3. REVIEW OF LITERATURE

Human Immunodeficiency Virus:  

History

The history of HIV and AIDS is a short one and was reported just over thirty years ago; it has spread to every corner of the world and has become one of the leading causes of death worldwide. In 1981, scientists in the United States reported the first clinical evidence of a disease known as AIDS. The first indication of this syndrome was reported from New York and Los Angeles (USA), in a sudden unexplained outbreak of two very rare diseases-Kaposi’s sarcoma and *Pneumocystis carinii* pneumonia among homosexuals and/or addicted to inject narcotics. These patients appeared to have lost the immunocompetence and this condition was given the term AIDS.\(^4^9\)

In 1983 Luc Montagnier and colleagues from the Pasteur Institute, Paris isolated a retrovirus from a patient with persistent generalized lymphadenopathy, and called it lymphadenopathy associated virus (LAV). In 1984 Robert Gallo and colleagues from the National Institute of Health, USA, reported the isolation of retrovirus, demonstrated clearly to be the causative agent of AIDS and called it human T-cell lymphotrophic virus-III. In 1986, the International Committee on Virus Nomenclature gave the name HIV. There are two types of HIV - HIV-1 and HIV-2. In 1985, a sensitive enzyme linked immunosorbent assay (ELISA) was developed to detect HIV infection by demonstrating antibodies in the patient’s serum.\(^4^9,^5^0\)

HIV is the etiologic agent of AIDS; it belongs to the family Retroviridae and the subfamily Lentiviruses. The most common cause of HIV disease throughout the world is HIV-1, which comprises several subtypes with different geographic
distributions. In 1986, HIV-2 was first isolated from West African patients. Molecular epidemiology data indicate that HIV-1 arose from simian immunodeficiency virus – SIVcpz retrovirus harboured by the chimpanzee subspecies Pan troglodytes troglodytes (Ptt). The deduced evolutionary sequence suggests that SIVcpz ancestors recombined when they crossed between several species of nonhuman primates, then into chimpanzees, and finally into humans. HIV-2 could have originated from sooty mangabeys. Scientists believe that the virus most likely would have transmitted to humans and mutated into HIV when humans hunted these chimpanzees for meat and came into contact with their infected blood. Over decades, the virus slowly spread across Africa and later into other parts of the world. The AIDS pandemic is primarily caused by the HIV-1 M group viruses. Although HIV-1 group O and HIV-2 viruses have been found in numerous countries, including in the developed world, they have caused much more localized epidemics.\textsuperscript{50,51}

**Morphology of Virus:**

HIV is a spherical enveloped virus measuring 90-120nm in size. The nucleocapsid has an outer icosahedral shell and an inner cone shaped core, enclosing the ribonucleoproteins. The HIV-1 envelope contains 72 external spikes formed by the two major envelope proteins, the external gp120 and the transmembrane gp41 (gp - glycoprotein). The HIV -1 lipid bilayer is also studded with various host proteins including class I and class II major histocompatibility complex molecules, acquired during virion budding. The cone of HIV-1 contains four nucleocapsid proteins (p24, p17, p9, p7). The phosphorylated p24 polypeptide forms the chief component of the inner shell of the nucleocapsid, whereas the p17 protein is associated with the inner surface of the lipid bilayer and stabilizes the exterior and interior components of the
The p7 protein binds directly to the genomic RNA through a zinc finger structural motif and together with p9 forms the nucleoid core. The retroviral core contains two copies of the single stranded HIV-1 genomic RNA that is associated with the various preformed viral enzymes, including the reverse transcriptase, integrase, ribonuclease and protease.\textsuperscript{50,51}

\textbf{Viral genes and antigens:}

The genome of HIV has genes that encode the structural proteins of the viruses: \textit{gag} encodes the proteins that form the core of the virion (including p24 antigen). It is expressed as a precursor protein p55 which is cleaved into three proteins, p15, p18 and p24 that make up the viral core; \textit{pol} encodes the enzymes responsible for protease processing of viral proteins, reverse transcription and integration. It is expressed as a precursor protein that is cleaved into p31, p51 and p66; and \textit{env} encodes the envelope glycoprotein gp160, that is cleaved into gp120 which forms the surface spikes and gp41, the transmembrane anchoring protein. However, HIV-1 is more complex than other retroviruses particularly those of the
nonprimate group, which contains six other regulatory genes (tat, rev, nef, vif, vpr and vpu) which code for proteins involved in the modification of the host cell to enhance virus growth and the regulation of viral gene expression. Several of these proteins are thought to play a role in the pathogenesis of HIV disease. Flanking these genes are the long terminal repeats, which contain regulatory elements involved in gene expression. The major difference between the genomes of HIV-1 and HIV-2 is that the latter lacks the vpu gene and has a vpx gene.

![Internal structure of HIV](image)

**Fig 2: Internal structure of HIV**

Nonstructural and regulatory genes: \(^{49,50,51}\)

1. **tat** (trans activating gene)- Transcriptional activator
2. **nef** (negative factor gene)- Downregulation of cellular CD4 and MHC I proteins
3. **rev** (regulator of virus gene)- Promotes transport of unspliced mRNAs.
4. **vif** (viral infectivity factor gene)- Increases viral infectivity in certain cell types.
5. **vpu** (in HIV-1) and **vpx** (in HIV-2)- Facilitates virus assembly and release
6. **vpr**- stimulates the promoter region of the virus. Facilitates nuclear entry in nondividing cells.
7. LTR (long terminal repeat) sequence, one at either end, containing sequences giving promoter, enhancer and integration signals.
Molecular heterogeneity of HIV:

Genome analysis indicates that HIV-1 is grouped into Main (M), Non-M-Non-O (N) and Outlier (O). The M group comprises nine subtypes/clades, designated A-K (except E and I). Similarly HIV-2 has six subtypes A-F. However HIV-2 has only 40% genetic identity with HIV-1 and is more closely related to SIV than to HIV-1. All HIV groups and subtypes are present in Cameroon, West Africa, which may perhaps be the site of origin of the virus. Subtype A is most prevalent and found worldwide, while B is more common in America and Europe. A, C and D in Africa while, E, C and B in Asia. In India and China subtype C is prevalent.49,52

AIDS remain one of the world’s most serious health challenges. Globally 34.0 million [31.4 million–35.9 million] people are living with HIV and an estimated 0.8%
of adults aged 15-49 years worldwide are living with HIV at the end of 2011. The burden of the epidemic varies considerably between countries and regions. Sub-Saharan Africa remains most severely affected, with nearly 1 in every 20 adults (4.9%) and accounting for 69% of the people living with HIV worldwide. Although the regional prevalence of HIV infection is nearly 25 times higher in sub-Saharan Africa than in Asia, almost 5 million people are living with HIV in South, South-East and East Asia combined. After sub-Saharan Africa, the regions most heavily affected are the Caribbean and Eastern Europe and Central Asia, where 1.0% of adults are living with HIV in 2011. Worldwide, the number of people newly infected continues to fall: the number of people (adults and children) acquiring HIV infection in 2011 (2.5 million [2.2 million–2.8 million]) was 20%. Every day nearly 7,000 people contact HIV—nearly 300 every hour. In 2011, 1.7 million [1.5 million–1.9 million] people died from AIDS-related causes worldwide. Since the beginning of the epidemic, more than 60 million people have contacted HIV and nearly 30 million have died of HIV-related causes. In India, the first known case of HIV was diagnosed by Dr. Suniti Solmon in 1986 amongst female sex workers in Chennai.

**Routes of transmission:**

HIV is transmitted primarily by

1. Sexual Transmission (vaginal, anal, oral)
   - Heterosexual (most common in India)
   - Homosexual

2. Blood Contact
   - Blood and blood products transfusions
   - Intravenous drug use
   - Occupational exposure (needlestick, cuts etc.)
3. Mother-to-Child

During pregnancy
During delivery
Breast Feeding

Infectiveness and Pathogenicity of HIV:

The body’s normal defenses are

- B cell: Humoral immunity antibodies
- T Cell: Cell Mediated Immunity
  
  CD4 – helper T
  
  CD8 – cytolytic T cell

HIV targets these defenses, primarily attacking CD4 cells. CD4 cells are progressively lost during the course of HIV disease (in the absence of treatment) leading to continuous viral replication and increased OI.\(^{55}\)

HIV enters host cells by binding the viral gp120 to the CD4 receptor (like Th cells, monocytes, dendritic cells and microglia) with one of the chemokine co-receptor namely CCR5 and CXCR4 on the host cell surface. The CCR5 beta-chemokine co-receptor is important in establishing the infection whereas CXCR4 alpha-chemokine receptor for disease progression. Entry into the host cells begins when the envelope fuses the plasma membrane and the viral RNA is released and transcribed into DNA by the enzyme reverse transcriptase. This viral DNA is then integrated into the host chromosomal DNA forming provirus i.e. latent infection. Time to time, lytic infection is initiated, releasing progeny virions which infect other cells. The virus also evades immune defenses by undergoing rapid antigenic changes. Retroviruses during the reverse transcription have a high mutation rate compared to DNA viruses and also lack “proof reading” capacity of DNA viruses. As a result,
mutation is probably introduced at every position in the HIV genome many times. These illustrate the potential problem of drug resistance and obstacles to the development of vaccines.

![Viral replication cycle](image)

**Fig 4: Viral replication cycle**

The primary pathogenic mechanism in HIV infection is in the first few months where virus specific CD8+ T cells are formed and reduce the viremia - viral load. This is followed by the appearance of neutralizing antibodies and production of infectious virus particles. The immune system gradually gets damaged and CD4+ T lymphocyte steadily falls and viral load rises, T4:T8 (helper: suppressor) cell ratio is reversed. Polyclonal activation of B lymphocytes leads to hypergammaglobulinemia, delayed type hypersensitivity responses and reduced natural killer (NK) cell and cytotoxic T (Tc) cell activities. Functional changes in T lymphocytes occur such as reduced
responses to mitogens, reduced interleukin 2 (IL-2) and interferon-
 gamma production (IFN-γ). Viral replication eventually outpaces the host immune system and results in clinical AIDS.49,51,55

HIV disease staging and classification systems are critical tools for tracking and monitoring the HIV epidemic and for providing clinicians and patients with important information about HIV disease stage and clinical management. Two major classification systems currently in use are the U.S. Centers for Disease Control and Prevention (CDC) classification system and the WHO Clinical Staging and Disease Classification System. In 1993, the CDC revised the definition of AIDS to include all HIV-infected individuals who have fewer than 200 CD4+ T cells per microliter or CD4 percentage <14% of blood because the development of OI is related to the CD4+ T cell concentration (Table 1, Graph 1). The diseases or conditions most commonly associated with HIV infection and AIDS are summarized (Table 2).

**Graph 1: Association between opportunistic infections and CD4+ lymphocyte count**

![Graph showing the association between opportunistic infections and CD4+ lymphocyte count.](image)
<table>
<thead>
<tr>
<th>Stage</th>
<th>Laboratory evidence*</th>
<th>Clinical evidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 1</td>
<td>Laboratory confirmation of HIV infection and CD4+ T-lymphocyte count of ≥500 cells/μL or CD4+ T-lymphocyte percentage of ≥29</td>
<td>None required (but no AIDS-defining condition)</td>
</tr>
<tr>
<td>Stage 2</td>
<td>Laboratory confirmation of HIV infection and CD4+ T-lymphocyte count of 200–499 cells/μL or CD4+ T-lymphocyte percentage of 14–28</td>
<td>None required (but no AIDS-defining condition)</td>
</tr>
<tr>
<td>Stage 3 (AIDS)</td>
<td>Laboratory confirmation of HIV infection and CD4+ T-lymphocyte count of &lt;200 cells/μL or CD4+ T-lymphocyte percentage of &lt;14†</td>
<td>or documentation of an AIDS-defining condition (with laboratory confirmation of HIV infection)†</td>
</tr>
<tr>
<td>Stage unknown§</td>
<td>Laboratory confirmation of HIV infection and no information on CD4+ T-lymphocyte count or Percentage</td>
<td>and no information on presence of AIDS-defining conditions</td>
</tr>
</tbody>
</table>

Source: CDC. 2008 Revised surveillance case definitions for HIV infection among adults, adolescents and children aged <18 months and for HIV infection and AIDS among children aged 18 months to <13 years- United states, 2008. MMWR 2008;57 [No. RR-10].)\(^7\)
### Table 2: AIDS-Defining Condition

<table>
<thead>
<tr>
<th>Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacterial infections, multiple or recurrent</td>
</tr>
<tr>
<td>Candidiasis of bronchi, trachea, or lungs</td>
</tr>
<tr>
<td>Candidiasis of esophagus</td>
</tr>
<tr>
<td>Cervical cancer, invasive</td>
</tr>
<tr>
<td>Coccidioidomycosis, disseminated or extrapulmonary</td>
</tr>
<tr>
<td>Cryptococcosis, extrapulmonary</td>
</tr>
<tr>
<td>Cryptosporidiosis, chronic intestinal (&gt;1 month’s duration)</td>
</tr>
<tr>
<td>Cytomegalovirus disease (other than liver, spleen, or nodes), onset at age &gt;1 month</td>
</tr>
<tr>
<td>Cytomegalovirus retinitis (with loss of vision)</td>
</tr>
<tr>
<td>Encephalopathy, HIV related</td>
</tr>
<tr>
<td>Herpes simplex: chronic ulcers (&gt;1 month’s duration) or bronchitis, pneumonitis, or esophagitis (onset at age &gt;1 month)</td>
</tr>
<tr>
<td>Histoplasmosis, disseminated or extrapulmonary</td>
</tr>
<tr>
<td>Isosporiasis, chronic intestinal (&gt;1 month’s duration)</td>
</tr>
<tr>
<td>Kaposi’s sarcoma</td>
</tr>
<tr>
<td>Lymphoid interstitial pneumonia or pulmonary lymphoid hyperplasia complex</td>
</tr>
<tr>
<td>Lymphoma, Burkitt (or equivalent term)</td>
</tr>
<tr>
<td>Lymphoma, immunoblastic (or equivalent term)</td>
</tr>
<tr>
<td>Lymphoma, primary, of brain</td>
</tr>
<tr>
<td><em>Mycobacterium avium</em> complex or <em>Mycobacterium kansasii</em>, disseminated or extrapulmonary</td>
</tr>
<tr>
<td><em>Mycobacterium tuberculosis</em> of any site, pulmonary, disseminated, or extrapulmonary</td>
</tr>
<tr>
<td><em>Mycobacterium</em>, other species or unidentified species, disseminated or extrapulmonary</td>
</tr>
<tr>
<td><em>Pneumocystis jirovecii</em> pneumonia</td>
</tr>
<tr>
<td>Pneumonia, recurrent</td>
</tr>
<tr>
<td>Progressive multifocal leukoencephalopathy</td>
</tr>
<tr>
<td><em>Salmonella</em> septicemia, recurrent</td>
</tr>
<tr>
<td>Toxoplasmosis of brain, onset at age &gt;1 month</td>
</tr>
<tr>
<td>Wasting syndrome attributed to HIV</td>
</tr>
</tbody>
</table>

Source: CDC. 2008 Revised surveillance case definitions for HIV infection among adults, adolescents and children aged <18 months and for HIV infection and AIDS among children aged 18 months to <13 years- United states, 2008. MMWR 2008;57 [No. RR-10].
WHO Clinical Staging of HIV/AIDS for Adults and Adolescents with confirmed HIV

Clinical Stage 1:
- Asymptomatic.
- Persistent generalized Lymphadenopathy

Clinical Stage 2:
- Unexplained moderate weight loss (<10% of presumed or measured body weight).
- Recurrent bacterial upper respiratory tract infections (current event plus one or more in last six months like Sinusitis, Tonsillitis, Otitis Media, Pharyngitis)
- Herpes zoster
- Angular cheilitis
- Recurrent oral ulceration (two or more episodes in last six months).
- Papular pruritic eruptions.
- Seborrhoeic Dermatitis.
- Fungal nail infections.

Clinical Stage 3:
- Unexplained severe weight loss (>10% of presumed or measured body weight).
- Unexplained chronic diarrhea for longer than one month.
- Unexplained persistent fever (above 37.5°C intermittent or constant for longer than one month).
- Persistent Oral Candidiasis.
• Oral Hairy Leukoplakia.

• Pulmonary tuberculosis (current).

• Severe Bacterial infections (e.g. Pneumonia, Empyema, Pyomyositis, Bone or Joint infection. Meningitis, bacteraemia, severe pelvic inflammatory disease).

• Acute Necrotizing ulcerative gingivitis or periodontitis.

• Unexplained anaemia (<8g/dl). Neutropenia (<0.5x10^9/L) and or chronic (more than one month) thrombocytopenia (<50x10^9/L).

**Clinical Stage 4:**

• HIV wasting syndrome [loss>10% of body weight and either a chronic (>1 month) fever of diarrhea in the absence of concurrent illness].

• *Pneumocystis carinii* Pneumonia.

• Recurrent bacterial Pneumonia (current episode plus one or more episodes in last six months).

• CNS Toxoplasmosis.

• Cryptosporidiosis with diarrhea>1 month.

• Isosporiasis with diarrhea >1 month.

• Cryptococcosis, extrapulmonary.

• Cytomegalovirus disease of an organ other than liver, spleen or lymph node.

• Chronic Herpes simplex infection, mucocutaneous (>1 month) or visceral (any duration).

• Progressive Multifocal Leukoencephalopathy.

• Any disseminated endemic Mycosis (e.g., Histoplasmosis, Coccidioidomycosis).

• Candidiasis of oesophagus, trachea, bronchi.
Atypical Mycobacteriosis, disseminated.

Recurrent septicemia including non-typhoid Salmonella bacteremia.

Extrapulmonary tuberculosis.

Lymphoma (cerebral or B cell non-Hodgkin) or other solid HIV-associated tumours.

Invasive cervical carcinoma.

Visceral Leishmaniasis.

Kaposi’s Sarcoma.

HIV encephalopathy.

HIV associated Nephropathy.

HIV associated Cardiomyopathy.

(Standard procedures of HIV/AIDS care., March 2010) developed by International Training and Education Center for Health, India) Implemented at Government Hospital of Thoracic Medicine (GHTM), Tambaram Sanatorium, Chennai, Tamil Nadu)

In contrast to the CDC system, the WHO Clinical Staging and Disease Classification System (revised in 2007) can be used readily in resource-constrained settings without access to CD4 cell count measurements or other diagnostic and laboratory testing methods. The WHO system classifies the HIV disease on the basis of clinical manifestations that can be recognized and treated by clinicians in diverse settings, including resource-constrained settings, and by clinicians with varying levels of HIV expertise and training.
Comparison of the WHO and CDC Definitions: Both the WHO and CDC surveillance case definitions for HIV infection now require laboratory confirmation of HIV infection. Differences between the WHO and CDC definitions and staging systems include the following:

WHO recommends reporting cases of HIV infection as

1. HIV infection or AHD (including AIDS), whereas CDC recommends reporting cases of HIV infection by stage (i.e. stage 1, stage 2, stage 3, or stage unknown). WHO presents four clinical stages for disease classification.

2. To reflect the WHO ART treatment guidelines, whereas CDC presents three, combining WHO stages 2 and 3 into CDC stage 2. Because of increased, although not universal, availability.

3. Of CD4+ T-lymphocyte testing, WHO recommends using clinical and immunologic criteria for clinical staging. CDC recommends using only immunologic criteria for staging, with the exception of stage 3, for which the cases must have a CD4+ T-lymphocyte count of <200 cells/μL or a CD4+ T-lymphocyte percentage of <14 or one of 26 AIDS-defining conditions.57

Despite differences in disease classification and clinical staging, and because CDC recommends reporting all CD4+ T-lymphocyte counts, CDC and WHO stages can still be compared (Table 3).
Table 3: Comparison of World Health Organization and CDC stages of HIV infection, by CD4+ T-lymphocyte count and percentage of total lymphocytes

<table>
<thead>
<tr>
<th>WHO stage</th>
<th>WHO T-lymphocyte count and percentage§</th>
<th>CDC stage</th>
<th>CDC T-lymphocyte count and percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 1 (HIV infection)</td>
<td>CD4+ T-lymphocyte count of ( &gt;500 ) cells/( \mu L )</td>
<td>Stage 1 (HIV infection)</td>
<td>CD4+ T-lymphocyte count of ( \geq 500 ) cells/( \mu L ) or CD4+ T-lymphocyte percentage of ( \geq 29 )</td>
</tr>
<tr>
<td>Stage 2 (HIV infection)</td>
<td>CD4+ T-lymphocyte count of 350–499 cells/( \mu L )</td>
<td>Stage 2 (HIV infection)</td>
<td>CD4+ T-lymphocyte count of 200–499 cells/( \mu L ) or CD4+ T-lymphocyte percentage of 14–28</td>
</tr>
<tr>
<td>Stage 3 (advanced HIV disease [AHD])</td>
<td>CD4+ T-lymphocyte count of 200–349 cells/( \mu L )</td>
<td>Stage 2 (HIV infection)</td>
<td>CD4+ T-lymphocyte count of 200–499 cells/( \mu L ) or CD4+ T-lymphocyte percentage of 14–28</td>
</tr>
<tr>
<td>Stage 4 (acquired immunodeficiency syndrome [AIDS])</td>
<td>CD4+ T-lymphocyte count of &lt;200 cells/( \mu L ) or CD4+ T-lymphocyte percentage of &lt;15</td>
<td>Stage 3 (AIDS)</td>
<td>CD4+ T-lymphocyte count of &lt;200 cells/( \mu L ) or CD4+ T-lymphocyte percentage of &lt;14</td>
</tr>
</tbody>
</table>

Based on the kinetics of virologic and immunologic events three dominant patterns of HIV disease are described.

- 80-90% of HIV infected are typical progressors with survival time approximately 11 yrs.
- 5-10 are “rapid progressors” with a median survival time of 3-4 yrs.
- 7-10% of HIV-infected individuals do not experience disease progression for an extended period of time and are called “long term non-progressors” (LTNPs). \(^5^6\)
Though at present there is no cure for AIDS, research, investment and commitment in understanding HIV and AIDS led in the development of highly-effective antiretroviral drugs. These chemotherapeutic agents used for the treatment of HIV have increased the outcome of people living with HIV around the world. The antiviral drugs currently approved for use are of four types (Table 4).  

Fig 5: Life cycle and antiretroviral agent’s site of action

Source: NACO PPT: Site of action of different groups of antiretroviral agents during the cycle. Fusion inhibitors act at fusion step. NRTI & NNRTI act on reverse transcriptase. Protease inhibitors act on enzyme protease. Integration inhibitors act on the enzyme integrase.
### Table 4: Drugs for chemotherapy of HIV.

<table>
<thead>
<tr>
<th>Generic Name</th>
<th>Alternative or Brand Name</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nucleoside analog reverse transcriptase inhibitors</strong></td>
<td></td>
</tr>
<tr>
<td>Abacavir</td>
<td>ABC, Ziagen</td>
</tr>
<tr>
<td>Didanosine</td>
<td>ddl, Videx</td>
</tr>
<tr>
<td>Emtricitabine</td>
<td>Emtriva</td>
</tr>
<tr>
<td>Lamivudine</td>
<td>3TC, Epivir</td>
</tr>
<tr>
<td>Stavudine</td>
<td>d4T, Zerit</td>
</tr>
<tr>
<td>Zalcitabine</td>
<td>ddc, Hivid</td>
</tr>
<tr>
<td>Zidovudine</td>
<td>AZT, ZDV, Retrovir</td>
</tr>
<tr>
<td>Tenofovir (Nucleotide analog reverse transcriptase inhibitor)</td>
<td>Viread</td>
</tr>
<tr>
<td><strong>Nonnucleoside reverse transcriptase inhibitors</strong></td>
<td></td>
</tr>
<tr>
<td>Delaviridine</td>
<td>Rescriptor</td>
</tr>
<tr>
<td>Efavirenz</td>
<td>Sustiva</td>
</tr>
<tr>
<td>Nevirapine</td>
<td>Viramune</td>
</tr>
<tr>
<td><strong>Protease inhibitors</strong></td>
<td></td>
</tr>
<tr>
<td>Atazanavir</td>
<td>Reyataz</td>
</tr>
<tr>
<td>Amprenavir</td>
<td>Agenerase</td>
</tr>
<tr>
<td>Indinavir</td>
<td>Crixivan</td>
</tr>
<tr>
<td>Nelfinavir</td>
<td>Viracept</td>
</tr>
<tr>
<td>Ritonavir</td>
<td>Norvir</td>
</tr>
<tr>
<td>Saquinavir</td>
<td>Invirase, Fortovase</td>
</tr>
<tr>
<td>Lopinavir* plus ritonavir</td>
<td>Kaletra</td>
</tr>
<tr>
<td><strong>Fusion inhibitors</strong></td>
<td></td>
</tr>
<tr>
<td>Enfuvirtide</td>
<td>T-20, Fuzeon</td>
</tr>
</tbody>
</table>
The most successful treatment approach in combining HIV/AIDS is to use a drug combination which is referred to as HAART (highly active anti-retroviral therapy).\textsuperscript{51,55}

Owing to the weakened immune state, PLWHA are more vulnerable to wide spectrum of infections depending on the severity of their immune defect and on the opportunistic organisms in their normal flora or with which they come in contact. These so-called OI may not be fatal in a person without HIV infection; however, they are a major cause of morbidity and death among patients with low CD4 counts or who are in the advanced stages of AIDS. Some clinical manifestations of AIDS may thus vary by locale.\textsuperscript{58} Opportunistic infections are caused by bacteria (e.g. TB, bacterial pneumonia), fungus (e.g. candidiasis, cryptococcosis, \textit{Pneumocystis jiroveci} pneumonia), protozoan parasites (e.g. toxoplasmosis, cryptosporidiosis) and viruses (e.g. herpes simplex, herpes zoster). In addition to OI, PLWHA are susceptible to a number of HIV/AIDS - associated malignancies (cancers) such as Kaposi’s sarcoma, lymphoma, and squamous cell carcinoma. These, too, can have a devastating impact on the health status of people with HIV/AIDS, even in those being treated with ART.

\textbf{Tuberculosis:}

The HIV epidemic has led to a huge rise in the incidence of TB. TB is the commonest opportunistic infection and the number one cause of death in HIV patients in developing countries, and accounts for about 40\% of all manifestations seen in HIV patients.\textsuperscript{60} The incidence of TB in HIV infected individuals is 60-100 fold more than in the general population HIV infection promotes the progression of recent and old TB infection to active disease. On the other hand there is also convincing evidence that TB too accelerates the course of HIV infection. \textit{M.tuberculosis} probably increase
viral replication by inducing macrophages to produce tumor necrosis factors, IL-1 and IL-6.\textsuperscript{59}

Tuberculosis is a disease of great antiquity and likely to have existed in prehominids and has caused more suffering and death than any other infection worldwide. The clinical features of both respiratory and spinal TB were well described by Hippocrates in about 400BC. Accounts of this disease appeared in the Vedas and other ancient Hindu texts, in which it was termed rajayaksha- the king of diseases. It was often called as “White Plague” and “The Captain of all men’s death”. The transmissible nature of TB was established by Villemin in 1868. In 1882, Robert Koch succeeded in cultivating the bacillus, staining with an alkaline solution of methylene blue for 24 hrs. Ehrlich used hot solution of carbol fuchsin which was later modified by Ziehl Neelsen and is still widely practiced. TB, declared as a global health emergency by WHO in 1993 is still a major global health problem. The problem has been further complicated with the emergence of HIV infection.\textsuperscript{61}

\textbf{Etiologic agent:}

TB is caused by the bacterium \textit{Mycobacterium tuberculosis} (MTB) complex. Mycobacteria belong to the family Mycobacteriaceae and the order Actinomycetales. On the surface of liquid media, their growth appears mold like, so the genus gets the term Mycobacteria. MTB complex includes \textit{M.tuberculosis}, \textit{M.bovis}, \textit{M.africanum}, \textit{M.canetti} and \textit{M.microti}. Mycobacteria are non-motile, non-spore forming, weakly gram positive, aerobic or microaerophilic, slender, straight/slightly curved rod (0.2-0.6\textmu m x 1-10\textmu m). The cell wall contains peptidoglycan which contains N-glycolyl muramic, rather than N-acetyl muramic acid. These organisms have distinguishing characteristic property of acid-alcohol fastness and slow growth. Acid-alcohol
fastness is the resistance to decolorization by acidified alcohol after being stained with a basic fuchsin dye; this is mainly due to high content of mycolic acids, long chain (C_{78}-C_{90}) cross-linked fatty acids and other cell-wall lipids. Slow growth, is due to hydrophobic cell surface with prolonged replication time ranging from 2 to 22 hrs. In cell wall, lipids (e.g. mycolic acids) are linked to underlying arabinogalactan and peptidoglycan that confer very low permeability of the cell wall reducing the effectiveness of most antibiotics. Another important molecule in the cell wall is lipoarabinomannan involved in the pathogen-host interaction that facilitates the survival of *M.tuberculosis* within macrophages. 61,62

**Fig 6: Cell wall structure of Mycobacteria**

At present Mycobacteria are grouped into two major divisions ‘slow growing’ and ‘rapidly growing’ mycobacterial species other than MTB complex are included under group Nontuberculous Mycobacteria (NTM). These are present everywhere in the environment and sometimes colonize healthy individuals in the skin, respiratory and gastrointestinal tracts. In 1959, Runyon classified NTM into 4 groups based on phenotypic characteristics i.e. growth rate and colonial pigmentation. These divisions have however, no formal taxonomic standing but are useful for the preliminary identification. 62
**Photochromogens:** These are slow growing NTM whose colonies become pigmented when exposed to light and take more than 7 days to appear on solid media. Ex: - *M.kansasii, M. marinum, M.intermedium M.asiaticum.*

**Scotochromogens:** These are slow-growing NTM whose colonies are pigmented when grown in the dark or the light and take more than 7 days to appear on solid media. Ex:-*M.scrofulaceum, M.szulgai, M.scrofulaceum and M.gordonae.*

**Nonphotochromogens:** These are slow-growing NTM whose colonies produce no pigment whether they are grown in the dark or the light and take more than 7 days to appear on solid media. Ex:- *Mycobacterium avium* complex (MAC- frequently isolated from HIV patients), *M.paratuberculosis* and *M.haemophilum.*

**Rapid-growers:** These NTM grow on solid media in less than 7 days. Ex:- *M.fortuitum, M. chelonae and M.abscessus.*

Among the NTM, MAC is frequently encountered and were first recognized as human pathogens in 1950. M. avium complex comprises *M.avium, M. intracellulare, M.paratuberculosis.* These organisms closely resemble each other and cannot be distinguished in routine tests or on clinical grounds. Therefore these organisms are sometimes referred to as M.avium intracellulare and to include *M.paratuberculosis* it’s called *M.avium complex.*

Some of the commonly cultivable Mycobacteria encountered in clinical specimens with their distinctive properties are listed in table 5.
Table 5: Distinctive properties of commonly cultivable Mycobacteria encountered in clinical specimens.
Pathogenesis:

TB is most commonly acquired by inhaling the bacillus transmitted from a person with infectious pulmonary TB to others by droplet nuclei, which are aerosolized by coughing, sneezing or speaking. The most infectious patients produce sputum with as many as $10^5$-$10^7$ AFB/ml, than patients who are sputum smear negative. Some of the inhaled bacilli reach the alveoli and are phagocytosed by macrophages in the lungs. Infection also occurs infrequently by ingestion through infected milk and rarely by inoculation. The engulfed bacilli multiply and give rise to subpleuralfocus of tuberculous pneumonia. Inside the macrophage, the intracellular Mycobacteria employ variety of survival strategies, which include: (1) prevention of an oxidative burst in phagocytosing cells and inhibition of phagosome-lysosome fusion; (2) resistance to lysosomal enzymes and reactive oxygen intermediates and secretion of superoxide dismutase; and (3) escape from the phagosome into the cytoplasm. If the bacilli are not destroyed, they replicate and kill the cell. A local area of inflammation is formed and more phagocytes are attracted to the site. Some bacilli are transported to the regional lymph nodes, where they are engulfed by antigen-presenting cells (APC). MHC class II molecules present epitopes of Mycobacteria within APC to CD4+ helper T–cells, these in turn produce a range of cytokines, including interferon γ (IFN-γ) that activate macrophages.

Activated macrophages aggregate around the lesion’s center and effectively neutralize tubercle bacilli without causing further tissue destruction to form the characteristic lesion of TB called granuloma. Some of these activated macrophages fuse to form multinucleated giant cells, characteristic but not exclusive to, granulomas of TB. The palisade of metabolically active macrophages consumes oxygen diffusing
into the granuloma, so that the center becomes anoxic and undergoes necrosis producing material similar like cheese and is termed caseation. The anoxia and free fatty acids in the caseous center provide highly unfavorable environment to the tubercle bacilli making them to die. If these caseous lesions calcify, they are called Ghon complexes.\(^5\),\(^6\)

The lesion tends to enlarge further, and the surrounding tissue is progressively damaged. The caseous material at the center of the lesion liquefies and form air-filled tuberculous cavities. From these cavities the bacteria spread into the environment through cough, talk and sneeze. In early stages of infection, bacilli reach the regional lymph nodes, from which they gain access to central venous; from there they reseed the lungs and disseminate to other organs via the lymphatics and the bloodstream. Hematogenous spread occurs in primary TB with implantation of bacilli in many organs such as central nervous system (meninges), kidney, bones and joints and pleurae. Other organs involved apart from lung are genitourinary tract, lymph nodes, peritoneum, pericardium and larynx. This spread is often called miliary TB. The ghon focus with enlarged, infected regional lymph nodes is termed the ‘primary complex’/Ghon complex.\(^5\),\(^6\)

Approximately 95% initially infected patients enter latent phase from which there is life-long risk of reactivation. In ~5%, the initial infection may progress directly to pulmonary, military, meningeal or other extra-pulmonary involvement.\(^6\)

TB is classified as pulmonary or extrapulmonary depending on involvement of the organs. If TB is confined only to lungs- it is pulmonary TB. If history/clinical findings consistent with active TB of any part of the body other than the lungs it is called extrapulmonary TB. Amongst Extra-pulmonary TB, TB lymphadenitis (lymph
node swelling), pleural effusion (collection of fluid between lung and its outer covering), abdominal TB, bone and joint TB, miliary TB (numerous TB lesions in the lungs and throughout the body) and meningeal TB (Brain TB) are commonest. Pulmonary TB can be categorized as primary or postprimary TB (adult-type, secondary). Disease occurring in a person never previously exposed to a tubercle bacillus is termed ‘primary TB.’

Post-primary TB: Post-primary TB develops in previously infected people either as a result of endogenous reactivation of latent disease or of exogenous reinfection. It is also called as reactivation or secondary TB. 61,63

The most common symptoms of pulmonary TB are persistent cough with expectoration for 2 weeks/more, rise of temperature in evening, chest pain for 3 weeks or more, night sweats, lethargy, lassitude, weight loss, loss of appetite and haemoptysis. In extrapulmonary TB, symptoms depend on the organs involved, for e.g. TB of the lymph nodes presents with swelling of the lymph node, when TB affects the pleura (an outer lining of the lungs), there is fluid collection between the lung and pleura. Such patients present with breathlessness and the severity varies depending on the amount of fluid present. TB of the joints presents with swelling and pain of the affected joints, meningeal TB (TB affecting the brain) presents with headache, fever, neck stiffness and mental confusion.

Features commonly associated with different forms of extra-pulmonary TB include:

- Lymph node swelling
- Ongoing pain in back or joint
- Breathlessness
- Headache, neck stiffness, confusion
Before the advent of HIV infection, 80% of all new cases of TB were limited to the lungs. However, up to two-thirds of HIV infected patients may have both pulmonary and extrapulmonary TB or extrapulmonary TB alone. Extrapulmonary TB is found to be more common among HIV infected patients. In various series extrapulmonary TB alone or in association with pulmonary disease-has been documented in 40-60% of all cases in HIV coinfected individuals. The most common forms are lymphatic, disseminated, pleural and pericardial. Mycobacteremia and meningitis are also frequent, particularly in advanced HIV disease.⁶,⁶³
Recent Diagnostic Algorithms for pulmonary TB as per RNTCP guidelines:

Cough for 2 weeks/more

2 sputum smears

1 or 2 Positives

2 Negatives

Antibiotics 10-14 days

Cough persists

Repeat 2 sputum examination

1 or 2 Positives

2 Negatives

X-ray chest

Sputum positive PTB, Suggestive of TB, Negative for TB

Anti TB treatment

Sputum negative PTB, Non TB

Anti TB treatment

Lab diagnosis:

The diagnosis of TB in late stages of HIV infected patients is difficult because of the increased frequency of sputum smear negativity, atypical radiographic findings, lack of classic granuloma formation and a negative tuberculin skin test (TST).
Radiological features: It is the cornerstone of diagnosis of pulmonary TB in which abnormal chest radiographic findings with respiratory symptoms are noted. Here upper-lobe disease with infiltrates and cavities or a solitary pulmonary nodule to diffuse alveolar infiltrates in a patient with adult respiratory distress syndrome may be observed. In AIDS patients radiographic pattern are inconclusive.\textsuperscript{61,64}

Tuberculin skin testing: In 1941, tuberculin purified protein derivative (PPD) developed by Seibert and Glenn is most widely used in screening for latent MTB infection (LTBI) which measures DTH to tuberculoprotein. The test is of limited value to diagnose active TB because of its relatively low sensitivity, specificity and its inability to discriminate between latent infection and active disease. False negative reactions are common in immunosuppressed patients and in those with overwhelming TB.\textsuperscript{61}

TB MPB-64 skin patch test: MPB64 is a specific mycobacterial antigen secreted by MTB, \textit{M. bovis} and some strains of \textit{M. bovis} BCG to diagnose LTBI.\textsuperscript{64}

IFN-γ release assay (IGRA): The assay is based on in vitro blood test in which T cells of sensitized individuals produce IFN-γ when encountered with antigens of MTB. These assays are available commercially in ELISA format, e.g. QuantiFERON-TB (QFT) and QuantiFERON-TB Gold assay and ELISPOT format, e.g. T-SPOT-TB assay.\textsuperscript{64}

Different types of clinical specimens may be collected for mycobacterial analysis such as respiratory tract (sputum, tracheal/bronchial aspirates, bronchoalveolar lavage fluid), urine, gastric aspirates, tissue, biopsy specimens, pus and normally sterile body fluids (such as cerebrospinal fluid (CSF), pleural and pericardial aspirates). Also blood, bone marrow or feces are used to diagnose TB.
Majority of the specimens used for the recovery of AFB are contaminated with organic debris, such as mucin, tissue, serum and other proteinaceous material. Commonly used digestion-decontamination methods are the sodium hydroxide (NaOH) method, the Zephiran-trisodium phosphate method and the N-acetyl-L-cysteine (NALC) - NaOH method. NaOH is a commonly used decontaminant that is also mucolytic. Tissues or body fluids collected aseptically do not require the digestion and decontamination methods. After digestion and decontamination, Mycobacteria are concentrated by centrifugation to enhance their detection by acid-fast stain and culture.  

**Direct detection methods:**

Microscopy provides a simple, sensitive, inexpensive and rapid means of detecting AFB by smear examination of sputum, body fluids or tissues. In many developing countries it is the only technique used to confirm the diagnosis of TB. To be detected microscopically there must be $5 \times 10^3$-$5 \times 10^4$ AFB/ml of sputum, while $10^6$ AFB/ml of specimen usually results in positive smear. Only 60% of the smears are positive if $10^4$ AFB/ml are present (European Society for Mycobacteriology 1991). Three types of staining procedures are used in laboratory for rapid detection and confirmation of AFB: fluorochrome, Kinyoun and Ziehl Neelsen (ZN). Acid fast stained smears of concentrated clinical specimens require at least $10^4$ AFB/ml for AFB detection. Mycobacteria stained with either the ZN or Kinyoun’s technique appear as bright red to pink rods against a blue background. Fluorochrome stained bacteria are bright yellow (auramine) or orange-red (rhodamine) against a dark background. The results of acid fast organisms observed on a smear must be quantified because it estimates the number of bacilli excreted and the extent of patient’s infectiousness. The recommended interpretations and ways to report smear results are given in table 6.
### Table 6: Acid fast smears reporting

<table>
<thead>
<tr>
<th>No. of AFB seen</th>
<th>No. of AFB seen</th>
<th>Report</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fuchsin stain</td>
<td>Flucorochrome stain</td>
</tr>
<tr>
<td></td>
<td>(1000X magnification)</td>
<td>(450X magnification)</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>(-) No AFB seen</td>
</tr>
<tr>
<td>1-2/300 fields</td>
<td>1-2/70 fields</td>
<td>(±) Doubtful; request another specimen</td>
</tr>
<tr>
<td>1-9/100 fields</td>
<td>2-18/50 fields</td>
<td>1+</td>
</tr>
<tr>
<td>1-9/10 fields</td>
<td>4-36/10 fields</td>
<td>2+</td>
</tr>
<tr>
<td>1-9/field</td>
<td>4-36/field</td>
<td>3+</td>
</tr>
<tr>
<td>&gt;9/field</td>
<td>&gt;36/field</td>
<td>4+</td>
</tr>
</tbody>
</table>


Approximately 0.01ml of specimen is smeared over a 2 cm area of the slide and heat fixed. About 5ml of carbol fuchsin–phenol solution is added and the bottom of slide is heated until the stain begins to steam. After heating for 5 min, the smears are rinsed with water and drained. The smears are then flooded with an acid-alcohol solution for at least 2 min. The smears are again rinsed, drained and counter stained with methylene blue for 1-2 min. The smears are examined under light microscope for about 100 fields at a 1000X magnification for the presence of AFB.\(^{62,65}\)

**Antigen protein detection:** Detection of mycobacterial products / components. Ex: Tuberculostearic acid.
**Molecular techniques:** Nucleic acid amplification tests (NAAT)/direct amplification tests (DAT) are designed to amplify nucleic acid regions specific for MTB complex and to use directly on the clinical samples. All the NAATs rely on the amplification of specific DNA/RNA targets but principles and techniques differ from each other. Commercially available NAAT has been listed out in table 7.\(^{64}\)

**Table 7: Commercial nucleic acid amplification test (NAAT) for TB.**

<table>
<thead>
<tr>
<th>NAAT</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplified <em>M.tuberculosis</em> Direct Test (AMDT)</td>
<td>Transcription-mediated amplification of rRNA</td>
</tr>
<tr>
<td>Amplicor MTB</td>
<td>PCR amplification of 16sRNA</td>
</tr>
<tr>
<td>Cobas Amplicor</td>
<td>PCR amplification of 16sRNA</td>
</tr>
<tr>
<td>BD-Probe Tec Direct (SDA)</td>
<td>Strand displacement amplification of IS6110 and 16sRNA</td>
</tr>
<tr>
<td>Loop-mediated Isothermal Amplification (LAMP)</td>
<td>Isothermal amplification and visual readout with UV fluorescence</td>
</tr>
</tbody>
</table>

**Loop-mediated isothermal amplification (LAMP):** It amplifies DNA with high efficiency under isothermal conditions using six set of primers specifically designed to recognize six different regions on the target gene.\(^{64}\)

**Fluorescence in situ hybridization (FISH) using Peptide Nucleic Acid (PNA) probes:** PNA is a novel DNA mimic in which the sugar-phosphate backbone of DNA is replaced with polyamide backbone. In this test PNA binds to selected
regions of mycobacterial 16s rRNA sequences. This technique identifies MTBC and NTM from culture media.  

**Line probe assay (LPA):** These are novel DNA strip based tests that use PCR and reverse hybridization methods for simultaneous detection, identification and drug resistance. This is used for identification of Mycobacteria from clinical samples and liquid cultures. It is based on amplification of the 16s-23s ribosomal DNA spacer region followed by hybridization with 16s-specific oligo-nucleotide probes.


Maureen Morgan et al (2005) and Cengiz et al (2002) in their studies found that Genotype MTBDR assay appears to be highly sensitive and specific in detection of rifampicin resistant TB when used on culture isolates and there is paucity when used directly on clinical specimens. The test could be useful in patient population in which MDR-TB is strongly suspected.

Hetero-resistance is a preliminary stage towards full resistance; LPA plays a significant role in hetero-resistance detection, i.e. both wild and mutant type genes are detected within the same specimen simultaneously. Also the technique offer advantages due to its rapid detection capacity compared to conventional DST which is labour intensive.

GenoType MTBDR plus assay: It includes 3 steps: DNA extraction, PCR amplification and reverse hybridization. Barnard reports that the assay is of limited use when used on smear negative samples. Mutation in the rpoB gene confers
rifampicin resistance, in katG gene for high-level isoniazid resistance and in inhA gene for low-levels of isoniazid resistance.

The band in TUB zone indicates that the tested Mycobacteria belong to MTB complex and the absence indicates atypical Mycobacteria. The absence of a band in at least one of the wild type probes and/or the presence of a band in mutation probe regions of the locus control zones of rpoB, katG and inhA indicates drug resistance. Each Hain’s strip has 27 reaction zones (Fig 7)
Wei- Lun Huang et al in 2009 assessed GenoType MTBDR plus test with three extra WT rpoB probes for RIF, two extra WT INHA regulatory probes and four extra mutated probes for INH the test has advantage of a short turnaround time for drug resistant MTB with high sensitive to RIF but less accuracy for INH resistance detection. He suggests that the new alleles of INH resistance genes should be evaluated to improve the sensitivity, especially for different geographic areas with genetically diverse MTB strains.  

Suhail Ahmad et al in 2009 compared two DNA line probe assays for rapid detection of MDR isolates of MTB i.e. GenoType MTBDR and INNO-LiPA and found that GT-MTBDR assay offers advantage of detecting resistance to both INH and RIF simultaneously. Ling et al (2008) demonstrated excellent accuracy for RIF resistance, even when used on clinical specimens.

Serological diagnosis of TB:

Detection of antibodies: ELISA techniques employing various mycobacterial antigens such as glycolipids from M.bovis BCG, antigen 5 & 6 from M.tuberculosis etc have been attempted to diagnose TB.

Detection of antigens: Capture ELISA test to detect liproalbumin (LAM) in urine specimens and dip stick method (semi-quantitative) for the detection of LAM in both pulmonary and extrapulmonary specimens.

Culture: Culture remains the gold standard for diagnosis of MTB infection. Cultural confirmation of TB diagnosis is essential for identification and susceptibility testing. Cultures on solid media usually take 3 weeks or longer to show visible colonies but it is about 10-12 days in liquid media. Growth of Mycobacteria in liquid media, regardless of the type, requires 5% to 10% CO₂.
**Solid media:** Egg based media such as Lowenstein-Jensen (LJ), Ogawa or Stonebrink and agar-based media like Middlebrook 7H10 or 7H11 media.

**Lowenstein Jensen Medium:** Among the solid media, LJ is the most commonly used egg based medium. It contains glycerol as carbon source, L-asparagine as nitrogen source, salt solutions and malachite green as an inhibitor of contaminants.

Potential advantage: LJ medium is less expensive than agar-based media. The medium has a good buffering capacity and materials in the inoculum toxic for Mycobacteria are neutralized. It has a strong shelf life when stored in refrigerator (several weeks) and allows direct visual recognition of colonies characteristic of mycobacterial species and growth of the contaminants.

The main disadvantage of the LJ medium is need for a long incubation time, especially in the clinical specimen which contains few bacilli.\(^{75}\)

Morphological and biochemical properties used for identification of Mycobacteria are well established, standardized, reproducible and relatively inexpensive. Mycobacteria are preliminarily identified by growth rate, colonial morphology, colonial texture and pigmentation. Additional biochemical tests help for further characterization but currently molecular techniques used are more sensitive and have simplified the detection.

Biochemical tests include niacin accumulation, nitrate reduction, catalase activity, growth inhibition by thiophene 2-carboxylic acid hydrazide, tween 80 hydrolysis, arylsulfatase activity, pyrazinamidase test, growth on MacConkey agar, urease activity, growth in 5% sodium chloride and iron uptake. Also analysis of mycobacterial lipids by chromatographic techniques including thin-layer
chromatography, gas-liquid chromatography, capillary gas chromatographic methods and high-performance liquid chromatography has been used to identify Mycobacteria.

Among liquid media, apart from Middlebrook 7H9 or Dubos Tween Albumin media - biphasic septi-chek, radiometric system, semiautomated BACTEC 460 TB and Mycobacteria growth indicator tube (MGIT) systems represent most efficient and rapid technique to culture Mycobacteria. Other continuous growth monitoring systems include ESP Culture System II, BACTEC 9000 MB and BACTEC MGIT 960. Some of the commonly used liquid media systems with their principle are listed in table 8.62
Table 8: Commonly used liquid media systems to culture and detect the growth of Mycobacteria.

<table>
<thead>
<tr>
<th>System</th>
<th>Basic principles of detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>BACTEC 460 TB</td>
<td>Culture media contains 14C-labeled palmitic acid. If present in the broth, Mycobacteria metabolize the 14C-labeled substrates and release radioactively labeled 14CO2 in the atmosphere, which collects above the broth in the bottle. The instrument withdraws this CO2-containing atmosphere and measures the amount of radioactivity present. Bottles that yield a radioactive index, called a growth index, greater than or equal to 10 are considered positive.</td>
</tr>
<tr>
<td>Septi-Chek AFB System</td>
<td>Biphasic culture system made up of a modified Middlebrook 7H9 broth with a three-sided paddle containing chocolate, egg-based and modified 7H11 solid agars, the bottle is inverted regularly to inoculate the solid media. Growth is detected by observing the three-sided paddle.</td>
</tr>
<tr>
<td>Mycobacteria Growth Indicator Tube (MGIT)</td>
<td>Culture contains Middlebrook 7H9 broth and a fluorescent compound embedded in a silicone sensor. Growth is detected visually using an UV light. Oxygen (O2) diminishes the fluorescent output of the sensor; therefore O2 consumption by organisms present in the medium are detected as an increase in fluorescence.</td>
</tr>
<tr>
<td>Continuous Growth Monitoring Systems</td>
<td></td>
</tr>
<tr>
<td>ESP Culture System II</td>
<td>Organisms are cultured in a modified Middlebrook 7H9 broth with enrichment &amp; a cellulose sponge to increase the culture's surface area. The instrument detects growth by monitoring pressure changes that occur as a result of O2 consumption/gas production by the organisms as they grow.</td>
</tr>
<tr>
<td>BACTEC MGIT 960</td>
<td>The instrument detects growth by monitoring O2 consumption by means of a fluorescent sensor.</td>
</tr>
</tbody>
</table>
TK Medium Culture System: Colorimetric system that indicates growth of Mycobacteria by changing its color.\(^6\)

MODS: The microscopic observation drug susceptibility assay which relies on light microscopy to visualize the characteristic cording morphology of Mycobacteria in culture.\(^6\)

Bacteriophage-based assays: Amplification of bacteriophages after their infection of MTB, followed by detection of progeny phages as lytic plaques on a lawn of \textit{M. smegmatis}. FAST Plaque- TB assay can be used on sputum specimens, FAST Plaque-TB-MDR to detect RIF resistance in culture isolates and FAST Plaque-TB-Response for direct use of clinical specimens.\(^6\)

Luciferase reporter phage-based test: Detection of fluorescence produced by action of Luciferase enzyme on Luciferin substrate. Here mycobacteriophage containing firefly Luciferase gene enters Mycobacteria, multiply and express Luciferase gene leading to production of Luciferase enzyme.\(^6\)

Antimicrobial Susceptibility Testing and Therapy:

Susceptibilities may be performed by either the direct or the indirect method. The direct method uses the inoculum-smear positive concentrate containing more than 50 acid fast bacilli per 100 oil immersion fields; the indirect method uses a culture as the inoculum source.

Four general methods used for determining susceptibility of Mycobacteria are absolute concentration, resistance ratio, proportion and commercial systems cleared by FDA (Table 9).\(^6\)
Table 9: Overview of conventional methods to determine susceptibility of mycobacterial isolates to antimicrobial agents.

<table>
<thead>
<tr>
<th>Method</th>
<th>Principle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absolute Concentration</td>
<td>For each drug tested, a standardized inoculum is inoculated to control (drug-free) media and media containing several appropriate graded drug concentrations. Resistance is expressed as the lowest concentration of drug that inhibits all of the growth, that is, the minimum inhibitor concentration.</td>
</tr>
<tr>
<td>Proportion</td>
<td>For each drug tested, several dilutions of standardized inoculum are inoculated onto control and drug-containing agar medium. The extent of growth in the absence or presence of drug is compared and expressed as a percentage. If growth at the critical concentration of a drug is &gt;1%, the isolate is considered clinically resistant.</td>
</tr>
<tr>
<td>Radiometric</td>
<td>Employing the principle of the proportion method, this rapid methods uses liquid medium containing $^{14}\text{C}$-labeled growth substrate. Growth is indicated by the amount of $^{14}\text{C}$-labeled-carbon dioxide (CO$_2$) released, as measured by the BACTEC 460 instrument. For each drug tested, a standardized inoculum is inoculated into a drug-free and drug-containing vial. The rate and amount of CO$_2$ produced in the absence or presence of drug is then compared.</td>
</tr>
</tbody>
</table>

Drugs: Four major drugs are considered the first-line agents for treatment of TB: isoniazid, rifampin, pyrazinamide and ethambutol.

These above mentioned agents are recommended on the basis of their bactericidal activity, sterilizing activity and their low rate of induction of drug resistance. Because of lower degree of efficacy and a higher degree of intolerability and toxicity, 6 classes of second line drugs are generally used for TB patients who are
resistant to first line drugs. The injectable aminoglycosides streptomycin, kanamycin and amikacin; the injectable polypeptide capreomycin; the oral agents ethionamide, cycloserine and PAS; and the fluroquinolone antibiotics. Of the quinolones, third generation agents are preferred: levofloxacin, gatifloxacin and moxifloxacin.\textsuperscript{61,63}

\textbf{Isoniazid} (INH - isonicotinic acid hydrazide): It was first synthesized in the early part of the 20\textsuperscript{th} century and has been the mainstay of anti-tuberculosis therapy for more than 40yrs. It is a critical drug used to treat both TB disease and LTBI. It has bactericidal activity against both intracellular and extracellular, actively dividing MTB. The drug is bacteriostatic against slowly dividing organisms. INH is used in treatment of LTBI as first-line agent due to its tolerance, well established efficacy and inexpensive. The drug is taken daily or intermittently (i.e. twice weekly) as DOT for 9 months. A 6 month course of daily or intermittent INH is considered second-line therapy. INH is used in combination with other agents to kill actively dividing MTB and slow growing persister organisms.

\begin{center}
\includegraphics[width=0.2\textwidth]{isoniazid.png}
\end{center}

\textbf{Mechanism of action:} INH is a prodrug activated by the mycobacterial KatG catalase/peroxidase (encoded by katG) and is coupled with reduced nicotinamide adenine dinucleotide (NADH). The resulting isonicotinic acyl - NADH complex blocks the mycobacterial ketoenoylreductase InhA, binding to its substrate and inhibiting fatty acid synthase and ultimately mycolic acid synthesis (an essential component for the mycobacterial cell wall).\textsuperscript{76} KatG activation of INH also releases free radicals that have antimycobacterial activity, including nitric oxide. Initially
Middlebrook et al demonstrated that a loss of catalase activity result in INH resistance. The MIC of INH for wild-type susceptible strains are <0.1µg/ml for MTB and 0.5-2 µg/ml for M.kansasii.

**Pharmacology:** It is a small water soluble molecule. The usual adult oral daily dose of 300 mg results in peak serum levels of 3-5 µg/ml within 30 min to 2 hrs after ingestion. Both oral and IM preparations of INH achieve good levels and diffuses well throughout the body, reaching therapeutic concentrations in body cavities and fluids, with concentrations in CSF compared to those of serum. INH is metabolized in the liver via acetylation by N-acetyltransferase 2 (NAT2) and hydrolysis; patients who are “fast acetylators” have lower serum levels of INH, whereas “slow acetylators” have higher serum levels of INH. Activated INH is encoded by inhA and is an enoyl-acyl carrier which is the primary target for INH resistance and ethionamide.

**Dosage:** Recommended daily dose for TB treatment is 5 mg/kg for adults and 10-20 mg/kg for children with a maximal daily dose of 300 mg for both. For intermittent therapy in adults (usually twice per week), the dose is 15 mg/kg with a maximal daily dose of 900 mg.

**Resistance:** Primary INH resistance is significantly higher in many populations among untreated patients. Four pathways have been elucidated for INH resistance. Most strains have amino acid changes in either the catalase-peroxidase gene (katG) or in the mycobacterial ketoenoylreductase gene (inhA). Less frequently, alterations in kasA, the gene – an enzyme involved in mycolic acid elongation and loss of NADH dehydrogenase 2 activity. Approximately 70-80% of INH resistance in MTB isolates can be due to mutations in the katG and inhA genes.
Adverse effects: Drug induced liver injuries and peripheral neuropathies are significant adverse effects. It may cause asymptomatic transient elevation of aminotransferase levels in up to 20% of recipients. Other adverse reactions include rash, fever, anemia, acne, arthritic symptoms, systemic lupus erythematosus-like syndrome, optic atrophy, seizures and psychiatric symptoms.  

Rifampicin (RIF): It was first introduced in 1968 for the treatment of TB and is the most active antimycobacterial agent used in first–line drug along with INH, PZA and EMB. It’s a good marker for MDR-TB and a good predictor of poor treatment outcome. It is a semisynthetic derivative of Amycolatopsis rifamyanica broad spectrum antibiotic that has bactericidal activity against both dividing and non-dividing MTB with sterilizing activity. The drug is active against *M. leprae, M. kansasi, M. haemophilum* and *M. marinum* as well as gram-positive and gram-negative bacteria in other genera. RIF, administered for 4 months, is also an alternative agent to INH for the treatment of LTBI.

**Mechanism of action:** RIF exerts both intracellular and extracellular bactericidal activity. It binds and inhibits mycobacterial DNA-dependent RNA polymerase, blocking RNA synthesis. RNA polymerase β subunit encoded by the rpoB gene. RIF resistance is associated with a 507-533. **Susceptible strains of MTB, M. kansasi** and **M. marinum** are inhibited by RIF concentrations of 1 µg/ml.
Pharmacology: RIF is a fat soluble, complex macrocyclic molecule readily absorbed after oral administration. Serum levels of 10-20 µg/ml are achieved 2.5 hrs after the usual adult oral dose of 10mg/kg (given without food). It has a half-life of 1.5-5 hrs, and is primarily excreted through the bile and enters the enterohepatic circulation; <30% of a dose is renally excreted.

Dosage: The daily dosage of RIF is 10 mg/kg for adults and 10-20 mg/kg for children, with a maximum of 600 mg/d for both. The drug is given once daily, twice weekly, or three times weekly.

Resistance: Resistance to RIF is the consequence of spontaneous, mostly missense point mutations in a core region of the bacterial gene coding for the β subunit of RNA polymerase (81-bp of rpoB). Altered RNA polymerase is no longer subject to inhibition by RIF.

Adverse events: Adverse effects of RIF are infrequent and generally mild. Hepatotoxicity alone is uncommon in the absence of preexisting liver disease and often consists of isolated hyperbilirubinemia rather than aminotransferase elevation. Other hypersensitive reactions include fever, chills, malaise, rash, and in some instances renal and hepatic failure may occur with intermittent therapy.61,63

Ethambutol (EMB): It is a bacteriostatic antimycobacterial agent first synthesized in 1961. Among first-line drugs it is the least potent pathogen against MTB and has a synergistic effect in combination with other agents in continuation phase of treatment in patients who cannot tolerate INH/RIF/patients infected with resistant strains to either of the latter drugs.
Mechanism of action: It is bacteriostatic and inhibits arabinosyltransferase (embB) involved in cell wall synthesis, which probably inhibits the formation of arabinogalactan and lipoarabinomannan. Three genes embC, A and B encode this enzyme and five mutations are associated with 70-90% of EMB resistant isolates.61,63,79

Pharmacology and dosing: A single dose of EMB is absorbed (75-80%) within 2-4 hrs of administration. Serum levels peak at 2-4µg/ml after the standard adult daily dose of 15 mg/kg. EMB is well distributed throughout the body except in the CSF; a dosage of 25 mg/kg is necessary for attainment of a CSF level half of that in the serum. For intermittent therapy, the dosage is 50 mg/kg twice weekly. To prevent toxicity the dosage must be lowered and the frequency of administration reduced for patients with renal insufficiency.

Adverse effects: It is usually well tolerated and has no significant interactions with other drugs. Optic neuritis is the most serious effect reported; due to effect on the amacrine and bipolar cells of the retina. The risk of ocular toxicity is dose dependent and can be increased by renal efficiency. Routine use of EMB for children is not recommended but if drug resistant TB is suspected it can be used. All patients prior to initiate therapy with EMB should undergo baseline test for visual acuity, visual fields, color vision and examination of the optic fundus. Other effect like Peripheral sensory neuropathy occurs in rare instances.
Resistance: EMB resistance in MTB and NTM isolates is associated primarily with missense mutations at codon 306 in the embB gene that encodes for arabinosyltransferase.\textsuperscript{61,63}

**Pyrazinamide** (PZA): It is a synthetic derivative of nicotinamide and is used in the initial phase of TB therapy. Its administration for the first two months of therapy with INH and RIF shortened from 9 to 6 months and decrease rates of relapse.

![Pyrazinamide structure](image)

Mechanism of action: It is an important bactericidal drug that is more active against slowly replicating organisms than actively replicating organisms. PZA is a prodrug that is converted by the mycobacterial pyrimidase to the active form- pyrazinoic acid (POA), which is active only in the acidic pH<6 and are found within phagocytes/granulomas.

Pharmacology and dosing: It is well absorbed after oral administration with peak serum concentrations of 20-60 µg/ml at 1-2 hrs after ingestion of the recommended adult daily dose of 15-30 mg/kg. It distributes well to various body compartments, including CSF. The serum half-life of the drug is 9-11 hrs with normal renal and hepatic function. PZA is metabolized in the liver to POA, 5-hydroxypyrazinamide and 5-hydroxy-POA. A high proportion of PZA and its metabolites (~70%) are excreted in the urine.
Adverse effects: Hepatotoxicity occurs less commonly when PZA is administered with INH and RIF in TB treatment, but the risk is increased with older age, active liver disease, HIV infection and low albumin level. Use of PZA with RIF for LTBI treatment is no longer recommended because of unacceptable rates of hepatotoxicity and death. Hyperuricemia is a common effect and clinical gout is rare.

Resistance: Lack of pyrazinamidase activity and PZA resistance is correlated with mutations in pncA gene that encodes the enzyme which converts the prodrug to active POA.\textsuperscript{61,63}

Regimens: Standard short-course regimens are divided into an initial/bactericidal phase and a continuation/sterilizing phase.\textsuperscript{61} During the initial phase, the majority of the tubercle bacilli are killed, symptoms resolve and usually the patient becomes noninfectious. The continuation phase is required to eliminate persisting Mycobacteria and prevent relapse. The treatment regimen of choice for all forms of TB in adults consists of a 2-month initial phase of INH, RIF, PZA and EMB followed by a 4-month continuation phase of INH and rifampin. In retreatment cases the treatment period is for 8 months.\textsuperscript{63}

If the disease is caused by MTB that is resistant to both RIF and INH- the most efficacious first-line TB drugs it is referred to as MDR-TB. Treatment regimens for MDR-TB generally include a late generation fluoroquinolone and an injectable second-line agent (such as capreomycin, amikacin or kanamycin). Both standardized and optimized/customized regimens are in use around the world. Extensively drug-resistant TB (XDR-TB) is MDR-TB with additional resistance to any fluoroquinolone and at least one of the second-line injectable agents. Now WHO defines TDR-TB has TB caused by virulent strain that is resistant to all known treatments.\textsuperscript{63}
Prevention by Vaccination: BCG (bacille Calmette - Guerin) –

It is a live vaccine derived from an attenuated strain of *M. bovis*. It is recommended and has been used for routine prophylaxis at birth in countries with high TB prevalence. This offers protection against serious forms of primary infection, especially those due to dissemination of bacilli from the primary complex and also against cervical lymphadenitis caused by environmental Mycobacteria. But it is less effective in preventing tubercle bacilli from persisting in the tissues and becoming reactivated later in life.\(^6\)

Another common opportunistic infection studied in HIV patients is candidiasis caused by *Candida* species.

**Candidiasis:**

**History**

Hippocrates made the first description of *Candida* infection, oral candidiasis (thrush) in two patients with underlying diseases in ‘Epidemics’ in the 4\(^{th}\) century BC. In modern medicine thrush was first described by Rosen von Rosenstein (1771) and Underwood (1784). Lagenbeck (1839) demonstrated a yeast-like fungus in oral thrush; Gruby in 1842 gave an exact association between oral thrush and the fungus and classified it as Sporotrichum.

The fungus was isolated by Bennett (1844) in sputum of TB patient, by Wilkinson (1849) from vagina, by Robin (1853) in systemic infection, by Zenker (1861) from a brain infection, in whom the fungus spread haematogenously. Hausemann established the possibility of infant infection during birth by demonstrating the analogy between the causative agent of oral and vaginal thrush. Grawitz noticed the dimorphic nature of the thrush fungi in 1877.
Dubendorfer (1904) described onychomycosis, Jacobi (1907) - dermatitis, chronic mucocutaneous candidiasis by Forbes (1923) and cystitis by Rafin (1910), Conner described Osteomyelitis (1940), Joachim and Polayeshich – endocarditis. Castellani was the first to suggest the possibility that Candida species other than C.albicans is involved in pathological processes.

The genus Candida was named by Robin (1923) as Oidium albicans and Monilia albicans by Zoph. Kreger – Van Rij lists 100 synonyms for C.albicans in the 1984 edition of The Yeasts. The generic name Candida was suggested by Christine Marie Berkhout to fungi which reproduce by budding and developed pseudomycelia. This was officially accepted in the Eighth Botanical Congress held in Paris in 1954. Vishwanathan and Randhawa (1959) isolated C.vishwanathii and Dublin from Ireland reported Candida dubliniensis closely related to C.albicans as new species in 1995; particularly in HIV infected and other immunocompromised individuals.81

**Taxonomy:**

The recent changes in nomenclature are based on molecular methods, which have been applied in search of a better understanding of taxonomic relationship between yeast species.81 In modern classification scheme, Candida is placed as ascomycetous yeast based on the characteristic features like negative urease reaction, absence of capsule, fermentation of carbohydrates except inositol. They possess β-glucans in their cell walls and do not produce starch or carotenoid pigment. Two serotypes A and B have been described based on the differences between the mannan components of the cell wall in C.albicans.82

The analysis of small ribosomal subunit sequence has shown that C.albicans, C.tropicalis, C.parapsilosis and C.viswanathii form one subgroup with a more distinct
connection to *C. guilliermondii*, *C. kefyr*, and *C. glabrata*. Development of molecular technologies in the use of taxonomy has resulted in changes of the status of certain *Candida* species and the recognition of the new species such as *C. dubliniensis*. Both classic and molecular researches into *Candida* species has steadily led to the discovery of teleomorphic stage in different species of *Candida*. *Candida* species exhibiting a teleomorphic stage are considered as ascomycetous fungi. On the basis of physiological characteristics, they are divided into 12 groups. Genus *Candida* is represented by 163 anamorphic species with teleomorphs in atleast 13 species. It is grouped under order Saccharomycetales.

The more important pathogenic species such as *C. albicans*, *C. tropicalis*, *C. parapsilosis* and *C. glabrata* have not been found to produce teleomorphs, even though they do not have phylogenetic affinities with ascomycetes.

The genus *Candida* is defined by the following key characteristics:

A: Colony: Carotenoid or melanin pigment absent.

B: Cell shape: Variable (globose, elliptical, cylindrical, triangular or lunate).

C: Cell wall: Two layers.

D: Whole cell hydrolysate: Contains no xylose.

E: Diazonium Blue B colour test: Negative

F: Starch like compound: Absent

G: Pseudo or true hyphae: Present or absent.

H: Budding: Holoblastic, not phialidic.

I: Blastospore: Absent

J: Arthroconidia: Absent
Several medically important species have been reduced to synonyms on the basis of DNA relatedness. *C. stellatoidea, C. clausenii* and *C. langeronii* are treated as synonyms of *C. albicans*. *C. pseudotropicalis* is considered a synonym of *C. kefyr*, whereas *C. paratropicalis* is a synonym of *C. tropicalis*.

Currently, the views on the taxonomy of anamorphic *Candida* as described by Kurtzman and Fell are as follows:

**Kingdom:** Fungi

**Phylum:** Ascomycota

**Class:** Hemiascomycetes

**Order:** Saccharomycetales

**Family:** Candidaceae

**Genus:** Candida

**Epidemiology:**

Candidiasis is a major fungal disease encountered in AIDS patients. It is generally caused by the fungus *Candida albicans* and sometimes by non-albicans *Candida* (NAC). Two major medical events revived the interest in *Candida* infections- first is introduction of antibacterial drugs, in the second half of the 20th century, which acted as predisposing factors for mycotic infections causing an imbalance of the host’s natural microflora in favor of fungi, upon which they have no inhibitory activity. Second event is increase in the prevalence of AIDS which led to parallel increase in the incidence of *Candida* infections in general and less pathogenic NAC species in particular.

At present there are 163 anamorphic *Candida* species with teleomorphs in at least 13 genera which are found in different habitats. *Candida albicans* was generally
considered as major pathogen among the *Candida* species but now NAC species have also increased during last decade. Most common NAC species recorded are *C.tropicalis, C.parapsilosis, C.glabrata* and *C.krusei*. Other NAC species occurrence varied with geographical region (Hazen 1995, Kremery and Barnes 2002).

*Candida* species appear as typical buds, round, or oval thin-walled, yeast cells measuring 4-6µm in diameter and reproduce by budding. Organisms of this genus occur in three forms in tissues: blastospores, pseudohyphae and hyphae, in certain infectious conditions they form hyphae. Some species form chlamydoconidia in culture. *Candida* species identification is based on a combination of biochemical, enzymatic, and morphological characteristics, such as carbohydrate assimilation; fermentation; and the ability to produce hyphae, germ tubes, and chlamydoconidia. The primary identification procedure involves differentiation of *Candida albicans* from other species, because it is the most frequent cause of disease. *C. albicans* is a common member of the oropharyngeal, gastrointestinal, and female genital flora. Infections are endogenous except in cases of direct mucosal contact with lesions.

**Structure:**

The main components of the cell wall are phosphorylated mannans, glucans, mannoprotein, chitin, and proteins. Polysaccharides in *Candida* species are primarily in the cell walls. Polypeptides and proteins are intimately bound with cell wall polysaccharides and the fine structure of the various cell wall phosphor-glycopeptide oligomers and polymers accounting for differences in antigenic structures, gross hydrophobic properties, and specific adhesions to host cells and other surface between *Candida* species. Yeast cells and germ tube are similar in their cell wall composition, although the relative amount of β-glucans, chitin and mannan may
Cell wall is composed mainly of carbohydrates (80-90%), and the majority of this carbohydrate is found in branched polymers of glucose \([\beta-1,3 \text{ and } \beta-1,6 \text{ glucose polymers (\(\beta\)-glucan)}\], unbranched polymers of \(\beta-1,4 \text{ acetyl-D glucosamine (chitin)}\) and polymers of mannose (mannan) with or without glucose covalently bound to protein. Chitin located primarily at bud scars diffuse throughout the cell wall. Protein (6 to 25%) and lipids (1 to 7%) are present as minor cell wall constituents.

Mannoprotein comprises 40% of the total cell wall polysaccharide (\(\beta\)-glucans) accounting for 47 to 60% by weight of the cell wall; chitin and lipid represent the remaining components of the wall. Mannose polymers are found covalently associated with proteins and are designated as mannoprotein. These are homopolymers in N-and O-glycosidic covalent linkages with polypeptide residues of asparagines.

The fungal protoplast is same as the structure of eukaryotes. The nucleus contains a single nucleolus rich in RNA. Double layered nuclear membrane has characteristic pores. Plasma membrane that surrounds cytoplasm contains ergosterol. Cytoplasmic organelles and inclusions present are mitochondria, vacuoles, vesicles, endoplasmic reticulum, microtubules, ribosome, and glycogen crystal; golgi apparati are absent.

The cell wall of *Candida* is now recognized as a dynamic, constantly changing structure that contains enzymatically active proteins such as enolase and N-acetyl glucosaminidase, ubiquitin like epitope and a protein related to the hsp 70 (heat shock protein) family. The nature of mannan structure in the walls can be altered by changes in the external pH and temperature.
Physiology:

In *Candida* species morphogenesis is influenced by temperature. At a temperature around 25°C, chlamydomospores are formed in *C. albicans*; at 37°C pseudohyphae are formed. Temperature requirements of *Candida* range from 20-40°C, pH from 2-8.

The organism can grow on a simple defined medium containing carbon (sucrose, glucose) nitrogen (e.g. ammonium salts) and phosphate. *Candida* species metabolize glucose via the hexose monophosphate shunt pathway under aerobic condition (assimilation) or via the Embden Meyerhoff Pathway under the anaerobic condition (fermentation). Additional metabolic mechanisms are mitochondrial oxidative phosphorylation, Kreb’s cycle and protein synthesis by 80S ribosome (composed by a 60S and 38S subunit).

*Candida* enzymes such as proteases are involved in pathogenesis and serve as targets for antifungal drugs. Catabolites secreted by *Candida* species are acids, fatty acids and alcohols including ethanol. Extracellular aspartyl proteinases are secreted by *C.tropicalis* and *C.parapsilosis*, but not by *C.guilliermondii*, *C.krusei* or *C.kefyr*. This indicates that obvious correlations exist between proteolytic ability and the degree of virulence among *Candida* species. Mycelial formation is accompanied by a suppression of the pentose phosphate pathway and the diversion of hexoses for cell wall biosynthesis.

*Candida albicans* (Robin) Berkhout 1923.
It is the most common cause of candidiasis, which is an acute, subacute or chronic infection involving any part of the body. It ranges from commensal through life-threatening disseminated diseases.

**Rate of growth:** Rapid; mature in 3 days

**Colony Morphology on SDA:** Cream-colored, pasty, smooth; after a month of incubation - creamy, glistening, waxy, soft, smooth to reticulated; old stocks gets wrinkled and folded with spicules.

**Microscopic Morphology:** On CMA - Tween 80 agar at 25°C, for 72 hrs, form pseudohyphae with clusters of round blastospores at the septa and large, thick-walled terminal chlamydospores that are characteristic of this species.

**Germ-tube:** Positive

**On CHROMagar:** Light green to bluish-green.

Other NAC which are usually nonpathogenic cause infections in susceptible individuals. Some of the common species are:

**Candida tropicalis** (Castellani) Berkhout 1923.\(^{91,92}\)

**Rate of growth:** Rapid; mature in 3 days.

**Colony morphology on SDA:** Cream-colored to off white, glistening to dull, soft, smooth or wrinkled with mycelial fringe; old stocks become hairy and tough.

**Microscopic morphology:** On CMA - Tween 80 agar form blastospores singly or in small groups all along the pseudohyphae. A few chlamydospores may rarely be produced.

**On CHROMagar:** Blue colonies (sometimes pink).

**Candida kefyr** (Castellani) Basgal 1931: Earlier it was known as *C. pseudotropicalis*. 
Rate of growth: Rapid; mature in 3 days.

**Colony morphology on SDA:** Colonies are creamy, dull, soft, smooth and after prolonged incubation it becomes cream to yellow.

**Microscopic morphology:** On CMA - Tween 80 forms pseudomycelia with elongate blastospores that often fall apart and line up in parallel to give the appearance of “logs in a stream.”

*C. tropicalis* is usually misidentified with *C. albicans*, because it produces “pseudo-germ tubes” which are constricted at the base/point of germ-tube origin from the yeast cell. 91,92

**Candida krusei** (Castellani) Berkhout 1923: 91,92

Rate of growth: Rapid; mature in 3 days.

**Colony morphology on SDA:** Colonies are flat, dull and dry; after incubation for a month they become greenish-yellow, dull, soft, smooth or wrinkled and develop a mycelial fringe.

**Microscopic morphology:** On CMA - Tween 80 they form pseudomycelia with elongate blastospores forming a “cross-match-sticks” or tree-like appearance.

**On CHROMagar:** Large, spreading pink colonies with matt surface.

**Candida parapsilosis** (Ashford) Langeron et Tallioce 1959: 91,92

Rate of growth: Rapid; mature in 3 days.

**Colony morphology on SDA:** Creamy, soft, smooth, white, sometimes developing a lacy appearance; and after incubation for a month they are cream to yellowish, glistening, smooth or wrinkled.
Microscopic morphology: On CMA - Tween 80 blastospores are produced singly or in small clusters, and are seen along the pseudomycelium. Outstanding characteristic is presence of large mycelial elements called “giant cells.”

On CHROMagar: Colonies cream-colored.

*Candida guilliermondii* (Castellani) Langeron et Guerra 1938: 91,92

Rate of growth: Rapid; mature in 3 days.

Colony Morphology on SDA: Colonies are thin, flat, glossy, smooth-edged, cream to pinkish, after a month yellowish cream to pink, glistening, smooth or dull, wrinkled.

Microscopic morphology: On CMA - Tween 80 pseudohyphae are short with small cells, sometimes bearing ramified chains of small ovoid blastospores.

*Candida glabrata* (Anderson) Lodder et de Vries 1938: It is the fourth leading cause of nosocomial bloodstream infections in the hospitalized patients. It accounts for 11-16% of all candidemia cases and is associated with severe complications among than other *Candida* species. This is the only species of genus *Candida* that does not produce pseudohyphae.

Rate of growth: Rapid; mature in 3 days.

Colony morphology on SDA: Colonies are glistening, smooth, cream-colored.

Microscopic morphology: On CMA - Tween 80 they appear as branched chains of round cells formed in some strains but no pseudomycelium.

On CHROMagar: Colonies are pink to purple.

*Candida lusitaniae* (van Uden et do CarmaSousa) 1959: 91,92

This is the anamorph of Clavispora lusitaniae (Rodrigues de Mirinda 1979).
Colony morphology on SDA: white to cream, glistening, soft and smooth.

Microscopic morphology: Cells are ovoid in pairs and chains. Short distinctly curved pseudomycelium with occasional blastoconidia, at the septa.

*Candida dubliniensis*: ⁹¹,⁹²

It was named “dubliniensis” because first isolation was from AIDS patients of Dublin, Ireland. The organism shares closely phenotypic and genotypic characteristics with *C. albicans*. They produce germ-tube and chlamydomspores.

On CHROMagar: Colonies are dark green compared to *C. albicans*.

Virulence Factors:

*Candida* species especially *C. albicans*, has been attributed with the virulence traits which play a role in the pathogenesis.⁸¹ Key factors like adherence, morphogenesis, production of degradative enzymes and phenotypic switching contribute to virulence.⁹³ The ability of the fungus to bind (adhere) to host tissues is the initial step in the recognition and interaction with the host. It has been shown that the organisms bind to exfoliated human epithelial, mucosal cells and human tissue lines. Adherence varies with species. Factors such as fungal surface, hydrophobic growth medium, growth conditions, hormonal and immune status of the host affect the adherence.⁸¹

There are fungal surface molecules involved in the binding process and several putative adhesins: mannan-the mannoprotein, primarily their proteinaceous moiety and chitin were recognized. MiyaKawa et.al, (1992) suggested that antigen 6 of the mannan might be associated with adhesion activity. The counterpart receptor molecule on the host cell depends on the type of cells, apparently fucosyl glucosamine, fibro-nectin or arginine-glycine-asparagines (RGD).⁸¹
Biofilm production is seen among *Candida* species. The biofilm contain extracellular materials, composed of proteins, carbohydrates and other substances. Biofilms are formed on inert surfaces of medical devices, such as catheters, artificial dentures, prosthetic valves and the use of these are known to be associated with increased risk for development of systemic infections.\(^8\) The overall proportion of non-*C. albicans* species isolated from the blood that produced biofilms were significantly higher than that of non-*C. albicans* isolates obtained from other sites.\(^9\) The next step involves interconversion of yeast form to a pseudohyphal or hyphal form, an event referred to as morphogenesis.\(^5\)

The production of specific hydrolytic enzymes such as Secreted Aspartyl Proteinases (SAP) and phospholipases facilitate tissue penetration and invasiveness.\(^8\) The aspartyl proteinase activity is seen with *C. albicans*, *C. tropicalis* and *C. parapsilosis* and *C. glabrata* to some extent. There are at least 10 secreted aspartyl proteinases isoenzymes, which are encoded by the SAP gene family. The SAP proteins can hydrolyze structural host proteins, proteins of host immune systems, cascade systems, proteinase inhibitors or regulatory polypeptides to support colonization, persistence, and invasion. Enhanced SAP production is correlated with the over production of a gene encoding a MDR efflux pump occurring in isolates that are exposed to subinhibitory concentrations of fluconazole.\(^6\)

*C. albicans* undergoes phenotypic switching which helps to adapt to different changing conditions in the host’s defenses. The switching process regulates expression of a number of phenotypic characteristics implicated in pathogenicity like antigenicity, sensitivity to neutrophils and oxidants, adhesion, susceptibility to common antifungals and bud to hyphal transition.\(^7\)
Pathogenesis:

As Candida is regularly present on mucosal surfaces, disease implies a change in the organism, the host, or both. The change from the yeast to the hyphal form is strongly associated with enhanced pathogenic potential of C. albicans. In histologic preparations, hyphae are seen only when Candida starts to invade, either superficially or in deep tissues. Morphologic changes occur with a number of factors associated with tissue adherence and digestion. C. albicans hyphae have the capacity to form strong attachments to human epithelial cells through a surface hyphal wall protein (Hwp1), which is found only on the surface of germ tubes and hyphae. This novel pathogenic strategy makes use of host enzymes to bind the pathogen to epithelial cells. Other mannoproteins that have similarities to vertebrate integrins may also mediate binding to components of the extracellular matrix (ECM), such as fibronectin, collagen, and laminin. Hyphae also secrete proteinases and phospholipases that are able to digest epithelial cells and probably facilitate invasion. In C. albicans there is evidence that it induces its own phagocytosis by endothelial cells and has protein surface receptors that bind the C3 component of complement in a manner similar to that of the receptors on neutrophils. C3 bound to the candidal surface by these receptors makes it unavailable for opsonization. Enhanced production of these receptors under various conditions, for example, elevated glucose concentration, is associated with resistance to phagocytosis by neutrophils. Antibacterial therapy and immunocompromised capacity of the host allow Candida to increase its relative proportion of the flora and are often associated with local and invasive infection. The disruptions of the mucosa associated with chronic disease and their treatments may enhance the invasion process by exposing Candida binding sites in the ECM.\textsuperscript{58,91}
Immunity:

Both humoral immunity and cell-mediated immunity are important in defense against *Candida* infections. Neutrophils are the primary first-line defense. *Candida* are readily phagocytosed and killed when opsonized by antibody and complement. In the absence of specific antibody, the process is less efficient, but a naturally occurring antimannan IgG is able to activate the classical complement pathway and facilitate the alternate pathway. Hyphal forms may be too large to be ingested by polymorphonuclear neutrophils (PMNs), but they can still kill the fungi by attaching to the hyphae and discharging metabolites generated by the oxidative metabolic burst. A deficit in neutrophils or neutrophilic function is the most common correlate of serious *C. albicans* infection. The increased frequency of oral and vaginal candidiasis in AIDS patients suggests that superficial infections involve lymphocyte–mediated immune responses (cell-mediated immunity [CMI]). A possible explanation for the association between AIDS and *Candida* infection is the upregulation of CD4 receptors on monocytes by *Candida* products. As with other fungi, cytokine activation of macrophages enhances their ability to kill *C. albicans*. A favorable outcome appears to require the proper balance between TH1- and TH2-mediated cytokine responses. The cytokines associated with TH1 (interleukin-2 [IL-2], IL-12, interferon, tumor necrosis factor) are correlated with enhanced resistance against infection where TH2 responses (IL-4, IL-6, and IL-10) are associated with chronic disease.\(^{58}\)

The macroscopic and microscopic appearance of lesions caused by *Candida* species is primarily influenced by the interaction of three factors: \(^ {81}\)

1. The site of infection
2. The pathogenicity of the infecting microorganisms to a lesser extent its species
3. The competence of the host’s immune system.
In the most serious form of *Candida* infection, the organisms disseminate hematogenously and form microabscesses and small macroabscesses in major organs. HIV-infected individuals with low CD4+ T cell counts and diabetic patients eventually develop into the disseminated form. Innate immunity is the most important defense mechanism against hematogenously disseminated candidiasis, and the neutrophil is the most important component of the defense.

The clinical manifestations of disease are extremely varied depending on the organ involved (Table 10) ranging from acute, subacute and chronic to episodic. It may be superficial manifestations and mucosal surfaces to deep-seated infections involving various internal organs and to disseminated disease. Involvement may be localized to mouth, throat, skin, scalp, vagina, fingers, nails, bronchi, lungs or the gastrointestinal tract, or become systemic as in septicemia, endocarditis and meningitis. *Candida* species can cause a range of clinical manifestations, most frequently involved is mucosal surfaces i.e. oral and vaginal.\(^{81}\)
Table 10: Clinical manifestations are classified depending on the primary organ system involved.91

<table>
<thead>
<tr>
<th>I. Infectious Diseases</th>
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<tbody>
<tr>
<td>A. Mucocutaneous involvement</td>
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<tr>
<td>1. Oral: thrush, glossitis, stomatitis, cheilitis, perleche</td>
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<td>2. Alimentary: esophagitis, gastritis, peritonitis, enteric and perianal disease</td>
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<tr>
<td>3. Bronchial and Pulmonary</td>
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<td>4. Vulvovaginitis, balanitis and balanoposthitis</td>
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<td>5. Chronic mucocutaneous candidiasis</td>
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<td>B. Cutaneous involvement</td>
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<tr>
<td>1. Intertriginous and generalized candidiasis</td>
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<td>2. Paronychia and onychomycosis</td>
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<tr>
<td>3. Diaper (napkin) candidiasis</td>
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<tr>
<td>4. Candidal granuloma</td>
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<td>C. Systemic involvement</td>
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<tr>
<td>1. Urinary tract</td>
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<td>2. Endocarditis</td>
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<td>3. Meningitis</td>
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<td>4. Septicemia</td>
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<tr>
<td>5. Iatrogenic candidemia (barrier break Candidaemia)</td>
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<tr>
<td>6. Dissemination to other organ systems.</td>
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<td>II. Allergic Diseases</td>
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<tr>
<td>a. Candidids</td>
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<td>b. Eczema</td>
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<td>c. Asthma</td>
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<td>d. Gastritis</td>
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</table>

In superficial invasion of the mucous membranes, *Candida* produces a white, cheesy plaque that is loosely adherent to the mucosal surface. The lesion is usually painless, unless the plaque is torn away and the raw, weeping, invaded surface is exposed. Oral lesions, called thrush, occur on the tongue, palate, and other mucosal
surfaces as single or multiple, ragged white patches. Symptoms include burning pain, altered taste sensation and difficulty with swallowing contribute to poor intake and weight loss. However, most patients are asymptomatic. Oral thrush is of 4 forms- Pseudomembranous, erythematous, angular cheilitis and hyperkeratosis (Candidal leukoplakia).\(^9\)

Esophageal candidiasis is often an extension of lesions from the oral cavity. It involves the esophagus and intestine. The clinical symptoms most often observed are dysphagia with retrosternal pain, gastrointestinal bleeding, nausea and vomiting. The frequency of this syndrome increased since the appearance of AIDS. As per estimate 20-30% of AIDS patients with OC also suffer from *Candida* esophagitis. Endoscopy should be performed for a definite diagnosis.

A similar infection in the vagina is vaginal candidiasis, produces a thick, curd-like discharge and itching of the vulva. Patients may be asymptomatic or may complain perianal pruritus or dysuria, vaginal soreness and vulvar burning.\(^9\)

**Laboratory Diagnosis:**\(^8\)

Lab diagnosis of candidiasis depends upon the nature of the different clinical entities, specifically whether they are mucocutaneous or deep seated. Diagnosis generally includes two steps:

1. Direct examination of pathological specimen to demonstrate fungal presence (KOH mount and gram staining)
2. Isolation of the fungus in culture and its identification (morphology & biochemical).
**KOH mount:**

KOH preparation: 10 gms of potassium hydroxide is mixed with 10 ml of glycerol and 80 ml of distilled water.

Slide KOH procedure: Specimen is spread over the slide. A drop of 10% KOH is added and the coverslip is placed on it. The slide is gently heated for few seconds and observed under the microscope.

**Gram staining:** Originally devised by Sir Hans Christian Gram (1884). The technique includes four steps:

- Primary staining done by crystal violet/gentian violet.
- Diluted with iodine mordant.
- Decolorized with organic solvents such as acetone/ethanol.
- Counterstained with carbol fuchsin/ safranine/ neutral red.
- Washed, dried and observed under the microscope.

**Germ Tube Test:** It is the presumptive identification of *Candida albicans* isolates from clinical samples and has been successfully used from many years. This is also known as Reynolds-Braude phenomenon or hand mirror forms.

**Growth on SDA:** Designed by the French dermatologist Raymond Sabouraud. For isolation from samples with bacterial contamination SDA with antibiotics is preferred. In this study SDA with Chloramphenicol was used to culture *Candida*.

**Morphology on Cornmeal agar (CMA):** It is a nutritionally deficient medium which suppress the vegetative growth of *Candida*. Pollack and Benham reported the use of CMA to study the morphology of *Candida*. Walker and Huppert modified the base formulation of CMA by adding polysorbate 80, which stimulated rapid, and abundant
chlamydospore formation and better morphology of yeasts. Kelly and Funigello reported that the addition of 1% Tween 80 enhances chlamydospore formation.\textsuperscript{99}

Factors enhancing chlamydospore production – slightly alkaline pH, slight anaerobic conditions, absence of inorganic ions, use of solid medium, absence of condensation on the medium surface, decrease surface tension, addition of Tween 80 (Polysorbate 80).\textsuperscript{100}

Factors inhibiting chlamydospore production: Heavy inoculum, pH 3.3-4.5.\textsuperscript{101}

\textbf{Biochemical tests:}

\textbf{Carbohydrate fermentation:} Yeast metabolizes carbohydrates via Embden Meyerhoff pathway in anaerobic conditions with production of acid (indicated by change in color through indicator) and carbon dioxide (detection by gas collected in Durham’s tube) as end products.

\textbf{Sugar assimilation:} This was first introduced in 1948 by Wickerham LJ, Burton RA. In classic Wickerham method, it was done using set of defined liquid media supplemented with different carbohydrates. This was precise but laborious and time consuming.\textsuperscript{102} In 1995, Auxanographic technique was introduced, in which paper discs impregnated with different carbohydrates were placed on agar plates. The ability of yeast to grow around the disc indicates the carbohydrate to be assimilated.\textsuperscript{103}

\textbf{CHROMagar Candida:} It is the presumptive identification of yeast-like organisms based on colony morphology. It is a product that utilizes enzymatic reactions to differentiate \textit{C.albicans} and several other yeast species. \textit{C.albicans} produce β-galactose aminidase and L-proline aminopeptidase but other species may produce one enzyme but not both.
Other diagnostic methods like serology i.e. by immunodiagnosis - detection of antigens/antibodies; candidal antigen can be detected by counter-immunoelectrophoresis, haemagglutination inhibition, latex agglutination, ELISA, immunoblotting, dot-immunoassay and liposomal immune assay and RIA. The antigens detected include Mannan Mannoproteins, non-characterized Cand-Tec antigens HSP 90, enolase and other immune dominant cytoplasmic antigens.\textsuperscript{104}

Other non-culture methods include detection of non-antigenic fungal metabolites released during infections into patient’s body fluids. E.g. Arabinitol released by most of the pathogenic Candida species, (except \textit{C.krusei} and \textit{C.glabrata}) is detected by Gas liquid chromatography and enzymatic fluorometric measurements. Cell wall component (1, 3-\(\beta\)-D glucan) metabolite is detected by commercial kits.\textsuperscript{81} Commercially available identification system such as - API 20c (Auxanogram) strip, API 32c, Vitek yeast biochemical card, Minitek, Uni-yeast-Tel plate, Candifast, Candidachek kit, Mycotube, Auxodisc, Microscan yeast identification panel- Enzyme based systems and Fungichrome, Rapid ID yeast plus, Iatron \textit{Candida} check. Among the commercially available kits API 20C, Abbott Quantum and Vitek yeast identification system are power tools and have gained wide acceptance.

Molecular technique based detection of \textit{Candida} DNA probe have become more promising as a possible diagnostic tool with the development of DNA amplification technique such as polymerase chain reaction (PCR). Possible probes such as the actin gene, the gene encoding cytochrome P-450, 14-lanosterol demethylase, the chitin synthetase gene, SAP genes, mitochondrial DNA or the \textit{Candida} DNA Repetitive Elements (CARE). Fluorescent insitu hybridization using fluorescent-labelled DNA probes to detect \textit{Candida} cells is a non-PCR based molecular method.\textsuperscript{105,106}
In spite of using a sole method for identification to reach an unequivocal identification traditionally used yeast morphology on corn meal agar and physiological tests were carried in this study in conjunctions with Vitek 2 automated system to characterize *Candida* species.

**VITEK® 2 identification System (BioMerieux):**

The VITEK 2 is an automated microbiology system utilizing growth-based technology. The system accommodates the colorimetric reagent cards that are incubated and interpreted automatically.

**Reagent Cards:** The reagent cards have 64 wells that can each contain an individual test substrate. An optically clear film present on both sides of the card allows for the appropriate level of oxygen transmission while maintaining a sealed vessel that prevents contact with the organism-substrate admixtures. Each card has a pre-inserted transfer tube used for inoculation. Cards have barcodes that contain information on product type, lot number, expiration date, and a unique identifier that can be linked to the sample either before or after loading the card onto the system.

**Suspension Preparation:** A sterile swab or applicator stick is used to transfer a sufficient number of colonies of a pure culture and to suspend the microorganism in 3.0 mL of sterile saline (aqueous 0.45% to 0.50% NaCl, pH 4.5 to 7.0) in a 12 x 75 mm clear plastic (polystyrene) test tube. The turbidity is adjusted to 1.8-2.20 Mac Farland, accordingly and measured using a turbidity meter called the DensiChekTM.

**Inoculation:** Identification cards are inoculated with microorganism suspensions using an integrated vacuum apparatus. A test tube containing the microorganism suspension is placed into a special rack (cassette) and the identification card is placed in the
neighboring slot while inserting the transfer tube into the corresponding suspension tube. The cassette can accommodate up to 15 tests. The filled cassette is transported automatically into a vacuum chamber station. After the vacuum is applied and air is re-introduced into the station, the organism suspension is forced through the transfer tube into micro-channels that fill all the test wells.

**Card Sealing and Incubation:** Inoculated cards are passed by a mechanism, which cuts off the transfer tube and seals the card prior to loading into the carousel incubator. The carousel incubator can accommodate up to 30 or up to 60 cards. All card types are incubated on-line at 35.5 ± 1.0°C. Each card is removed from the carousel incubator once every 15 min, transported to the optical system for reaction readings, and then returned to the incubator until the next read time. Data are collected at 15-min intervals during the entire incubation period.

**Optical System:** A transmittance optical system allows interpretation of test reactions using different wavelengths in the visible spectrum. During incubation, each test reaction is read every 15 min to measure either turbidity or colored products of substrate metabolism. In addition, a special algorithm is used to eliminate false readings due to small bubbles that may be present.

**Test Reactions:** Calculations are performed on raw data and compared to thresholds to determine reactions for each test.

**Database Development:** The databases of the VITEK 2 identification products are constructed with large strain sets of well-characterized microorganisms tested under various culture conditions. These strains are derived from a variety of clinical and industrial sources as well as from public (e.g., ATCC) and university culture collections.
Analytical Techniques

Test data from an unknown organism are compared to the respective database to determine a quantitative value for proximity to each of the database taxa. Each of the composite values is compared to the others to determine if the data are sufficiently unique or close to one or more of the other database taxa. If a unique identification pattern is not recognized, a list of possible organisms is given, or the strain is determined to be outside the scope of the database.

YST Card: The YST card is used for the automated identification of 49 taxa of the most significant yeasts and yeast-like organisms. Of the 49 taxa, three are grouped taxa in either slashline (2) or genus (1) designations. When representative species of these collective designations are included, the total number of taxa claimed by YST is 54. As with low discrimination identifications, grouped taxa can be separated into their component taxa by supplemental testing and/or observation. The YST identification card is based on established biochemical methods and newly developed substrates (Atlas 1993, Barnett et al. 2000, Kreger-van Rij 1984, Larone 1995, Lodder 1971, McGinnis 1980, Murray et al. 1999). There are 46 biochemical tests measuring carbon source utilization, enzymatic activities and resistance.

This system delivers fast, accurate microbial identification and antibiotic susceptibility testing for a wide range of organisms from environmental sources or final products. The automated platform provides rapid results with increased operation safety while eliminating repetitive manual operations. Unique technology features user-friendly intuitive software, on-line result validation software, innovative test card format, and a comprehensive identification database. It is ideal for bacteria and yeast identification and monitoring, antibiotic susceptibility testing and reporting, and quality assurance testing.
The test card is automatically filled, sealed, read, and disposed of in a final waste container.\textsuperscript{107}

**Antifungal susceptibility:**

Marked increase in the incidence of candidiasis, increasing number of drugs and an innate or acquired resistance of certain yeast species to several drugs has insisted for AST.

The most commonly used antifungal agents are azoles (fluconazole, itraconazole, and ketoconazole) and polyenes (amphotericin B). Some *Candida* species have intrinsic resistance and some develop resistance to azoles. The widespread use of fluconazole and itraconazole as therapeutic or prophylactic doses has increased recently and is most often associated with the HIV infected with oropharyngeal candidiasis. This has led to the increase reports of resistance.\textsuperscript{81}

The efficacy of antifungal therapy is dependent on several factors:

a. Drug factors such as potency of the drug, tissue penetration and distribution within the body.

b. Organism factors such as virulence, susceptibility to a given drug and development of resistance.

c. Host factors such as the underlying immune status including the alteration of the normal mucosal flora, neutropenia and humoral as well as cell-mediated immunity.

**Classification of Antifungal drugs:**

Ergosterol in the cytoplasmic membrane is the most important site of action of many antifungal drugs. There are four classes of antifungals polyenes, azoles,
allylamines and morpholines and rest of the antifungals act at other sites of fungal cell. Based on the source, the antifungal agents are divided in two broad groups and others are clubbed in a miscellaneous group as shown in the table 11:

A. Antifungal Antibiotics

B. Synthetic Antifungals

C. Miscellaneous Antifungals

They are further classified as topical or systemic antifungal agents on the basis of route of administration.\(^{108}\)

**Table 11: Classification of Antifungal Drugs**

A. Antifungal Antibiotics

1. Polyene Antibiotics
   - **Amphotericin B**
     - (i) Conventional amphotericin B
       - Amphotericin B deoxycholate
     - (ii) Liposomal Formulations of AmB
       - Amphotericin B lipid complex
       - Amphotericin B colloidal dispersion
       - Liposomal-encapsulated AmB

   - Nystatin
   - Pimaricin
   - Hamycin

2. Other Antibiotics
   - Griseofulvin
   - Pradimicin
B. Synthetic Antifungal Agents

1. Thiocarbamates
   Tolnaftate

2. Allylamines and Benzylamines
   Naftifine
   Terbinafine
   Butenafine

3. Azoles
   (i) Imidazoles
   Bifonazole   Butoconazole
   Clotrimazole   Econazole
   Fenticonazole   Ketoconazole
   Miconazole   Omoconazole
   Oxiconazole   Sulconazole
   (ii) Triazoles
   Fluconazole   Itraconazole
   Voriconazole   Terconazole
   Posaconazole   Ravuconazole

C. Miscellaneous Antifungal Agents
   Flucytosine
   Ciclopiroxolamine
   Amorolfine
   Whitfield’s ointment
   Potassium iodide
   Selenium sulfide
Some of the commonly used antifungal agents are described below.\textsuperscript{108}

**Amphotericin B:**

It is a broad spectrum antibiotic, which is obtained from *Streptomyces nodosus*. The drug was discovered in 1957 and has greater affinity for ergosterol than cholesterol. It is available in four different parental formulations, including amphotericin B deoxycholate and three lipid formulations. The active compound is identical but the pharmacokinetics and toxicity profiles vary from formulation to formulation.\textsuperscript{108}

**Mode of action:** The pores formed by amphotericin B (AmB) increase permeability so that the essential molecules of cell leak from the cytoplasm and the fungal growth is blocked.

It is insoluble in water and available for infusions by complexing with bile salt deoxycholate and unstable at 37\degrees C and potentially an effective fungicidal drug. It is
active against most of the fungal pathogens of both yeast-like and mycelial types and intravenous infusions remains the gold standard of therapy for the invasive *Candida* diseases.\(^{108}\)

**Azoles:** The azole derivatives are the most frequently used antifungal agents in clinical practice. These have basic 5-member azole ring attached by carbon nitrogen bonds to other aromatic rings.

**Mode of action:** These are fungistatic drugs and inhibit cytochrome P-450 dependent C\(_{14}\) demethylation (sterol 14-\(\alpha\)-demethylase enzyme) in the biosynthesis of ergosterol in the fungal cell membrane. This results in impaired ergosterol biosynthesis, accumulation of 14-methylated sterols - a defective cell membrane and consequently fungal cell death. They also interact with mammalian P-450 enzyme systems that are responsible for synthesis of “endobiotics” and result in side effects.\(^{108}\)

**Ketoconazole:** This was first successful orally used azole agent (1980). The oral availability is critically dependent upon gastric pH. The side effects are anorexia, nausea, constipation, headache, hepatitis, pruritus, exanthema and inhibition of steroid hormone synthesis. It is a potent inhibitor of adrenal androgen biosynthesis.\(^{108}\)

![Ketoconazole structure](image)

**Fluconazole:** This triazole was described in 1990 and has water solubility, oral absorption, extensive bio-availability independent of food or gastric pH and a sufficiently long half-life to allow once-a-day administration. It penetrates readily into
CSF and is excreted unchanged in urine and feces, being metabolically stable with recovery of over most of the administered dose.

The drug is effective in treating infections caused by *C. albicans*, *C. tropicalis* and *C. parapsilosis* but ineffective for treating *C. krusei* which is naturally resistant. The drug is fungistatic when given in lower doses and fungicidal when given in higher doses.

Side effects are nausea, headache, skin rash, vomiting, abdominal pain and diarrhea. Some *Candida* species show fluconazole resistance. 108

**Itraconazole:** This is found to be superior to other azoles and was first synthesized in 1980 and finally introduced for clinical use in 1993. It has better distribution in tissues except CSF due to blood brain barrier and its expanded half-life is 15-24 hrs. It is a lipophilic compound characterized by good absorption after meals. The drug is highly bound to plasma protein especially albumin and is degraded into several inactive metabolites and excreted primarily in bile and urine. This broad spectrum antifungal agent cause side effects like headache, nausea and raised liver enzymes.
**Voriconazole:** It is a novel triazole with potent broad-spectrum activity against some NAC which are inherently resistant to fluconazole. This is more effective than fluconazole in blocking *Candida* sterol biosynthesis. In addition to inhibition of sterol 14-α-demethylase enzyme in the fungal cell biosynthesis, it is inhibitory to methylene dihydro-lanosterol de-methylation in some of the yeasts.\(^{108}\)

![Voriconazole chemical structure](image)

**Flucytosine (5- Fluorocytosine):** It is one of the commonly used miscellaneous antifungal agents. It is a synthetic fluoropyrimidine which is water-soluble drug and can be administered orally. It is converted by fungal cytosine deaminase to antimetabolite, 5-flourouracil which inhibits thymidylate synthetase and consequently DNA synthesis.

![Flucytosine chemical structure](image)

It can also serve as a companion drug to amphotericin B with synergistic effects against most of the *Candida* isolates. Primary and secondary resistance against flucytosine is frequently observed. Therefore, amphotericin B in combination with flucytosine prevents the development of secondary drug resistance during the course of therapy. The side effects include gastrointestinal disturbances, neutropenia, thrombocytopenia, bone marrow depression and alopecia.\(^{108}\)
Echinocandin (Caspofungin): These are potential fungicidal agents and are the first inhibitor of β-(1,3)-D-glucan synthesis to be approved by the United States Food and Drug Administration.

These drugs prevent cell wall synthesis by blocking and results in osmotic stress, lysis and death of the organism. This drug when combined with azole or polyene antifungal agents they indicate an absence of antagonism. It is a water-soluble amphipathic lipopeptide with a molecular mass of 1213 kDa that is a semisynthetic derivative of pneumocandin B0, a fermentation product of *Glarea lozoyensis*.108,109

Caspofungin has an excellent safety profile. Alvaro et al and Arathoon et al (2002) in their studies demonstrated that drug-related adverse effects occurred significantly less frequently among the caspofungin recipients and it is as efficacious and tolerated as that of fluconazole.110,111 Eduardo G Arathoon et al concluded that in HIV patients any dose of caspofungin experienced drug-related adverse events compared to patients given doses of conventional amphotericin B.111

Some fungi do not respond to the antifungal therapy in many diseases due to the development of drug resistance. The antifungal resistance can be divided into two broad categories:

(a) Clinical Resistance

(b) In vitro Resistance
(a) **Clinical Resistance:** This indicates lack of a clinical response to the antifungal drugs used in the particular disease. Clinical failure is more often due to low levels of the drug in serum and/or tissue for several reasons, mainly non-compliance with medication regimen. A significant reason for clinical failure/resistance in AIDS patients is the presence of a severely immunosuppressive state, where the antifungal agents alone, including high-dose fungicidal agents, are unable to eradicate the fungi.

(b) In **vitro resistance:** This can be divided into primary (intrinsic/innate) and secondary (acquired) resistance. Primary resistance occurs when the organism is naturally resistant to the antifungal agent e.g. *C.krusei* to fluconazole. Secondary resistance is when the isolate producing infection becomes resistant to the antifungal agent during the course of treatment. This was rare in past but now most frequently reported in AIDS patients who suffer from recurrent azole - resistant oropharyngeal or esophageal candidiasis.\(^\text{108}\)

Antifungal Susceptibility testing is done by:

- Disc Diffusion method
- Agar Dilution method
- Broth Dilution method involves
  - Broth macro-dilution method
  - Broth micro- dilution method
- E-Test, commercial method for determination of MIC

**Disc Diffusion Method:** It is an easy, quick and cost effective method that could be used to various classes of antifungal agents. The M44-A, Clinical and Laboratory Standards Institute (CLSI) method has provided disc diffusion susceptibility testing of yeasts with an established methodology for *Candida* species that includes the zone
interpretative criteria. It recommends the use of Mueller-Hinton agar supplemented with 2% glucose and 0.5µg/ml methylene blue dye which enhances the zone edge recognition.\textsuperscript{112,113} Fresh colonies of yeast suspension adjusted to 0.5 McFarland Standard is inoculated onto the surface of agar plate with swab. Antifungal discs are applied to the surface and incubated at 35\textdegree{}C for 24 to 48 hours. Calipers are used to measure the diameter of zone of inhibition.\textsuperscript{114,115}

**Agar Dilution Method:** In test tubes, serial two fold dilutions of antifungal drugs are prepared in YNB Agar medium. The antifungal solution is mixed well in 1:10 dilution with 2% difco agar and poured in 90 mm diameter petri dish. Each plate is spot inoculated with 20µg of prepared suspension of each isolate. Results read after incubation of the plates at 37\textdegree{}C for 48 hours\textsuperscript{116}.


**Macro Broth Dilution Method:** Susceptibility testing is performed in RPMI 1640 medium, with L-glutamine and without Sodium bicarbonate, and buffered at pH 7.0 with 0.165 mol/L morpholine propane sulfonic acid (MOPS). Drug dilutions are prepared at 10 times the strength of the final drug concentration with consecutive drug dilution schemes for minimizing systematic pipetting errors. Yeast suspension is adjusted to 0.5 McFarland Standard, and this working suspension is made by a 1:100 dilution followed by 1:20 dilution in RPMI 1640 broth medium in sufficient volume to directly inoculate each tube with 0.9ml of inocula\textsuperscript{117} to 10x drug dilutions, bringing the drug dilutions to the final test concentrations. Drug free tubes with the inocula, without inocula and drug are used as controls for each isolate. All tubes are incubated without agitation at 35\textdegree{}C and read after 48 hours.
**Micro broth dilution method:** Broth micro dilution testing is performed on sterile, flat bottomed 96 - well microtitre plates. The working suspension of the inoculum is made by a 1:50 dilution followed by a 1:20 dilution with RPMI 1640 to obtain a 2x final suspension. 100µl of this suspension is added to each well, resulting in the final drug concentration. The plates are then incubated at 35°C and read after 24 - 48 hrs. The micro dilution wells are scored with the aid of a reading mirror. The growth in each well is compared with that of the control (drug free) well. A numerical score is given to each well by using the following scale: 0-optically clear, 1-slightly hazy, 2-prominent decrease in turbidity (~50% inhibition), 3-slight reduction in turbidity and 4- no reduction in turbidity.

**Epsilometer-Test:** On RPMI-Glucose agar E-strip is placed onto the surface of the inoculated agar plates and zone diameter is measured after incubation.

A novel microtitre assay for AST was developed and called as Rapid Susceptibility Assay (RSA). This method has many advantages over CLSI-M-27A assay of CLSI. This gives the MIC results within 6 to 8 hours, a graphical display of data, and the availability of objective quantitative end points. RSA is based on substrate utilization by fungi in the presence of antifungal drugs. Substrate uptake is determined by a colorimetric method, which can be scored by analysis of data obtained from a microtitre plate reader.

Peterson JF et al reports (2011) Vitek 2 system as an excellent quantitative and qualitative agreement with reference to broth dilution method. Estrella M C et al in his comparative study of CLSI and European Committee on Antimicrobial Susceptibility Testing (EUCAST) revealed Vitek 2 to be a more rapid, easier and an alternative technique to determine AST of yeast species compared to other two. Also
it is reliable for identification of azole such as voriconazole, amphotericin B, and 5-flucytosine resistance in vitro.\textsuperscript{120,121}

Opportunistic Infections and co-infections are the major cause of deaths amongst HIV infected individuals and this mostly depends upon the risk factors, type of exposure and geographic region. The commonest types of infections reported are TB, chronic diarrhea, OC, herpes simplex virus-2, cytomegalovirus, hepatitis B virus and hepatitis C virus. Due to the scarcity of OIs data available from this region, we designed a study to determine the frequency of TB and candidiasis - the most common OIs amongst HIV seropositive patients and their DST susceptibility pattern in Mysore, Karnataka, Southern India.