wounds were cultured.

There are several possible mechanisms which contribute to the increased incidence of candidiasis. *C. albicans* is a ubiquitous organism and can be grown in the stool in a small percentage of routinely admitted hospital patients (McGovern et al., 1953). Further it has been shown that there is a sharp increase in incidence of recovery of these organisms in stool cultures after treatment of these patients with broad spectrum antibiotics. Prophylactic penicillin has been administered routinely in all patients with large burns to
prevent the development of streptococcal septicemia. Indication for the use of additional broad spectrum antibiotic for such treatment include septicemia, burn wound sepsis, pneumonia and urinary tract infections. However patients with large burns who survive the immediate post burn period usually develop one or more of these complications. As indicated by the review of the subject by Seelig (1966) the use of broad spectrum antibiotics is a definite predisposing factor to the development of candidiasis. This is also true of patients treated by hyperalimentation or by any long term intravenous therapy especially with concomitant antibiotic therapy.

All the burned patients are treated with one of the several topical agents designed to suppress bacterial growth. Majority of these agents with the possible exception of silver nitrate and betadine have no activity against fungal infection (Manu et al. 1988). The work of Louria et al., 1960 in animals indicate that renal seeding of Candida organisms can occur from the blood stream. Krause et al., 1969 indicate that human volunteers who swallowed a culture of C.albicans, promptly developed candidemia and candiduria. Therefore, oral mycostatin is prescribed in these patients in whom candiduria is present. It is recognised however that the majority of patients with large burns have indwelling catheters during part of their hospital course and retrograde infection may occur in such patients, but in most of these cases blood borne origin of lesions seemed likely (Law et al., 1972).

During the early period it was felt that C.albicans represented the only species pathogenic to human beings but other Candida species were sometimes proved to be lethal in burned patients. For example in a study by Edward
et al., 1972 autopsy of some patients showed blood cultures positive for *Candida* species other than *C. albicans*. The association of fungus infections with other organisms is of considerable interest. According to a study by Neely et al., 1990 it has been proved that proteolytic environment is required for the lethality of infection. In this study on a burned mice model a pre challenge with proteinase producing *Pseudomonas aeruginosa* strain and a subsequent challenge of *C. albicans* was necessary for the development of lethal candidiasis. On the contrary prechallenge with non-proteinase producing *P. aeruginosa* and then challenge with virulent *C. albicans* did not produce any lethal infection. Hence it appeared that the level of proteolytic activity in the environment in which the *C. albicans* was found determined whether *Candida* challenge would be lethal. It was also found that the challenge of *C. albicans* along with proteinase producing *P. aeruginosa* strain and enzyme augmentation given to an unburned mice did not produce lethal infection (Neely et al., 1986). This phenomenon may be explained that the burn itself increases the net proteolytic activity in the circulation of patients with burns (Neely et al., 1988). Thus the total proteolytic environment in which the *C. albicans* is found might be a major factor in determining the lethality of the infection.

Known Chung et al., 1988 suggested that there is a threshold level of specific *Candida* proteinase needed to contribute to *C. albicans* virulence. Moreover a burn activates the homeostasis and complement cascade systems and *Candida* triggers the coagulation and angiotensinogen angiotension cascades (Ruchel et al., 1983, 1984). It has been found that different strains of *Candida* produce different amounts of acid and neutral proteinase which may
be active in the host. It has been postulated by Neely et al. (1988 and 1990) that the influx of proteinase caused by the burn and infection by *Candida* greatly increases the proteolytic activity by causing an imbalance in the proteinase-proteinase inhibitor balance in the host. This increased proteolytic activity consumes elements important for host defense. Hence the burned infected host becomes susceptible to lethal candidiasis. Spebar and Pratt (1981) have reported that *Candida* colonization of the colon and subsequent invasive candidial sepsis eventually leads to the death of these patients.

A study conducted by Neely et al., in 1988 provided detailed information about *Candida* epidemiology. In the study of the pathogenesis in paediatric burn patients, out of 113 patients studied for 3 years a total of 85% of patients were colonized by *C.albicans*, 15% by *C.tropicalis*, 11% by *C.parapsilosis*. In this study *C.albicans* biotype 57 was the major isolate.

Candidiasis is difficult to treat and so an active program of prevention of infection may be an effective way to attack this disease. The formulation of such a program required detailed information about *Candida* pathogenesis and epidemiology. Detailed study of the sources, modes of transmission and types of *Candida* species involved, has not been possible due to absence of a method for fine differentiation of strains within the species.
2.2 DIFFERENTIATION OF SPECIES

2.2.1 Conventional methods

With the increased use of cytotoxic and immuno suppressive drugs in the treatment of neoplastic diseases, infections caused by *Candida* have become an important clinical problem. In many cases these infections limit successful anti cancer therapy (Gunasekaran et al., 1983). The development of simple and rapid methods for differentiation of *Candida* species in clinical specimen is needed. In order to predict appropriate antimycotic therapy and to monitor development of resistance and spread of infection methods of differentiation that are rapid and reliable are needed.

Conventional and commercial systems (API 20 C) for the identification of yeasts are based on different properties that include colony morphology, microscopical appearance and fermentative and assimilative reactions (Lodder and Kreger Van Rij 1967) But these methods are labour intensive, time consuming and expensive. Yeasts are known to be sensitive to dyes an observation affording a possible basis for differentiating species (Sobczak 1985).

2.2.2 *Candida* Strains Typing

Despite the variety of typing methods for *Candida* described in recent years none has particulary good discrimination and some suffer from poor reproducibility (Hunter and Gaston 1988; Odds et al. 1989). Furthermore, the choice of the most appropriate method is not obvious, since different authors have used different populations on which to validate their methods. Even using
the same killer strains, two sets of workers (Polonelli et al. 1983) found rather different discriminations when methods were applied to different populations. There is, therefore need to compare existing typing methods on the same population.

The poor discrimination of available typing methods may be due to deficiencies in the methods themselves; alternatively, it may be that strains of *C.albicans* isolated from clinical material are highly homogeneous. If the reason for poor discrimination is the former, then there are two possible approaches to increasing discrimination. The first is to attempt development of a new typing method that is highly discriminatory, although the diversity of typing methods already described for *C.albicans* would suggest that this approach would be fruitless. The other approach is to use more than one typing method either in parallel or in a hierarchial system. Warnock et al. (1979) reported that the resistotyping has the best combination of discrimination and reproducibility of the four methods (resistotyping, morphotyping, biotyping by extra cellular enzyme production and carbon source assimilation) examined.

Physiological tests to differentiate biotypes of *C.albicans* have been used widely in epidemiological studies of *Candida* infections. Although no evidence has been obtained to associate particular *C.albicans* biotypes with symptomatology (Warnock, et al., 1979), biotyping procedures have supported the concept of sexual transmissibility of the fungus (Odds et al., 1983) and shown that recurrence in vaginal candidiasis is most often the result of relapse due to same strain of *C.albicans* rather than reinfection with a different strain (O’conner and Sobel 1986).
In several studies, most female patients who harbour *C. albicans* were found to carry the same biotype in different anatomical sites, and usually the same biotype was retained over a period of weeks and months (Warnock et al. 1979). They also found that patients frequently harboured more than one *C. albicans* biotype.

2.3 PROTEIN PROFILES OF CANDIDA SPECIES

Polyacrylamide gel electrophoresis (PAGE) is an electrophoretic technique where the proteins are separated under continuous electric field. Laemmli (1970) reported that the separation of proteins by PAGE in the presence of anionic detergent Sodium deodecyl sulphate (SDS) is dependent on the molecular weights of their peptide chains.

Separation of native proteins on polyacrylamide gel is dependent not only on size of the molecule but also on the charge. The binding of SDS ions to proteins has been shown for several protein molecules and was assumed to be the basis of the separation of the denatured proteins upon SDS electrophoresis on poly acrylamide gel.

Weber and Osborn (1969) showed that SDS- gel electrophoresis can be used with great confidence for a wide variety of proteins. It appears that by this technique polypeptide molecular weights may be determined with an accuracy of at least ± 10%.
Several workers have demonstrated the usefulness of electrophoretic separation of proteins in defining variations within or between fungal species. Many groups of fungi have been studied taxonomically by electrophoresis including dermatophytes (Schechter et al., 1972) Candida (Schechter 1973) Aspergillus (Sorenson, et al., 1971) and Fusarium (Glynn and Reid 1969) using comparative electrophoresis. Isoelectric focusing and numerical taxonomy of some isolates of Fonssasaea pedrosoi and allied fungi were also carried out by Schechter (1973).

Protein patterns analysed by SDS-PAGE successfully applied to bacterial classification and epidemiology have been used in the retrospective analysis of 7 systemic C.albicans infections in a neonatal intensive care unit. 2 strains were found to be involved, the result was confirmed by restriction endonuclease digestion of whole cell DNA by EcoR I (Naudry. 1988). Cross infections was suspected as the strains were isolated from patients linked spatially and temporally in the unit. Others have found PAGE patterns to be too variable for use in their taxonomic or epidemiological studies (Jones et al. 1982; Cunningham et al. 1989).

Isoenzyme patterns of Candida species by PAGE enabled 23 biotypes to be recognised amongst 37 strains of C.albicans and also revealed biotype information for C.tropicalis and C.pseudotropicalis (Lehmann et al 1989). The epidemiology of several outbreaks of Microsporum canis has been examined using protein PAGE patterns (Tucker 1990). This study showed that genetically diverse strains had different PAGE patterns.
The cellular protein profiles and enzyme profiles from whole cell extracts of *Candida* spp were studied with PAGE (Lehmann *et al.*, 1989). They reported that the protein and enzyme patterns of *C. tropicalis* and its sucrose negative variant *C. pseudotropicalis* were indistinguishable (Ahearn *et al.* 1977). In addition the *C. stellatoidea* isolates were found to be separable on the basis of their enzyme profiles into the same two types that have been reported by Kwonchung *et al.* (1988). Similar investigations have been used to a limited extent to distinguish *Candida* spp. (Schechter 1973).

Isoenzyme profiles were obtained following PAGE of crude extracts of *C. albicans* and *C. tropicalis* (Lehmann *et al.* 1989). The two species were separated by distinct isoenzyme patterns. Within each species, variations were found for several isoenzymes. This allowed the development of a method for biotyping these fungi.

Cell surface proteins from eight *Candida* spp were compared by SDS-PAGE (Kobayashi *et al.* 1984). This study concluded that the cell wall protein of those species were similar in their electrophoretic patterns. On the other hand differences were detected among the patterns of membrane proteins, although *C. albicans* serotypes A and serotype B gave similar electrophoretic patterns.
2.4 CANDIDA DNA TYPING

Among nosocomial infections, fungal infections especially those produced by *C.albicans* have emerged as increasingly important causes of morbidity and mortality.

Most methods used to examine the epidemiology of *C.albicans* rely on phenotypic expression of one or more characteristics such as biotyping, antigen expression or isoenzyme profile (Lehmann *et al.* 1989) as the basis for determination of strain relatedness. The usefulness of these methods can be hampered by various factors, including insufficient strain discrimination and phenotypic variability. Recently RFLP (restriction fragment length polymorphism) patterns of highly conserved r DNA regions have been used to study strain relatedness among *C.albicans* isolates (Carle *et al.* 1984; Stevens *et al.* 1990). Because of the stability of these patterns, this method has been proposed as a useful and rapid adjunct to strain discrimination. Additional methods of DNA comparison such as electrophoretic karyotype (Chung *et al.* 1988), RFLP analysis of mitochondrial DNA and southern blotting probing with cloned fragments of DNA have also been used and show promise as tools for the study of *C.albicans* epidemiology. While a thorough comparison and correlation of some phenotypic methods and DNA typing remains to be done, a recent comparison of DNA typing and biotyping indicated neither complete nor random association between the two methods (Ganesan *et al.*, 1991). Clemens *et al.* (1991) have investigated the application of DNA typing scheme to the epidemiology of an out break of candidiasis in heroin addicts in whom one biotype of *C.albicans* appeared to predominate. Unlike the biotyping
results, of their study results showed that no single DNA type was prevalent among 13 isolates obtained from seven patients. Hence, DNA typing has been used to identify the focal outbreak of candidiasis with common source of origin.

An outbreak of *C. albicans* infection was reported in 1985 from an intensive care unit in London (Burnie *et al.* 1985). Thirteen patients in the unit developed systemic candidiasis caused by a single strain. The authors attributed the outbreak to cross infection, with hands acting as the major route of transmission.

Later, four outbreaks of *C. albicans* were reported in four British Hospitals (Burnie *et al.* 1987). Overall, 13 systemic cases occurred, six of whom died. A single strain was isolated in each of the four outbreaks and the authors concluded that cross contamination had occurred, although the mode of transmission was not entirely clear. *C. albicans* frequently colonizes the skin of debilitated patients, but systemic infection can occur only if other conditions are present as well, such as an IV catheter providing access to blood or urinary catheter leading to a urinary tract infection and consequently to funguria.

Two reports have been published on the application DNA finger printing to the analysis of *C. albicans* outbreak (Moro *et al.* 1990, Vaundry *et al.* 1988). Vaundry, *et al.*, (1988) investigated a cluster of systemic *C. albicans* infections in a neonatal intensive care unit typing the five available clinical strains with the electrophoresis pattern and DNA profiles. These methods gave similar results and divided the strains into the same two groups identified by the
epidemiological data. Mathews and Burnie (1989) assessed the validity of DNA finger printing with 45 previously characterized C.albicans isolates from five different outbreaks and with 96 unrelated isolates from a mixed control population. DNA analysis discriminated better than any of the other typing methods used, including serotyping (Hasencler, et al., 1961) biotyping and morphotyping (Odds and Abbott 1980, 1983).

2.5 SUSCEPTIBILITY TO ANTIFUNGAL AGENTS

2.5.1 Antifungal agents

The current therapy of candidiasis depends mainly on antifungal agents belonging to three different chemical groups, the imidazoles, thiocarbamates and antibiotics such as the polyenes, or griseofulvin (Georgopoulos, et al., 1981 and Odds et al. 1986).

Amphotericin B (AMB) is a haptene macrolide antibiotic produced by Streptomyces nodosus. It is efficacious in the treatment of systemic mycoses. However nephrotoxicity and other adverse effects are encountered with the use of this agent.

A water soluble derivative of AMB, amphotericin B methyl ester (AME) is less toxic in vivo than AMB (Gadebresch, et al., 1976). Although pathogenic yeasts and molds have been shown to be susceptible to AME in vitro (Bannatyne, et al., 1977) variable results regarding the therapeutic effectiveness of AME have been reported (Bonner, et al., 1975).
Furthermore, although published guidelines exist for fungal susceptibility testing, these are not well standardized, particularly with respect to incubation temperature. This parameter has previously been shown to influence the antimicrobial susceptibility of bacteria (Mackowiak, et al., 1982) and some yeasts (Shadomy et al. 1982).

The fungistatic agent 5-fluorocytosine (5FC) has repeatedly proven effective in the treatment of infections due to the pathogenic yeast *C. albicans* (Hoeprich, et al., 1974). This agent is relatively non-toxic to humans and is a desirable anti-fungal agent for that reason.

*C. albicans* has been increasingly noted as a cause of systemic fungal infection in immunocompromised hosts. In 1973, flucytosine (5FC) was introduced in the United States for the treatment of such infections. However, several earlier surveys of clinical isolates have demonstrated a 4.5% - 11.7% rate of *in vitro* resistance to 5FC in Europe (Speller, et al., 1972) and a 21% rate of *in vitro* resistance in Canada (Auger, et al., 1979). A survey of 402 clinical isolates from five widely separated geographic centres in the United States revealed an *in vitro* resistance rate of 11.5% - 15.5% in four centres and 35% at the fifth centre (Stiller et al., 1982).

Enthusiasm for the use of 5FC in serious *Candida* infections has been hampered by these reports of primary *in vitro* resistance as well as by the development of secondary resistance after initiating 5FC therapy (Hoeprich, et al., 1974).
Since the discovery of the antifungal properties of 5-fluorocytosine (5FC) and of the low toxicity of the drug for mammalian cells, many studies have been directed towards identifying the activity spectrum of this product, particularly with regard to species of *Candida* (Stillier, *et al.*, 1983). The discovery of Hasenclever and Mitchell (1961) of two serotypes A and B in the species *C. albicans* was followed by epidemiological studies that showed a close relationship between the serotype of this yeast and its susceptibility to 5FC.

In general, serotype A strains are sensitive to 5FC, while most serotype B strains are resistant. Moreover, the latter strains have been isolated mainly from black persons living in Africa (Drouhet, *et al.*, 1975). Subsequent research has failed to reveal either an active site or an antigenic determinant that would associate serotype with susceptibility to 5FC in *C. albicans* (Montplaisir, *et al.* 1976). Although, many mechanisms of resistance to 5FC have been demonstrated (Neuhard, *et al.*, 1968) Montplaisir, *et al.*, (1976) also studied the ultrastructure of the *C. albicans* cell wall with electron microscopy. They described a fourth protein like layer inside the wall of cell sensitive to 5FC.

Resistant strains occur at significant frequency and limit the clinical usefulness of 5FC. Although the mechanism of action of 5FC on *C. albicans* has been studied extensively the genetic basis of the resistance has not been demonstrated. Five genes determine 5FC resistance in another yeast, *Saccharomyces cerevisiae* (Montplaisir *et al.* 1976).
2.5.2 *In vivo* study

Many mouse models have been used for the study of disseminated candidiasis. Usually the mouse is infected by intravenous injection of the fungal organisms (Bistoni *et al.* 1984 and 1985). Samonis *et al.* (1990) have established colonization of the gastro-intestinal tract of healthy adult mice without altering the host by feeding the mice chow containing *C. albicans* for 14 days. The fecal concentration of *C. albicans* was high at the end of the feeding period.

Gastrointestinal colonization by *C. albicans* has been difficult to maintain in healthy adult mice without use of immunosuppressive agents (Ekenna and Sherertz 1987). Prolonged colonization without use of such agents has been achieved in infant and new born mice. (Pope and Cole 1981) Most of the reported animal experiments used a inoculum suspension rather than a solid form of food containing *C. albicans* to feed the animal (Ekenna and Sherertz 1987). But Saminis *et al.* (1990) established gastro intestinal infection by using solid chow, which partially protects the *Candida* from gastric acidity, and this could have accounted for ability to achieve long term *C. albicans* colonization. The role of gastric acidity in the prevention of *Candida* colonization and infection has long been recognized (Saminis *et al.* 1990).

The inoculum size needed to induce disseminated candidiasis in animals was shown to be greater when the suspension containing *C. albicans* was delivered into the stomach rather than small intestine. The activity of the gastric secretions might explain this difference (Stone *et al.* 1974).
In Samonis et al. 1990 studies one additional factor that may have contributed to colonization was the prolonged duration of feeding with the chow containing *C. albicans*. Other studies used substantially shorter duration of feeding (≤ 3 days) (Ekenna and Sherertz 1987).

Persorption of *Candida* spp, through the intact intestinal mucosa and subsequent dissemination have been found to be related to inoculum concentration (Stone et al. 1974).

Samonis et al. 1990 reported that the *Candida* dissemination did not occur when the *C. albicans* inoculum concentration was ≤ 10⁸ CFU/g in solid food.

Infant mouse model of candidiasis also lends itself to a study of how compromising agents contribute to the severity of the infection (Pope et al. 1979; Cole et al. 1988 and 1990). Chronic mucocutaneous candidiasis, a unique form of the disease, is often associated with T-cell deficiencies or with combined T-cell and B cell defects (Lee and Balish 1983). Although patients with this form of disease rarely, if ever, suffer from disseminated candidiasis, the defense mechanism of greatest importance against *C. albicans* is commonly thought to be T-cell mediated immunity (Pearsal et al. 1978).

Macrophages play an important role in host immune and non-immune responses to *Candida* infection. Lee and Balish 1983 have explored the contribution of macrophages in resistance to I.V. infected *C. albicans*. Macrophage activity was disrupted in both euthymic and athymic mice by I.V.
infection of silica. The results presented by Lee and Balish (1983) also demonstrated that normally functioning macrophages play a crucial role in *Candida* infections in mice but not through a direct effector mechanism.

Hector *et al.* 1990 have developed models of candidiasis and aspergillosis using DBA/2N mice, which are known to be deficient in the C5 complement. These models require less infecting inoculum and follows a less acute course than previous models with outbred strains of mice. Hector *et al.* and Ye (1990) enabled differentiation between fungistatic effects of several azoles and fungicidal effect of amphotericin by using DBA/2N animal model.

Guentzel and Herrera (1982) have demonstrated in immunosuppressed mice, that *C.albicans* can disseminate from the gut to the liver, kidney and other organs. Kennedy and Volz (1985) have found that in Syrian hamsters pretreated with antibiotics, *C.albicans* can disseminate similarly. Factors identified that facilitate this dissemination include suppression of the intestinal bacterial flora and high levels of *C.albicans* in the gut.

Ekenna and Sherestz (1987) reported that a combination of two antibiotics was required to achieve high level and prolonged colonization of the gut with *C.albicans*. Individually clindamycin and gentamicin had an equal effect on *Candida* gastrointestinal colonization.

Bayer *et al.* (1981) have developed a rabbit model of intra abdominal candidiasis, for use in evaluating a variety of treatment modalities for this disorder and delineating the regiments of choice.
Bodey and Anaissie (1989) have suggested that certain leukemic patients treated with arabinosyl cytosine may be at a particularly high risk for the development of hepatic candidiasis because of the gastrointestinal toxicity of this antileukemic drug. *C. albicans* is a common component of the gastrointestinal microflora of humans. Prolonged administration of arabinosyl cytosine results in an extended period of granulocytopenia (Cole *et al.* 1990) in patients with pre-existing *C. albicans* colonizing their gastrointestinal tract. Immunosuppressive effects of chemotherapy combined with damage to the gastrointestinal mucosa are suggested to result in the localized invasion of the mucosal tissue by *Candida* hyphae and ultimate dissemination of the pathogen via, the hepatic portal vein to the liver and spleen. Cole *et al.* (1989) have described a murine model of chronic systemic candidiasis which simulates aspects of this disease reported in humans.

The intraperitoneal administration of cyclophosphamide and cortisone acetate to 3 to 4 week-old mice with G.I. candidiasis established by oral - intragastric inoculation at infancy results in the systemic spread of *C. albicans* and the formation of yeast and hypha containing abscesses in the liver, lungs and kidney (Cole *et al.* 1989) Cyclophosphamides is known to be toxic to dividing cells in the G.I. tract and can alter normal integrity of the mucosal epithelium of the gut. The effects of toxicity combined with severe leukopenia induced by administration of cyclophosphamide plus cortisone acetate probably account for the enhanced infectivity of *C. albicans*. It has been suggested by Bistoni *et al.*, 1984 and Cole *et al.*, 1990 that this animal model stimulates conditions in leukemic patients who have *C. albicans* as a recently acquired or
previously established component of their indigenous gut flora and who are subjected to G.I. tract toxic and chemotherapeutic regimens e.g., arabinosyl cytosine treatment (Bodey and Anaissie 1989).

In both the human and animal model, G.I. mucosal damage apparently leads to Candida proliferation and invasion followed by dissemination of this opportunistic pathogen to other organs of the body (Cole et al. 1988).

Oral candidiasis is a frequent complication of patients with HIV infection and has been associated with progressive immune suppression (Glatt et al. 1988). Treatment is usually successful with either ketoconazole (200-400 mg/day) or clotrimazole (30-50 mg/day). In a comparative trial of fluconazole (50 mg/day) verses ketoconazole (200 mg/day) 17 out of 17 patients responded to fluconazole, while 12 out of 16 patients responded to ketoconazole (De Wit et al. 1989). Relapse after cessation of therapy was observed in both treatment groups. Fluconazole used as a single 150 mg dose was found to be mycologically and clinically effective in 20 (87%) of 23 patients at 7 days and in 9(41%) of 22 patients at 14-21 days; however there was no control treatment in this trial (Chave et al. 1989). From such limited data, fluconazole appears to be as efficacious as currently available imidazoles for the treatment of oropharyngeal candidiasis but more expensive than ketoconazole or clotrimazole. Possibly intermittent dosing of fluconazole could reduce its overall cost to be comparable to those other agents, but such dosing regimens have not been tested (Mattie et al. 1989).
There is no comparative trials of the efficacy of itraconazole for oral candidiasis in patients with AIDS (Larsen 1990). There are several less expensive alternatives to the newer triazole compounds for the treatment of oral candidiasis. Toxic side effects of fluconazole are infrequent and usually not severe. Overall both fluconazole and itraconazole appear to be generally well tolerated with minimal serious toxic side effects although there are several important drug to drug interactions.

A comparison of the efficacy of amphotericin B, fluconazole and itraconazole in an animal model of systemic candidiasis revealed that in normal mice the maximum effect that can be achieved with amphotericin B is greater than that which can be obtained with the triazoles (Van Twout et al. 1989). However, in neutropenic mice the efficacy of amphotericin B was greatly diminished, despite the use of very high doses (upto 4 mg/kg). Because of its toxicity, higher doses of amphotericin B could not be administered. The relative efficacy of amphotericin B during neutropenia in both experimental animals and patients (Lopez - Berestein et al. 1984) is disappointing in view of the highly fungicidal activity of the drug in vitro. This suggests that atleast part of the antifungal effect of amphotericin B in vivo must be explained by a synergism between the drug and cellular host defense mechanisms. This hypothesis was supported by divergence of regression lines of the dose-response curves for amphotericin B in normal and neutropenic mice. One would expect these regression lines to be parallel if the effects of amphotericin B and cellular host defense mechanisms on systemic candidiasis are additive (Van Twout et al. 1989).
The slopes of the dose-response curves for normal and neutropenic mice treated with fluconazole or itraconazole were not significantly different. After correction for the higher number of *C. albicans* in the kidney of neutropenic mice before treatment, both theconazole and itraconazole were equally effective in normal and neutropenic mice. Thus they found (Van'Twout *et al.* 1989) no evidence for synergistic activity between the triazoles and cellular host defense mechanisms against systemic candidiasis. Flucanozole proved to be more potent than itraconazole. However since the kidney was the main target organ in the animal model, the renal excretion of fluconazole may have favoured the efficacy of this drug compared with those of amphotericin B and itraconazole (Hughes *et al.*, 1986).
Scope and Plan
SCOPE AND PLAN

3.1 INCIDENCE

Over the past three decades, the incidence of candidiasis has risen dramatically. The predisposing conditions are immunosuppression, surgery, patients with indwelling catheters, prolonged antibiotic therapy, burns and trauma.

Candidiasis is world wide in distribution and equally common in both sexes. About 20 million cases in leukemia patients are diagnosed world wide each year. These type of systemic and mucocutaneous candidiasis is steadily increasing in prevalence especially in patients with predisposing conditions. Effective prevention and control of these health problems requires proper epidemiologic characterization, species identification, pathogenic role, suitable treatment and documentation of base line information. Since such a comprehensive picture about Candida infections is lacking in this part of the country, Tamil Nadu, this study was aimed at clinico-epidemiological characterization and incidence of Candida infection among the hospital attending population of Madras.

Most of the reported studies on candidiasis have included patients with various underlying diseases, especially cancer. There has not however been a comparative study of different clinical conditions and these types of severe
infections have not been adequately analysed. We report our experience of *Candida* infection in Madras during the study period 1988-91.

Our goal was to study the epidemiology and compare the extent, changing patterns and outcome of *Candida* infection episodes in patients with various underlying diseases using statistical methods. We studied the influence of microbiologic and clinical variables on outcome in patients with candidiasis and different types of underlying diseases.

*Candida* species adhere more readily to buccal and vaginal epithelial cells from some donors than others suggesting phenotypic differences in susceptibility of colonization. Correlation between ABO blood group and infectious diseases including mycotic infections are well documented, and blood group determinants have been shown to function as *Candida* receptors. In Western countries O blood group subjects have a greater risk in the development of infection. In our country the relation between ABO blood group and oral carriage rate in normal subjects have not been analysed by well designed population-based studies. Hence we examined the relation between ABO blood group and oral carriage of *Candida* species in normal healthy subjects.

The rate of recovery and density of *Candida* spp. from the gastrointestinal tract of healthy individuals varies with the site, sample and with the age of the subject. *Candida* has a definite pathogenic potential in the gastrointestinal tract of patients with a depressed immune status as in those with acquired immune deficiency syndrome (*Candida* stomatitis and
esophagitis). In such patients *Candida* becomes an invasive organism, causing inflammation and ulceration. However, whether *Candida* causes diarrhoea remains controversial. Neither reviews of infectious diarrhoea nor those of antibiotic associated diarrhoea mention Candida as a possible pathogen. Publications on *Candida* diarrhoea in the adult consist of occasional case-reports and mention the disorder in a review of gastrointestinal disease caused by fungi. Hence we have planned this study to find out evidence for a specific relation between intestinal overgrowth by *Candida* and antibiotic associated diarrhoea (AAD).

### 3.2 SPECIATION OF CANDIDA

Conventional and commercial systems for the identification of yeast are based on different properties that include colony morphology, microscopic appearance and fermentative and assimilative reactions. These methods are laborious, time consuming and expensive. In order to predict appropriate antifungal therapy, to monitor development of resistance and spread of infection, methods of differentiation that are rapid, reliable and easy are needed. Yeasts are known to be sensitive to dyes, an observation affording a possible basis for differentiating species. Hence a disk diffusion method for the identification of yeasts was developed that depends on their different but distinct susceptibilities to different chemicals and dyes.
3.3 SDS-PAGE PROTEIN PROFILES OF CANDIDA

The identification of fungal genera and species is based on a body of morphologic and physiologic characters. Variability in these characters is expected and accepted by most mycological taxonomists. Though C.albicans is defined, in part by its ability to form germ tubes under specific environmental conditions, a small percentage of isolates of this yeast may fail to form these distinctive structures and in addition, may fail to assimilate one or more carbohydrate sources. Consequently, such variability in the characters used to differentiate these species does not in itself negate the entire body of information accumulated for individual taxa, nor does it invalidate the taxonomic status of a given species. Hence we have planned to study the cellular protein patterns of some of our isolates of Candida species by using SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) to find out the inter and intra species differences.

3.4 DNA TYPING

Various methods have been attempted to type Candida isolates, with the objective of developing an epidemiologic tool. In phenotypic methods, problems encountered have included reproducibility, cumbersome test methods and a variety of variables. Hence we have worked on DNA extraction, digestion of Candida genome with a restriction endonuclease and electrophoresis under standard conditions. This study provides a strong base for the epidemiological investigations.
3.5 SUSCEPTIBILITY TO ANTIFUNGAL AGENTS

Significant cure rates have been claimed by different investigators studying therapy of candidiasis, yet the spontaneous, unpredictable and ill understood resolution, provides an uncertain base upon which to judge the efficacy of any individual drug treatment. The number of antifungal agents for candidiasis is small and most are toxic. Fluconazole is a new triazole antifungal with a novel pharmacokinetic profile. Hence we have done a study to evaluate the effectiveness of the drug fluconazole (Pfizer, U.K.) by in vitro, in vivo methods and by using scanning electron microscopic (SEM) studies.

3.5.1 In vitro studies

Imidazole antifungal compounds have generated substantial interest as new therapeutic agents for many of the mycoses. Our investigation with these compounds using in vitro systems indicate that they may offer the advantages of relatively broad spectrum activities without excessively severe side effects or toxicity to the hosts. The purpose of this study was to compare the in vitro antifungal activity of fluconazole with amphotericin B, 5-fluorocytosine and miconazole against clinical isolates of Candida species.

3.5.2 In vivo studies

The lack of a suitable animal model for the study of candidiasis as it occurs in humans has hampered understanding of the host-pathogen interrelationships in the diseases and impeded development of antifungal therapy. Oral intragastric inoculation of infant mice leads to systemic spread,
lethality or long term colonization of the gastrointestinal tract. These characters make mice a useful model for the study of gastrointestinal and systemic candidiasis. Hence we studied the efficacy of different antifungal agents using mice as the animal model.

3.5.3 Effect of fluconazole on germ tube formation

Fluconazole has a pronounced effect on *Candida* species. The mechanism of action of many imidazole antifungals have been much studied and are fairly well understood. There are many different intracellular targets and those which are affected depends upon the concentration and specific imidazole used. At high concentrations imidazoles have direct effect on membrane integrity and cause deposition of membranous elements near cell wall. Another related effect of the imidazole at high concentration is to make membranes leaky. This interaction of imidazole with the plasma membrane, may explain the fungicidal properties of the drug. There are however, only few reports on the morphological changes caused by fluconazole. Hence the present study was undertaken to further previous observations with the aid of scanning electron microscope.