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2. REVIEW OF LITERATURE
PART - I

2.1. HISTORICAL BACKGROUND

2.1.1 Preamble.

The clinical entity thrush has been described since antiquity. Hippocrates (1839) in his "Epidemics" has described "Ophthae", "thrush" (white patches) in debilitated patients. The term "thrush" is derived from ancient Scandinavian or Anglo-Saxon. The term "Torsk" is Swedish equivalent of this word. The ubiquitous nature of thrush was evidenced by various vernacular names like "Soor" in Germany, "Afta" in Italian and Spanish. In French the term used for this condition is "le maguet" which means "Lily of valley". Galen and Samuel Pepys (1665) described 'thrush' as of common occurrence in sick children. Rosen van Rosenstein (1771) and Underwood (1784) described the occurrence of this condition in new borns. Veron in 1835 postulated that it was acquired during passage through the womb. He has described the first case of esophageal candidiasis. Berg (1846) has noticed that the fungus is transmitted by unhygienic bottle feeding practices. Bennet (1844) and Robin (1853) proposed that debilitation was the most important prelude to candidal infection. 3,4,5

In 1839 Lagenback described this fungus as "cryptogamic plant" from the lesion of thrush. Berg (1841) and Bennett (1844) have conclusively demonstrated the fungal etiology of thrush, while Bennet has accurately illustrated the fungus in lung and sputum of patient with pneumothorax due to tuberculosis. Berg (1841) reproduced the disease in healthy babies by inoculating "the ophthous membrane" material. David Gruby in 1842 placed this fungus in the genus "Sporotrichum". Robin (1853) documented that the thrush fungus could lead to systemic disease as a terminal event of illness. He renamed the organism as "Oidium albicans" a bewildering array of names for the fungus were proposed by Quinquand in 1868, and 'Saccharomyces albicans' by Reiss in 1877. The dimorphic nature of the fungus was noted by
Grawitz in 1877, while Audrey (1887) noted conversion of yeast form into mycelial form in response to environmental stimuli.\textsuperscript{3,4,5}

2.1.2 Origin of Monilia and Moniliasis.

The term "monilia" with which Candida is often confused, was first used by Hill in 1751 to describe the fungus from rotting vegetation. While Gmelin (1791) used the term "monilia" to describe the genus, in which some species of Mucor and Aspergillus were also included. After the dimorphic nature of yeast causing thrush was described in 1844 by Bennet, Robin (1847) placed it in the genus "Oidium". The name "\textit{Oidium albicans}" was proposed to indicate oval morphology of yeast cells.\textsuperscript{3}

A fungus isolated from rotting woods and revealing yeast and mycelial forms was described by Hansen (1868) as "\textit{Monilia albicans}". The name "\textit{Monilia albicans}" became popular and remained in use for several decades and the name "moniliasis" was proposed for the infections caused by this fungus. In 1923 Berkhout erected the genus "\textit{Candida}" to encompass asporogenous yeasts forming conidia and hyphae. The Eighth Botanical Congress at Paris accepted this name in 1954.\textsuperscript{3}

The word "\textit{Candida}" was derived from Latin name "toga, Candida" for specific white robe worn by candidates for the senate and the word "albicans" was also derived from Latin word "albicare" means, "to whiten". The combination Candida albicans essentially means "Whitening White" – a tautology of meaning.\textsuperscript{3}

2.1.3 Candidosis Versus Candidiasis.

Since 1923 when Candida replaced "monilia" the term "Candidosis" and "Candidiasis" emerged gradually into common use to replace the term "moniliasis". Emmons objected that suffix "osis" was Greek and therefore should not be attached to a Latin term. Both "osis" and "iasis" as medical suffixes denote a state or condition and were derived from Greek source. The word "Candidosis" is preferred over "candidiasis" for simple reason that "osis" suffix is consistent with the ending used for most of the fungal disease names and therefore systematically superior, it conveys sense of "Candida mycosis", 

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while most parasitic diseases end with suffix-"iasis". The International Society for Human and Animal Mycology (ISHAM) recommends "Candidosis" and the council of International Organisation of Medical Sciences recommended "Candidiasis". At present the use of term "Candida Infection" is more pertinent.\textsuperscript{3,37}

2.1.4. Different Species of Candida.

The genus comprises more than 150 species, the main common feature among the species is the absence of any sexual form. Medically significant species frequently encountered in deep seated infections and which fulfil Koch's postulates are \textit{C. albicans}, \textit{C. kefyr}, \textit{C. parapsilosis}, \textit{C. tropicalis}, \textit{C. vishwanathi}, \textit{C. glabrata}, \textit{C. guilliermondii} and \textit{C. krusei}. These eight species are potential pathogens particularly in situations of severely depressed anti microbial defenses, \textit{Candida albicans} is frequently encountered in most of the forms of candidiasis. Some species apart from these eight species that have been unequivocally demonstrated in human infections include \textit{C. vishwanathi}, \textit{C. lusitaniae}, \textit{C. clausenii}, \textit{C. intermedia}, \textit{C. lambica}, \textit{C. macedoniensis}, \textit{C. robusta}, \textit{C. norvengensis}, \textit{C. rugosa}, \textit{C. inconspicua}, \textit{C. catelunata}, \textit{C. famata}, \textit{C. ravautii}, \textit{C. lipolytica} and more recently \textit{C. ciferrii} and \textit{C. chiropetrum}.\textsuperscript{2.7}

2.1.5 Clinical Forms of Candida Recorded for the First Time.

Table 2.1.1 Showing clinical forms of Candida recorded for first time.

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<td>and thrush</td>
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<td>Vaginal candidiasis</td>
<td>Wilkinson(1849)</td>
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<td>Zenker(1881)</td>
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<td>Parrot(1870)</td>
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<td>Association between vaginal candidiasis</td>
<td>Hausmann(1875)</td>
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<td>Onychomycosis</td>
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<td>Jacobe(1907)</td>
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<tr>
<td>Osteomyelitis</td>
<td>Conner(1928)</td>
</tr>
<tr>
<td>CMC as endocrine disease</td>
<td>Sutpin(1943)</td>
</tr>
</tbody>
</table>
Since 1940, association between steroid therapy, treatment with immunosuppressive drugs, cytotoxic drugs, immune defect and candidal infection became apparent.\textsuperscript{3,4,5}

2.2. TAXONOMY: CHANGING CONCEPTS IN MEDICALLY IMPORTANT YEASTS.

2.2.1 Preamble.

The initial classification of fungi into four subdivisions viz \textit{Mastigomycotina} (sexual spores are motile oospore) \textit{Ascmoyatina} (sexual spores are ascospores) \textit{Zygomycotina} (sexual spores are zygospores) was \textit{Basidio mycotina} (sexual spores are basidio spores) was based on criteria of sexual spore formation. The fungi which do not form sexual spores were grouped into a formed division called "\textit{Fungi imperfectii}" or "\textit{Deuteromycotina}".\textsuperscript{3,5}

In view of recent developments in the classification of fungi on basis of ultrastructural studies of cell wall, septation manner and molecular sequences it is now possible to allocate all the "\textit{fungi imperfectii}" in to either of the above mentioned four divisions. Thus a formal division of imperfect fungi is removed and a term "\textit{mitosporic fungi}" is introduced to indicate the fungi which donot form sexual spores and do not have meiotic reproduction.

Medically important Candida species were considered as polyphyletic i.e. belonging to ascomycetes as well as basidiomycetes group. However, Aheran (1978) laid clues to characterise any yeast either into ascomycetes or basidiomycetes group, these are: no colour change with diazonium blue B (DBB), absence of xylose but a high ratio of mannann – chitin in the cell wall, the DNA base composition (G+C) in the range of 30-60 moles %, failure in urease production, and ability to form holoblastic, multilateral buds with the nucleus located at the neck of bud during mitosis. Application of these criteria to pathogenic Candida species has strongly suggested that these are ascomycetes rather than basidiomycetes.\textsuperscript{5,37,38,39}

2.2.2 Genus Candida.

Candida are the fungi with unicellular mode of development. The principle unifying characteristics of nearly 200 species genus. Candida as...
mentioned in recent review of Meyer et al (1982) and Barnett et al (1983) are as follows, They have no sexual forms, produce variety of cell shapes, possesses varying ability to ferment or assimilate carbohydrate, do not produce carotinoid pigment, ascospores, teliospores, ballistospores or arthospores. Within the genus, various species are characterised on the basis of physiological properties, wherein; similar properties indicate a close relationship between species.37

2.2.3 Systemic Classification of Genus Candida.

The medically important yeasts are classified as ascomycetes. The characteristic feature of fungi included in this group is the presence of ‘ascus’ a sac like structure containing ascospores born following karyogamy i.e. fusion of gametes and meiosis. The supposed biological classification of phylum ascomycota highlighting only the few species in the genus Candida is as given below.5

Table 2.2.1 Showing systemic classification of genus Candida

<table>
<thead>
<tr>
<th>Phylum</th>
<th>Class</th>
<th>Family</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascomycota</td>
<td>Endomycetes</td>
<td>Saccharomycetaceae</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Saccharomyce cervisiae</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Candida krusei</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Endomycetaceae:</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Candida famata, Candida pelliculosa</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(teleomorph Pichia anamola)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Candida norvengensis</td>
</tr>
<tr>
<td>Dipodaceae:</td>
<td></td>
<td>Candida lusitaniae</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Geotricum Candida</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Geotricum capitatum</td>
</tr>
<tr>
<td>Lipomycetaceae:</td>
<td></td>
<td>Candida kefyr</td>
</tr>
</tbody>
</table>

2.2.4. Perfect State in Candida Species.

The discovery of sexual stages in some Candida species isolated from clinical material has resulted into major taxonomical changes and confusion. The Candida species was named binomially using anamorph and teleomorph state for example Candida kefyr (anamorph kluyvyro maxicanum). However, in view of confusion with use of binomial names the original dual or binomial
nomenclature system (based on anamorph or telemorph state) is replaced with use of only holomorphic i.e teleomorph name for medically important fungi. The Nomenclature committee has permitted use of familiar (anamorphic ) names of Candida a species. Names of sexual and asexual forms of Candida a species invariably found in clinical material are as listed below.\textsuperscript{5,37}

Table 2.2.2 Showing names of asexual and sexual forms of Candida.

<table>
<thead>
<tr>
<th>Name of asexual forms</th>
<th>Name of sexual forms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Candida famata</td>
<td>Debaromyces hansenii</td>
</tr>
<tr>
<td>Candida guilliermondii (Var. guilliermondii)</td>
<td>Pichia guilliermondii</td>
</tr>
<tr>
<td>Candida guilliermondii (Var. membraneefacens)</td>
<td>Pichia ohmeri</td>
</tr>
<tr>
<td>Candida kefyr</td>
<td>Kluyveromyces maxicanus</td>
</tr>
<tr>
<td>Candida krusei</td>
<td>Issatchenkia orientalis</td>
</tr>
<tr>
<td>Candida lusitaniae</td>
<td>Clavispora lusitaniae</td>
</tr>
<tr>
<td>Candida norvengensis</td>
<td>Pichia norvengenis</td>
</tr>
</tbody>
</table>

2.2.5 Perfect /Sexual State in Candida albicans.

In 1967, Van der Walt, has described a sexual state in C. albicans, obtained from the chlamydospires and found to contain twice the DNA per cell of the budding haplophase and in 1970, the perfect state was named as "Syringospora albicans". However, the evidences regarding the concepts of sexual state of C. albicans and C. tropicalis await confirmation.\textsuperscript{37}

2.2.6. Nomenclatural Changes Among Medically Important Candida Species.

Taxonomy is not a static discipline, the genus and species undergo changes in the light of newly discovered characteristics. Within the genus Candida, various species were initially separated primarily on basis of morphological, physiological, sexual states, nutritional profiles viz patterns of assimilation of carbohydrates, temperature range for growth and metabolism, vitamin requirement and gelatin liquefaction. Afterwards, these criteria were found to be less discriminatory therefore were replaced by cellular and biochemical parameters such as presence or absence of xylose in cell wall, the
The development of molecular techniques has further resolved the taxonomic confusions among species of Candida. Among the various techniques used 5.8 s ribosomal RNA (r RNA) sequencing, southern blotting with conserved genes, DNA base composition (G + C mole %) and degree of DNA relatedness are found to be powerful tools for understanding phylogenetic and taxonomic relationship.  

The analysis of small ribosomal subunit sequences has shown that C. albicans, C. tropicalis, C. vishwanathi, form one group with more distant connection to C. guilliermondii, C. kefyr and C. glabrata. These findings are in agreement with those of co-enzyme Q analysis, which assessed the number of isoprene units per ubiquinone molecule, accordingly C. albicans, C. tropicalis and C. guilliermondii share the same system (Q 9), the C. kefyr and C. glabrata share the Q 6 system, whereas C. lusitaniae and C. krusei have Q8 and Q7 system respectively.

Among medically important yeasts, the nomenclatural changes from new taxonomic concepts are moderate in the genus Candida. On the basis of DNA homology data, Candida stellatoidea Jones et al Langer and Guerr Candida clausenii Lodder and Kregger–Van Rij and Candida langergonii Dietrichson ex van Uden were judged conspecific. These species were originally separated from Candida albicans (Robin.) Berkhout on the basis of a few morphological differences e.g. C. clausenii and C. langergonii were differentiated from C. albicans by the lack of sucrose assimilation. Electrophoretic karyotyping and DNA finger Printing of C. stellatoidea merited a varietal status of C. albicans var stellatoidea (Jones et al) Diddens and Lodder is not merely a sucrose negative mutant of C. albicans but is a variant resulting from a significant genetical rearrangement in the species.

The conspecificity of Candida tropicalis (Castell) Berkhout with Candida paratropicalis (Castell) Basgal, and the proposed conspecificity of Candida krusei (Castell) Berkhout with Issatchenkia orientalis Kudryavtsev, has been
confirmed by molecular and biological methods (based on DNA reassociation values), and is also supported by karyotyping as well as southern blot analysis methods.\textsuperscript{6,37}

The justification of division of \textit{C. guilliermondii} into two varieties viz \textit{C. guilliermondii var. guilliermondii} and \textit{C. guilliermondii var. capnophilic} on biochemical differences is not decisive because they show a high degree of DNA relatedness.\textsuperscript{6,37}

\textit{Kluyveromyces fragilis} (the teleomorph of \textit{C. pseudotropicalis}) was found to be conspecific with \textit{K. maxicanus} (the teleomorph of \textit{C. kefyr}) and subsequently both anamorphs have also been found to be conspecific (Kwon Chung and Bennett). As \textit{K. maxicanus} and \textit{C. kefyr} have precedence over \textit{K. fragilis} and \textit{C. pseudotropicalis} respectively, their nomenclature has been accepted.\textsuperscript{37,7}

Various taxonomic revisions affecting familiar names of clinically important Candida species are as shown in table.

\textbf{Table No.2.2.3: Showing former and current names of different species of Candida}

<table>
<thead>
<tr>
<th>Former name</th>
<th>Current name</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{Candida brumptii}</td>
<td>\textit{C. catenulata}</td>
</tr>
<tr>
<td>\textit{Candida clausenii}</td>
<td>\textit{C. albicans}</td>
</tr>
<tr>
<td>\textit{Candida parakrusei}</td>
<td>\textit{C. parapsilosis}</td>
</tr>
<tr>
<td>\textit{Candida paratropicalis}</td>
<td>\textit{C. tropicalis}</td>
</tr>
<tr>
<td>\textit{Candida pseudotropicalis}</td>
<td>\textit{C. kefyr}</td>
</tr>
<tr>
<td>\textit{Candida ravautii}</td>
<td>\textit{C. catenulata}</td>
</tr>
<tr>
<td>\textit{Candida stellatoidea}</td>
<td>\textit{C. albicans}</td>
</tr>
<tr>
<td>\textit{Torulopsis Candida}</td>
<td>\textit{C. famata}</td>
</tr>
<tr>
<td>\textit{Torulopsis glabrata}</td>
<td>\textit{C. glabrata}</td>
</tr>
</tbody>
</table>

\textbf{2.3. CANDIDA GENETICS}

The genetic constitution of Candida was a matter of controversy. Based on various lines of evidence there is an agreed consensus that natural Candida isolates are diploid.\textsuperscript{41}
2.3.1 Genetics of *Candida albicans*.

The imperfect nature of *Candida albicans* has hampered its genetic studies. However, it is stated that, the imperfect state provides a system in which an eukaryotic microorganism is freed from the influences that a sexual cycle imposes on the genetic biology of most eukaryotes. The reason for the imperfect state could only be speculated upon as if commensal population in individual hosts are clonal, the sexual cycle have no value to the yeast and therefore gets eliminated either by chance or by selective pressure.41

Developments in molecular techniques has facilitated progressive genetic analysis of *Candida albicans* and other Candida species, and it facilitated studies related to the process of colonisation, adaptation and mechanism of disease production at molecular level.39,40,41

Molecular studies have provided the precise nature of genetic markers within *C. albicans* for example a gene designated as ADE⁵ is found to be associated with block in conversion of imidazole ribotide, while FCY₁, and FCY₂ gene are responsible for flucytosine resistance in variants deficient in Uridine mono phosphorylase (UMP) and cytosine deaminase respectively. The products of these gene helped in correlating genetic markers among *C.albicans* and other non albicans Candida species.39,42

A genetic study in *C.albicans* has revealed that the haploid number of chromosomes in *C. albicans* is eight. The chromosome number 2 contain repetitive sequences. It is highly variable in size and carries r DNA and change in number of repeat units of r DNA results into variation in chromosome number two. The chromosome number six and other chromosomes show randomly repeat units and changes in these sequences results into genomic variation in *C. albicans*. This effect is also noted in other Candida species.39,40

Other studies reported presence of 6-9 separable large DNA elements in *C.albicans*. The remarkable feature noted was unusually large size of DNA element i.e. 5-10 Mbp for the largest chromosome.39,42
2.3.2. Genetics of Other Candida Species.

Among medically important Candida species, extensive genetic studies were also done with *C. tropicalis* and *C. glabrata*. The "lactose negative variants" of *C. kefyr* were successfully transformed with DNA from "lactose – positive strains". Genetic studies of *C. guilliermondii* have shown that, its genome is haploid and contains six chromosomes. While studies on *C. tropicalis* has shown that like *C. albicans*, *C. tropicalis* produces variants on exposure to mutagens and on protoplast fusion produce heterokaryones. Gene coding for paroxysmal proteins in *C. tropicalis* have been cloned and sequenced, it is reported that RNA of *C. tropicalis* contain two thionucleotides. 42

2.3.3 Genetic Analysis of Drug Resistance.

Molecular studies on mechanism of azole resistance has revealed the presence of multidrug efflux transporter of ATP-binding cassettes (ABC) super family and major facilitators (MF) which are are responsible for the low level of accumulation of azole antifungal agents. Two genes for these transporters, the ABC transporter gene CDR_1 and the MF gene BEN^R_ (also called Ca MDR_1) were shown to be over expressed in resistant isolates. Recent studies suggest that the over expression of BEN^R_ is responsible for the specific resistance of clinical isolates of *C. albicans* to fluconazole. 41

Other multidrug efflux transporter genes of both classes exist in *C. albicans* and some of them have been cloned recently. These are ABC – transporter genes : CDR_2, CDR_3, CDR_4, CDR_5 and the MF gene FLU_1. Over expression of CDR_2 gene in *C. albicans* showing cross-resistance to azole derivatives was reported. The expression of other CDR_2, CDR_3, CDR_4 and CDR_5 FLU_1 genes are not correlated with resistance yet. 41

2.3.4. Genetic Analysis of Commensal Candida.

Genetic studies of *C. albicans* population in oral cavity and in digestive tract has revealed that Candida population at these sites is clonal and of the same type as the individual's oral cavity strain. Further, it is also reported that *C. albicans* strain isolated from vaginitis patient are clonal and are of the same
heterozygote type as the strain carried in the women's oral cavity and digestive tract.41

2.3.5. Variants of *Candida albicans*.

Whelan et al (1981) reported "hereditary variants" of *C. albicans*, which arise by de novo mutation or by mitotic recombination and segregation. Some variants recorded and reported in early experiments are colony form variants, sucrose negative variants, peptide transport variants, and cytochrome deficient variants, variants with abnormal cell wall and cell membrane.40

2.3.5.1. Colony Variants.

Several studies have established that genetic rearrangement results into aberrant colony morphology of *C. albicans*. The occurrence of natural variants such as WO-1 (White opaque-1) are reported in *C.albicans* and *C.stellatoidea*.39,40

2.3.5.2. Sucrose Negative Variants.

*Candida stellatoidea* is considered as sucrose negative variant of *C. albicans* and was previously classified into type I and type II based on genetic and physiological characters. A transfer of regulatory gene CASUC1 that code for alpha - glucosidase when transferred into type II *C. stellatoidea* resulted into correction at sucrose utilization defect. However, it was later observed that CASUC1 is not essential for sucrose utilisation but it encodes a transcriptional activator.39

2.3.5.3. Peptide Transport Variant.

In fungi the proton translocating ATPase (H+-ATPase) drives the uptake of essential nutrients (peptides) required for maintenance of cellular function. In *C. albicans* PMA1 gene is located on chromosome 3 of Candida genome. Existence of plasma membrane ATPase variants has been reported in *C.albicans*.39

2.4. GROWTH CHARACTERISTICS AND NUTRITIONAL REQUIREMENT OF DIFFERENT CANDIDA SPECIES

The commonly encountered pathogenic Candida species grow aerobically on nutritionally rich or poor medium at pH range 2.5 - 7.5 and the
temperature range 20-30°C. They all assimilate and ferment glucose as a
carbon source and none of the Candida species assimilate nitrate as a nitrogen
source. Different Candida species vary in their abilities to utilize carbon and
nitrogen source. Among Candida species only C. krusei can grow in vitamin
free media, most of the species require biotin for growth and some require
additional vitamins.

All C. albicans strains and few isolates of other Candida species grow at
pH below 2. All the pathogenic Candida species grow at 37°C as the optimum
growth temperature of virulent Candida species e.g. C. tropicalis and
C. albicans is near to 37°C, when compared to less pathogenic species such as
C. guilliermondii which grow better below 37°C. 39,43

It is reported that all species of Candida except C. guilliermondii and
C. parapsilosis grow in partial anaerobic conditions. C. albicans produces
"stellate" colonies under anaerobic condition. Strict anaerobiosis prevents the
growth of Candida species. Growth rates of Candida species show substantial
variation according to the strain and growth conditions used. It is reported that
under optimal growth condition species such as C. albicans, C. glabrata,
C. tropicalis, achieve maximum doubling time within one hour. 39,43

2.4.1. Effect of Environmental Factors on Candida Species.

The pathogenic Candida species are killed within minutes at temperature
above 50°C but in presence of rich nutrients require one hour at 70°C. Candida
species are killed rapidly by exposure to ultraviolet radiation. Where as only
C. glabrata, C. kefyr and C. parapsilosis can be photoreactivated and recovered
after ultra violet damage. In C. albicans, C. tropicalis and others recovery from
the effects of ultraviolet light is less easy. 39,43

Candida albicans grow under hyperbaric oxygen used in medical
therapeutics, but pure oxygen at pressure of 2 atmosphere and above inhibits
its growth.

Candida albicans is very resistant to the plasmolytic effects of high
concentration of glucose and sucrose and to media with very low water content.
It can survive for very long periods in seawater and distilled water and is more
resistant to variation of humidity. Though ultrasonication for brief period has negligible effect on Candida viability, gamma radiation and high magnetic field is inhibitory to their survival.\textsuperscript{39,43}

2.4.2. Effect of Microbiota Presence on Candida Species.

Presence of other microbial forms like bacteria for example Staphylococci, members of Enterobacteriaceae family namely E.coli, Salmonella, Shigella, Proteus species and anaerobic bacteria exhibit either promotional or inhibitory influence on Candida. \textit{Candida albicans} promotes growth of staphylococci and inhibit growth of N.gonorrhæae. Growth of \textit{C.albicans} is inhibited by presence of Enterobacteria, anaerobic bacteria, and \textit{Pseudomonas aeruginosa}.\textsuperscript{39,43}

Similar phenomenon occurs among the yeast like fungi and other fungi. \textit{C.albicans} inhibits the growth of dermatophytes and Histoplasma.\textsuperscript{43}

Bacteriocine like substances producing inhibition among different species of Candida have been reported. These killer factors are mannoprotein in nature.\textsuperscript{43}

It is stipulated that like bacteria, Candida too shows susceptibility to viruses and are likely to have the presence of phage in similar temperate and lytic cycles. However, its presence and its association with virulence is not dogmatically proved like in bacteria.\textsuperscript{43}

2.4.3. Killer Phenomenon in Candida Species.

Very few strains of medically important Candida species naturally express "yeast killer factors". These molecules are secreted into the medium and exhibit pH and strain specific ability to kill other yeast strain. \textit{C.albicans} exhibits high frequency of susceptibility to killer factor of other yeast species, \textit{C.glabrata} expresses killer properties more often than other species. The effects are reported to be mediated through mannoproteins that are products of chromosomal genes rather than of cytoplasmic DNA as in \textit{S. cerevisiae}. Two plasmids coding for the killer property in \textit{kluyveromyces lactis} are reported which can be transferred to \textit{C.kefyr} by protoplast fusion techniques.\textsuperscript{43}
2.4.4. Interaction With Viruses.

Reports of viruses naturally infecting pathogenic Candida species are rare. Kozlova (1973) found viruses like particle in strains of *C. parapsilosis*. Mehta et al (1982) demonstrated spherical virus like particles producing lytic plaques in lawns of *C. albicans* when aceuleacin A was present in growth medium. Virus like particle is also demonstrated in *C. tropicalis*. 43

2.4.5. Cell Wall and Other Structural Components of Candida Cells.

Electron microscopy of pathogenic Candida species revealed multilayered cell wall sometimes surrounded by outer fibrillar layer. The plasmalemma and the intracellular organelles are typical of eukaryotes such as bilayered phospholipid plasma membrane, the ribosomes, double membrane mitochondria, the true nuclei with double membrane and porous nuclear membrane, glycogen granules and vacuoles. 43

The dry weight of yeast cell contains 20-40% protein, 30-50% polysaccharide and 1-7% lipids. Lipid fractions vary with environmental condition and the phospholipids and sterols represent the dominant lipids extractable from *C. albicans*. Among phospholipids neutral lipids, fatty acids like oleic, linoleic, plasmatic and palmitoleic are predominant lipids which help in differentiation of different Candida species. Ergosterol is the predominant sterol (40-94%) of all the sterol found in the cell wall of Candida. 44,45

2.4.5.1. The Cell Wall and Cell Wall Polymers.

The cell wall of Candida serves unique function namely cells shape maintenance, establishment of contact between the fungus and the environment, in pathogenesis and serodiagnosis. Under the influence of different physiological conditions *C. albicans* changes its cell shape and cell wall components and expresses adhesion proteins. The cell wall components of Candida species are normally absent in host, hence detection of these components and the enzymes involved in their biosynthesis are the future potential targets for antifungal agents and serodiagnosis of Candidasis. 42,43,45

The polysaccharide mannans, glucans and chitin are the important constituents of the cell wall of Candida. The mannan polysaccharides are distributed throughout the cell
wall, while inner cell wall layers are made of chitin and glucan. The percent composition of cell wall of yeast cell and germ tube are relatively similar but the alkali soluble and insoluble glucan are exclusively synthesized during early stage of germ tube formation, while higher proportion of chitin is present in hyphae. 40,45

2.4.5.2. Mannoprotein.

Mannoproteins constitute the major cell surface antigen of Candida and the side chain determines the serospecificity. The mannan polymers are covalently linked to cell wall protein through O – linkage or N – linkage, these linkages are different in different genera and therefore are of taxonomic relevance. The major epitope of serotype A of C. albicans predominantly contain straight chain of α-1,3 linked mannose residue. While in serotype B of C. albicans these epitopes are shorter, more complex and contain α-1,6 linked mannosyl residue and lack terminal α-1,3 linked mannose residue.

Various serofactors have been detected in different Candida species. as shown in table (Table 2.4.1). In C. albicans serotype A factor 6 and occasional factor 13b is present. The sero factors are serologically most active factors. In serotype B C. albicans “factor 13b” and occasional “factor 7” is present. Presence of “serofactor 6” distinguishes serotype A from serotype B. 43,45

On account of antigenic similarity a cross reactivity between and among C. albicans and different Candida species is reported. For example C.albicans serotype A cross-reacts with C.tropicalis, while C. albicans stereotype B cross-reacts with C.stellatoidea. 43

Table 2.4.1. Showing serofactors (antigenic structure) of the main pathogenic Candida species

<table>
<thead>
<tr>
<th>Species</th>
<th>Antigenic component (Factor) numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>C.albicans type A</td>
<td>+</td>
</tr>
<tr>
<td>C.albicans type B</td>
<td>-</td>
</tr>
<tr>
<td>C.glabrata</td>
<td>+</td>
</tr>
<tr>
<td>C.guilliermondii</td>
<td>+</td>
</tr>
<tr>
<td>C. kefyr</td>
<td>+</td>
</tr>
<tr>
<td>C.krusei</td>
<td>+</td>
</tr>
<tr>
<td>C.parapsilosis</td>
<td>+</td>
</tr>
<tr>
<td>C.tropicalis</td>
<td>+</td>
</tr>
</tbody>
</table>

V= Variable reaction with factor sera.
2.4.5.3. Glucans.

Presence of three distinct types of glucans are reported in *C. albicans* viz. a highly branched (1-6) β-glucan, a highly branched (1-3) β-glucans and an insoluble mixed (1-3) β-glucans complexed to chitin. In the germ tube, the newly synthesized glucans predominantly consists of (1-3) linked polymer.  

2.4.5.4. Proteins.

About twelve proteins are found to be associated with cell wall. Ultrastructural studies reported presence of outer fibrillar layer, also described as "mucous coat" or "capsule". On account of presence of recepters and adesins in this layer its role in mediating adhesion of pathogenic Candida species to epithelial cell is reported. The fibrillar protein vary between species to species between individual isolates and the growth stages of Candida.  

2.4.5.5. Cytoplasmic Inclusions.

Under electron microscope mitochondria in *C. albicans* appear as a double membrane, cigar-shaped structure. The variation in mitochondrial DNA sequence permits the differentiation of *C. albicans* on basis of restriction fragment length polymorphism.  

The nucleus of *C. albicans* is like the nucleus of other eukaryotes with double layered nuclear membrane. Other organelles described in cytoplasm of *C. albicans* are ribosome, endoplasmic reticulum, Mg++dependent cytoplasm microtubules and storage granule vacuole.  

Cell membrane of *C. albicans* is a bilayer containing protein and enzymes required for cell wall biosynthesis which also represent the site for attack by antifungal agents. Presence of electron microscopically visible fimbrae on Candida has also been reported.  

2.4.5.6. Reproduction in Candida Species.

New growth in *C. albicans* is initiated by a temperature dependent mechanism of site selection, which favours budding from sites adjacent to previous buds. But this mechanism is not operative in hyphae. Where the daughter cells, either bud/ hyphae expand with new cell wall material primarily
synthesized at the apex. At the time of mitosis, nucleus migrates to the junction between mother and daughter cells, divides, and a septum is laid down at a site determined by a band of filamentous rings, which are formed early in the stage of development of the daughter cells. Septation occurs in daughter cells when they have achieved diameters approximately 60-70% of parent diameter i.e. (20-30% of parent volume). The normal life span of an individual C. albicans yeast cell may involve not more than three or four generations. 39, 42, 43

All the Candida species multiply primarily by the production of blastospores (yeast cells). The size and shape of blastospores (elongated, ovoid or spherical) is characteristic of a species. The gross morphological appearance in all the species is similar, but some Candida species can form chains of elongated, unseparated blastospores (pseudohyphae), while C. albicans can form true hyphae as well as large refractile chlamydospores. 39, 42, 43

2.4.5.7. Occurrence of Pathogenic Candida Species in Nature.

Yeasts are found to be distributed ubiquitously in terrestrial and aquatic habitat, they are found commonly in association with plants and insects. The yeast species principally associated with human candidosis show restricted natural distribution and are found to be primarily associated with man and other warm-blooded animals. Occasional reports of isolation of Candida from amphibians are documented. Soil serves as a reservoir of different yeast species. The type of yeast species found in soil is related to source, temperature, composition and moisture content. 25, 39, 43

Number of Candida species were isolated from bark sap exudate, leaves, trees, pasture grains, flowers and fruits etc. Spenner (1995) isolated C. famata and Rhodotorula graminis from exudate of Algabrobo trees from North West Argentina. 25, 39, 46

Morais et al. (1994) isolated cactophilic yeast including Pichia barkeri, Candida sonorensis and Geotrichum species from crop the external surface of fly Drosophila and insects are reported as an important factor for spreading of yeast. 25, 39, 47

Toads, Frogs and fish are also reported to colonise by different Candida species. 25, 39, 46
2.4.5.8. Occurrence of Pathogenic Candida Species in Animals.

The digestive tract, especially the esophagus and the crop was the most frequent source of yeast isolation and in the majority of animals it is also the source of infection. The spectrum and type of Candida infections in animals resemble that in humans, with superficial, deep seated and disseminated forms. Among the various Candida species isolated, *C. albicans* is recovered from a wider range of animal hosts indicating that *C. albicans* is the principal opportunistic yeast pathogen in most warm-blooded animals.25

Carmo-sousa (1969) reported that *C. albicans* and *C. glabrata* are obligatory animal saprophytes, while *C. guillermondii, C. krusei, C. parapsilosis* and *C. tropicalis* are facultative saprophytes. *C. albicans* are isolated from soil, plants atmosphere and water, these isolates are restricted to human or animal habitat.25

2.4.5.9. Carriage of Candida Species in Normal Subjects.

*C. albicans* and few other yeast species are common, harmless commensal of the mucus membranes and digestive tract of normal individuals. These Candida species are the most important sources of endogenous candidiasis.

2.4.5.9.1. Carriage of Candida on the Skin.

Prevalence of Candida on skin of healthy individuals varies with geographical location and occupation. Carriage is more common in intertriginous sites than on glabrous skin. The skin of hospital patients showed higher carriage rate than the skin of those from normal subjects. Highest carriage of Candida was noted on the skin of cancer patients, who are highly predisposed to candidal infections. Association of *C. parapsilosis* with leg ulcers and of other Candida species with tinea pedis is commonly reported.25

2.4.5.9.2. Carriage of Candida in Mouth.

Overall prevalence of yeasts in the mouth of normal subjects varies between the range 17.7% to 40.8% respectively. The tongue is most populated with Candida followed by the palate, cheeks and other sites. The variation in Candida carriage is attributed to variation in predilection and adherence of
Candida to epithelial cells. Higher carriage rates are reported in denture wearers and subjects with poor oral hygiene.25

2.4.5.9.3. Carriage of Candida in the Gastrointestinal Tract.

The Candida occurs as commensal in the gut. Higher carriage rates in stomach and intestine are reported. The median percentage frequencies ranging from 46-51% from stomach and 45-47% from intestine are recorded.25

2.4.5.9.4. Carriage of Candida in the Vagina.

The higher prevalence of Candida in the vagina in many types of patients without clinical evidence of vaginitis or vaginal discharge is reported. Further higher carriage rate as compared to patient with vaginitis is reported in patients with candidal vaginitis.25

2.4.5.9.5. Carriage of Candida In the Faeces and Urine.

Prevalence of yeasts in the faeces increases when the patient is on antibiotic treatment. From urine of various types of subjects carriage rate of Candida varies from 1.4% to 60 percentage.25

2.4.5.10. Relative Occurrence of Candida Species in Different Clinical Material.

Subject to the yeast identification methods, the relative occurrence of yeast species may vary from different clinical material. However, it is reported that C.albicans accounts 60-80% of oral isolates, C.glabrata and C.tropicalis are found with moderate frequency (approximately 7% of all samples) in the mouth, while other species occur only rarely. A slightly lower occurrence of C.albicans from Gl tract and anorectal tract is reported. From the stomach and lower digestive tract the prevalence of C.glabrata and C.tropicalis is higher accounting for 16% and 10% respectively. Occurrence of C. parapsilosis is higher in the gut sample (6%) than in the oral sample (2%). In the vaginal sample from patients with vaginitis higher prevalence of C.albicans is reported.

The other common species isolated from vaginal samples are C.glabrata and C.tropicalis. Extremely low prevalence of C.albicans on skin of normal subjects and higher prevalence on skin of subjects with cutaneous candidiasis is documented. C.guilliermondii and C.parapsilosis are particularly common isolates from the skin of healthy individuals.25
The prevalence of *C. albicans* in blood samples is 50% of all Candida isolates, which is close to the prevalence of this species in stomach, intestine and faeces and is compatible with the concept that gut is the source for dissemination of *C. albicans* into blood stream.25

Recent reports of higher occurrence of *C. tropicalis* and *C. parapsilosis* from blood indicated existence of selection process in favour of enhancing pathogenicity of these organisms over the other Candida species. *C. glabrata* is another common species isolated from blood culture. Thus *C. glabrata*, *C. parapsilosis* and *C. tropicalis* accounts for more than 90% of Candida species isolated from blood culture. The frequency and distribution of Candida species from urine is closer to distribution of Candida in the gut than in blood, suggesting ascending route of spread to produce infection. 25

2.4.5.11. Factors Favouring Candida Carriage in Human.

Many factors such as natural factors, dietary factors, mechanical factor, hormonal and iatrogenic factors influence the overall prevalence and distribution of Candida. Most important and thoroughly studied factor is pH. It is observed that pathogenic Candida species grow over a pH range of approximately 3-8. The vaginal pH of 4.3-4.6 and the 3.4 pH of gastric secretion favours Candida carriage at these sites. Reports suggest that pH exerts its significant influence in the pathological status of clinically important Candida species by altering morphological forms, increasing adherence and invasive properties rather than influencing the growth rate or survival. 25

2.4.5.12. Distribution of *C. albicans* Serotype and Biotypes in Clinical Specimen from Human Sources.

Higher prevalence of *C. albicans* serotype A than serotype B from clinical specimen is reported and is attributed to either tendency toward selection of serotype A cells or environmental pressures associated with *C. albicans* pathologies favoring expression of serotype A antigenic epitopes only. In USA the higher prevalence of serotype B was reported and was attributed to the resistance of serotype B, *C. albicans* to fluocytosine. 25, 48
Phenotyping studies has shown that same biotype is present in different anatomical sites over a period of time. The study also revealed that some *C. albicans* phenotypes are frequently associated with the pathogenic conditions than others.  

### 2.4.5.13. Transmission and Source of Candida Species.

Biotyping of *C. albicans* suggested that the oral Candida act as a source of infection in cancer patients. Oral – cutaneous spread has been implicated in cases of nipple candidosis in India. Oral spread of Candida is also reported in cases of Candida paronychia, chronic vaginal candidiasis and infection in burn patients.  

Fecal Candida act as source of infection for vaginal candidiasis, cystitis, diaper dermatitis etc have been reported.

The vaginal Candida gets transmitted through sexual route or gets maternally transmitted to cause neonatal oral thrush. The concept that Candida gets transmitted by persorption from gastrointestinal was proved by Krause (1969) by self experimentation, which is also proved by other workers in experimental models.

Hospital ward surfaces and various fomites can act as a reservoir of Candida and thus introduce Candida into the blood stream or cause wound infections. Among drug addicts a lemon juice contaminated by Candida has been documented as a source of infection.

### 2. 5. VIRULENCE ATTRIBUTES OF CANDIDA

The difference in pathogenicity of different Candida species is attributed to difference in cellular and molecular determinants of virulence. No single dominant character representing “the virulence factor” of Candida is documented, in contrast multiple factors each with low propensity to cause infection are reported.  

Finite number of genes called "virulence trait" genetically codes the virulence phenotype of a species or strain. Qualitative and quantitative differences in expression of similar or dissimilar traits results into variation in...
virulence properties of different Candida species.\textsuperscript{52,53} During commensal association, Candida are present in yeast phase and express "the variant trait" critical for their commensal existence on mucosal surfaces where, a constant and dynamic interplay between innate and acquired host defense exist. Studies indicated that in immunocompromised host, "the variant trait" becomes "the virulence trait" leading to expression of multiple virulence related phenotypic character. The ability to produce hyphae, different enzymes, adhesins and toxins and phenomenon of dimorphism are reported as the few and distinct virulence attributes of Candida species.\textsuperscript{15,52}

\section*{2.5.1. Ability to Produce Hyphae.}

Production of hyphae and multiple blastoconidia on hyphae potentially represent multiple infectious elements or "a megamolecule" whereby the phagocytes that are chemotactically attracted are unable to phagocytose this molecule, thus providing a scope for colonisation by the pathogenic Candida species. Therefore, hyphae formation precedent to multiple blastoconidia formation is considered as an important event in the pathogenicity of medically important Candida species. However, it is now reported that phagocytosis is not prerequisite for hyphal killing, mere attachment of phagocytes followed by release of toxic products into hyphae can also result into death of hyphae.\textsuperscript{15,54}

However, the germination of yeast cell after phagocytic engulfment leads to destruction of phagocyte and therefore is an important virulence attribute of Candida.\textsuperscript{15,52,54}

Mutants producing only yeast cells are reported to be less virulent as compared to those mutants producing abundant hyphae when injected into mice intravenously. While in other studies it is reported that the yeast cells grown at 25\textdegree C become more hydrophobic, germinate quickly and are more virulent in mice indicating that hyphal committed yeast cells are more virulent than other yeast cells.\textsuperscript{15,54,55,56}

Loss, degeneration or reorganization of pre-existing cell wall components, their denovo synthesis during yeast to mycelial conversion expression of adhesins and receptor molecules, synthesis of hyphae specific
antigens determine the virulence characters of hyphae. Binding of $C_3d$ and $C_3$ complement conversion product, "fibrinogen and laminin", to the hyphae but not to the yeast cells contribute in determining the virulence of *C. albicans* hyphae.\textsuperscript{56}

2.5.2. Ability to Produce Different Enzymes.

Enzymes such as proteinases, lipases and phospholipases contribute in causing damage to host cell.\textsuperscript{57}

2.5.2.1. The Proteinases.

The extracellular proteinases found in *C. albicans* are classified as aspartyl proteinase, which possess broad substrate specificity over a broad range of pH. Candida proteinase is a mannoprotein; its activity is inhibited by pepstatin. Proteinase from different species and strains of Candida exhibit differences in substrate specificity, molecular weight, pH optima and other properties.\textsuperscript{57,58,59,60}

The low pathogenic potential of some Candida species is correlated with low extracellular proteolytic enzymes production, for example *C. parapsilosis* produce negligible amount of enzyme during fungemic episode.\textsuperscript{61} Production of intracellular proteinase by virulent species of Candida is also documented. In acidic milieu extracellular proteinase and in alkaline milieu intracellular proteinase help in invasion of host tissue.\textsuperscript{57,61}

Genes coding Candida aspartic proteinases have been cloned and it is shown that a family of seven genes SAP\textsubscript{1}-SAP\textsubscript{7} regulates proteinase synthesis.\textsuperscript{57} Phenotypic switching affects quantity of acid proteinases production and virulence trait of infecting Candida species. In addition to other virulence factors proteinases are essential for adherence and invasion before Candida colonization. The proteinase deficient Candida species are less virulent to mice in causing systemic candidiasis.\textsuperscript{57} A relation between resistance to intracellular killing by phagocyte and Candida proteinase is documented where proteinase inhibit the generation of microbicidal oxygen radical and resist intracellular killing by phagocytes. It is reported that
blastoconidia but not hyphae express the proteinase following phagocytic engulfment.\textsuperscript{54,62}

2.5.2.2. The Phospholipases.

In \textit{C. albicans} three types of phospholipases viz. lysophospholipase, lysophospholipase transcyclase and phospholipase B are reported.\textsuperscript{63,64} These phospholipases are concentrated at tips of hyphae indicating their role in tissue invasion.\textsuperscript{63, 64, 65}

2.5.3. Adherence as a Virulence Mechanism in Candida.

Adherence plays an important role in colonisation of \textit{C. albicans} to appropriate epithelial surfaces and seeding of various tissues during fungemia. It is reported that pathogenic Candida species show greater adherence in experimental animals and among the pathogenic species, \textit{C. albicans} is more adherent than other species, moreover, the hyphal forms are more adhesive to epithelial cells.\textsuperscript{15, 66}

2.5.3.1. Cell Surface Components Determining Adherence.

Mechanism of adhesion of \textit{C. albicans} to mucosal surfaces is complex and multi factorial and ultrastructural studies of \textit{C.albicans} adhesion revealed three morphologic classes of Candida adhesins.\textsuperscript{67}

i) Floccular Adhesins: A “fuzzy” amorphous, 100-300nm thick cell wall coat, located only at adhesive junctions of the \textit{C.albicans}.

ii) Fibrillar Adhesins: It is also called fimbrilar layer and is distributed evenly around the cell.

iii) Third Category of Adhesins: The cells possessing this category of adhesins lack obvious adhesive appendages but are still capable of invading intestinal mucosal surface.

2.5.3.2 Adhesion - Receptor Molecules.

Calderon (1991)\textsuperscript{66} and Hazen (1991)\textsuperscript{52} typed adhesin - receptor molecules into three different categories.

i) The mannoproteins on surfaces of \textit{C.albicans} participating in most of adhesive reactions, these are CR\textsubscript{2} like, CR\textsubscript{3} like and β\textsubscript{1} integrin like molecules which binds to host glycoproteins with arginine - glycine -
aspartic acid (RGD) sequences on iC3b, fibronectin, laminin, type I and IV collagens, fibrinogen and fibrin.

ii) The second category includes molecules involved in lectin activity in which protein portion of *C. albicans* surface molecule recognizes sugar moieties such as fucose or N-acetyl glucosamine of the host cell glycoproteins.

iii) The third category encompasses the carbohydrate portion of a molecule that recognises yet unidentified host cell membrane receptor ligand the "Factor - 6" of *C. albicans* is typified in this adhesion molecule. 52

In addition to these adhesin-receptor molecules, cell surface hydrophobicity (CSH), secreted acid proteinases, chitin and lipid components of outer surface of Candida also mediate adhesion. 52,67

It is observed that in *C. albicans* CSH is increased prior to morphological transition / germination on account of expression of hydrophobic protein molecules. Interaction between these protein molecules and yet unknown molecules on epithelial surface and plastic devices mediate adherence. The specificity and selectivity of binding of Candida (yeast phase or hyphal phase) to specific regions of each tissue indicates the expression of unique type of ligand by host cells. 52,53,68

### 2.5.3.3. Regulation of Adhesion.

The putative mechanism, which regulates adhesion of *C. albicans* in various host environments, is not reported. However, the role of morphologic and phenotypic variation and occurrence of regulatory mechanism, enabling the organism to discriminate various host environment and expression of genes necessary for production of adhesin and other virulence properties, are reported.

High concentration of galactose, sucrose, glucose or maltose affect adherence of *C. albicans*, by synthesising extracellular polymeric (EP) material, and promoting stickiness and binding of cells. The galactose-grown cells produce more EP, than glucose or sucrose grown cells. The EP of galactose-
grown cell contains higher antigenic determinants and this effect is observed only in *C. albicans* and *C. tropicalis*.\(^{66,68}\)

It is reported that Candida adhesin ligand is a mannoprotein fraction and strains with defective mannoprotein synthesis exhibit reduced adherence. Structural alteration such as changes in number and size of mannose side chains attached to the mannan backbone via phosphate bonds also result in the reduced level of adherence.\(^{66,69,70}\)

### 2.5.4. Toxins Produced by Candida.

Henrieiin (1940) reported presence of toxin in *C. albicans* for the first time. Salvin (1952) found that formalin killed blastospores of several Candida species and supernatant from mechanically disrupted *C. albicans* were lethal to mice on intraperitoneal inoculation. These are less potent, acidic, heat labile, intracellular proteins, with molecular weight 74kD and four subunits. The lethal effect is an acute anaphylactic shock. LD50 of canditoxin to mouse is 0.3 mg. The exact role of this toxin in virulence is not yet proved.\(^{69}\)

### 2.5.5. Other Virulence Attributes of Candida.

#### 2.5.5.1. Phenotypic Variation.

On account of alternation in virulence traits, cell surface antigens, susceptibility towards antifungal drugs, increase in epithelial cell trophism and resistance towards phagocytic killing, phenotypic variation bestow an opportunity to Candida to get established in changing hosts environment.\(^{71,72}\)

#### 2.5.5.2. Immunomodulation.

During systemic candidiasis the candidal antigens are released into the circulation, which modulate the hosts immune response. These antigens are mannoproteins in nature and their molecular size and charges regulate the immune response. For example the small size i.e. \(< 6\) mannan residue molecules suppresses immune response, while neutral or less charge molecules are reported to enhance the immune response.\(^{15,73}\)

#### 2.5.5.3. Antigenic Variability.

Ability to change surface antigenic components leads to evasion of hosts immune mechanism. Various factors such as nutritional sources, growth phase,
temperature of incubation, morphogenic form of the infecting strain influences antigenic expression in Candida species. The gene regulating surface antigen such as PEP₁, OP4 and Wh 11 have been reported to affect virulence of Candida species.¹⁵,⁶⁴,⁷¹

2.5.5.4. Phenotypic Switching.

Occurrence of several switching systems affecting colony and cell morphology in different Candida species have been documented. Among the Candida species C.albicans and C.tropicalis are reported to display heritable and reversible phenotypic variability at a very high frequency of 10⁻² to 10⁻³. It is reported that this mechanism provides a means for rapid adaptation to host’s changing environment and also affect the virulence trait of commensal candida into the pathogenic type. The white to opaque (WO-1) phenotype is commonly used for studies related to phenotypic switching.⁷¹,⁷³

2.5.6. Phenomenon of Dimorphism in Candida Species.

The morphological transition is extensively studied in Candida species and like other pathogenic fungi it is reported to be associated with pathogenicity of Candida. The distinguishing feature of morphological transition are: i) the transition takes place from yeast to mycelium, ii) there is no multinuclear intermediate form as is found in many other fungi or there is no further developmental stage and iii) the transition is reversible.⁷⁴,⁷⁵

2.5.6.1. Cell Wall Associated Changes During Dimorphism.

Structural analysis of cell wall during dimorphism has revealed changes in the glucan rich layer of the parent and daughter cell wall, distinctly at the junction between the cells. The mycelial cell wall is thinner and possesses more fibrillar outer layer than the yeast cells.³⁹

2.5.6.2. Chemical Composition of Cell Wall in Yeast – Mycelium Phase.

Chemically the cell wall of C. albicans hyphae is found to contain two to three fold higher chitin than the cell wall of blastospores. Which is correlated with the increased activity of chitin synthase. Quantitative differences in glucan, mannan and protein components in the two morphological forms is correlated with difference in susceptibility to various hydrolytic enzymes.⁶⁹
A higher proportion of β 1-3 linkages and relatively low proportion β1-6 linkage glucans are noted. This increase has been correlated with increased activity of β-D-glucan synthase during Y-M transition.\textsuperscript{12,15}

2.5.6.3. Regulation of Cellular Morphology.

It is hypothesised that a quantitative and temporal differences in apical and general expansion during bud formation results into spherical / ovoid blastospores, pseudohyphae and hyphae formation.\textsuperscript{39,76,77}

2.5.6.4. Genetic Mechanism.

Several genes differentially expressed between morphological forms in \textit{C. albicans} have been cloned and studied. These are described as 'chitin synthase gene', 'aspartic proteinase gene', extent of cell elongation gene, 'hyphal – specific gene' and 'pH responsive genes'.\textsuperscript{74,75}

2.5.6.4.1. Role of Chitin Synthase Gene.

Chitin synthase enzyme plays a major role in cell morphology. The two chitin synthase genes, CHS\textsubscript{1} and CHS\textsubscript{2} are studied in yeast as well as mycelial forms of \textit{C. albicans} and over expression of CHS\textsubscript{2} and not CHS\textsubscript{1} gene in mycelial forms is reported, indicating its role in morphogenesis.\textsuperscript{74,75}

2.5.6.4.2. Role of Aspartic Proteinase Gene.

Expression of aspartic acid proteinase SAP1-SAP2 genes under various conditions of growth are studied. It is observed that SAP1 and SAP3 genes regulate the white to opaque (W-0) colony morphology change, while the SAP2 gene is highly expressed in yeast forms but not in mycelial forms. The SAP 4 and SAP5 genes are expressed during hyphae formation at neutral pH. A chitin switch affects the production of secreted aspartyl proteinase and determine the distribution and behavior of the organism in the host tissue and the severity of infection thus indicating interdependence of chitin synthase and aspartyl proteinase genes.\textsuperscript{39,53,67}

2.5.6.4.3. Expression of Other Genes.

Various other genes differentially expressed in cells undergoing yeast to mycelial transition include "extent of cell elongation (ECE\textsubscript{1}) gene", Wh\textsubscript{11} and HYR\textsubscript{1} is gene. Expression of ECE\textsubscript{1} is observed when cells are induced to form
The gene Whu is expressed in yeast cells, while HYR1 gene is transcribed during morphogenesis.\textsuperscript{77, 74}

It is stated that differential expression of phenotype specific gene is not a major factor in the regulation of morphology in \textit{C. albicans}. But the regulation of morphotype in \textit{C. albicans} is at transcriptional or transnational level of gene expression and is under the direct effect of environmental factors such as pH, temperature, nutritional factors etc as shown in model.\textsuperscript{77, 74}

2.5.6.4.4. Yeast – Mycelial Transition Model.

Changes in wall structure and patterns of cell wall biosynthesis and site of new cell growth are observed during germ tube formation (Y-M conversion). In yeast growth at 28\textdegree{}C, budding is almost entirely polar, while emergence of bud is almost equatorial and multiple in germ tube. The cytoskeleton is involved in maintenance of tube like mode of mycelial growth. In glucose starved cells, deprivation of calcium ions prevented mycelial transition, indicating the role of Ca\textsuperscript{++} ions in dimorphism of \textit{C. albicans}.\textsuperscript{74}

Factors like glucose starvation, presence of nitrogen sources, variation in temperature, pH and availability of suitable nutrients induce yeast cells to form germ tube or vice versa, supporting a view that candidal growth from is a phenotypic response to environmental stimulus aided by specific genetic (structural or transcriptional control gene) responses.\textsuperscript{39, 74}

\begin{center}
\textbf{Model for Y-M transition in Candida albicans.}
\end{center}

\begin{itemize}
\item Environmental induction
\item Induction stimuli
\item Intra cellular messages
\item Altered Cytoskeleton
\item Altered cytoplasmic constituents
\item Altered pattern of wall biosynthesis
\item Altered wall composition
\item Altered wall thickness
\item Altered site of evagination
\end{itemize}

Underlined changes are observed during transition.
2.5.6.4.5. Occurrence of Morphogenesis in Vivo.

Occurrences of various morphological forms such as yeast, pseudohyphae and true hyphae in clinical material are documented frequently. However, clear-cut correlation between particular morphological form of Candida and its clinical significance is not clearly stated. 

Experimental studies on *C. albicans* dimorphism and relevance in pathogenicity have suggested that either yeast or hyphae consistently play a superior role per se in the pathogenesis of candidiasis. Both the morphological forms of the fungus possess the ability to initiate and sustain pathological response in specific ecological microniches in vivo. Interconversion between blastospores and hyphae or vice versa offers an opportunity that confers a heightened degree of pathogenicity on *C. albicans*. In early days results of studies on morphogenesis are documented as "Langeron effect". 

2.5.6.4.6. Morphological Forms Demonstrable in Vitro.

a) Blastospore.

These are unicellular forms of Candida, formed by a process of mitotic cell division called budding. Process of budding involves growth of new cellular material from a small selected site on mother cell surface. The new growth in form of bud enlarges for a period of time, the cross wall or septum formation proceeds the nuclear division to separate parent and daughter cell resulting into two blasto-spore formation. 

b) Hyphae.

Hyphae arise as branch of existing hypae or from blastospores. The blastospores produce a new cellular material in a cylinder shape called a 'germ tube', which grows continuously by apical extension. The newly evaginating hyphae till the time of formation of first septum are called as 'germ tube'. In *C. albicans*, the germ tube formation is not a process of germination. As in mycology, germination means emergence of hyphal growth from a resting spore, with little or no continuity between the cell wall of spore and hyphae. In *C. albicans* a hyphae is a tube that contain multiple fungal cell units. The mycelium refers to the entire fungal growth including hyphae, branches and lateral buds. Only *C. albicans* and rare isolates of *C. tropicalis* produce true hyphae. 

- 40 - Review of Literature
c) Pseudohyphae.

Though it resembles true hyphae, its mode of formation is different. Pseudohyphae arises from a blastospore or from a hyphae by a process of budding which differs from process of blastospore formation in two respect; each generation of buds remain attached to its parent cell and the budding progeny assumes a narrow elongated shape. This results into an end to end aggregation of blastospores and when the cells are very elongated these are microscopically indistinguishable from the true hyphae. Only careful observation of constriction at the septal junction helps to identify the pseudohyphae.12

The pseudohyphae is a morphogenetic development intermediate between budding and hyphal growth.12,15

2.5.6.4.7. Environmental Factors Inducing Yeast to Mycelium Transition.

A sole "mophogen" capable of provoking one morphological form is not identified and documented, but many substances and environmental factors are documented as inducers of Y - M transition.12

Table 2.5.1. Showing the different environmental factors favoring hyphae / blastospores formation in C. albicans.

<table>
<thead>
<tr>
<th>I] Factors favoring filament forms:</th>
<th>II] Factors favouring blastospores forms:</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. General factors:</td>
<td>A. General factors:</td>
</tr>
<tr>
<td>• CO₂/O₂ ratio &gt; 2:1.</td>
<td>• Glucose a carbon source</td>
</tr>
<tr>
<td>• High molecular weight/non fermentable carbon source.</td>
<td>• Inoculum &gt;10⁶ ml⁻¹ p&lt;6.4-7.0</td>
</tr>
<tr>
<td>• Inoculum of yeast in critical metabolic condition</td>
<td>• Temperature &lt;35°C</td>
</tr>
<tr>
<td>• Liquid media</td>
<td>Visible light</td>
</tr>
<tr>
<td>• Inoculum ≤ 10⁶ ml⁻¹</td>
<td>B. Particular chemicals</td>
</tr>
<tr>
<td>• Partial anaerobiosis</td>
<td>• Ammonium salts.</td>
</tr>
<tr>
<td>• pH ≥ 6.5-7.0</td>
<td>• Azole antifungals</td>
</tr>
<tr>
<td>• pH ≤ 6.5-7.0</td>
<td>• Biotin conc &gt; 1 µg ml⁻¹</td>
</tr>
<tr>
<td>• Temperature: ≥35°C/C ≤ 35°C</td>
<td>• Cerulenin.</td>
</tr>
<tr>
<td>B. Particular chemicals:</td>
<td>• Ergosterol</td>
</tr>
<tr>
<td>• Alcohol / Diols.</td>
<td>• Glycerol Monoleate</td>
</tr>
<tr>
<td>• Various aminoacids</td>
<td>• High P₆₄</td>
</tr>
<tr>
<td>• L-α - aminobutyric acids</td>
<td>• Polyene antifungals</td>
</tr>
<tr>
<td>• Biotin con. &lt; 1 µg ml⁻¹</td>
<td>• Sodium butyrate</td>
</tr>
<tr>
<td>• Fe⁺⁺ salts</td>
<td>• Polyoxene antifungals</td>
</tr>
<tr>
<td>• Extracts of various tissues / cells</td>
<td></td>
</tr>
<tr>
<td>• Galactose, lactose, maltose, xylose, trehalose</td>
<td></td>
</tr>
</tbody>
</table>
2.6. HOST DEFENCE MECHANISM AGAINST CANDIDA

Candida species are the part of normal microbial flora and in an immunocompetent host, innate immunity in the form of delayed hypersensitivity (DHS), and acquired immunity in the form of antibody mediated immunity (AMI) and cell mediated immunity (CMI) prevent mucosal colonization and further progression into infection.\textsuperscript{17,78,79,80}

In immunocompromised host as well as in patients with recurrent or persistent Candida infection CMI is defective, and the levels of IgG, IgA and IgE are increased. Based on this observation it is proposed that to establish persistent infection \textit{C.albicans} have ability to down regulate CMI and stimulate antibody production.\textsuperscript{79}

In different Candida infections, the two major CD\textsubscript{4}Th subsets produce different cytokines, which mobilise and activate appropriate anti Candida effector cells such as mononuclear phagocytic cells (MPC), neutrophils and natural killer cells (Nk) cells and provide prompt and effective control against infection. The cell-mediated immunity is crucial in defense against mucosal candidosis while neutrophils play a pivotal role in host defense against invasive or disseminated candidiasis.\textsuperscript{78, 81} The immunity to Candidal infection and candida host interaction is a complex phenomenon and is governed under bi-directional influence of innate as well as adaptive immune system.\textsuperscript{14, 17

2.6.1. Non Specific Mechanism at Muco Cutaneous Interface.

2.6.1.1. Mechanical Barrier.

The dry, firm surface of keratinised epithelium normally act as mechanical barrier for invasion by Candida and the lipid components present on skin, the 300 KDa protein and a 49 KDa protein of comeocyte inhibit the growth of Candida. The epithelial lining of the respiratory tract possess a mucociliary cleansing mechanism that help to eliminate the particle like Candida.\textsuperscript{78,82,83

2.6.1.2. Non Specific Anticandidal Substances.

Various secretions in the body act as non-specific inhibitors of Candida viz. amniotic fluid, saliva, etc. Histidine rich polypeptide from saliva is reported as the potent inhibitor of \textit{C.albicans}.\textsuperscript{76,83,84}
Basic proteins in saliva, seminal plasmin, protamine and cytochrome C inhibit the *C. albicans* growth. Serum directly is not Candidacidal but through activation of alternate complement pathway it helps in phagocytosis and killing of *C. albicans*.\(^{14,83,84}\)

Mannose binding protein (MBP) a lectin like substance, secreted by liver containing serine protease subunit identical to the first component of complement pathway, stimulate release of chemotaxins and opsonins that help in phagocytotic killing of candida\(^ {15}\)

### 2.6.1.3. Defence Against Gastrointestinal Colonisation.

Both aerobic and anaerobic bacterial flora, the nutritional competition, the toxic metabolites and normal gastric acidity restrict Candida proliferation in GI tract.\(^ {50,78}\)

Direct penetration of endothelial cells of GI tract and modulation of the host immune response by releasing protacydine and PGE2 results in down regulation of superoxide production by PMN.\(^ {78,85}\)

### 2.6.2. Immunoregulatory Role of Polymorphonuclear Leukocytes (PMN).

Candida reacts safely in an immune competent host. Search in a candidal component, which turns the beneficial association into infection, has reported that cell wall and secretory mannan component of candida are constanly released into the circulation, these mannan fractions serves various important functions such as immunomodulation, immunoevasion and adhesion to host cell surface.\(^ {20,78,80}\)

A mannoproteint fraction designated as MPF\(_2\) in the range of 34-36 KDa and 60-64 KDa is reported to stimulate PMN and peripheral blood mononuclear cells respectively. Apart from the phagocytic function of PMN, recently it is discovered that PMN secretes two important cytokines the IL\(_{12}\) and IL\(_{10}\). During progressive infection IL\(_{10}\) and during resolving infection IL\(_{12}\) are synthetized. The occurence of neutrophilia in vivo and the presence of abundant neutrophils at site of infection thus indicate the role of PMN in TH\(_1\)/TH\(_2\) response production.\(^ {20,73,78}\)

It is speculated that, since the PMN activating components such as lipopolysacharides, mannoproteins etc are found across wide range of
microorganisms, these components bestow the PMN with immunoregulatory function. Thus neutrophils are not only the ultimate effector cells in systemic candidiasis but also play an important role in immunoregulation of immunomodulation against candidiasis.\textsuperscript{20,78,80}

2.6.3. Role of Cytokine in Protection against Candidiasis.

The activation of T-lymphocytes by candida and release of cytokines requires the presence of human leucocyte antigen DW\textsubscript{1} i.e HLA – DW\textsubscript{1} and DR\textsubscript{1} - DW\textsubscript{12} DR\textsubscript{2} antigen on macrophages. A significant association between low responses and HLA B\textsubscript{15}, the high responses and HLA B7 and DW\textsubscript{1} are documented.\textsuperscript{81,86}

The extent and effectiveness of cytokine responses depends on the route of infection, the tissue infected, the local immune response, the genetic make up of host and infecting strain/organism.\textsuperscript{81}

In immunocompetent host \textit{C.albicans} preferentially induces Th\textsubscript{1} cells to produces IL\textsubscript{12}, IFN\textsubscript{r} and TNF of which IFN \textsubscript{r} and IL\textsubscript{12} are produced regardless of infective load. While a substantial increase in load of infection in susceptible organ induces Th\textsubscript{2} response, consisting of IL\textsubscript{4}, IL\textsubscript{5}, IL\textsubscript{6} cytokines. Studies indicated that IL\textsubscript{6} also plays important role in induction of both humoral and cellular immunity in candidiasis.\textsuperscript{19,80,81}

2.6.3.1. Th1/Th2 Paradigm in \textit{C.albicans} Infection: Implication for Therapy.

In view of observations that Th\textsubscript{1}cytokines are associated with protection against candidiasis, possible strategies for preventing candidal infection in severely immuno-comprised host is to induce protective immunity against \textit{C.albicans} by exogenous administration of Th\textsubscript{1} cytokines, or neutralisation of Th\textsubscript{2} cytokines. Concomitant administration of antifungal drugs and neutralisation of IL\textsubscript{4} into animal models has resulted in reduction of fungal load and induction of protective (IL12 associated) response.\textsuperscript{80,81}

2.6.4. Role of Mononuclear Phagocytic Cells (MPC).

Diverse population of MPC in their different states of differentiation and activation phagocytise candida via an array of opsonic and non-opsonic mechanism, kill blastosrorses as well as hyphae using both oxygen dependent
and oxygen independent mechanisms. Anti-Candida antibodies particularly IgG in serum and complement components of both the pathways mediate phagocytosis. Identification of such antibody is the future candidate for therapy in high-risk individuals.  

Direct recognition of candida by macrophages assumes important role in tissue, which are poor in opsonins such as lung, renal medulla, CSF particularly during initial phase of infection. The mannose receptors on monocytes activate the candidacidal activity by coupling these receptors with microbicidal mechanism. Increase in intracellular Ca++ concentration in mononuclear phagocytic cell leads to activation of multiple biochemical reactions to bring about killing of C. albicans.

2.6.4.1. Candidacidal Mechanism of Macrophages.

Mononuclear phagocytic cells kill C. albicans by oxygen dependent and oxygen independent mechanism under aerobic and anaerobic conditions. The macrophage and neutrophil has greater Candidacidal activity under aerobic than under anaerobic condition and kills C. albicans by respiratory burst. The various mediators of oxygen dependent killing mechanism of mononuclear phagocytic cells are superoxide anions, myeloperoxidase -hydrogen peroxide - halide system and the reactive nitrogen intermediate. Superoxide is a product of reactive oxygen intermediate (ROI) and is involved in oxidative killing of Candida by macrophages.

Super oxide exert its cidal effect either by oxidation of essential metabolites or by generation of other oxidative metabolites such as hydroxyl radicals and/or superoxide anion which combine with myeloperoxidase-hydrogen peroxide- halide and reactive nitrogen intermediate. The Candidacidal capacity of macrophages is dependent on different population and their state of activation as well as on species of Candida.


Monocytes and neutrophils generate myeloperoxidase and myeloperoxidase – hydrogen peroxide halide system to kill C.albicans. Hydrogen peroxide serves as a substrate for generation of strong microbicidal
oxidant from myeloperoxidase. The hydroxyl radical generated from hydrogen peroxide acts with halides and forms various oxidizing agents. Macrophages which are deficient in myeloperoxidase assimilate it through mannose receptors, which get incorporated in lysosomal granule to become enzymatically active and to exert its cidal effect on *C. albicans*. The difference in virulence of different Candida species is due to differences in sensitivity to various effector molecules generated by phagocytic cells.\textsuperscript{86,88}

### 2.6.4.1.2. Reactive Nitrogen Intermediate Mediated Killing.

Using arginine, oxygen and NADPH as substrate nitric oxide synthase (NOS) synthesises nitric oxide (NO) which on combination with super oxide anion generate a potent Candidacidal molecule – the peroxynitrite which is responsible for higher Candidacidal activity of macrophages producing nitric oxide and super oxide anion.\textsuperscript{86}

### 2.6.4.1.3. Oxygen Independent Candidacidal Mechanism.

It is reported that the lysosomal enzyme reduces the ability of *C. albicans* to incorporate methionine, valine, lysine phenylalanine and leucine and thus affect the viability of the fungus. Lysosomal extract contain cationic protein which are associated with two defensins, called as MCP –1 and MCP-2, these proteins damages the cytoplasmic membrane of *C. albicans* and suppresses oxygen consumption by fungus.\textsuperscript{86}

### 2.6.4.1.4. Mechanism of Killing of Hyphae.

The growth of Candida hyphae but not blastospore is inhibited by the extracellular mechanism of macrophages that involve the production of stable nitrogen containing compounds. The mechanism of killing by PMN involves chemotaxis, attachment, progressive spread and release of oxidants and granule contents into the hyphae and mediate lethal effect. PMN also release potentially toxic U-cytoplast capable of releasing sufficient oxidants.\textsuperscript{54,86}

The PMN induces fungal cell wall injury, but the DNA injury is the actual cause of lethal effect. The oxidants alter *C. albicans* gene expression and increase the rate of phenotypic switching. The \(\text{H}_2\text{O}_2\) induces nucleotide base oxidation and DNA strand breakage. It is not only highly toxic but when it diffuses and reacts with

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free Fe+++ /Ca++ metal ion cofactors, it releases highly toxic OH radicals which mediate instant damage of DNA strands. DNA associated Fe+++/Ca++ react with H2O2 and yields OH and other radicals thereby mediating damage of an adjacent molecule. Cell death occurs when DNA damage outpaces endogenous repair mechanism accompanied by depletion of ATP/NAD levels. Evidences suggest that immediately after attachment of PMN. DNA fragmentation begins by digestion at single strand breaks sites. When single strand breaks become prominent, double strand break commences and increases further slowly, in result eventually terminating into irreversible cell damage.54,86

2.6.5. Antibody Mediated immunity in Candidiasis.

Though the role of antibody in protection against candidiasis is controversial, recently, in patient recovering from disseminated candidiasis a specific antibody against a 47 KDa protein of C.albicans has been demonstrated, which is a degradation product of human heat shock protein hsp 90. It is reported that the human – anti – hsp 90 antibodies and monoclonal antibodies against 47 KDa protein both protect mice against disseminated candidiasis.18

2.6.6. Naturally Occurring Anti- Candidacidal Antibodies.

Presence of anti – Idiotype antibodies (anti – Ids), which mimicked killer toxin (KT) like antibodies have been demonstrated in vaginal fluid of patients with candidal vaginitis. The killer toxin react with specific receptor killer toxin receptors on Candida cells (KTRs). The KTRs are homologous evolutionary conserved receptors found on various eukaryotic cells including C.albicans thus anti KT antibodies, which are antidiotype – antibodies cross react with KTRs on Candida and brings about Candidacidal effect. Animals immunised with Candida also produce similar type of antibody. These results emphasise the importance of antibody mediated immunity in candidiasis.18,78 Using hybridoma and DNA recombination technique the single chain fragment variable anti – Idio antibodies i.e. a ScFV are produced which reacted with KTRs on Candida and exhibited in vitro Candida cidal activity, thus indicated its potential therapeutic use in future.18,20

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2.7. PREDISPOSING FACTORS.

Hippocrates and other workers documented that Candidal illnesses are the indicators of hosts impaired immune function. The impairment may be in the form of immune function, alteration in antimicrobial defences, and phagocytic functions. However, some Candida infections like endocarditis, liver abscess, meningitis etc. occur in healthy, post operative or non-immunocompromised hosts. The range of predisposing factors that promote Candida invasion are as follows. ⁴, ⁵, ²³, ⁸⁷, ⁸⁸

Table 2.7.1. Showing the range of predisposing factors which promote Candida infection.

<table>
<thead>
<tr>
<th>Predisposing factor</th>
<th>Explanation</th>
<th>Examples</th>
</tr>
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</table>
| 1. Natural factor   | 1. Infections, idiopathic, congenital or other debilitating diseases and disorders.  
2. Digression from normal physiological status. | • Microbial infections, endocrine dysfunctions, lymphocyte defects, phagocyte abnormalities.  
• Pregnancy, infancy, old age. |
| 2. Dietary factors  | 1. Excess or deficiency of foodstuffs alters the composition of endogenous microflora. | • Carbohydrate rich diets, vitamins deficiencies. |
| 3. Mechanical factors | 1. Trauma  
2. Local occlusion or maceration of tissues. | • Burns and other accidental wounds.  
• Wearing dentures, thumb sucking and occlusive clothing. |
| 4. Iatrogenic       | 1. Treatment with drugs that alter the composition of the endogenous microbial flora or alter host defences against infection.  
2. Surgical procedures or introduction of mechanical devices and prostheses into vessels or tissues. | • Antibiotics, corticosteroids, other immunosuppressive drugs.  
• Bowel resections, heart valve replacements, intravascular catharses. |

The severity and extent of acquiring Candida infection increases with increase in number and severity of predisposing factors. The exhaustive range of predisposing situations in which candida invasion may occur are as follows.
2.7.1. Diabetes mellitus.

It predisposes to all kinds of candidosis and significant increase in concentration of Candida in oral samples from diabetic patients is reported. The predisposing mechanisms by which diabetes increases hosts susceptibility to candidiasis is high blood and residual tissue glucose levels or low skin lactate levels that favour the growth of Candida in diabetes. Conversion of glucose to sorbitol reduces the available concentration of NADPH and thus reduces the natural oxidative killing by neutrophils.\textsuperscript{87,89,90,91,92}

2.7.2. Endocrine disorders.

Multiple endocrinopathies are documented to be associated with candidosis viz. hypothyroidism, hypo-parathyroidism and hypo-adrenocorticism. Exact mechanism by which endocrine disorders increase susceptibility to Candida infection is not known but it is proposed that endocrine influences intracellular steroid – binding proteins in \textit{C. albicans} thus affecting the behavior of fungi in host.\textsuperscript{87,93}

2.7.3. Immunopathological Disorders.

These disorders include hypo-gammaglobulinemia, severe combined immuno deficiency, T-cell deficiency and myeloperoxidase deficiency etc.

Chronic mucocutaneous candidosis is most often associated with defect in Tcell immunity. In AIDS patients oral yeast carriage increases with decrease in T-helper to T-suppressor lymphocyte ratio, the oral and esophageal candidiasis are the common infections among these patients and indicate lowered hosts immune status. Vaginal candidosis is also common in AIDS affected women.\textsuperscript{87,94,95}

2.7.4. Malignant Diseases.

Cancer patients are at higher risk of systemic Candida infections, hematological malignancies are major predisposing factors than the solid tumors. Changing trend in therapeutic management of malignancies such as more vigorous use of cytotoxic and immunosuppressive agents has resulted in rising frequency of Candidal infections in these patients.\textsuperscript{87, 96}
2.7.5. Genetic Factors.

Major racial or genetic factors predisposing to candidosis are not documented till today but, a close epidemiological review may reveal a genetic predisposing factor that might influence variation in susceptibility among individuals. The non-secretors of blood – group ‘O’ substance show higher predisposition to superficial candidiasis than their normal counter parts.87

2.7.6. Age Factor.

Oral carriage of Candida in neonates is exceptional and oral infection arises primarily because of natural contamination from birth canal. The mucocutaneous candidal infections in contrast to systemic infection are common in neonates. Other important sources of neonatal candidal infections are cross- infection in neonatal ICUs. The immaturity of antimicrobial defenses predisposes to Candida infection. Old age itself is not susceptible to candidosis but oral candida carriage rate increases with age. 4,5,87

2.7.7. Pregnancy.

Vaginal carriage of Candida is higher in pregnant women than non-pregnant women the physiological factors predisposing to vaginal candidoses are corticosteroid, vaginal pH, glycogen concentration and receptor status of the vaginal epithelia etc. The tendency to adhere to vaginal epithelial cell has profound effect on occurrence of Candida vaginitis in pregnant women than in non- pregnant women.87

2.7.8. Dietary Factors.

Carbohydrate diet-promote oral colonization of Candida. Presence of high carbohydrate favour multiplication of Candida over bacteria.97

Iron deficiency is an important predisposing factor in etiology of chronic mucocutaneous candidosis. Increased susceptibility of rats and mice to candidiasis was reported with vitamin B1 and B2 deficiency.87

2.7.9. Mechanical Factors.

Accidental injury or surgical and medical procedures leading to Candida peritonitis or septicemia are documented as the predisposing factor among the non immuno comprimied patients.87, 88
Severely burnt patients are particularly susceptible to wound infection and deep-seated infection by Candida. Improved methods of burn patient management and better diagnostic techniques have emerged as a plausible cause for increased incidence of Candida infection among burn patients. A patient on intravenous hyper-alimentation and extensive treatment with antibiotic further increases the risk. It is reported that aggressins such as elastases and thermolysin released from *Pseudomonas aeruginosa* potentiate *C.albicans* infection in mice. 98, 99, 100-106

2.7.10. Situations Leading to Occlusion or Maceration of Skin or Moist Membranes raises the local humidity and promote colonisation and super infection by Candida. Common occupations leading to local maceration and predisposition of the skin to candidal infection include bar tenders, fishmongers, cloth washers etc, who are prone to acquire candidosis of the nails, nailfolds and finger clips. Candida dermatitis is reported in bed-ridden patients. Angular chelitis and denture stomatitis due to over growth of candida in the occluded spaces between denture and palate is reported in old age group patients. 87, 88

2.7.11. Iatrogenic Factors.

Iatrogenic predisposition alone has greatly increased clinical awareness in candidosis. Candida endocarditis, an extremely rare disease exclusively found in drug addicts is now increasingly noted in patients under going open heart surgery. Carriage of potentially pathogenic Candida is more frequent in patients with prolonged stay in hospital, treated with antibiotics and steroids than in healthy individuals. 87, 107

2.7.12. Antibiotic or Cytotoxic Drugs and Corticosteroids.

Treatment with antibiotics disrupts the ecology of intestinal flora therefore allows overgrowth and subsequent passage through intestinal mucosa to initiate invasive infections. 107, 108

Antibiotics like tetracycline, penicillin and erythromycin lead to moderate to two folds increase in carriage of Candida in gut. Vancomycin, clindamycin reduces
anaerobic bacterial flora in the gut, and allows candidal proliferation and dissemination of Candida in the gut to cause disseminated candidiasis.\textsuperscript{107,109}

Higher vaginal carriage of Candida among hormonal contraceptive users is reported. The estrogen component has greater influence on Candida carriage than the progesterone component.\textsuperscript{87}

Cytotoxic drugs used in treatment of cancers induce neutropenia and predispose patients to systemic candidiasis, on account of increase in colonisation and dissemination of Candida from gut.\textsuperscript{87}

Corticosteroid reduces resistance of host towards Candida, combinations of corticosteroids are reported as statistically significant predisposing factors. The immunosuppression is not the only means by which corticosteroid predisposes to candidoses, but it is reported that these drugs affect expression of intracellular Candida binding proteins on the cell surface.\textsuperscript{87}

2.7.13. Catheter / Foreign body.

Use of indwelling catheter is associated with high risk of systemic candidosis. The risk of intravascular catheter associated sepsis rises, with the length of time. The pathogenic Candida species readily colonise plastic tips of catheters and lead to septicemia. Indwelling urinary catheters, heart valve, silicon voice prostheses intrauterine devices, shunts predispose patients to acquire Candida infections.\textsuperscript{87}


A strong association between chronic candidiasis and oral epithelial neoplasia is recently documented and is attributed to production of nitrosamine compound which directly or indirectly activates specific proto oncogenes and initiate oral neoplasia.\textsuperscript{110}

2.8. CLINICAL DISEASES CAUSED BY CANDIDA

Candida infections of all the tissue and organ systems are documented, the pathology evoked and the clinical syndromes are variable. Among the various Candida species \textit{C. albicans} is the most protean infectious agent. Infections are classified as mucocutaneous infections, cutaneous infections, subcutaneous infections and systemic or disseminated candidiasis.
2.8.1. Mucocutaneous Candidiasis.

2.8.1.1. Oral Candidiasis.

This is the most common form of candidal infection produced by overgrowth of *C. albicans*. The oral cavity of newborn has a low pH that promotes the proliferation of *C. albicans* and the condition is most commonly associated with mothers having high vaginal Candida colonization. The lesions are white patches or sometimes a pseudomembrane formation on buccal mucosa, the tongue and other oral surfaces. The membrane lesions are masses of fungi both in yeast and mycelial form. Among children, the clinical condition is identical to that seen in newborn but it usually indicate anatomic defect or immuno-compromisation.\(^{87}\)

In adult, oral candidiasis results due to mild avitaminosis or diabetic condition, advanced neoplasia, administration of steroids, antibiotics or endocrine disorder. *Candida albicans* is major species involved in thrush on accounts of its ability to adhere to oral epithelial cells and secrete inflammatory aggressin.\(^{87}\)

*Candida* leukoplakia is chronic, discrete, raised lesions are usually found on inner surface of cheeks and is associated with tobacco chewing. Speckled leukoplakia is commonly associated with malignancy. Denture stomatitis by *Candida* usually results due to badly fitting dentures or poor oral hygiene. Adherence of *C. albicans* to buccal mucosa and to the acrylic of dentures are the pathogenic attributes of *Candida albicans*.\(^{111,112}\)

Chronic oropharyngeal candidiasis occurs as complications of inhaling steroids for respiratory diseases, which may occasionally lead to bronchopulmonary candidiasis. Oropharyngeal as well as esophageal candidiasis is noted as first time clinical sign in AIDS patients. Persistent oropharyngeal candidiasis in an immune-suppressed patient may lead to disseminated candidiasis.\(^{87,95}\)

2.8.1.2. Vaginitis and Balanitis.

These diseases are characterized by the presence of a thick and yellow milky vaginal discharge and pseudomembrane on the vaginal mucosa. The rate
of occurrence of disease is high in pregnant woman, particularly in third trimester than non-pregnant woman. Alteration in the normal physiological state of health such as due to hormonal changes in pregnancy, diabetes, antibiotic treatment and use of contraceptive increases the chances of acquiring Candida infections etc. Vaginal glycogen, pH, and hormonal level favours Candida colonisation, while deficiency in local (vaginal) T- lymphocyte response is recently documented as the risk factor in vaginal candidiasis as well as chronic or recurrent vaginal candidiasis. Recently it is reported that the C. albicans surviving deep in the stratum corneum on account of temporary acquisition of polyene resistance due to alteration in membrane sterol composition, causes recurrent vaginitis. Better adherence to vaginal epithelial cells accounts for higher occurrence of vaginitis due to C. albicans. Other species reported to cause vaginitis are C. glabrata, C. tropicalis, C. albicans var. stellatoidea etc. In women with AIDS, defective CMI predisposes to recurrent vaginal candidiasis. Sexual transmission leads to balanitis. As no yeasts are demonstrable in these lesions, balanitis is considered as an allergic or toxic condition. It is also seen in patient with diabetes.

2.8.2. Bronchial and Pulmonary Candidiasis.

Bronchial candidiasis is a chronic bronchitis with cough, expectoration. The etiologic significance of C. albicans is difficult to assign. Candida readily colonise patients with preexisting pulmonary pathologic conditions, but pulmonary candidiasis as a primary disease is extremely rare. The newborn infant and children with cystic fibrosis are prone to acquire primary pulmonary candidiasis. Other predisposing factors are prolonged treatment with antibiotics, use of steroids, intravenous fluids etc. Pulmonary candidiasis following septicemia or disseminated candidiasis is wide spread and miliary, the lesions are in form of micro-abscesses or necrotic areas containing yeasts and mycelial form of Candida.
2.8.3. Alimentary Candidiasis.

2.8.3.1. Esophagitis.

Involvement of esophagus and intestine occur as an extension of lesions from the oral cavity, especially in thrush of the newborn. Chronic infection in older children is associated with genetic defects and polyendocrine deficiencies. In adults, Candida, infection of esophagitis is associated with antibiotic therapy, treatment with corticosteroids, diabetes mellitus, AIDS and radiation therapy, various neoplasias, endocrinopathies, and other debilitating conditions. Diagnosis is made by culture method and radiology findings. In AIDS Candida esophagitis is a useful clinical parameter for diagnosis.\(^{121,122}\)

2.8.3.2. Gastritis.

It usually occurs during the final stages of disseminated candidiasis. Candida super infection is also common in surgical patient. The drug cimetidine raises the gastric pH, and therefore encourages colonisation by Candida.\(^{121}\)

2.8.3.3. Peritonitis.

It can result from perforated ulcers, surgery or colonisation of indwelling catheter or intra abdominal neoplasm that permit transmission of fungus through intestinal wall. If untreated the disease is fatal. Common causative agents are \textit{C. parapsilosis, and C. tropicalis}.\(^{123}\)

2.8.3.4. Enteric Candidiasis.

This condition follows the administration of tetracycline and other antibiotic therapy. The diagnosis is difficult, requires demonstration of Candida in mucosal ulcerative lesions in intestine. Mycelial invasion can also be demonstrated in fecal smear.\(^{121}\)

2.8.4. Chronic Mucocutaneous Candidiasis (CMC).

This condition is common in persons with various genetic defects, which include dysgenesis of thymus, polyendocrine dysfunction and defective immune response. The disease manifests usually in children. The skin lesion principally appear on face, ears, neck and shoulders, which in severe cases develop into hyperkeratotic crusts known as "Candida granuloma". The most striking feature is the absence of dissemination of the Candida into deep sites.\(^{124,125}\)
2.8.5. Cutaneous Candidal Infections.

2.8.5.1. Intertriginous Candidiasis.

Cutaneous candidiasis involves the intertriginous areas of the glabrous skin primarily or secondarily to pre-existing infections. It is most commonly seen in the axilla of groin, infra-mammary folds, intergluteal folds, interdigital spaces, glans penis and umbilicus. Candidiasis of the skin is associated with metabolic disorders such as diabetes, chronic alcoholism, obesity or with those occupation where continuous moist condition favour proliferation of Candida growth.114

2.8.5.2. Paronychia and Onychomycosis.

These are most common forms of cutaneous candidiasis. The paronychial folds are readily colonised, particularly in people whose occupation requires frequent immersion of their hands in water e.g. orange sorters, fish mongers etc.4,87

Other cutaneous Candidal infections are seen in infants e.g. diaper candidiasis. Candida granuloma is a rare condition among children with defective immune system.4,87

2.8.6. Systemic Candidiasis.

It is a relatively rare condition that occurs as a terminal event of a debilitating illness. Continuous seeding of yeast into blood stream, on repeated pricks among drug addicts, presence of indwelling catheters, use of long-term antibiotics or steroid therapy result into systemic candidiasis.87,126,127

2.8.7. Urinary Tract Candidiasis.

Involvement of urinary tract is reported in association with disseminated candidiasis, diabetes, pregnancy, and administration of antibiotics and use of unclean catheters. The condition is more common in women than in men. In more than 80% cases C. albicans is reported as the causative species.

_Candida albicans_ is also the predominant species causing cystitis, other species are C. _glabrata_, C. _guilliermondii_ C. _kefyr_, C. _lusitaniae_ and _C. tropicalis_.87
2.8.8. Endocarditis.

It arises in patients with pre-existing cardiac pathology. The etiological agents are usually species of Candida other than \textit{C. albicans}. Three categories of patients are susceptible to Candida endocarditis. The first include patients with preexisting valvular disease, second group of patients include drug addicts and third category of Candidal endocarditis is seen in patients who have undergone heart surgery.\textsuperscript{128,129}

Common species of Candida involved are \textit{C.parapsilosis}, \textit{C. guilliermondii}, \textit{C. albicans var. stellatoidea} than \textit{C. albicans}. Peripheral vein Candida phlebitis and thermbophlebitis occur at site of insertion of I.V. catheters. \textit{C. tropicalis}, \textit{C.glabrata} are the species incriminated as a causative agent.\textsuperscript{128,129}

2.8.9. Meningitis.

It is relatively rare and occur by dissemination of Candida from foci in gastrointestinal tract, respiratory system or as septic emboli from infected heart valves or by introduction of Candida during intravenous therapy.\textsuperscript{187} The CNS lesions vary from large, solitary abscesses to widespread micro abscesses to granuloma like lesions. Usually it is indolent and caused by \textit{C. albicans}, \textit{C.guilliermondii}, \textit{C.tropicalis}, \textit{C. vishwanathii}, but almost all the cases are caused by \textit{C. albicans}.\textsuperscript{13,131}

2.8.10. Septicemia.

Candida septicemia is increasingly noted as a terminal event to underlying disease. The \textit{C. albicans} and \textit{C. tropicalis} are most commonly involved. There is candidemia, followed by micro abscess formation.\textsuperscript{132}

The clinical signs of Candida septicemia include, fever, chills and impaired renal function. \textit{Candida albicans} is the species involved in most cases, followed by \textit{C. tropicalis} and \textit{C. parapsilosis}.\textsuperscript{132}

Occurrence of Candida arthritis and osteomyelitis is reported as a result of fungemia or drug abuse. Knee joints are commonly affected. The \textit{C.guilliermondii} and \textit{C.parapsilosis} are the commonly involved species.\textsuperscript{132,133}
2.8.11. Allergic Diseases Involving Candida.

Allergy to cell wall components, polysaccharide of Candida is well established phenomenon and the clinical condition is called as "candidids". Many individuals without clinical symptoms of infection or allergy react to the intradermal injection of a culture filtrate of C. albicans called "Oidiomycin", indicating that antigen can pass through mucosal wall.\textsuperscript{134}


Candida species are noted as seventh most common nosocomial pathogens in intensive care units and accounted for occurrence of candidemia and urinary tract infections in hospitalized patients. Although C. \textit{albicans} is the most common cause of fungemia, infections by other Candida species viz. \textit{C. tropicalis, C.glabrata, C. parapsilosis, C. krusei} and \textit{C. lusitaniae} are increasingly documented. Many of these infections arise from an endogenous source, but the patient population, various treatment regimens, antibiotics or other supportive care measures also influence the frequency of occurrence of nosocomial candidiasis. Infections may arise from exogenous source via contaminated hands of health care workers, contaminated infusates, biomaterials and inanimate environment.\textsuperscript{28}

2.9 LABORATORY DIAGNOSIS OF CANDIDIASIS: SIGNIFICANCE AND IMPORTANCE OF LABORATORY DIAGNOSIS.

In a case of suspected candidiasis complete cultural confirmation is necessary. Though a negative culture result is significant but a positive one is not unusable proof of involvement of Candida. Isolation of yeasts from any specimen which is in contact with the mucocutaneous surfaces is of no pathological significance unless the number and morphology of the organism present as well as the possibility of other etiological agents is taken into consideration, before implicating the Candida as inciting agent. However, isolation of Candida from normally sterile specimen such as blood, CSF, joint aspirates etc gives absolute diagnosis of Candida.\textsuperscript{135,136}

Many clinical laboratories avoid yeast isolation and identification on account of cost and pragmatism. The isolation and identification of Candida species help in
better understanding and management of patient on account of knowledge of newer species involved and their changing sensitivity pattern. \textsuperscript{135,136}

2.9.1. Isolation of Candida from Muco-Cutaneous Surfaces.

For collection of specimen from these areas, cotton swabs are commonly used. To yield positive culture a clinical specimen should contain more than \(10^3\) cells/ml. Use of enrichment media to increase the sensitivity of isolation of Candida is recommended. For vaginal candidiasis at least four vaginal swabs yielding similar colony counts are required to achieve significant result. For skin samples adhesive tape strips and cyano acrylate biopsies offer greater sensitivity of Candida recovery. For oropharyngeal sample dry swabs are less sensitive, while mouthwash gives higher recovery and the imprint culture is also sensitive method. \textsuperscript{135,136}

2.9.2. Isolation of Candida from Sputum.

Candida infections of the bronchi and lungs are difficult to diagnose definitely, as the pathological significance of yeast in sputum is doubtful. On account of occurrence of Candida in the respiratory tract, mere isolation of Candida from sputum or identical specimen indicates contamination of sputum with commensal yeast from throat or mouth, so also the methods for processing of sputum for Candida isolation are exactly not standardized. Trypsinisation and liquefaction of sputum to increase sensitivity of yeast isolation is recommended, however, processing of freshly collected sputum sample on selective or non-selective media is widely accepted. \textsuperscript{135,137}

The collected data indicate that concentration of yeast vary in relation to symptoms of pulmonary candidosis. The concentration of Candida greater than \(10^4\) cellc per ml is significant. Evidences suggesting the presence of particular yeast in sputum and its pathogenic association are not documented. \textsuperscript{135,137}

2.9.3. Isolation of Candida from Urine.

Occurrence of Candida in urine is regarded as an abnormal finding and it signifies urinary tract infection that requires treatment. The significant candiduria count varies from \(10^5\) / ml to \(10^4\) / ml depending on presence or absence of catheters and the general status of patient. Candida counts are
useful in assessing prognosis of the patient. Microscopically, detection of hyphal forms of candida in urine in contrast to budding yeast cells donot resolve the pathological significance. Detection of Candida in urine sample obtained by direct aspiration is of greater significance than the clean catch midstream urine specimen. Cystosomy tubes, nephrostomy tubes, and catheters provide favorable surface for colonization by Candida, while catheterisation beyond twelve days induces candiduria, where the Candida count reaches upto $10^5$/ml in hospitalized patients.  

2.9.4. Isolation of Candida from Blood.

In many clinical situations candidemia result endogenously due to persorption or penetration Candida across intestinal wall. As much as more than two weeks incubation is required for yielding blood culture positive result for Candida. Use of brain heart infusion broth, hypertonic media such as 0.3 molar sucrose solution are recommended to increase blood culture positivity. Advance techniques involve intermittent ventilation and agitation of blood culture bottles, use of biphasic media, lysis centrifugation and concentration by filtration etc. Published data reported, increase in sensitivity of blood culture when biphasic system or isolator system is used. It is also reported, that incubation temperature at $35^0$ C also provide better results. Species such as C. krusei, C. glabrata take many days to grow, while C. albicans, C. parapsilosis and C. tropicalis take 1-3 days. Isolation and identification of the species recovered from blood provides important clues regarding antifungal sensitivity pattern of the species isolated and prognosis of disease, the source of infection, e.g. isolation of C. krusei warrants its relative resistance to fluconazole, while fungemia due to C. glabrata warrant fatal outcome of infection.

2.9.5. Isolation of Candida from Faeces and Body fluids.

Isolation of Candida from stool species is not difficult and untreated specimen can be cultured. Mycelial forms of Candida are commonly seen in watery or mucoid stool specimen from neonates. To distinguish pathogenic
species from non-pathogenic, presence of mycelial form should be looked on
direct microscopic smear examination.\textsuperscript{135,136}

2.9.6. Isolation and Identification of Candida Species.

2.9.6.1. Microscopic Examination of Yeast in Clinical Specimens.

Direct microscopic examination of clinical specimens such as skin scrapings, biopsy sections and other samples from patients is useful diagnostics procedure, but is less sensitive than culture for detection of Candida in clinical specimen. Routinely Gram stain and potassium hydroxide preparations are useful for demonstration of Candida directly in clinical specimen. In tissue sections stained by H and E or Giemsa’s stain, Candida are difficult to demonstrate and special stains such as Methnamine silver, Girdley’s and PAS stains are used to increase the chances of demonstration of Candida in tissue section. Other dyes which can be used for staining of Candida are periodic and acridine yellow, acridine orange, woolfast pink, RL-methylene blue, fluorescein conjugated lectins and crystal violet etc. Incorporation of Parker’s ink with potassium hydroxide in wet mount is reported to improve Candida detection. Phase contrast microscopy is simplest method for detection of Candida.\textsuperscript{135}

Rao (1989)\textsuperscript{141} and Graham (1983)\textsuperscript{142} reported that Candida can be easily detected by viewing the material under UV illumination as Candida exhibit florescence when stained with eosin.

2.9.6.2. Identification of Candida Species Based on Microscopic Appearances.

\textit{C.albicans} yeast cells are usually ovoid but not conspicuously elongated, \textit{C. parapsilosis} yeast cells are commonly more ovoid than those of \textit{C. albicans}. Blastospores of \textit{C. kefyr} are large, ovoid or elongated yeast cells. Blastospores of \textit{C. krusei} are elongated, but are more parallel-sided than \textit{C. kefyr}.\textsuperscript{136}

2.9.6.3. Culture Media for Candida Isolation.

The most popular and useful agar media for the primary isolation of pathogenic Candida species are versions of the peptone - glucose or peptone – maltose agar first described in 1894 by Sabouraud and therefore known as “Sabourauds Agar”. These media suppress the growth of bacteria because of acidic pH (less than 6). Bacterial growth can also be suppressed by an addition
of antibacterial antibiotics to the medium such as cholramphenical, penicillin and streptomycin. Cycloheximide inhibit growth of Candida species, such as *C. glabrata*, *C. krusei* and *C. parapsilosis*. 136,143

Various media which can be used for isolation of pathogenic Candida species are Nickerson's (1954) medium containing bismuth polyhydroxy polysulphite, on which *C. albicans* and few other species produce distinct dark brown to black colour. 138

Pagano Levin and Trejo (1958) devised a variant of Sabouraud agar in which triphenyl tetrazolium chloride (TTC) is used as an indicator. *Candida albicans* is unable to reduce tetrazolium dyes as a result it produces white colonies, while other Candida species gives various degrees of pink to red coloration. This medium is recommended as a primary isolation medium for Candida species as it facilitates detection, tentative identification of candida species in specimens containing mixture of species. Nickerson's agar and Pagano Levin agar in combination are used for isolation and identification of *C. albicans* from water samples. 136,144,145,

Bromocresol green with neosporin is used for a mixed Candida culture, species of yeast are differentiated by colour, which is a function of pH and the morphologic characteristic of the colony. 145

On phosphomolybdate containing agar, *C. albicans* produces green colonies, while other species produce white to blue colonies. Fleming et al in 1987 used a media for presumptive identification of *C. albicans* and other fungi on mustard oil-cake agar. Fischer and Kane 1974 devised a selective agar that prevented overgrowth of *C. albicans* over dermatophytes in specimens. 136

Commercial systems for *C. albicans* isolation are Microstix-Candida and Orient-N, which are dip slides used for superficial candidiasis. Till-U test, plastic culture envelopes (PEM-C), and Vagicult are used for isolation of vaginal yeasts. 136
2.9.7. Medically Important Candida Associated With Human Infections.  

2.9.7.1. Candida albicans (Robin) Berkhout 1923.

2.9.7.1. a. Synonymy.

- *Oidium albicans* Robin 1853 *Monilia albicans* (Robin) Zopf 1890;
- *Syringospora robinii* Quinquaud 1869; *Endomyces pinoiyi* Castellani 1912;
- *Mycotoruloides triadis* Langron et Talice 1932; *Candida genitalis* Batista wt Silveria 1959; Kreger-van Rij lists 90 synonyms. Grub described the organism in 1842 as a *Sporotrichum* spp.

2.9.7.1. b. Colony Morphology.

- On SDA 25°C, after three days - creamy smooth; one month-creamy glistening, waxy, soft, smooth to reticulate.

2.9.7.1. c. Microscopic Morphology.

- These are globose, short ovoid (5 to 7 μm) Sometimes elongate cells (4 to 6 x 6 to 10 μm). Smaller and larger cells are also seen.

2.9.7.1. d. Fermentation.

- Glucose+, galactose +, Sucrose +/-, maltose +, cellobiose -, lactose -, melibiose -, raffinose -, melizitose -, insulin -, trehalose +/- w/ -

2.9.7.1. e. Assimilation.

- Glucose+, galactose+, sucrose+, maltose+, cellobiose-, trehalose+, lactose -, melibiose -, raffinose-, ribose-, L-rhamnose -, ethanol +/-, glycerol +/-, erythritol-, ribitol +/-, D-manintol+, L-arabiniose +/-, D-arabinose -, inositol -, potassium nitrate -, L-sorbose +/-, inulin +/-

2.9.7.1. f. Teleomorph state.

- As yet unknown, Syringospora with affinities to the Basidiomycetes has been proposed as the teleomorph stage.

2.9.7.2. Candida guilliermondii (Castellani) Langeron et. Guerra 1938.

2.9.7.2. a. Synonymy.

2.9.7.2. Colony Morphology.
After three days at 25 °C thin, flat, glossy, and cream to pinkish.

2.9.7.2.c. Cell Morphology.
After 3 days short ovoid cells (2 to 5 x 3 to 7 μm) sometime cylindrical cells.

2.9.7.2.d. Fermentation.
Glucose +, galactose +/w, sucrose +/w, maltose-, cellobiose -, trehalose +/w, lactose -, melibiose +/-, raffinose +/-, melezitose -, inulin +/w.

2.9.7.2.e. Assimilation.
Glucose + galactose +, L-sorbose+, sucrose+, maltose+, cellobiose+, trehalose+, lactose-, melibiose+, raffinose+, melizitose+, inulin+, D-xylose+, L-arabinose+, ethanol +/-, D-ribose+/-w/-, soluble starch+/-w/-, L-ramnose +/w/-, ribitol-, glycerol+, erythritol -, D-mannitol+, inositol-, potassium nitrate -.

2.9.7.2.f. Teleomorph state.
Mating strains are Pichia guilliermondii. Many strains do not mate. The organism is found on normal skin of human in sea water feces of animals, butter milk, leather, fish and beer, it is also isolated from many human infections.

2.9.7.3. Candida krusei (Castellani) Berkhout 1923.

2.9.7.3.a. Synonymy.
Saccharomyces krusei Castellani 1910; Endomyces krusei Castellani 1912; Monilia krusei Castellani et Chalmers 1913; Candida lobata Batista et Silveria 1959.

2.9.7.3.b. Colony Morphology.
After three days at 250°C on SDA colonies are flat, dull dry after one month dull soft smooth wrinkled dense growth of mycelium extending as a lateral fringe around the colony.

2.9.7.3.c. Cell Morphology.
These are cylindrical sometimes ovoid cells (3 to 5 x 6 to 20) some become very long. A thick pellicle develops across the broth and crawls up the side of tube.

2.9.7.3.d. Fermentation. Only glucose is fermented.
2.9.7.3.e. Assimilation.


2.9.7.3.f. Telemorph state. *Pichia kudriavezii.*

It is regularly isolated with some forms of infant diarrhea and occasionally with systemic disease. Found in beer, milk products, skin, feces of animals and birds, and pickle brine.


2.9.7.4.a. Synonymy.


2.9.7.4.b. Colony Morphology.

After three days colonies are soft, smooth, white, sometimes lacy.

2.9.7.4.c. Cell Morphology.

Short - ovoid to long - ovoid cells (2.5 to 4 x 2.5 to 9μm).

2.9.7.4.d. Fermentation.

Glucose +, galactose +/w-, sucrose -, maltose-, cellobiose -, trehalose -, lactose -, Melibiose -, Raffinose -, Melezitose -, Inulin -.

2.9.7.4.e. Assimilation.

Glucose +, galactose +, L-sorbose+/w, sucrose+, maltose+, cellobiose -, trehalose+, lactose-, melibiose-, raffinose-, melizitose+, inulin-, D-xylose+, L-arabinose+/-, ethanol +, D-arabinose-, D-ribose +/w/-, soluble starch w/-, L-rhamnose-, ribitol+, erythritol -, D-mannitol+, inositol-, potassium nitrate -.

2.9.7.4.f. Telemorph State.

*Loddermyces elongisporus.* Regularly found in nail disease and endocarditis. Found in pickle brine, normal skin and feces.

2.9.7.5. *Candida albicans* Var. *stellatoidea* (Jones et Martin) Langeron et Guerra 1939.

2.9.7.5.a. Synonymy.

*Monilia stellatoidea* Jones et Martin 1938; *ProCandida stellatoidea* Novak et Zsolt 1961,
2.9.7.5.b. Colony Morphology.

After six days slow growing, small creamy smooth colonies. After one month, dull soft verrucose or lightly folded colonies appear. On blood agar it grows as small stellate colonies hence the name.

2.9.7.5.c. Cell Morphology.

After three days short to ovoid or long – ovoid cells (4 to 8 x 5 to 10 μm) elongate or apiculate cells.

2.9.7.5.d. Fermentation.

Glucose +, galactose -, sucrose -, maltose +, cellobiose -, trehalose -, lactose-melibiose -, raffinose -, melezitose -, inulin -.

2.9.7.5.e. Assimilation.

Glucose +, galactose +, L-sorbose-, sucrose -, maltose +, cellobiose -, trehalose +, lactose -, melibiose -, raffinose -, melizitose -, inulin -, D-xylose +, L-arabinose +/-/w, ethanol +, D-arabinose -, D-ribose -, soluble starch +, L-rhamnose-, ribitol -, glycerol +/-, erythritol -, D-manintol +, inositol -, potassium nitrate -.

Most isolates are from vaginal discharge. The organism is considered as mutant of C. albicans. Both have GC ratios at 35 (C. albicans 35.1, C. stellatoidea 35.7).

2.9.7.6. Candida tropicalis (Castellani) Berkhout 1923.

2.9.7.6.a. Synonymy.

Oidium tropicalis Castellani 1910; Monilia tropicalis, Castellani et Chalmers 1913; Kreger –van Rij lists 57 synonyms.

2.9.7.6.b. Colony Morphology.

After 3 days creamy white smooth, after one-month dull, soft, smooth, reticulate or wrinkled colonies appear.

2.9.7.6.c. Cell Morphology.

After 3 days globose ovoid or short ovoid cells (4 to 8 x 5 to 11 μm). A thin film with air bubbles form over the broth.

2.9.7.6.d. Fermentation.

Glucose +, galactose +/-/w, sucrose +, maltose +, cellobiose -, trehalose +/-/w, lactose -, melibiose -, raffinose -, melezitose -/+ , inulin -.
2.9.7.6.e. Assimilation.

Glucose +, galactose +, L-sorbose-/+, sucrose -, maltose +, cellobiose +/-/w, trehalose +, lactose -, melibiose -, raffinose -, melizitose +, inulin -, D-xylose +, L-arabinose +/w/-, ethanol +, D-arabinose -, D-ribose -, soluble starch +, L-rhamnose-, ribitol +, glycerol +/w/-, D-mannitol -, inositol -, potassium nitrate -.

This organism is closely related to C. albicans it is isolated from feces, shrimp, kefir, and soil.

2.9.7.7. Candida pseuduotropicalis (Castellani) Basgal 1931.

2.9.7.7. a. Synonymy.

Endomyces pseuduotropicalis 1911, Monilia pseuduotropicalis Castellani et Chalmers 1913.

2.9.7.7. b. Colony Morphology.

After three days creamy smooth, and after one month dull, soft, smooth, or slightly reticulate colonies appear.

2.9.7.7. c. Cell Morphology.

After 3 days short – ovoid with few elongated cells (2.5 to 5 x 5 to 10μm). Pseuduomycelium is abundant in most strains, in rare isolates non is formed. The cells are very long, fall a part and lie parallel like "logs in a stream."

2.9.7.7. d. Fermentation.

Glucose +, galactose +, sucrose +, maltose -, cellobiose -, trehalose -, lactose +, melibiose -, raffinose +/w, melezitose -, inulin +.

2.9.7.7. e. Assimilation.

Glucose +, galactose +, L-sorbose -, sucrose +, maltose -, cellobiose +, trehalose -, lactose +, melibiose -, raffinose +, melizitose -, inulin +, D-xylose +, L-arabinose +, ethanol +, D-arabinose -, D-ribose +/w/-, soluble starch -, L-rhamnose -, ribitol -, glycerol +/w, D-mannitol +/w/-, inositol -, potassium nitrate -.

2.9.7.7. f. Telemorph State.

Kluyveromycetes fragilis. Commonly isolated from nails and lung infection. Found in cheese and dairy products.

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2.9.7.8.a. Colony Morphology.

After 3 days soft, creamy, glistening. After one-month creamy soft to membranous, wrinkled colonies appear.

2.9.7.8.b. Cell Morphology.

After 3 days ovoid to cylindrical (2.5 to 7 x 4 to 12 μm).

2.9.7.8.c. Fermentation.

Glucose +, galactose +/-, sucrose -, maltose +, cellobose -, trehalose +, lactose -, melibiose -, raffinose -, melizitose +, inulin -.

2.9.7.8.d. Assimilation

Glucose +, galactose +, L-sorbose +/-, sucrose +, maltose +, cellobose +, trehalose +, lactose -, melibiose -, raffinose -, melizitose +, inulin -, D-xylose +, L-arabinose +/-, ethanol +, D-arabinose - D-ribose - soluble starch +, L-rhamnose -, ribitol +, glycerol +, D-mannitol +, inositol -, potassium nitrate -.

Isolated from spinal fluid and sputum.


This is the anamorph of *Clavispora lusitaniae* Rodrigues de Miranda 1979, an ascosporogenous yeast. The cells are ovoid, (1.5 to 6 x 2.5 to 10 μm) in pairs and chains. On PDA it forms pseudomycelium with verticilate chains of blastoconidia. It ferments glucose and sucrose and assimilates variety of sugars.

It has been isolated from several cases of opportunistic infection and is resistance to AmB.

2.9.8. Germ Tube Test.

It is a traditional test for identification of *C. albicans*, where *C. albicans*, produces hyphal overgrowth from blastospores when incubated at 37°C in serum for 2-3 hours. The term "germ tube" is used to indicate newly evaginating hyphae up to the time of formation of the first septum. The germ tube produced by Candida species other than *C. albicans* are called pseudohyphae and show marked constriction at the junction between the germ tube and the parent blastospores. Reynolds and Braude in 1956 for the first time discovered the ability of blood components to stimulate hyphae formation.
in *C. albicans*, while Taschdjian et al in 1960 for first time described its use in identification of yeast.136

2.9.8.1 Factors Affecting Germ tube Development.

Temperature, pH and nutrition are the three external variables affecting germ tube formation.39,146,147 Decrease in germ tube positivity is noted with stored *C. albicans* cultures. It was observed that in Lee’s medium (amino acid salt agar) germ tube formation is poor unless the yeast inoculum consists of starved, stationary-phase cells. In a defined medium, germ tube formation is influenced by two different variable viz. 1) Yeast growth phase and 2) strain to strain variation. Many isolates undergo yeast-mycelium transition when stationary phase yeast cells are transferred to fresh medium containing amino acids or N-acetyl glucosamine. (GlcNAC). 39,146, Recently it is observed that a starvation period of 20 minutes is sufficient for exponential phase cells to acquire the capacity to undergo germ tube formation in serum and in buffered GlcNAC, exponential phase blastospores give almost as many germ tubes as cells in stationary phase. In Lee’s medium the proportion of germ tubes formed from stationary phase blastospores inoculum depends on the nature of the carbon source used to grow the inoculum, while with glucose and galactose, superior results are obtained than other carbohydrate.9,39 Divalent cations especially zinc play a significant role in initiation of growth in *C. albicans*, where as in buffered GlcNA, Ca++ is specially essential for germ tube induction.39

In all germ tube inducing media, initial blastospore concentration more than 10⁶/ml suppresses hyphal development. This effect of yeast cell ‘overcrowding’ depended on the metabolic history of yeast cells, and is much remarkable with cells grown in galactose rather than in glucose medium.12,39 It is reported that *C. albicans* produces a substance known as MARS (morphogenic auto regulatory substances) which supresses the germ tube production.148

Berardinelli and Opheim (1985)149 and Shepherd et al (1985)39 reported that inoculum size in the range of 10⁴-10⁷ cells per ml, is the optimum yeast cell
concentration, especially when animal serum is used, the germ tube formation is very sensitive to cell concentration more than $10^6$ per ml. Mackenzie et al (1962) found minor effect until the cell concentration is greater than $10^7$ cells per ml. A serum stored at $4^\circ$C for 15 days resulted in 50% reduction in germ tube positivity when compared to the freshly prepared or frozen serum.

2.9.8.2. Strain Variation in Germ Tube Formation.

Mattia et al (1992) and Shepherd et al (1985) proposed the terms high, low and non responders to distinguish the strains that respond to simple compound (such as GlcNAC, proline, glucose plus glutamine), that require serum and a germinative strains respectively. Buckley et al (1982) reported a variant which produced germ tube at 25°C on corn meal agar and on synthetic medium containing GlcNAC and Tween 80 but failed to produce germ tube on medium such as serum or serum substitute, egg white, various peptone containing media, Sabourauds medium (pH adjusted to 7.5) GlcNAC and synthetic medium.

2.9.8.3. Site Of Selection for Germ Tube Formation.

It is demonstrated that during growth at 25°C new buds emerge mainly in the narrow polar regions of the parent blastospores adjacent to the previous bud scar and when growth is re-initiated from the stationary phase a random pattern of budding was observed.

When stationary phase cells were transferred to GlcNAC at 37°C a random pattern of germ tube formation was observed and up to five protuberances arise on the surface in first 30 minutes most of which subside and only one or two persists and develops into the tube.

2.9.8.4. Various Inducers of the Germ Tube.

Such inducers are compounds that promote or trigger germ tube formation, but do not induce the expression of specific genes required for hyphal growth. Peptide fractions as well as aminoacids from serum and seminal plasma induce germ tubes, and the peptide fractions of amino acids are the preferred nitrogen sources for germ tube formation. L-prolines induce germ tube formation by stimulating specific proline permease enzyme.
Glucose at 2.5-5 Mmol is required for gratuitous germ tube formation. High concentration of glucose repressed induction of GlcNAC permease and germ tube formation.\(^39\)

In addition to serum other media inducing germ tube formation such as plasma, egg white, saliva, tissue culture media, sheep serum, tryptase soya broth, nutrient broth, peptone, tryptone, 0.5% glucose with L-leucine, DL-phenyl alanine, glycine, DL-aspartic acid, DL-isoleucine, histidine, tyrosine, glutamic acid, glutamine etc, are recommended for germ tube production.\(^153\)

Because of inherent safety problems non-human sera are increasingly recommended. Berrardinelli et al (1985)\(^149\) introduced a medium containing rabbit coagulase plasma with EDTA and tryptase soya broth. Media that can be used for production of germ tube in \textit{C. albicans} are Lee's medium with amino-acids/salts, Eagle's minimal essential medium containing glucose, salts, amino acids, and Imidazole-buffered N-acetyl D-glucosamine etc.\(^12,146,149\)

### 2.9.8.5. Germ Tube Positive \textit{Candida tropicalis}.

Though the ability to form germ tube is characteristic of \textit{C. albicans} Martin et al (1979)\(^154\) in their study noted germ tube formation in fresh isolates of \textit{C. tropicalis}. Joshi et al (1983)\(^153\) also reported germ tube producing strains of \textit{C. tropicalis}. Tiemo and Milstoc (1977)\(^155\) reported that, following changes in respiration and glycolysis, morphological changes occur in \textit{C. tropicalis} like in \textit{C. albicans}, for example an abrupt change from aerobic to fermentative metabolism of glucose may results into filament formation in \textit{C. tropicalis}. It is also stated that hypoxic state of the patients and association of fungus with other bacteria create a suitable ecological condition that induces morphological change (in vivo) in \textit{C. tropicalis}. Martin et al (1979)\(^154\) reported that during repeated subculture of \textit{C. albicans} and \textit{C. tropicalis} germ tube forming ability is lost, which is probably either due to dilution of germ tube stimulating substance found in surrounding milieu, specimen or due to variations among strains.
2.9.9. Chlamydospores Formation.

Chlamydospores are large, refractile, thick walled cells that are suspended from hyphae on specialized blastospores called "suspensor cell". The C. albicans C. dubliensis and saprophytic Candida are able to produce chlamydospore. Though chlamydospore production is only observed under in vitro condition, some workers report the presence of chlamydospores in host tissue (in vivo conditions). 12

Chlamydospores arise in nutritionally depleted medium thus represent a dormant growth form of C.albicans. Usually, chlamydospores arise singly and occasionally in pairs without suspensor cells, indicating the metamorphic conversion of suspensor cell into chlamydospore. On prolong incubation, chlamydospores get deteriorated. Structurally chlamydospore posses thick cell wall, outer layer of chlamydospore is continuous with suspensor, and is mainly made up of β-1-3-D-glucan, and small amount of chitin and polysaccharide. The inner wall is rich in protein. 12

Chlamydospores are produced on nutritionally poor culture medium with a low glucose concentration and prolonged incubation. There is universal agreement that addition of a detergent usually polyoxythenesorbitan monoleate (Tween 80) and sometimes bile salts induces chlamydospores formation. Other factors influencing chlamydospore formation are temperature at 25-50°C, and low density inoculum. Chlamydospore formation is inhibited by light. The ability to form chlamydospore is reduced or lost in C. albicans strains maintained by subculture for prolong periods, chlamydospore production is independent of germ tube producing ability of the strain.12,156

2.9.9.1. Media Used For Chlamydospore Production.

Different media have been recommended for chlamydospore production by C. albicans, namely N-acetyl glucosamine, Chitosan-trypticase-Tween 80, Corn meal agar, 0.1% glucose agar with Tween 80, Czapek-Dox agar, Liu-Newton agar, Milk based agars, oxgall agar, polysaccharide-tryphan blue agar, Potato–carrot-bile agar, rice based agars, sodium taurocholate agar, soil extract agar, Zein agar etc. 12
2.9.10. Fermentation and Assimilation Reaction.

Numerous investigators have attempted to classify Candida by determining the ability of fungi to ferment certain carbohydrates with or without formation of gas. Variations in the fermentation results of the sugars are noted by many workers. \(^{38,47,143}\) It was documented that strains of same species gave variable results when tested with certain carbohydrates and the same strain vary in their ability to ferment carbohydrate from time to time. \(^{38}\)

Consistent results were obtained by using fermentation reactions and the Candida species were classified into different species using glucose, sucrose, lactose, maltose and galactose. Fermentative ability of Candida species is usually tested in liquid medium with 1\% peptone, 2-4\% sugar and suitable pH indicator. Production of gas and change in color of indicator usually indicates fermentation of carbohydrate. All carbohydrates that are fermented are also assimilated, but all carbohydrates that are assimilated are not fermented.

Wickerham in (1957) reported that assimilation reactions are less variable than fermentation reaction and only 0.5\% carbohydrate is required as against 2\% to 4\% in fermentation reaction. \(^{4,7}\)

Assimilation profiles of Candida species were determined by auxanography method. Wickerham and Burton (1948) introduced liquid medium deficient in carbon source to increase the sensitivity and specificity, of the test result. Liquid assimilation test media's were prefered over auxanograms for their higher sensitivity and specificity, the only limitation is the prolong incubation period required.

Further modification of assimilation test is use of agar slants incorporated with sugars. Mickelson et. al. (1977) \(^{157}\) suggested use of heavy inoculum and shallow agar layers to obtain rapid results.

The choice of substrate for assimilation and fermentation tests varies with laboratories. Baker et. al. (1981) \(^{158}\) used melezitose and inulin for identification of sucrose negative and germ tube negative variants of \(C.albicans\). While Syverson et. al. (1981) \(^{159}\) used eleven carbohydrates for
identification of Candida isolates. Kamiyama et al. (1989)\textsuperscript{160} used number of carbohydrates to check fermentative and assimilation ability of \textit{C. albicans} to delinate them into \textit{C. stellatoidea} and classical \textit{C. albicans} strains.

Molina et al. (1992)\textsuperscript{161} suggested use of microfermentation test, for rapid identification of Candida species.

2.9.11. Identification of Candida Species Based on Mycelial Morphology.

Martin et al in 1937 for the first time noted that, commeal Tween 80 agar stimulates the production of abundant chlamydomospores in \textit{C. albicans}. While Dolan et al. (1971)\textsuperscript{162} and Joshi et al (1975 and 1993)\textsuperscript{163,164,165} recommended use of 0.1\% glucose agar for demonstration of the different mycelial forms of Candida species, as well as germ tubes and chlamydomospores of \textit{C. albicans}. Microscopic features namely pseudohyphae, hyphae, blastospores or arthroconidida produced by different Candida species serves as potential identification markers of different Candida species.\textsuperscript{159,166} The characteristic morphological features produced by different Candida species are as describrd below.

2.9.11.1. \textit{Candida albicans}.

It forms branched hyphae with characteristic cluster of blastospores along its course. Some times after 24 hrs. of incubation, short hyphae with terminal chlamydomospores are seen without cluster of blastospores. Which usually appear in 48 hrs.

2.9.11.2. \textit{Candida tropicalis}.

It forms long branched thin pseudohyphae with sparse lateral blastospores.

2.9.11.3. \textit{Candida pseudotropicalis}.

If forms pseudohyphae consisting of spindles cells with similar cells lying parallel to and along side of them.

2.9.11.4. \textit{Candida krusei}.

It forms pseudohyphae with short lateral branches bearing single or a few terminal blastospores giving them" match stick" appearance.
2.9.11.5. *Candida parapsilosis.*

It forms spidery, branched tree like mycelium radiating from small central colony of blastospores. Some other form large blastospores and giant hyphae. Some other isolates form only a few short hyphae.

2.9.11.6. *Candida guilliermondii.*

It forms a few short branched / unbranched pseudohyphae.

2.10. **EPIDEMIOLOGICAL TYPING OF *CANDIDA ALBICANS***

Increased in incidence of mucosal and systemic infection caused by Candida species during recent time has been well documented. Though *Candida albicans* is generally accepted as the most pathogenic and frequently encountered member of the genus Candida, recent increase in frequency of infections due to species other than *C.albicans* namely *C.tropicalis, C.krusei, C.lusitaniae* etc is documented. Irrespective of the reason for this rise, discrimination of the clinical isolates into subpopulation and types is assuming importance, as the resulting data helped in identification of subpopulation with definite pathogenic potential, and shall permit epidemiological investigations. Such data will also help in implementing adequate control measures to prevent spread of infection.34,35

Number of typing methods for strain differentiation has been documented. These are broadly grouped as phenotypic methods and genotypic methods as shown in table:

**Table 2.10.1 Showing different phenotypic and genotypic typing Methods for Candida strains.**

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<th>Phenotypic methods</th>
<th>Genotypic methods</th>
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<tr>
<td>• Serotyping</td>
<td>• Restriction fragment length polymorphism</td>
</tr>
<tr>
<td>• Morphotyping</td>
<td>• Southern blot hybridisation using species specific probes complementary to dispersed repetitive DNA element</td>
</tr>
<tr>
<td>• Resistotyping</td>
<td>• Random amplification of polymorphic DNA (RAPD)</td>
</tr>
<tr>
<td>• Biotyping</td>
<td>• Amplicon fragment length polymorphism</td>
</tr>
<tr>
<td>• Protein variability</td>
<td>• Electrophoretic karyotyping</td>
</tr>
<tr>
<td>• Sensitivity to yeast killer toxin</td>
<td></td>
</tr>
<tr>
<td>• Carbon assimilation reaction</td>
<td></td>
</tr>
<tr>
<td>• Isoenzyme types</td>
<td></td>
</tr>
</tbody>
</table>
Because of genetic heterogeneity and phenotypic switching observed within strains of Candida species, especially *C. albicans* has a very unstable karyotype and consequently an unstable phenotype, neither phenotypic nor genotypic method is an ultimate typing method. However, the optimal approach is to involve a combination of genotypic and phenotypic typing methods.\textsuperscript{34,35}

### 2.10.1. Serotyping.

In 1961 Hasenclever and Mitchell \textsuperscript{167} found that *C. albicans* strains could be divided by agglutination-absorption experiments into two serotypes, of which type A contained at least one antigen more than yeast phase cells of serotype B, which was later reported as factor six by Tsuchiya et al. (1975)\textsuperscript{168}

Subsequent studies conducted to find relatedness between *C. albicans* *C. stellatoidea* and *C. tropicalis* indicated a close antigenic similarity between *C. albicans* serotype A and *C. tropicalis* and between *C. albicans* serotype B and *C. stellatoidea*. Using cross immunoelctrophoresis technique, Tsuchiya et al. (1975)\textsuperscript{168} determined the third serotype of *C. albicans* i.e. serotype C which was later reported as an intermediate strain of *C. albicans* by other workers.\textsuperscript{167,169}

Using the cytoplasmic antigens in double diffusion gel electrophoresis about five to six antigens of *C. albicans* were observed, 15-17 antigens by classical immunoelectrophoresis and 68-78 antigens by cross immunoelectrophoresis.\textsuperscript{170,171}

Cross linked immunoelectrophoresis studies using soluble cytoplasmic antigens of serotype A and serotype B of *C. albicans*, revealed presence of large number of cross reacting antigens, differing qualitatively and quantitatively from each other and that *C. albicans* serotype A contain four additional antigens not found in serotype B *C. albicans*. Using monoclonal antibodies against immuno-determinants of *C. albican* five different serotypes types were obtained.\textsuperscript{170,171}

Epidemiological studies carried out in Europe, Africa and North America revealed higher prevalence of serotype A than serotype B in human infections. Differences in colonisation rate of serotype A and serotype B in animal...
innoculated with both serotypes were noted by Auger et al. (1983). Consistently higher recovery of serotype A from stomach and retrosigmoidal areas were recorded, suggesting the possibility of influence of host factors in selection of serotype A.

Auger et al. (1983), Poulain et al. (1983), Martin and Lamb (1982) indicated that in vivo condition or under influence of environmental factors there is tendency towards selection of serotype A cells or overexpression of serotype A epitopes by serotype B cells resulting into higher reports of serotype A recovery from various infections.

Dupont et al. (1992), Auger et al (1979), and Brawner et al. (1989) documented higher occurrence of serotype B in HIV patients and correlated it, with sensitivity of serotype A strains to 5 FC, the antifungal drug used in treatment of HIV cases. Treatment with 5FC results into removal of serotype A strains in HIV patients.

2.10.2. Morphotyping.

This typing scheme is based on observation that many C. albicans strains produce colonies that are obviously different from the typical, smooth, white, colonies and frequently show fringed borders or a wrinkled surface. Using this character C. albicans was typed into various morphotypes by Phongpaichit et al (1987), on malt extract agar by inoculating the single streak-line of inoculum containing 9 X 10^8 cells/ml.

The variation in morphology is now explained on the basis of presence of high frequency switching phenomenon in C. albicans.

Various morphological characters of streak line such as texture, surface topography, fringe distribution, width etc. were included for preparing a code (Phongpaichit et al 1987). However, to simplify the statistical analysis and to achieve better discrimination Hunter et al (1989) proposed a modified code, in which six different fringe characters and two surface topography states were taken in to consideration. The strain which do not produce fringe is coded as 000, while those strains with initial digits 1,2,3, or 5 as discontinuous fringes.
The strains producing wide fringes are labelled as 7,5,3, or 4 and 7,5,1 or 2 as wide coarse. Theoretically, the given morphotyping system could possibly distinguish over 100 morphotypes, and Hunter et al (1989) 179 reported 50 different morphotypes within 446 clinical strains of *C. albicans*. The discontinuous fringe type was found to be frequently associated with fatal deep infections.

Better discrimination among *Candida albicans* strains were reported when morphotype and resistotype technique was used in combination. Other combination like killer typing, API20C, APZYM or susceptibility to flucytosine, sodium arsenate or resistance to boric acid was reported to be less discriminatory when compared to morphotyping.34,35

Hunter et al (1990) 180 used combination of morphotyping and resistotyping technique to determine carriage of *C. albicans* strains among patients and staff in intensive care unit. The study revealed that all patients, except one, were colonised by a single strain, throughout their stay in the unit, while nurses were colonised by more than one strain type. The strains isolated from nurses hands were indistinguishable from those strains colonising the patient under their care. Where as strains recovered from the mouth of the nurses were distinct from that of the patients strain.

Borromeo et al (1992) 181 reported the occurrence of single morphotype in mouth of healthy non-smokers and multiple morphotypes in mouth of smokers and non-smokers with erythematous candidiasis.

Oliver et al. (1993) 182 reported that morphotypes of *C. albicans* recovered form oral cavity of patient in the early stage of HIV infection were different form morphotypes isolated form the advanced stage of infection. The association of particular morphotype (724) of *C. albicans* in patient with ARC and AIDS was reported, this 724 morphotype was not found in HIV Ab+ve patients.

Earlier reports have mentioned higher occurrence of 000 morphotype and thus were considered as wild and most virulent *C. albicans* types. Based on this assumption, higher rate of occurrence of this morphotype from clinical
specimen like blood, body fluids etc. from patients of deep-seated candidiasis is expected. But in practice the discontinuous morphotype was found to be frequently associated with fatal infection. Later it was found that over heating of the medium abolishes fringe formation. The possible association of discontinues narrow fringe formation can be explained in light of occurrence of phenomenon of "phenotypic switching" in \textit{C.albicans}. \textit{C.albicans} exists either in mycelial form or yeast form, a discontinuous fringe formation along the streak line results because only a small proportion of cells on margin of streak line grow into mycelial form. It is reported that, full virulence of \textit{C.albicans} is determined by cell committed to different morphological forms rather than cells in single phase. Thus a population of invading cells which are switching between the two morphological forms are likely to be more virulent. The demonstration of frequent occurrence of discontinuous fringe type in \textit{C.albicans} form oral site is attributed to the occurrence of variety of ecological environment in oral cavity which favour those strains that readily adapt changes in niche. Whether there is direct relationship between strains causing oral infection and deep infection is not clear.\textsuperscript{179, 180}

At present, morphotyping technique is the only technique, which indicate virulence of \textit{C.albicans}. This test can be used as first line test as it has obviously more value in assessing clinical significance and prognosis of a blood culture isolate of \textit{C.albicans}. Irrespective of association between virulence and morphotype, this technique is simple. Reproducibility of the test is reported at 86\% for the identical type and 96\% when difference of only one character is taken into consideration.\textsuperscript{34, 35}

2.10.3. Resistogram Typing.

Resistogram typing was first developed for bacteria in early 1970s. Bacterial species are delineated into strains based on their resistance or susceptibility to selected organic or inorganic compounds.

In 1979, Warnock et al. developed a resistogram typing method for distinguishing strains or types of \textit{C.albicans}. Originally six chemicals viz. malachite green, boric acid, sodium arsinate, copper sulphate, acrylamide, and
4-chlororesorcenol were tested at six concentrations. Initial data revealed that most patients harboured isolates of identical resistogram types. Warnock et al. (1979) characterised 420 C.\textit{albicans} isolates recovered from 30 patients with vulvo-vaginitis, 16 women were colonised or infected with single resistogram strain. The same strain was recovered over multiple times, and the vaginal tract was found to get recolonized with the same strain isolated from intestinal tract of women. Similarly sexual partners were found to be colonised by the same resistogram defined strain.\textsuperscript{183, 184}

In 1982 McCreight and Warnock\textsuperscript{185} screened 75 additional organic and inorganic compounds to make the procedure easier to perform and growth end points easier to interpret. Sodium selenite, boric acid, cetrimide, sodium periodate, and silver nitrate were tested at four concentration. Improved reproducibility over the original procedure was observed.

Using modified resistogram method the distribution of \textit{C.albicans} resistograms in patients with denture induced stomatitis was determined by McCreight et al. (1984).\textsuperscript{185} Eighteen different resistogram strains were detected, 10 types in single patient and eight types in multiple individuals. Similarly, the resistotype of \textit{C.albicans} recovered from oral and cutaneous sites of patients receiving radiation for oral and laryngeal cancer were compared. Total thirteen resistogram-defined types were recovered, one major type was found among both patient groups.

Ghannoum et al. (1985)\textsuperscript{186} also used the resistogram method to investigate whether specific \textit{C.albicans} are more frequently associated with cancer patients undergoing chemotherapy or radiotherapy than healthy control individuals, two strains predominated and were found to be equally distributed among the three study group population. The two strains predominated in this study, and the oral strain distinguished by McCreight et al. (1984)\textsuperscript{186} were also found to be different. These findings support the conclusion that no particular strain delineated by resistogram typing was associated with oral cavity.

Modified resistogram methods have been applied for epidemiological investigations. The epidemiology of vaginal candidosis was studied by Meinhof...
et al. (1982) using a resistogram system supplemented by proteinase activity, lipase activity and 5-FC susceptibility, the study revealed that 70% of the vaginal strains were identical to the oral or intestinal strains recovered from the same individual. In a similar study Hunter and Fraser et al. (1987) compared strains of *C. albicans* recovered from GI tract with strains recovered from patients of vaginitis. No particular resistogram types were found to be more frequently associated with vaginal infections, than any other strains.

The resistogram methods can be used for strain delineation, pathogenesis or epidemiological studies. The resistogram, however, do not correlate with the pathogenic potential of the strain. This method does not require any special equipment and the procedures are simple to perform in moderately equipped laboratory. Problems encountered with the procedure include standardisation of the inoculum, reproducibility of the results and end point designation. Addition of more tests to expand the variation and development of more objective end point results are needed to improve the value of resistogram typing method.

2.10.4. Biotyping.

In 1980 Odds and Abbott developed a nine-test scheme to biotype isolates of *C. albicans*. The tests were based on growth and no-growth end points under a variety of conditions and the generation of 3-digit code, that theoretically could distinguish 512 strains. The 9-test grouped in three's were growth at pH 1.4, proteinase activity, and 5-FC resistance; urea assimilation, sorbose assimilation, and NaCl tolerance; and citrate assimilation, glycine assimilation and safranine resistance. In the initial study, 45 biotypes were distinguished among oral and vaginal isolates of *C. albicans* recovered from 85 patients.

This biotyping methods have been applied to investigate several epidemiologic and virulence questions. In a study of 25 women, the biotypes of the vaginal isolates were the same as isolates recovered from their mouths, rectum or urethra supporting the conclusion that individuals usually harbour one strain of *C. albicans*. It also supports the conclusion that the GI tract may be an endogenous reservoir for development of recurrent vaginal candidiasis.
Analysis of the biotypes of 282 isolates of *C. albicans* recovered from 50 males and 181 females revealed that no specific biotypes were associated with Candida infected individuals, when compared with healthy control individuals. The above data indicates that the biotyping scheme may only be used for epidemiological studies and not for comparing virulence among different biotypes.\(^{34}\)

Odds and Abbott (1983)\(^{190}\) investigated variability of *C. albicans* biotypes from different geographic areas. The three-code biotype, serotype and 5-FC resistance of 247 isolates from United Kingdom were compared with 330 isolates recovered from five different geographical locations in the United States. Only 160 biotypes were distinguished among all isolates tested. Therefore it is concluded that all possible 512 biotypes may not occur in nature. Ten major clusters of related biotypes were found to be more prevalent in both populations. The isolates from United States tended to be more pH tolerant and more resistance to 5-FC or safranine and assimilated urea, sorbose or glycine more often than isolates from the United Kingdom. Overall no biotypes or cluster of biotypes was found to be associated with anatomic site(s) neither any association between biotype(s) from infected versus colonised individuals was found.

Odds and Abbott (1983)\(^{191}\) subsequently refined the original nine-test biotyping scheme to improve strain delineation of *C. albicans* and to allow species identification as well as strain delineation of other Candida species. The glycine test was replaced with boric acid resistance test. In addition, resistance to pH 1.55 and four inhibition tests viz. (cetrimide, tetrazolium, sodium periodate and MacConkey agar) permitted identification of species other than *C. albicans*.

Burnie et al. (1985)\(^{192}\) used biotyping to confirm an outbreak of systemic candidiasis in an intensive care unit. Strain delineation was done by serotyping, morphotyping and modified biotyping system. Isolates from 13 cases of systemic candidiasis and cases of superficial infections in the same hospital unit were found to be of the same strain type, although variation in tolerance to pH 1.4 and resistance to boric acid were noted. The four staff members in the
unit were also found colonised by the outbreak strain. These strains were more persistent in hand washings may be due to resistance to antiseptics.

Krogh et al (1987) determined the yeast species and biotypes from patients with oral leukoplakia and lichen planus, using modified biotyping system, association of specific biotype was not noted among these patients.

Biotyping scheme has potential to be used for epidemiological studies of *C. albicans* and other medically important yeast studies, but not to assess the pathogenic potential. The procedures are relatively simple and require no special equipment. The results are highly reproducible within laboratory, but reproducibility among laboratories is poor, therefore, this method can be applied for performing epidemiological studies within one laboratory.

2.10.5. Biotyping Based on Commercial Carbon Assimilation Patterns and Enzyme Profiles.

The API 20C yeast identification kits with 19 carbon assimilation reactions was used to biotype 61 isolates of *C. albicans* isolated from human immuno deficiency virus-infected patients. A single biotype was found to be dominant (64%) and eight other biotypes were detected. Problems in interpreting growth or no growth end points, lack of reproducibility and less strain variation limits the utility of this test for *C. albicans* strain delineation.

Biotyping based on enzyme profiles obtained with commercially available product the APIZYM system contains 19 hydrolytic enzyme reactions, and is used for biotyping of Candida on basis of variable enzyme profiles. Roman and Silica (1983) typed 126 isolates of *C. albicans* and found five enzymatic reactions. Williamson et al. (1986) used the same enzyme system to type 213 oral isolates of *C. albicans* and reported only eight types.

Commercial availability of both these kits makes them useful of epidemiological purpose. The only limitation is the narrow range of discrimination. However combination of these 2 kits will provide broader range of variation.
2.10.6. Typing Based on Protein Variability.

During the yeast cell cycle, thousands of proteins are synthesised within each cell. Non lethal mutations yield proteins with different physical properties, which can be distinguished from the native proteins. One or two dimensional gel electrophoresis methods have been used to separate proteins from cell extracts by their molecular weights. The protein bands can be visualised by Coomassie blue staining, and the protein profiles from the different strains can be compared, or the proteins can be detected by reactions with standard anti-sera.\textsuperscript{34,35}

Cytoplasmic antigens of yeast and mycelial phases of two strains of \textit{C.albicans} were compared by the use of two-dimensional gel electrophoresis by Manning and Mitchell et al. (1980).\textsuperscript{196} Approximately 168 cytoplasmic antigens were detected, none was found to be mycelial specific.

By immunoblotting, difference in protein profiles of isolates of \textit{C.albicans} serotype A and B were noted. Forty protein bands were detected for the serotype A and 31 for the serotype B strain.\textsuperscript{196}

Lehmann et al. (1989) \textsuperscript{197} has recently compared protein and specific isoenzyme difference among Candida species and strain. Eight specific enzyme patterns were noted in \textit{C.albican} strains.

Protein profile differences among \textit{C.albican} isolates permit strain delineation. However, the procedures are time consuming, and further, require special equipment and technical expertise.

2.10.7. Typing by Sensitivity to Yeast Killer Toxins.

Killer toxins are extracellular toxins first described in \textit{S. cerevisiae}. These toxins kill sensitive yeast strains but not the producer strain. Production of such toxins has been documented in many species of yeast and chemically has been shown to be associated with double stranded RNA, virus like cytoplasmic particles.\textsuperscript{34,198}

Middlebeek et al. (1980)\textsuperscript{199} found that 70 to 87 \textit{C.albicans} isolates were sensitive to at least one killer strain when tested by seeded agar/streak plate method.
Polonelli et al. (1983) expanded the killer toxin strain method to delineate medically important yeast and from among 100 isolates of *C. albicans* 25 types were delineated.

Typing of *C. albicans* isolates by determining their sensitivity to a panel of killer toxins is useful for strain discrimination.

2.10.8. Strain Delineation Based on Genotypic (DNA) Methods.

Molecular biology provides approaches to explore the structure and function of the genetic makeup of *C. albicans*. The molecular biologic approaches based on ability to determine variations in the structure of DNA have been applied to find differences both among and within species of medically important yeast. These approaches are: i) Electrophoretic karyotyping, ii) Restriction fragment length polymorphism and iii) PCR based typing.

Genetic studies with *C. albicans* to delineate the strains are limited because of absence of a sexual cycle and the difficulty of inducing mutants due to stable diploid nature. The diploid nature of *C. albicans* has only recently been confirmed through analysis of mitotic recombinants, DNA content and other mutagenesis studies. Pulsed-field electrophoresis studies has permitted the separation of chromosome sized DNA molecules of medically important yeast viz. *C. albicans, C. tropicalis, C. guilliermondii* etc.34,35

2.10.8.1. Electrophoretic Karyotyping.

Electrophoretic karyotyping methods based on the genetic structure of an isolate reveal sufficient variation for strain delineation. No association between a particular karyotype-defined strain(s) and virulence has been demonstrated. Limitations of the method include the need of specialised equipments, prolong turn around time, small number of isolates that can be tested and the technical expertise.34,35

2.10.8.2. Restriction Fragment Length Polymorphism.

This method for strain delineation is based on variations in DNA structure. The DNA extracted from the isolates is cleaved into fragments and these fragments are separated on the basis of molecular size using gel electrophoresis technique. The difference or similarities in the fragments are
detected by staining gel with ethidium bromide and visualising bands under UV light, or hybridising fragment with specific DNA probe. Based on differences in the patterns of major bands, multiple isolates of C. albicans or C. tropicalis, C. parapsilosis, C. krusei can be distinguished.

RFLP provide excellent means for strain delineation. The extent of variation detected with DNA probes is as good as fingerprinting. Limitations include need of specialised equipment for radiolabeled probes and limited number of strains tested at a time.\(^{34,35}\)

2.10.8.3. PCR Based Finger Printing.

PCR technology can aptly be used for amplification of polymorphic DNA with arbitrary sequences. PCR amplicon fragment length polymorphism analysis involves the digestion of polymorphic DNA (RAPD) amplimers with restriction endonucleases and separation on gel electrophoresis. Distinct RAPD profiles were obtained for C. albicans, C. tropicalis, C. stellatoidea, and C. dubliniensis.\(^{34,35}\) The PCR technology is particularly convenient for epidemiological investigations of Candida species. The limitations include need for specialised laboratories, special equipments and technical expertise.

2.11. ANTIFUNGAL SUSCEPTIBILITY TESTING

Fungal infections due to common pathogenic fungi such as candida have become more frequent as a result of increase in number of immunocompromised patients. An important change in the epidemiology of opportunistic and nosocomial candidal infections has been noted with an emergence of non-albicans candida species such as C. parapsilosis, C. krusei, C. tropicalis, some of which cause life threatening infections and exhibit decreased susceptibility to azole antifungals. Though prophylactic antifungal therapy is indicated in such infections, because of their intrinsic resistance, there is a problem in the management of such patients.\(^{200,201,202}\)

Although extensive research has resulted in the development of new antifungal agents, only few of them are in current use. These are the polyenes such as Amphotericin B (AmB), the imidazoles such as miconazole (Mcz) and
ketoconazole (Kcz), the triazoles such as fluconazole (Fcz) and itraconazole (Itcz) and the pyrimidine synthesis inhibitors e.g. 5-Flucocytosine (5-FC). \(^{200,201,203}\)

Until recently AmB was the "gold standard" antifungal agent available for treatment of systemic fungal infections, since this agent is associated with many adverse reactions, less toxic, systemic antifungal agents such as 5-FC and azoles have come into practice, and are in use successfully. The frequent use of these agents in treatment of immunocomprosied patients however, has resulted into emergence of drug resistance for example resistance of candida species to 5-FC during monotherapy. Higher doses of azoles for prolonged time also has resulted in the development of resistant Candida. But, primary resistance for these agents is rarely reported in Candida. \(^{201,203}\)

Thus in view of availability of several new effective antifungal agents and the reports of emergence of drug resistance in Candida, in vitro antifungal susceptibility testing is increasingly demanded for the better management of the cases showing candidal infections. \(^{201,202,204}\)

### 2.11.1. Methods of Antifungal Susceptibility Testing. \(^{200,201,202}\)

The national committee on clinical laboratory standards (NCCLS) in 1982 established a subcommittee to develop a reliable reference method for in-vitro susceptibility testing of yeasts and other fungi. In 1992, this subcommittee has proposed the reference macrodilution method for testing of yeast fungi such as Candida species, \(T. glabrata\) and for \(C. neoformans\).

Since many fungi are susceptible to AmB, susceptibility testing is not an urgent need and is preformed on rare occasions. Susceptibility testing of candida against azole is of value because of variations in the sensitivity pattern of different Candida species. Because of emergence of denovo resistance to 5-FC among candida species, susceptibility testing with 5-FC is indicated for all the pathogenic Candida isolates from patient receiving this drug. \(^{201,205}\)

### 2.11.2. NCCLS Macrobroth Dilution Method.

The macrobroth dilution method is most widely used technique for antifungal susceptibility testing and is the reference method for yeast cells and is adequate for testing all the antifungal agents against any fungi. The method
is performed using synthetic medium “RPMI 1640 cell culture broth” with L-glutamate, a pH indicator and without sodium bicarbonate. The medium is buffered to pH 7.0 at 25°C using morpholine propane sulfonic acid (MOPS) of 0.165 molarity of pH 7.0. Satisfactory results are obtained with MOPS. This medium is suitable for testing of drugs like AmB, 5-FC and azoles against the Candida species.201,202

Variety of solvent are recommended by NCCLS subcommittee to dissolve these antifungal agents for example Dimethyl sulfoxide (DMSO) for AmB, polyethylene glycol (PEG), DMSO i.e. dimethyl Formamide (DMF), ethanol and mixture of ethanol and HCl for azoles, and sterile distilled water for 5-FC and Fcz. The stock solutions should be prepared as 10 fold concentrations and stored at -60°C or below, in small aliquots.201,202

The inoculum is prepared by selecting 5 colonies of one-millimeter diameter and suspending in sterile saline (0.85% NaCl), which results in 1X10^8 to 5X10^8 yeast cells per ml (equivalent to 0.5 McFarland standard). For obtaining test inoculum in the range of 0.5 X 10^3 to 2.5 X10^3 cells/ml, the initial inoculum is further diluted 1:2000 with RPMI medium. MIC endpoints are visually determined at the end of incubation at 35°C for 48 hrs. For AmB, MIC is recorded as lowest concentration that prevented the growth of Candida. Due to trailing or partial inhibition effect of azoles, MIC is calculated using 80% diluted drug free growth control medium (8 part of medium and 2 part of yeast suspension) which provide good approximation of “prominent decrease in turbidity”. The quality control strains of Candida used and their MIC range is as shown in the table.201,202

Table 2.11.1 Showing MIC range in µg/ml for quality control ATCC strains of C. albicans.

<table>
<thead>
<tr>
<th>Antifungal agents</th>
<th>C. albicans (90028)</th>
<th>C. albicans (90029)</th>
<th>C. parapsilosis (90018)</th>
<th>C. parapsilosis (22019)</th>
<th>C. krusei (6258)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AmB</td>
<td>0.25-1</td>
<td>0.25-1</td>
<td>0.25-1</td>
<td>0.25-1</td>
<td>0.5-2.0</td>
</tr>
<tr>
<td>5-Fc</td>
<td>1-4</td>
<td>&gt;64</td>
<td>2-8</td>
<td>0.12-0.5</td>
<td>4-16</td>
</tr>
<tr>
<td>Ktcz</td>
<td>&lt;0.03-0.12</td>
<td>&lt;0.03-0.12</td>
<td>0.12-0.5</td>
<td>0.06-0.25</td>
<td>0.12-0.5</td>
</tr>
<tr>
<td>Fcz</td>
<td>&lt;0.03-0.5</td>
<td>&lt;0.12-0.5</td>
<td>1-4</td>
<td>2-8</td>
<td>16-64</td>
</tr>
</tbody>
</table>
2.11.3. NCCLS Microdilution Method.

This method is similar to macrodilution method except the volume used is less. This method is easy to perform and the MIC values obtained are comparable to the values obtained by macrobroth dilution method. 201,202,205

2.11.4. Modification of Reference Methods for Clinical Laboratory Use.

The reference method is essential for standardisation and to overcome inter laboratory discrepancies. But these are not convenient for routine laboratories. Thus modification of the reference method to serve the needs of laboratories are proposed these include colometric end point determination methods for MIC determination and agar diffusion MIC methods (E test). 182,183,186

2.11.5. Colorimetric Methods for MIC End Point Determination.

Use of colorimetric oxidation-reduction indicator to facilitate more accurate MIC end point determination is recommended. This approach is a reliable indicator of the number of viable yeast cells present, the end points can be determined visually, it also minimises the trailing effect frequently observed with azoles. 201,202

Modifications in temperature e.g. from 30-35°C and agitation of sealed microdilution plates are recommended as per the susceptibility testing of yeast species under question. 201,202

2.11.6. Agar Diffusion Method.

The agar diffusion methods for antimicrobial susceptibility testing are in common use, and it provides a quantitative (zones of inhibition) results and qualitative (sensitive or resistance) results.

Recently E test based on the diffusion of a continuous gradient of an antimicrobial agent from a plastic strip into an agar medium is proposed to determine MICs. The results are comparable to the current broth dilution method and is superior to disk diffusion test. The flexibility of E test makes it applicable to a wide range of antimicrobial susceptibility testing, including fungi. It is reported that E test is more valuable in testing organisms for which there is no routine method of quantitative testing and is suitable for testing of drugs such as azoles, that gives trailing end points with broth dilution methods. 201,202
2.11.7. Agar Disc Diffusion Method.

In this method plates containing yeast nitrogen base glucose (YNBG), medium with L-asparagine (1.5% for azoles) and without L-asparagine are used. Discs containing 1µg, 2µg, 5µg, and 10µg concentration of each antifungal agent and inoculum containing 10^6 yeast cells/ml are used. The strain is labelled as sensitive (S), Intermediate (I) and resistant (R) depending on MIC values as shown in table.

Table 2.11.2 Showing concentration of antifungal agents in µgm /ml for categorising a strain sensitive, intermediate or resistant.

<table>
<thead>
<tr>
<th>Antifungal agent</th>
<th>Concentration of drug in µgm /ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sensitive</td>
</tr>
<tr>
<td>AmB</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>&lt;1</td>
</tr>
<tr>
<td>5-FC</td>
<td>≤ 4</td>
</tr>
</tbody>
</table>

The strain is considered sensitive when the zone diameter is ≥ 80% of zone diameter of control strain, intermediate when zone diameter is < 80% provided there is visible zone of inhibition and resistant when there was no zone of inhibition.

When only discs of 10 µg concentrations are used. The strain is reported as sensitive, intermediate or resistant as shown in table.

Table 2.11.3 Showing diameter of zones of inhibition in mm for designating a strain sensitive, intermediate and resistant.

<table>
<thead>
<tr>
<th>Antifungal agents</th>
<th>Zones of diameter in mm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sensitive</td>
</tr>
<tr>
<td>AmB</td>
<td>≥ 15</td>
</tr>
<tr>
<td>Azoles</td>
<td>≥ 20</td>
</tr>
<tr>
<td>5- FC</td>
<td>≥ 30</td>
</tr>
</tbody>
</table>

The limited application of this technique is attributed to poor diffusibility of certain antifungal agents and slow growth rate of some fungi. However, a good
correlation between MICs and diameter of zone of growth inhibition around discs has been documented with water-soluble agents such as flucytosine and fluconazole.\textsuperscript{206}

2.11.8. Factors that Influences Antifungal Susceptibility Testing.\textsuperscript{201,202,205}

In vitro susceptibility testing is influenced by number of technical variables, including inoculum size, nature of medium, pH of medium, duration and temperature of incubation and the criteria used for MIC end point determination.

In addition, problems unique to fungi such as slow growth rate, ability of candida to produce various morphological forms e.g. hyphae, pseudohyphae etc. at different pH and temperature. The basic properties of the antifungal agent themselves such as solubility, chemical stability, mode of action, tendency to produce partial inhibition of growth also influences the in vitro susceptibility test result.

2.11.8.1. End Point Determination.

It is most significant factor for interlaboratory variation with azoles and 5-FC. This is due to partial inhibition of growth or trailing effect. It is reported that trailing effect is due to occurrence of growth for a period of time prior to the onset of complete drug effect, resulting into variation in MIC values. The suggested methods for reducing this effect includes use of, 80% diluted drug free growth control medium (2ml of yeast suspenson plus 8 ml of medium) which approximates 80% inhibition standard.

2.11.8.2. Inoculum Size.

Recently it is reported that an inoculum in the range of $0.5 \times 10^3$ to $2.5 \times 10^3$ per ml gives reproducible interlaboratory results hence, has been recommended in NCCLS reference method. However, with most drugs MIC increases with increase in the inoculum density.

2.11.8.3. Inoculum Preparation.

Among the various methods used for inoculum preparation the Wickerhams card method, hemocyto meter method and the prompt inoculation method often fails to produce inoculum of desired density. Adjustment by
matching the turbidity at 530 nm of 0.5 McFarland standard is the best method to obtain inoculum containing 1x10^6 cells per ml. Based on these results the NCCLS reference method has recommended the spetrophotometric method for preparation of standard inoculum.

2.11.8.4. Incubation Time and Temperature.

MIC for Candida are usually stable by 4 days. Temperature changes in the range from 22-37°C causes rise and fall in MIC values. The proposed NCCLS reference method specifies incubation at 35°C for 48 hrs for Candida species.

2.11.8.5. Media.

The presence of purine and pyrimidine derivatives present in Sabouraud's agar, the Tris-80 in synthetic amino acid medium fungal (SAAMF), and the acidic pH affect the MIC of antifungal agents. On basis of these observations the NCCLS method recommended a cell culture medium RPMI1640, bufferd to 7.0 with MOPS for antifungal susceptibility testing by broth dilution method.


2.11.9.1. 5-Fluorocytosine.

Resistance of candida species to 5-FC is acquired during monotherapy. Combination of 5 FC and AmB reduces the occurrence of resistant in C. albicans isolates, the acquired resistance results on account of failure to metabolise 5 FC into 5 F UTP and 5FdUMP, or from the loss of feed back control of pyrimidine biosynthesis. Deficiency of enzymes involved in the uptake or metabolism of 5 FC or deregulation of pyrimidine synthesis pathway are the factors leading to development of intrinsic resistance to 5 FC.

2.11.9.2. Resistance of Candida to Polyene.

Studies using polyene resistance strains revealed a marked decrease in membrane ergosterol content; the ergosterol, which is the favoured sterol target of polyenes, is replaced by biosynthetic precursors such as lanosterol, fecosterol, lichesterol and episterol. The change in sterol composition is frequently associated with an overall increase in the membranie sterol content and some changes in phospholipids. In some polyene resistant strains no
apparent alteration in their membrane sterol content, and possibly changes in cell wall permeability to polyenes is the mechanism proposed. Another mechanism thought to mediate AmB resistance is the increased catalase activity, which diminishes oxidative damage caused by this agent.

Drutz and Lehrer (1978)\textsuperscript{207} have proved that candidal resistance to polyene can develop in patients with very low host resistance to infection and resistance to such drugs is a negligible problem in patients with moderate host defence.

Most of the clinically isolated polyene-resistant Candida are the species other than \textit{C.albicans} notably \textit{C.tropicalis} and \textit{C.lusitaniae}. The potential for polyene resistance is reported to be high in \textit{C.glabrata} and \textit{C.parapsilosis}. In view of its haploid nature \textit{C. glabrata} can mutate frequently, and develop resistance faster than \textit{C.albicans} and in \textit{C.parapsilosis}, which is inhibited readily as other Candida species by polyenes but is less readily killed by them.\textsuperscript{208}

2.11.9.3. Resistance of Candida to Azoles.

Horseburgh et al (1982)\textsuperscript{209} and Wamock et al (1983)\textsuperscript{210} reported two instances of resistance developing in patients with chronic mucocutaneous candidiosis who were treated for a very long period with ketoconazole, and concluded that \textit{C.albican} resistance to azoles is an unusual event developing only with protracted period of exposure to the drug. The reported mechanism of resistance is lowered permeability to azoles and alteration in their membrane sterols. The resistant strains also show cross-resistance to otherazole compounds.

Azole – resistant strains of Candida species other than \textit{C.albicans} have not been reported, but in-vitro studies documented high MICs for these species. The development of resistance in haploid species of Candida such as \textit{C.glabrata} and \textit{C.guilliermondii} is anticipated with frequent clinical use of azoles, comparing to the diploid species of Candida.\textsuperscript{208}

Lynch et al. (1994)\textsuperscript{211} reported that \textit{C.albicans} isolates from patients with vaginosis were uniformly sensitive to the low concentration of AmB, 5FC and azoles. But non-albican species viz. \textit{C.glabrata} and Sacchromyces cerevisiae revealed higher MICs to azoles, hence they recommended the
sensitivity testing of all non-albican Candida isolate in patients with clinical failure.

2.11.9.4. Molecular Mechanism of Azole Resistance.

Molecular studies has revealed multidrug efflux transporters of ATP– binding cassette (ABC) superfamily and of the class of major facilitators (MF) are responsible for the low level of accumulation of azole antifungal agents. Two genes for these transporters, the ABC transporter gene CDR and the MF gene BENR (also called CaMDR1) were shown to be over expressed in resistant isolates. Most recent studies suggests that the over-expression of BENR is responsible for the specific resistance of clinical isolate of C. albicans to fluconazole.203,212

Some of other multidrug efflux transporter genes of both classes existing in C. albicans have been cloned recently. These are ABC – transporter genes: CDR2, CDR3, CDR4, CDR5 and the MF gene FLU1. Over expression of CDR2 gene in C. albicans isolates showing cross-resistance to azole derivatives is reported. The expression of other (CDR2, CDR3, CDR4 & CDR5 and FLU1) genes are yet to be correlated with resistance.203,212

2.12. NON-CULTURE TECHNIQUE FOR DIAGNOSIS OF CANDIDIASIS

Invasive candidal infections are frequently encountered in severely immunocompromised patients and are an important cause of morbidity and mortality. Clinically, no specific signs indicative of invasion by candida are present and the diagnosis depends on a combination of microbiological (microscopy and culture), histological and serological results. Microbiological results are helpful in diagnosis of some cases and the results are often difficult to interpret. Positive results are only available in advanced stage of infection and depend on the nature of specimen under examination and the negative result does not rule out the diagnosis of fungal infections. Though histological techniques confirm the diagnosis, the invasion procedures are sometimes not practicable in seriously ill patients.213,214

The serological tests can provide the most rapid means of establishing the diagnosis of fungal infection, enabling prompt therapy to be initiated. The
test can be used to monitor the course of disease and response to therapy by serial detection of antigens and or antibody titers. Most of the currently used serological tests are based on detection of antibodies to candida, but the tests for candidal antigens are used with increasing frequency.

The serological tests suffer from various shortcomings. With some tests, specificity is the problem, mainly because the large number of Candida antigens show cross-reaction with unrelated pathogenic fungi. Some tests lack specificity, either due to inadequate sensitivity of the method used in reagent preparation or failure of the host to produce anticipated immuné response to the infecting agent. 18, 29, 30,  

Nevertheless, there have been developments and improvement over recent year in antigen extraction techniques, more attention has been focused on the antigens produced by different growth phases of candida, and on antigens likely to be associated with the infection process such as the acidic carboxyl proteinase, aggression and enolase antigen of candida. Methods of detection of these components in clinical specimen have also been improved. The introduction of ELISA and immuno blotting assay has enabled the determination of class-specific antibody response to individual antigens and also the detection of specific antigens. Recent developments in the diagnostic serological tests for invasive candidiasis include characterization and production of monoclonal antibodies. 29, 18

Antibody tests are widely used for serodiagnosis of fungal infections but they also have their own demerits. Although some tests are reliable, false positive results are often obtained in healthy individuals because of exposure to environmental fungi. The antibody tests are frequently unable to distinguish between active and past infection, colonisation or transient fungemia. However, immunoblot assays offer better qualitative analysis of the antibody response in invasive candidal infections e.g. Ab specifically associated with infection can be distinguished from cross-reacting antibodies or antibodies due to commensal organisms using immuno blotting assays. 18, 29, 30
In innate deficiency of all antibody tests is that they are dependent on host’s immune status. The potential markers used in Ag detection and methods used for Ab detection for diagnosis of candida infections are as shown in the table.\textsuperscript{213}

Table 2.12.1. Showing different markers used for Candida antigen detection and tests used for anti-Candida antibody detection.

<table>
<thead>
<tr>
<th>Potential markers used to detect diagnosis of invasive candidiasis:</th>
<th>Serological tests used for antigenemia</th>
</tr>
</thead>
<tbody>
<tr>
<td>♦ Candida cell wall Ag. (CWMP &amp; β-D-glucans)</td>
<td>♦ Agglutination tests</td>
</tr>
<tr>
<td>♦ Candida metabolites such as D-arabinitol</td>
<td>♦ Complement fixation tests</td>
</tr>
<tr>
<td>♦ Candida cytoplasmic Ags viz. Enolase.</td>
<td>♦ Precipitation test</td>
</tr>
<tr>
<td>♦ Secreted aspartyl proteinases.</td>
<td>♦ Hemagglutination test</td>
</tr>
<tr>
<td>♦ Candida DNA.</td>
<td>♦ Latex agglutination test</td>
</tr>
<tr>
<td>♦ Secreted aspartyl proteinases.</td>
<td>♦ Indirect fluorescent test</td>
</tr>
<tr>
<td>♦ Candida DNA.</td>
<td>♦ ELISA</td>
</tr>
</tbody>
</table>


2.12.1.1 Detection of Candida Cell Wall Mannoprotein.

Among the various antigens that circulate during infection with Candida albicans and other related Candida species, the cell wall mannoprotein is predominant antigen, which is also called as mannan or phosphomannoprotein. It is immuno dominant antigen in serotype A and B of C. albicans and consists of four epitopes, two are serotype specific and other two are common to both serotypes. In an immunocompromised patient with invasive candidiasis the CWMP circulate in blood (mannanemia) in form of immune complex following dissociation, it can be detected by latex agglutination (LA), enzyme immuno assay (EIA), radio immuno assay (RIA) and reverse passive latex agglutination (RPLA) test and sandwich ELISA. The sandwich ELISA test is highly sensitive. By using antibodies against non-albicans Candida species the specificity of these tests is further increased to detect invasive candidiasis caused by them.\textsuperscript{31,213,215}

In view of rapid clearance from blood, the serum levels of CWMP are very low ≤ 100 ng/ml, and this necessitate frequent sampling. The stage of disease, period of sampling, immunological status of the patient and the species of Candida causing infection are the variables influencing sensitivity of
the test. The highest sensitive results are recorded in severely immunocompromised patients e.g. patients with acute leukemia, as these patients are less likely to produce antibodies against these antigens.31,213,215

2.12.1.2. Detection of β-D-glucan by Tachypleus assay.

Using Tachypleus lysate assay the cell wall component of Candida i.e. β-D-glucan is detected. The "Tachypleus tridentatus" is a Japanese horseshoe crab and is a source of amoebocyte lysate assay. The tachypleus-clotting cascade has two pathways, which are differentially activated by fungal cell wall product and by endotoxin. The gelation reaction of the lysate assay, the one that is activated by a fungal cell wall i.e. β-D-glucan is useful in detection of fungemia. The tachypleus cascade become activated in the presence very low concentration >1 ng/ml of β-D-glucan. The diagnostic kit commercially available for detection of β-D-glucan is 'Fungitech Kit'. This test (factor G-test) is useful in diagnosis of invasive candidiasis even during initial stages of infection.213,216

2.12.1.3. Detection of Candida Species Metabolite D-Arabinitol.

The cyclopentol D-arabinitol is the most extensively studied metabolite of C. albicans, serum concentration of D-arabinitol are detected by gas liquid chromatography. D-arabinitol was first discovered in patients of disseminated candidiasis (DC). Yeast pathogens such as C. albicans, C. tropicalis, C. parapsilosis and C. pseudotropicalis produce arabinitol. The C. albicans produces the D-enantiomer of arabinitol. During DC the arabinitol is accumulated in urine of patients. Circulating levels of D-arabinitol are detectable in cases of invasive candidiasis. The complexity, time required and cost prohibit the routine use of GLC. Other methods of detection of D-arabinitol include enzymatic fluorometric assay using D-arabinitol dehydrogenase enzyme. Significantly increased serum D-ara / creatinine ratio are found in 2/3rd of patient with DC with heavier tissue burden of candida. Raised levels are also reported in patients with disseminated candidiasis caused by C. albicans.30,213,214,215
2.12.1.4. Detection of Candida Cytoplasmic Antigen.

2.12.1.4.a. Enolase (EC 4.2.1.11).

Stroekbine et al (1984) identified an immuno dominant cytoplasmic 48 KDa antigen of Candida. This was subsequently found to be "Candida enolase". Experimental studies of disseminated candidiasis in mice and rabbits demonstrated that antigenemia due to the 48 kDa antigen detected by ELISA was expressed in the absence of fungemia and correlated with degree of deep tissue infection. The levels distinguished superficial infections from deep infections and declined in response to antifungal therapy. Correlation between the level of circulating antigen and the extent of deep tissue infection was documented thus detection of enolase as a marker of deep visceral infection in the absence of fungemia is a useful adjunct to blood culture.

In non-immunocompromised patients, profound antibody response to enolase was detected by Western blot technique. Thus a combination of antigen and antibody detection will increase the diagnostic utility of Candida enolase detection. Detection of antigen is most useful in profoundly immunocompromised patients while antibody detection is useful in less compromised patients viz. surgical patients.

Detection of Candida enolase in the urine of high-risk patient is an another approach in diagnosis of invasive candidiasis. Detection of antigenuria is a non-invasive technique and is advantageous in detection of enolase antigenemia in the presence of neutralising antibody and complement in less immunocompromised patients.


Another important immunodominant cytoplasmic antigen of Candida is a 47kDa antigen. It has been identified as an immunodominant break down product of 90kDa heat shock protein (hsp) of C. albicans.

Heat shock proteins are highly conserved ubiquitous proteins found in all cell systems. Based on their molecular weight and a high degree of sequence conservation, are categorized into families such as hsp 60, hsp70 and hsp90. They represent the immunodominant antigens, that can be recognized in a
range of bacterial, parasitic and fungal infection e.g. hsp60 and hsp70 in histoplasmosis, whereas hsp70 and hsp90 in parasitic infection.\textsuperscript{30,213}

The 47 KDa antigen is identified as immunodominant component of hsp 90 of \textit{C. albicans} therefore, have a greater potential as a diagnostic marker in disseminated candidiasis. 47 KDa antigen can be detected using RPLA test. The test is negative in superficial candidiasis, but positive in DC. Using immunobinding assay an antibody against an immunodominant epitope "LKVI\textsubscript{R}K" on 47kDa hsp 90 antigens was detected in 705 patients with disseminated candidiasis caused by \textit{C. albicans}.\textsuperscript{30,213,215,217}

Candida enolase antigen and 47kDa antigen are distinguished by their pattern on western blots using monoclonal antibodies. Both Candida enolase and 47kda antigen are immunodominant cytoplasmic antigens and elicit an immune response especially in patients recovering from invasive candidiasis.\textsuperscript{30,213,215}

\textbf{2.12.1.5. Detection of Candida Aspartyl Proteinase EC (30.4.23.6).}

Secreted aspartyl proteinase (SAP) is an important antigenic marker of \textit{C. albicans} and \textit{C.tropicalis}. Other less pathogenic species of candida e.g. \textit{C.parapsilosis} secrete antigenically distinct aspartyl proteinase. Candida SAP is expressed by invasive strain of \textit{C. albicans} causing disseminated candidiasis, and facilitates the host tissue invasion. Results using rabbit model suggest that the SAP antigenuria can be detected by using EIA, within 24 hr. following intravenous infection and it helped to discriminate gastrointestinal \textit{C. albicans} colonisation and disseminated candidiasis. Further developments in this test are under investigation. Recent report of existence of family of seven genes (SAP 1 to SAP 7) encoding SAP has complicated the application of this marker in diagnosis of DC.\textsuperscript{30,213,215}

\textbf{2.12.1.6. Detection of Candida DNA Using Polymerase Chain Reaction (PCR).}\textsuperscript{30,213,215,218}

Over the last years, several reports have documented the feasibility of detecting nucleic acid sequences of pathogenic candida in clinical specimens obtained from invasive candidiasis. In most assays, DNA was detected using the PCR. PCR-based assays are highly sensitive and are able to identify candida species
and this feature of PCR assays help in overcoming the limitations of conventional culture method.

The specific target sequences used include the low copy gene sequences and multicopy gene sequences of *C. albicans*. It is reported that multicopy gene targets provide higher sensitivity by detecting very few numbers of *C. albicans* cells than single-copy DNA sequences.

The low copy gene targets sequence of *C. albicans* are actin, 14-α lanosterol demethylase on cytochrome P450, secreted aspartyl proteinase, heat shock protein 90 (hsp 90). Among these sequences first two are genus specific and later two are species (*C. albicans*) specific. In vitro sensitivity of the assays using low copy sequences ranges form 10-100 colony forming unit (c. f. u.) of candidal cells.

The multicopy sequences are 18s r RNA, 5s r DNA, r DNA from internal transcribed spacer regions (ITs), repetitive mitochondrial DNA (mt DNA), mt RNA etc. In vitro sensitivity of the assays using multicopy target sequences ranges from 1-20 c.f.u.of candida cells.

Miyakawa et al (1993) detected 3 cells per 0.1 ml of clinical sample, by targeting a mt DNA sequence. By targeting 158 bp fragment of actin gene, 79% sensitivity in patients with candidemia is reported. While 71% sensitivity was noted, when cytochrome P-450 was used as target sequence in nested PCR on a range of clinical specimens.

In view of availability of range of commercial kits with varying sensitivity for diagnosis of invasive candidiasis in high-risk patients, use of more than one type of test kits, capable of detecting different antigen is recommended. Because of the transient nature of antigenemia frequent testing is necessary for diagnosis of infection caused by opportunistic fungi such as candida.


2.12.2.1. Agglutination Tests.

Until recently this test was commonly used. Now its reliability is disputed when it was discovered that anti-candida antibodies detected by this test are mostly anti-mannan antibodies, which are also found in normal individuals. However, it is reported that using mycelial or cytoplasmic (internal) antigens of *Candida* rather than the blastospore
antigens the reliability of the agglutination test can be increased. Correlation between rising titer of anti-mycelial antibody and candidal infection is documented. 220,221

2.12.2.2. Complement Fixation Test.

Many workers220,222 commonly used this test. However in view of less sensitivity and complex methods of antigen preparation, the test was abandoned. Shivananda et al (1980) 221 used this test for detection of anti-candida antibody. According to them, a four fold rising titer is reliable indicator of deep-candidal infections.

2.12.2.3. Precipitation Tests.

In view of better discrimination between individual antibodies, this test is more valuable than other agglutination tests used for diagnosis of deep candidal infections. Single radial immunodiffusion (SRID) and double diffusion tests are reported to be reliable for quantitation and rapid detection of anti-candida antibodies respectively. Though, the counter immuno electrophoresis is rapid and reliable test for diagnosis of invasive candidiasis, its sensitivity is low when compared to ELISA, RIA and immunoblotting test 220,221,222,223,224

2.12.2.4. Haemagglutination Test.

Using human 'O' group RBCs and candida cytoplasmic antigen, this test is reported to be very useful in diagnosis of deep candidal infections. 220,222 Using polysaccharide antigens from C. albicans species, rapid and sensitive results are obtained in cases of pulmonary and urinary candidiasis. 224

2.12.2.5. Latex Agglutination Test.

In this test the latex particles coated with cell wall or cytoplasmic antigens are used for detection of antibodies in serum or body fluids of patients with disseminated candidiasis. So far the latex agglutination test for detection of antimannan antibodies have been successfully developed and used for rapid diagnosis deep candida infections. Trials using other candidal antigens are under progress. 220,224

2.12.2.6. Indirect Fluorescent Test.

These tests are available for detection of immune complexes deposited on erythrocytes and for detection of antibodies adhering to macrophages. This test is also used for detection of anti-candida antibodies in patients with invasive candidiasis.
Demonstration of rising titer of antibodies is a very reliable indication of invasive infection.\textsuperscript{224}

2.12.2.7. Enzyme Linked Immunosorbant Assay.

In view of higher sensitivity and specificity, these are increasingly used in diagnosis of invasive candidal infections. Detection of anti-enolase antibodies by ELISA is reported to be highly reliable indicator of invasive candidal infection.\textsuperscript{220,224}

2.13. DEVELOPMENT OF CANDIDA VACCINE AND THEIR USE IN PREVENTION OF CANDIDAL INFECTIONS


The eradication of Candidal infections is a major goal and challenge in 1) Immunocompromised patients, who are vulnerable to superficial and deep fatal Candidal infection,

2) HIV patients: As 70\% HIV patients under risk of developing oral candidiasis which is associated with significant morbidity due to repeated episodes emerging alongwith progressive worsening of immune system.

3) Fertile women: Since two third of healthy women experiences at least one episode of vaginal candidiasis during lifetime without obvious predisposing factors.

4) Non-neutropenic patients: Who are vulnerable to develop systemic candidiasis which is life threatening event, if not treated promptly.

5) Healthy individuals: As large number of subjects are affected by mucocutaneous form of candidiasis.

Though, variety of antifungal agents are available for treatment of candidal infections, most of these are associated with renal toxicity, adverse reactions. Due to recently noted problem of emergence of drug resistance continuous therapy is practically not feasible. Thus development of safe and reliable vaccine is required.\textsuperscript{225}

2.13.2. Experimental Studies.

Several Candida preparations were tested as protective immunogens usually in animal model, that involve intravenous challenge in mice, several studies revealed that live or killed whole \textit{C.albicans} cells given intraperitoneally or intracutaneously offered
some protection against subsequent intravenous challenge with live *C. albicans* in mice. The protective effect was also observed when components of *C. albicans* namely glucans, ribosomes, phenol extracts, of cell walls and broken-cells extracts were used. The protection is not absolute in terms of mortality or otherwise, but it is in the terms of higher number of survivors. Recently molecular techniques have facilitated in exploring new candidate antigens to be used as Candidate antigen for vaccine production viz:

2.13.2.1. Vaccinations Using Ribosomal Fraction.

Immunisation with ribosomal fraction of *C. albicans* is reported to confer protection in 60-70% mice challenged with lethal dose of homologous live *C. albicans* as well as heterologous Candida species for example *C. tropicalis*. The protective effect is due to stimulation of both humoral and cellular immune responses (Polonelli 1994).  

2.13.2.2. Idiotypic Vaccination.

A conceptually new prophylactic approach against candidiasis is idiotypic vaccination. Recently yeast killer toxin like anti-idiotypic antibodies (Anti-Ids) are produced in rabbits by immunising them with monoclonal antibody MabKT4. These antibodies are reported to exhibit a wide spectrum of antimicrobial activity including Candida when administered as a vaccine by parenteral or intravaginal routes. Rabbits immunized by intravaginal route with MabKT4 produced secretary antibodies in the vaginal fluid these anti bodies can be passively transferred to non vaccinated animal.  

2.13.2.3. Vaccination Against Heat Shock Protein-90.

The immundominant antigen of Candida heat shock protein is a 47 Kda antigen which is reported to contain seven epitopes "LKVIRRK" These epitopes are also found on human hsp 90 containing eight epitpe "KILKVIRK". When serum of patient with antibody against these epitope is injected prophylactically to mice challenged with lethal dose of *C. albicans*, the mortality reduced from 100% to 50%. These finding suggest the role of hsp 90 as a future Candidate for vaccination against Candidasis.
PART II

2.14. ANIMAL PATHOGENICITY AS A VIRULENCE MARKER IN CANDIDA SPECIES.

2.14.1. Introduction.

The clinical importance of Candida infections has stimulated much interest in elucidating the interaction occurring between the fungus and its host. However, clear understanding of the pathogenesis of fungal disease remains elusive. Technological advances in molecular biology and microbial genetics has provided various new insights into both microbial virulence factors as well as host susceptibility to infection, but at present there is no substitute for animal models in elucidating microbe-host interaction so also, the animal models are still useful in evaluation of effect of newly discovered antimicrobial agents. The single most important advancement in application of animal models is the availability of genetically unique strains of animal as an alternative to the animal models treated with immuno suppressive drugs, to be used in the studies of microbial virulence and host defense mechanism. These animals are also useful in understanding the modes of action and effectiveness of antifungal drugs.69,226

In experiments using laboratory animals, Candida infections can be established under controlled and reproducible conditions. Using this advantage many investigators studied animals models to investigate mechanism of pathogenesis of candidosis.69

Of the many types of animals used, the mouse is the most popular species, followed by the rabbit, the rat and the guinea pig.69

In 19th century, Berg, Grawitz, Hasten and others experimented with oral or vaginal inoculations of Candida in animals and human volunteers. Klepmpmerer in 1885, produced disseminated candidosis in guinea pigs by intravenous inoculation of C. albicans By the twentieth century the pathogenicity of Candida in a range of laboratory animals is well established and its effect is thoroughly studied and described.69
The Intravenous injection of suitable inoculum of *C. albicans* into an animal is sufficient to establish a disseminated visceral infection with multiple organ involvement. In mouse the intraperitoneal inoculation also leads to disseminated infection but the inoculum given by this route needs to be ten times higher than an intravenous inoculum to establish similar extensive pathology, but nowadays the intraperitoneal route of inoculation is of little use in research.\(^6^9\)

In mouse intravenous model, many workers tested yeast inocula ranging from $10^3$-10$^8$ cells per ml. For most *C. albicans* isolates the intravenous LD 50 in normal non pretreated mice is $10^4$-10$^6$ yeast cells/ml.\(^2^2^7\)

Hasenclever and Mitchell (1961)\(^1^6^7\) tested a large number of *C. albicans* isolates and reported that 94% gave an I.V. LD50 in mice in the range quoted above, with only 4% higher and 2% lower. Strains with relatively low virulence or with resistant strains of mouse the LD50 is above $10^8$ yeast cells per ml.\(^1^6^7,2^2^8\)

Many investigators have found that an I.V. inoculum greater than $10^6$ per ml. *C. albicans* yeasts is rapidly lethal to adult mouse (median survival time less than 7 days).\(^2^2^9,2^3^0,2^3^1\) While inocula less than $10^4$ per ml. produce a low-grade chronic infection with spontaneous resolution.\(^2^3^2\)

The effects of intravenously inoculated *C. albicans* can be modulated by pretreatment or co-treatment of animals with various antibiotics, immuno suppressing agents, adjuvant and trauma etc.\(^2^3^3\)

Rabbits, rats and guinea pigs are approximately equally as susceptible as mice to *C. albicans* when given intravenously. For all these species the LD50 is common of the order of 5000 yeast cells per gram body weight, 50,000 cells are very rapidly fatal and 500 cells per gram are rarely fatal.\(^6^9\)

2.14.2. The Course of Disseminated Candidasis in Experimental Animal.

In the first few hours after intravenous inoculation of *C. albicans* the highest fungal cell counts are found in the lungs and the liver, and lower concentration in the spleen, kidneys and other organs. One or two days after infection (the rate of progression varies with the size of the inoculum) the organ
with greatest and increasing population of live *C. albicans* is the kidney, while counts in the lungs, liver and spleen tend to decline with time. \(^{11,234}\)

Louria et al (1963)\(^{230}\) and Fransen et al (1984)\(^{233}\) reported disseminated candidiasis in guinea pigs and mice respectively. In all types of animals that survive the first week of disseminated infection, kidney is the most conspicuously affected organ, its surface is studded with white abscesses visible to the naked eye. Histopathologically, the kidney lesions and other visceral organ lesions reveal focal collections of *C. albicans* blastospore, pseudohyphae and hyphae, sometimes with little or no evidence of host inflammatory reaction, sometimes with massive infiltrates of polymorphs and macrophages and sometime granuloma formation. \(^{11,230}\) These types of host reaction are also noted in human visceral candidiasis. \(^{235}\)

Many workers\(^{229,230,236,237}\) have quantitatively determined the extent of kidney involvement, which is reported to vary with inoculum size and experimental protocol. The general consensus is that in healthy adult mice inoculated I.V. kidney counts usually range from \(10^2-10^4\) per gram during first two days and, rises up to \(10^5-10^7\) per gram by 10\(^{th}\) day. The high predilection of *C. albicans* to kidneys remains unexplained but it is speculated that it may be due to hypertonicity of renal medulla which enhanced grem tube formation in renal tissue. While in diabetic animals elevated urinary glucose concentrations is the factor proposed for particular predilection of the Candida. Moreover it is reported that small intravenous inocula in rodents resulted into right-sided (unilateral) renal involvement. \(^{69,234}\)

Following I.V. inoculation of mice, progressive infection is observed only in the kidney. Which is associated with passage of fungus through the wall of both cortical and medullary tubules or glomerular tufts into the lumen of the nephrons. Growth at these sites is relatively protected from hosts leukocyte response. After forming long pseudohyphae within the renal tubular lumen, the fungus penetrates back into the renal parenchyma, which is associated with marked polymorphonuclear infiltration. \(^{237}\) In chronic renal infections (small
intravenous inocula) cortical abscesses heal but the tips of the pyramids within
the renal pelvis become necrotic and support masses of obstructive filaments
often seen in human renal candidosis. 69,230

Intravenous C. albicans inoculation into mice sometimes induce changes
in platelet count, leukocyte counts but no changes in blood gases, pH, heart or
arterial pressure is reported. While circulatory collapse and changes in blood
urea nitrogen is recorded in experimental candidosis only shortly before the
death of an animal.69

2.14.3. Susceptibility of Different Strains of Mice to I.V. Challenge by C. albicans

Strains of mice differ considerably in their inherent susceptibility to I.V.
challenge with C. albicans. Investigators reported that C57 BL / 6 mice are
highly susceptible. BALB /C mice show intermediate susceptibility. These
differences are attributed to different propensities of the animals to release
lymphokines and to digression of natural killer cell activity. Nude mice (nu/nu)
are more resistant to I.V. C. albicans challenge than their conventional
counterpart, this property is attributed to their commensal microbial flora than to
their congenitally athymic state. 69,238

Development of severe infection more often in male mice than female
mice after intraperitoneal inoculation with C. albicans has also been reported. 69


Many investigators have modeled the gut-blood route of spread of
Candida, a phenomenon of persorption is the proposed mechanism by which
yeast passes into the blood. It is observed that protein free diet facilitate the
invasion of GI tract by C. albicans. 49

Kennedy and Volz (1983 and 1985) 239,240,241 achieved dissemination of
Candida from the gut, in mice and hamsters merely by pretreatment of the
animals with antibiotics inhibitory for strict and facultative anaerobic bacteria. In
infant mouse model, C. albicans reach the blood stream readily from the gut
especially when the animals are pretreated with cortisone or
cyclophosphamide. It is observed that persorption from gut via the thoracic
lymph duct is most likely route. Athymic mice require large yeast inocula and antibiotic pretreatment for dissemination of Candida through gut. The relative resistance of the rat gut to passage of yeast is attributed to the failure of \( C. \text{ albicans} \) to disseminate from closed loops of rat intestine. Detection of antibodies to Candida in animals challenged intra-oesophageally or orally with \( C. \text{ albicans} \) is corroborative evidence for systemically processing of Candida antigens released from the gut, but it does not prove that candidemia is due to live yeasts. 

Candida species vary in their predilection to colonise the gut and spread from it to cause candidemia and visceral candidiasis. It is reported that \( C. \text{ glabrata} \) colonise the mouse gut better than \( C. \text{ albicans} \) but do not spread viscerally. Kennedy et al (1983) \(^{239}\) found that isolates of \( C. \text{ albicans} \), \( C. \text{ parapsilosis} \) and \( C. \text{ kefyr} \) disseminate readily from the gut to the bloodstream than \( C. \text{ kefyr} \). Wingard et al (1980 and 1982) \(^{242,243}\) found that \( C. \text{ tropicalis} \) is more likely to disseminate than \( C. \text{ albicans} \) from the gastrointestinal tract in mice pretreated with cytarabine. Variation in the ability of different strains of \( C. \text{ albicans} \) to colonise the gut is noted in infant mice model. Dissemination of weakly virulent yeasts such as \( C. \text{ guilliermondii} \) is reported, but these are cleared from viscera within few hours, \( C. \text{ parapsilosis} \) is detectable even after 24 hours or more and \( C. \text{ albicans} \) for even longer periods. \(^{69}\)

Table 2.14.4.1 Showing comparative pathogenicity of seven Candida species in different animals

<table>
<thead>
<tr>
<th>Experimental model</th>
<th>( C. \text{ albicans} )</th>
<th>( C. \text{ tropicalis} )</th>
<th>( C. \text{ parapsilosis} )</th>
<th>( C. \text{ kefyr} )</th>
<th>( C. \text{ krusei} )</th>
<th>( C. \text{ guilliermondii} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lethality (mouse)</td>
<td>+++</td>
<td>++</td>
<td>+</td>
<td>±</td>
<td>±</td>
<td>-</td>
</tr>
<tr>
<td>Systemic spread from gut (mouse)</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Cutaneous penetration (mouse/rat/rabbit/human)</td>
<td>+++</td>
<td>+</td>
<td>±</td>
<td>-</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>Muscle damage during systemic infection (mouse)</td>
<td>+++</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Among the different species of Candida, *C. albicans* isolates are consistently reported as the most virulent Candida causing morbidity or mortality in all the experimental models, while other Candida species are also pathogenic but less so than *C. albicans*. Of the other species listed in table, *C. parapsilosis* emerges as a low grade pathogens, *C. kefyr* occasionally cause morbidity, while *C. glabrata*, *C. guilliermondii* and *C. krusei* seldom reveal pathogenic attributes in experimental animal. Several workers reported the slightly higher pathogenicity of *C. albicans* over *C. tropicalis*. Few studies have shown *C. tropicalis* to be disseminated from the gut more readily than *C. albicans* in mice treated with cytarabin. In some clinical survey association of *C. tropicalis* infection with higher morbidity and mortality is documented. *C. glabrata* and *C. guilliermondii* are noted as very low grade pathogens, while *C. parapsilosis* show some degree of morbidity. The ability of *C. guilliermondii*, but not *C. albicans* to cause coetaneous lesions in rabbit is also reported.

Among the other yeast species, *C. vishwanthii* has been reported to cause pathogenicity broadly equivalent to *C. parapsilosis*. *Candida lusitaniae* is also examined for its experimental pathogenicity. It is observed that none of the yeast is pathogenic for unpretreated hosts, although large inocula of a few species sometimes cause morbidity or mortality in cortisone immuno suppressed hosts.

Many investigators mention the differences in virulence of strains of *C. albicans*. Reports comparing *C. albicans* and *C. stellatoidea* have shown that *C. stellatoidea* is similar or slightly less pathogenic than *C. albicans*. Hasenclever and Mitchell (1962) failed to find difference in virulence of serotype A and B in mouse lethality tests. However, Auger et al (1983) and Martin and Lamb (1982) in different experimental settings have documented that serotype A of *C. albicans* are selected in vivo than serotype B. But a
general consensus is that serotype B strains express type A antigenic
determinants in vivo.

Depending on the basis of their lethality for mice C. albicans strains have
been grouped as high, moderate and low virulence, but reports of infant mouse
model suggested that the relative virulence of a strain is unstable in
character. 228

Buckley et al (1982) 249 reported a variant of C. albicans, which failed to
form a germ tube at 37° C and is non-lethal to mice in vivo. Morphological
variants with different virulence also has been described by Shepherd (1985) 39.

There is no evidence to associate particular biotypes of C. albicans, as
determined by physiological phenotypic tests with high or low virulence attributes. A
frequent finding of strains typed as serotype A, biotype 0/1/1/5 in pathogenic settings
such as an epidemic outbreak of disseminated candidosis and among heroin addicts
with disseminated candidosis suggests that their may be relationship between
biotype and virulence. It is also a possibility that particular phenotype of C. albicans
is more frequently expressed by genetically different strain of the fungus when it is
isolated from invasive Candida lesions. 69

2.14.6. Tissue Response to Blastospore and Hyphae of C. albicans In Mouse

The blastospores and filamentous forms of C. albicans are commonly
found together in the infected tissues of human and experimental animals,
therefore, the relative pathogenicity of the individual form of C. albicans is not
clear. However, it is frequently observed that yeast to mycelium transformation
precedes the invasive process. Hence mycelial form is frequently observed in
infected tissue and therefore, it is postulated as more virulent form of candida.
However, there are evidences, which suggest the higher virulence of yeast than
mycelial forms.

Evans (1980) 250 observed that blastospores are more virulent for mice
than hyphal forms grown from the same strain of C. albicans, and the degree of
virulence is reported to be independent of cellular mass or viable units. It is
reported that the increased virulence of blastospores resides in host defense
mechanism.
It is reported that the blood and lung clears yeast phase cells of *C. albicans* injected into peripheral vein, the liver cells clear those injected into a mesenteric vein. Lung tissue is more effective than other tissues in destroying blastospores as well as hyphae of *C. albicans*. The effective Candidacidal activity is also demonstrated by Damodaran et al (1973) in the lungs of rabbit. This observation explains why the Candida rarely produces primary pulmonary infections in the human host.

Liver tissue is reported to be least effective in killing organisms of either morphology, but because of greater mass it accounts for substantial organ for clearance. Injection of blastospores into mice elicited a prompt inflammatory response consisting of PMN leukocytes in liver and lung tissues. In contrast injection of hyphal cells resulted in a minimal infiltration of PMN cells in the lung and liver. These results support the finding that blastospores are highly chemotactic for PMN than hyphae.