PREVALENCE OF BACTERIAL AND FUNGAL AGENTS CAUSING LOWER RESPIRATORY TRACT INFECTIONS IN PATIENTS WITH HUMAN IMMUNODEFICIENCY VIRUS (HIV) INFECTION

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BY

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Recommendation

This is to certify that Ms Sanchita Dahal has completed this dissertation work entitled **"PREVELENCE OF BACTERIAL AND FUNGAL AGENTS CAUSING LOWER RESPIRATORY TRACT INFECTIONS IN PATIENTS WITH HUMAN IMMUNODEFICIENCY VIRUS INFECTION"** as a partial fulfillment of Master of science in Microbiology under our supervision. To our knowledge, this work has not been submitted to any other Universities/ Institutes for the same degree.

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ABSTRACT

Present study was carried out to study the prevalence of bacterial and fungal agents causing lower respiratory tract infection in HIV infected individuals. LRTI is a common problem among HIV positive patients and majority are due to bacterial infections. A total of 120 HIV infected individuals were included in this study. Gram stain, Ziehl-Neelsen stain and sputum culture were performed. The pattern of microbial distribution on direct microscopic examination was: Gram-positive bacteria-19, Gram-negative bacteria-37, AFB-6 and fungal isolates-36. Sixty-five percent samples showed growth of different bacteria in different culture media. Among them *K. pneumoniae* (28.6%) was most frequently isolated bacterial pathogen followed by *E. coli* (17.9%), *S. pneumoniae* (17.9%), *S. aureus* (16.1%), AFB (5.0%), *P. aeruginosa* (8.9%), *M. catarrhalis* (7.0%) and *H. influenzae* (3.6%). Among fungal pathogens, most frequently isolated was *Candida albicans* (44.4%), *Aspergillus* spp (30.6%) and *Penicillium* spp (8.3%). Antibiotic susceptibility pattern was examined for bacterial isolates. Among Gram negative bacteria, *K. pneumoniae* was most sensitive towards chloramphenicol (62.5%) whereas least sensitive towards co-trimoxazole (37.5%). *E. coli* showed 60% sensitivity towards chloramphenicol and gentamicin whereas 20% sensitivity towards ampicillin. *P. aeruginosa* was most sensitive towards gentamicin (60%) and least sensitive towards chloramphenicol. *M. catarrhalis* showed 50% sensitivity towards chloramphenicol, co-trimoxazole and gentamicin whereas 25% sensitivity towards ampicillin and tetracycline. *H. influenzae* showed 50% sensitivity towards chloramphenicol, ampicillin, gentamicin and tetracycline whereas least sensitive towards co-trimoxazole. *S. pneumoniae* showed 50% sensitivity towards chloramphenicol, gentamicin and tetracycline whereas 20% sensitivity towards ampicillin. *S. aureus* showed 55.5% sensitivity towards chloramphenicol, gentamicin,and tetracycline whereas 22.2% sensitivity towards ampicillin. Polymicrobial isolation was also observed. *Pneumocystis carinii* pneumonia was not documented in our stu

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ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
ARV	Antiretroviral Therapy
CDC	Centers for Disease Control
CMV	Cytomegalovirus
DNA	Deoxy ribonucleic Acid
DOTS	Directly Observed Treatment, Short Course
ELISA	Enzyme Linked Immunosorbent Assay
FDC	Follicularb Dendritic Cells
HAART	Highly Active Anti Retroviral Therapy
HIV	Human Immunodeficiency Virus
MAC	Mycobacterium avium Complex
MRSA	Methicillin Resistant *S. aureus*
NCASC	National Center for AIDS and SID Control
PCP	*Pneumocystis carinii* pneumonia
PCR	Polymerase chain reaction
RNA	Ribonucleic Acid
SAARC	South Asian Association for Regional Cooperation
STD	Sexually Transmitted Disease
TB	Tuberculosis
UN	United Nation
WHO	World Health Organization

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CHAPTER-I

1. INTRODUCTION

Human immunodeficiency virus (commonly known as HIV, and formerly known as HTLV-III and lymphadenopathy-associated virus is a retrovirus that is the cause of the disease known as AIDS (Acquired Immunodeficiency Syndrome), a syndrome where the immune system begins to fail, leading to many life-threatening opportunistic infections (Levy, 1993).

HIV primarily infects vital components of the human immune system such as CD4+ T cells, macrophages and dendritic cells (Knight *et al*, 1990). It also directly and indirectly destroys CD4+ T cells. As CD4+ T cells are required for the proper functioning of the immune system, when enough CD4+ T cells have been destroyed by HIV, the immune system functions poorly, leading to AIDS. HIV also directly attacks organs such as the kidneys, heart and brain, leading to acute renal failure, cardiomyopathy, dementia and encephalopathy. Many of the problems faced by people infected with HIV result from failure of the immune system to protect from opportunistic infections and cancers.

HIV is transmitted through direct contact of mucous membrane with body fluid containing HIV, such as blood, semen, vaginal fluid, preseminal fluid or breast milk. This transmission can come in the form of: penetrative (anal or vaginal) sex; oral sex; blood transfusion; contaminated needles; exchange between mother and infant during pregnancy, childbirth, or breastfeeding; or other exposure to one of the above body fluids.

Infection in humans is now pandemic. As of January 2006, the Joint United Nations Programme on HIV/AIDS (UNAIDS) and the World Health Organization (WHO) estimate that AIDS has killed more than 25 million people since it was first recognized on December 1, 1981, making it one of the most destructive pandemics in recorded history. In 2005 alone, AIDS claimed an estimated 2.4—3.3 million lives, of which

more than 570,000 were children (UNAIDS, 2005). A third of these deaths are occurring in sub-Saharan Africa, retarding economic growth by destroying human capital (Greener, 2002). Current estimates state that HIV is set to infect 90 million people in Africa, resulting in a minimum estimate of 18 million orphans (UNAIDS, 2006). As of June 30, 2006, 6990 HIV seropositive cases have been detected, with 1085 cases of AIDS in Nepal (NCASC, 2006). Antiretroviral treatment reduces both the mortality and the morbidity of HIV infection, but routine access to antiretroviral medication is not available in all countries (Palella *et al*, 1998).

The respiratory and gastrointestinal tracts are the two major connections between the interior of the body and the outside environment. The respiratory system can be divided into upper and lower tracts. The respiratory tract is the pathway through which the body acquires fresh oxygen and removes unneeded carbon dioxide. The upper respiratory tract includes the epiglottis and surrounding tissues, larynx, nasal cavity, and the pharynx. The lower respiratory tract comprises the structures including the trachea, bronchi, and bronchioles (Forbes *et al*, 2002).

Patients with HIV infection frequently present with a wide spectrum of pulmonary complications from opportunistic infections and neoplasm that may be associated a high mortality rate. Disease of respiratory tract accounts for half of deaths from AIDS. Bacterial pneumonias and AIDS can lead to significant morbidity and mortality and are second to *Pneumocystis carinii* pneumonia (PCP) as an immediate cause of death (Orenstein *et al*, 1985). Among the most frequent and severe opportunistic infections in patients with AIDS is PCP. Over half of all AIDS patient will have at least one episode of PCP at some point during their clinical course, with mortality from a single episode ranging from 10-30% (Klatt, 2004).

Overall bacterial organism accounts for more pulmonary infection than other infectious agent in person with AIDS (Mann *et al*, 1997). The defect in T-cells as well as B-cell mediated immunity in HIV infection result in pneumonia caused by large group of bacterial organisms, both Gram-positive and Gram-negative. The risk of HIV infected person is the highest when CD4 count is less than 200/mm^3. Among risk group injection

drug user were most likely to develop bacterial pneumonia (Salomon *et al*, 1997). When microbiological culture are performed the most common etiologic agent for bacterial pneumonias are *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*, *Haemophilus influenzae*, *Klebsiella pneumoniae* and enteric gram-negative organism. Bacterial bronchopneumonia may be present with opportunistic infections (Wallace and Hannah, 1987).

In addition to above-mentioned common bacterial pathogens, many other organisms can cause respiratory tract infections, including *Nocardia* spp, *Rhodococus equi*, and *Legionella* spp (Forbes *et al*, 2002).

S. pneumoniae is the commonest cause of community-acquired pneumonia with the second most common being *H. influenzae* (Janoff *et al*, 1993; Moreno *et al*, 1991). These two organisms account for two-third of community-acquired pneumonia. Community-acquired pneumonia may be recurrent but have low fatality rates. In comparison, nosocomial pneumonia occurs primarily in patient with AIDS and is usually due to *S. aureus*, *P. aeruginosa* and other gram negative bacilli (Hirschtick *et al*, 1995; Polsky *et al*, 1986). Nosocomial infection has high fatality rates. *S. aureus* is an important cause of morbidity and mortality in patients with AIDS and has emerged as a secondary opportunistic in lungs of patients with opportunistic disease.

Resistance of numerous bacterial pathogens to many antibacterial agents continues to increase globally. Frequencies, patterns, and distributions of resistant bacteria vary significantly with geographic regions and often reflect the usage patterns of antibiotics. Factors that increase in resource-poor and resource-rich nations include total antibiotic consumption as well as under use through lack of access, inadequate dosing, poor adherence, and substandard antimicrobial usage (Spector *et al*, 1994). These increases in bacterial resistance create barriers to treatment of severe and recurrent infections in HIV-infected children and adults, especially in resource-poor countries.

Mycobacterium tuberculosis (MTB) occurs commonly in many persons without AIDS but the risk of MTB is substantially higher in persons infected with HIV. In most part of the world, patient with TB were evaluated regarding HIV infection. HIV infection has

dramatically changed the epidemiology and clinical status of TB in the world. The incidence of TB in person with HIV infection is more than 500 times that of general population and patient dually infected with HIV and latent MTB progress to active TB at a rate of 8-10% per year (Havlir and Barnes, 1999; Waxman *et al*, 1995).

The incidence of TB among person infected with HIV is increased with CD4 lymphocytes count less than 200/mm^3. Tuberculosis and HIV has been closely linked since the emergence of AIDS. HIV infection has contributed to produce a progressive decline in cell-mediated immunity. HIV alters the pathogenesis of TB greatly leading to more frequent extra pulmonary involvement and atypical radiographic manifestations.

Although HIV infection continues to climb in developing nation where HIV infection and TB are endemic and resources are limited. Worldwide, tuberculosis is the most common opportunistic infection affecting HIV seropositive individual and it is the most common cause of death in patient with AIDS. The WHO estimates that one-third of the world's population is infected with *M. tuberculosis*, resulting in estimated eight million new cases of TB and nearly two million deaths each year (Small, 1996).

Fungi such as *Candida albicans*, *Cryptococcus neorformans*, *Histoplasma capsulatum*, *Coccidioides immitis*, *Aspergillus* spp, *Blastomyses dermatitidis*, *Penicillium marneffei*, *Sporothrix schenckii* can cause pulmonary infections in persons infected with HIV (Forbes *et al*, 2002).

CHAPTER-II

2. OBJECTIVES

2.1 General Objectives:

- General objective of this study is to establish the prevalence of bacterial and fungal infections among HIV positive individuals of Nepal.

2.2 Specific objectives:

- To isolate and identify the bacterial pathogen from the sputum sample of HIV infected individuals.
- To isolate and identify the fungal pathogens from the sputum sample of HIV infected individuals.
- To identify tubercle bacilli by Ziehl Neelsen staining technique.
- To identify the appropriate antibiotic profile for the isolated bacterial pathogens.

CHAPTER-III

3. LITERATURE REVIEW

3.1 HIV and AIDS

3.1.1 Origin and Discovery

The AIDS epidemic was discovered June 5, 1981, when the U.S. Centers for Disease Control and Prevention reported a cluster of Pneumocystis carinii pneumonia (now classified as Pneumocystis jiroveci pneumonia) in five homosexual men in Los Angeles.

In 1983, scientists led by Luc Montagnier at the Pasteur Institute in France first discovered the virus that causes AIDS (Barré-Sinoussi *et al*, 1983). They called it lymphadenopathy-associated virus (LAV). A year later a team led by Robert Gallo of the United States confirmed the discovery of the virus, but they renamed it human T lymphotropic virus type III (HTLV-III) (Popovic *et al*, 1984). In 1986, both the French and the US names for the virus itself were dropped in favour of the new term, human immunodeficiency virus (HIV) (Coffin *et al*, 1986).

HIV was classified as a member of the genus lentivirus (ICTV db, 61.0.6), part of the family of retroviridae (ICTV db, 61). Lentiviruses have many common morphologies and biological properties. Many species are infected by lentiviruses, which are characteristically responsible for long duration illnesses associated with a long period of incubation (Levy, 1993). Lentiviruses are transmitted as single-stranded, positive-sense, enveloped RNA viruses. Upon infection of the target cell, the viral RNA genome is converted to double-stranded DNA by a virally encoded reverse transcriptase which is present in the virus particle. This viral DNA is then integrated into the cellular DNA by a virally encoded integrase so that replication using cellular machinery may take place. Once the virus enters the cell, two pathways are possible: either the virus becomes latent and the infected cell continues to function, or the virus becomes active and

replicates, and a large number of virus particles are liberated which can infect other cells.

Two species of HIV infect humans: HIV-1 and HIV-2. HIV-1 is hypothesized to have originated in southern Cameroon after jumping from wild chimpanzees (Pan troglodytes troglodytes) to humans during the twentieth century (Gao *et al*, 1999; Keele *et al*, 2006). HIV-2 is hypothesized to have originated from the Sooty Mangabey (Cercocebus atys), an Old World monkey of Guinea-Bissau, Gabon, and Cameroon (Reeves and Doms, 2002). HIV-1 is more virulent, more easily transmitted and is the cause of the majority of HIV infections globally, while HIV-2 is less easily transmitted and is largely confined to West Africa (Reeves and Doms, 2002).

3.1.2 HIV structure and genome

HIV is different in structure from previously described retroviruses. It is around 120 nm in diameter (120 billionths of a meter; around 60 times smaller than a red blood cell) and roughly spherical.

HIV-1 is composed of two copies of single-stranded RNA enclosed by a conical capsid, which is in turn surrounded by a plasma membrane that is formed from part of the host-cell membrane. Other enzymes contained within the virion particle include reverse transcriptase, integrase, and protease.

HIV has several major genes coding for structural proteins that are found in all retroviruses, and several nonstructural ("accessory") genes that are unique to HIV. The *gag* gene provides the physical infrastructure of the virus; *pol* provides the basic enzymes by which retroviruses reproduce; the *env* gene supplies the proteins essential for viral attachment and entry into a target cell. The accessory proteins *tat*, *rev*, *nef*, *vif*, *vpr*, and *vpu* enhance virus production. Although called accessory proteins, *tat* and *rev* are essential for virus replication. In some strains of HIV, a mutation causes the production of an alternate accessory protein, Tev, from the fusion of *tat*, *rev*, and *env*.

The gp120 and gp41 proteins, both encoded by the *env* gene, enable the virus to attach to and fuse with target cells to initiate the infectious cycle. Both, especially gp120, have been considered as targets of future treatments or vaccines against HIV.

3.1.3 Epidemiology

UNAIDS and the WHO estimate that AIDS has killed more than 25 million people since it was first recognized in 1981, making it one of the most destructive epidemics in recorded history. Despite recent, improved access to antiretroviral treatment and care in many regions of the world, the AIDS epidemic claimed an estimated 2.8 million (between 2.4 and 3.3 million) lives in 2005 of which more than half a million (570,000) were children (UNAIDS, 2006).

Globally, between 33.4 and 46 million people currently live with HIV (UNAIDS, 2006). In 2005, between 3.4 and 6.2 million people were newly infected and between 2.4 and 3.3 million people with AIDS died, an increase from 2004 and the highest number since 1981.

Sub-Saharan Africa remains by far the worst-affected region, with an estimated 21.6 to 27.4 million people currently living with HIV. Two million [1.5–3.0 million] of them are children younger than 15 years of age. More than 64% of all people living with HIV are in sub-Saharan Africa, as are more than three quarters (76%) of all women living with HIV. In 2005, there were 12.0 million [10.6–13.6 million] AIDS orphans living in sub-Saharan Africa 2005 (UNAIDS, 2006). South & South East Asia are second worst affected with 15%. AIDS accounts for the deaths of 500,000 children in this region. Two-thirds of HIV/AIDS infections in Asia occur in India, with an estimated 5.7 million infections (estimated 3.4—9.4 million) (0.9% of population), surpassing South Africa's estimated 5.5 million (4.9-6.1 million) (11.9% of population) infections, making it the country with the highest number of HIV infections in the world (UNAIDS, 2006). In the 35 African nations with the highest prevalence, average life expectancy is 48.3 years—6.5 years less than it would be without the disease (UNAIDS, 2001).

The first case of AIDS in Nepal was reported in 1988. By mid 1990s, Nepal has entered the Concenterated epidemic stage with consistent HIV prevalence in female sex workers, injecting drug users and migrants. The HIV prevalence is estimated around 0.5% in the general adult population (NCASC, 2003 and WHO/UNAIDS, 2003). There were around 60,018 people living with HIV/AIDS and 2,598 AIDS related deaths by 2002. Yearwise data indicates that the cases of HIV/AIDS have increased sharply since mid 1990s. In 1992, more than double members of new cases were reported than the formerly reported cumulative cases. Similarly, higher numbers of cases were reported in 2004 as compared to other years.

3.1.4 Pathogenesis

Pathogenesis, the ability of microorganisms to cause disease, starts with adherene of the microorganisms to the host cells, followed by colonization and growth resulting in damage to the host. Disease producing microorganisms elicit host changes using virulence, the relatie ability of a pathogen to cause disease.

3.1.4.1 HIV tropism

The term viral tropism refers to the cell type into which HIV may infect and replicate within. HIV can infect a variety of cells such as CD4+ T cells, macrophages, and microglial cells. HIV-1 entry to macrophages and CD4+ T cells is mediated not only through interaction of the virion envelope glycoproteins (gp120) with the CD4 molecule on the target cells but also with its chemokine coreceptors.

Macrophage (M-tropic) strains of HIV-1, or non-syncitia-inducing strains (NSI) use the β-chemokine receptor CCR5 for entry and are thus able to replicate in macrophages and CD4+ T cells (Coakley et al, 2005). The normal ligands for this receptor, RANTES, macrophage inflammatory protein (MIP)-1-beta and MIP-1-alpha, are able to suppress HIV-1 infection in vitro. This CCR5 coreceptor is used by HIV-1. Indeed, macrophages play a key role in several critical aspects of HIV disease. They appear to be the first cells infected by HIV and perhaps the very source of HIV production when CD4+ cells are markedly depleted in the patient. Macrophages and microglial cells are the cells

infected by HIV in the central nervous system. In tonsils and adenoids of HIV-infected patients, macrophages fuse into multinucleated giant cells that produce copious amounts of virus.

T-tropic isolates, or syncitia-inducing (SI) strains replicate in primary CD4+ T cells as well as in macrophages and use the α-chemokine receptor, CXCR4, for entry (Coakley *et al*, 2005; Deng *et al*, 1996; Feng *et al*, 1996). The α-chemokine, SDF-1, a ligand for CXCR4, suppresses replication of T-tropic HIV-1 isolates. It does this by down regulating the expression of CXCR4 on the surface of these cells. HIV that use only the CCR5 receptor are termed R5, those that only use CXCR4 are termed X4, and those that use both, X4R5. However, the use of coreceptor alone does not explain viral tropism, as not all R5 viruses are able to use CCR5 on macrophages for a productive infection (Coakley *et al*, 2005) and HIV can also infect a subtype of myeloid dendritic cells (Knight *et al*, 1990), which probably constitute a major reservoir that maintains infection when CD4+ T cell numbers have declined to extremely low levels.

3.1.4.2 Life cycle

The life cycle of HIV-1 can be considered in two distinct phases. The initial phase occur with in short time and include viral attachment, entry, reverse transcription, entry into the nucleus and integration of double stranded DNA (the provirus). The second phase occurs over the lifetime of the infected cell as viral and cellular proteins regulate the production of viral proteins and new infectious virions.

Viral entry to the cell: The interaction between the glycoprotein gp120 on the HIV virion and its receptor, CD4 on the target cell, provokes conformational changes in gp120. This exposes a region of gp120, the V3 loop, which binds to a cytokine receptor on the target cell, such as CCR5 or CXCR4 depending on the strain of HIV.

The change in gp120's shape also exposes a portion of the gp41 glycoprotein, which was previously buried in the viral membrane and loosely bound to gp120. A fusion peptide within gp41 causes the fusion of the viral envelope and the host-cell envelope, allowing the capsid to enter the target cell. The exact mechanism by which gp41 causes the fusion is still largely unknown (Chan and Kim, 1998; Wyatt and Sodroski, 1998).

Once HIV has bound to the target cell, the HIV RNA and various enzymes, including but not limited to reverse transcriptase, integrase and protease, are injected into the cell.

Viral replication and transcription: Once the viral capsid has entered the cell, an enzyme called reverse transcriptase liberates the single-stranded (+)RNA from the attached viral proteins and copies it into a negatively sensed viral complementary DNA of 9 kb pairs (cDNA). This process of reverse transcription is extremely error prone and it is during this step that mutations (such as drug resistance) are likely to arise. The reverse transcriptase then makes a complementary DNA strand to form a double-stranded viral DNA intermediate (vDNA). This new vDNA is then transported into the nucleus. The integration of the proviral DNA into the host genome is carried out by another viral enzyme called *integrase*. This is called the latent stage of HIV infection (Zheng *et al*, 2005).

To actively produce virus, certain transcription factors need to be present in the cell. The most important is called NF-kB (NF Kappa B) and is present once the T cells becomes activated. This means that those cells most likely to be killed by HIV are in fact those currently fighting infection.

The production of the virus is regulated, like that of many viruses. Initially the integrated provirus is copied to mRNA which is then spliced into smaller chunks. These small chunks produce the regulatory proteins Tat (which encourages new virus production) and Rev. As Rev accumulates it gradually starts to inhibit mRNA splicing (Pollard and Malim, 1998). At this stage the structural proteins Gag and Env are produced from the full-length mRNA. Additionally the full-length RNA is actually the virus genome, so it binds to the Gag protein and is packaged into new virus particles.

Interestingly, HIV-1 and HIV-2 appear to package their RNA differently; HIV-1 will bind to any appropriate RNA whereas HIV-2 will preferentially bind to the mRNA which was used to create the Gag protein itself. This may mean that HIV-1 is better able to mutate (HIV-1 causes AIDS faster than HIV-2 and is the majority species of the virus).

Viral assembly and release: The final step of the viral cycle is the assembly of new HIV-1 virions, begins at the plasma membrane of the host cell. The Env polyprotein (gp160) goes through the endoplasmic reticulum and is transported to the Golgi complex where it is cleaved by protease and processed into the two HIV envelope glycoproteins gp41 and gp120. These are transported to the plasma membrane of the host cell where gp41 anchors the gp120 to the membrane of the infected cell. The Gag (p55) and Gag-Pol (p160) polyproteins also associate with the inner surface of the plasma membrane along with the HIV genomic RNA as the forming virion begins to bud from the host cell. Maturation either occurs in the forming bud or in the immature virion after it buds from the host cell. During maturation, HIV proteases (proteinases) cleave the polyproteins into individual functional HIV proteins and enzymes. The various structural components then assemble to produce a mature HIV virion (Gelderblom, 1997). This step can be inhibited by drugs. The virus is then able to infect another cell.

3.1.4.3 The clinical course of HIV-1 infection

Infection with HIV-1 is associated with a progressive loss of CD4+ T-cells. This rate of loss can be measured and is used to determine the stage of infection. The loss of CD4+ T-cells is linked with an increase in viral load. The clinical course of HIV-infection generally includes three stages: primary infection, clinical latency and AIDS. HIV plasma levels during all stages of infection range from just 50 to 11 million virions per ml (Piatak *et al*, 1993).

3.1.4.3.1 Primary Infection

Primary, or acute infection is a period of rapid viral replication that immediately follows the individual's exposure to HIV. During primary HIV infection, most individuals (80 to 90 %) develop an acute syndrome characterised by flu-like symptoms of fever, malaise, lymphadenopathy, pharyngitis, headache, myalgia, and sometimes a rash (Kahn and Walker, 1998). Within an average of three weeks after transmission of

HIV-1, a broad HIV-1 specific immune response occurs that includes seroconversion. Because of the nonspecific nature of these illnesses, it is often not recognized as a sign of HIV infection. Even if patients go to their doctors or a hospital, they will often be misdiagnosed as having one of the more common infectious diseases with the same symptoms. Since not all patients develop it, and since the same symptoms can be caused by many other common diseases, it cannot be used as an indicator of HIV infection. However, recognizing the syndrome is important because the patient is much more infectious during this period.

3.1.4.3.2 Clinical Latency

As a result of the strong immune defense, the number of viral particles in the blood stream declines and the patient enters clinical latency. Clinical latency is variable in length and can vary between two weeks and 20 years. During this phase HIV is active within lymphoid organs where large amounts of virus become trapped in the follicular dendritic cells (FDC) network early in HIV infection. The surrounding tissues that are rich in CD4+ T-cells also become infected, and viral particles accumulate both in infected cells and as free virus. Individuals who have entered into this phase are still infectious.

3.1.4.3.3 The declaration of AIDS

AIDS is the most severe manifestation of infection with HIV and occurs when the CD4+ T cell count drops to below 200 cells per mm^3. Classical symptoms are often severe opportunistic infections, rare cancers, neurological complications, and malnutrition. These include:

1. Kaposi's sarcoma - a cancer of the blood vessels that accounts for extensive "bruising" sometimes seen in AIDS patients. It is aggressive, and can attack the mouth, lymph nodes, and internal organs, which may include the lungs or gastrointestinal tract.

2. Pneumocystis jiroveci pneumonia - a form of pneumonia caused by the yeast-like fungal microorganism *Pneumocystis jiroveci* (sometimes spelled jirovecii and formerly

classified as Pneumocystis *carinii*).

3. AIDS dementia complex - encephalopathy due to HIV-infected brain cells.

4. Toxoplasmosis, Progressive multifocal leukoencephalopathy, common bacterial infections (*Salmonella, Shigella, Listeria, Campylobacter*, or *E. coli*) and uncommon opportunistic infections such as cryptosporidiosis, microsporidiosis, Mycobacterium avium complex (MAC) and cytomegalovirus (CMV) colitis.

3.1.5 Transmission

Since the beginning of the epidemic, three main transmission routes of HIV have been identified:

3.1.5.1 Sexual route: The majority of HIV infections are acquired through unprotected sexual relations. Sexual transmission occurs when there is contact between sexual secretions of one partner with the rectal, genital or mouth mucous membranes of another. The probability of transmission per act is between 1 in 53 to 1 in 10,000 for the case of receptive vaginal sex (Pilcher *et al*, 2004), 1 in 8000 in the case of insertive vaginal sex, 1 in 1000 in the case of insertive anal sex, and between 1 in 100 to 1 in 30 in the case of receptive anal sex .

3.1.5.2 Blood or blood product route: This transmission route is particularly important for intravenous drug users, hemophiliacs and recipients of blood transfusions and blood products. It is also of concern for persons receiving medical care in regions where there is prevalent substandard hygiene in the use of injection equipment (e.g. reused needles in Third World settings). Health care workers (nurses, laboratory workers, doctors, etc) are also directly concerned, although more rarely. Also concerned by this route are people who give and receive tattoos and piercings, i.e. "scarification" procedures.

3.1.5.3 Mother-to-child route: The transmission of the virus from the mother to the child can occur *in utero* during the last weeks of pregnancy and at childbirth. Breast

feeding also presents a risk of infection for the baby. In the absence of treatment, the transmission rate between the mother and child was 20%. However, where treatment is available, combined with the availability of Cesarian section, this has been reduced to 1%.

HIV has been found at low concentrations in the saliva, tears and urine of infected individuals, but the risk of transmission by these secretions is considered to be negligible.

The use of physical barriers such as the latex condom is widely advocated to reduce the sexual transmission of HIV. Recently, it has been proposed that male circumcision may reduce the risk of HIV transmission (Siegfried *et al*, 2005), but many experts believe that it is premature to recommend male circumcision as part of HIV prevention programs (WHO, 2005).

3.1.6 Spectrum of Respiratory Illnesses in HIV-Infected Patients

Bacterial Infections

S. pneumoniae

H. influenzae

Gram-negative bacilli (*P. aeruginosa*, *K. pneumoniae*)

S. aureus

Mycobacterial Infections

M. tuberculosis

M. kansasii

M. avium complex

Fungal Infections

P. carinii

C. neoformans

H. capsulatum

C. immitis

Aspergillus

Candida species

Viral Infections

Cytomegalovirus

Herpes simplex virus

Parasitic Infections

Toxoplasma gondii

Strongyloides stercoralis

Neoplasms

Kaposi's sarcoma

Non-Hodgkin's lymphoma

Bronchogenic carcinoma

Upper Respiratory Illnesses

Upper respiratory tract infection

Sinusitis

Pharyngitis

Lower Respiratory Tract Disorders

Lymphocytic interstitial pneumonitis (LIP)

Nonspecific interstitial pneumonitis (NIP)

Acute bronchitis

Obstructive lung disease

Asthma

Chronic bronchitis

Bronchiectasis

Emphysema

Pulmonary vascular disease

Illicit drug-induced lung disease

Medication-induced lung disease

Primary pulmonary hypertension

Bronchiolitis obliterans organizing pneumonia (BOOP)

LRTI and CD4 Cell Count

CD4 cell count <500 cells/mm^3

Bacterial pneumonia (recurrent)

Pulmonary mycobacterial pneumonia (nontuberculous)

CD4 cell count <200 cells/mm^3

P. carinii pneumonia

C. neoformans pneumonia

Bacterial pneumonia (associated with bacteremia/sepsis)

Disseminated or extrapulmonary tuberculosis

CD4 cell count <100 cells/mm^3

Pulmonary Kaposi's Sarcoma

Bacterial pneumonia (Gram-negative bacilli and *Staph. aureus* increased)

Toxoplasma pneumonitis

CD4 cell count <50 cells/mm^3

Disseminated *H. capsulatum*

Disseminated *C. immitis*

Cytomegalovirus pneumonitis

Disseminated *Mycobacterium avium* complex

Disseminated mycobacterium (nontuberculous)

Aspergillus pneumonia

Candida pneumonia

3.1.7 Laboratory diagnosis

HIV infection can be detected in the laboratory either by detection of antibodies to HIV or by detection of the virus, its antigen and its DNA. Detection of specific antigens, viral nucleic acid, isolation/culture of virus are all confirmatory tests in that the presence of the virus is detected. But they are risky because of the danger of infection to laboratory workers, are very laborious and difficult to perform, require skilled expertise and hence are to be done only in laboratories specified research purposes.

The indirect predictors of HIV infection (CD4 cell count, β_2 microglobulin etc.) are monitors of immunity status of patients and are to be done at routine intervals to monitor the progression of disease.

Detection of HIV-specific antibodies

In HIV infection, there is an initial period of viral replication during which antibody is undetectable. This period of seronegativity, called window period, may last for 1-3 months, but in a small minority of patients for up to a year, and rarely for years.

This is done by performing initial screening tests, which if positive, are followed up by supplemental tests to confirm the diagnosis.

Screening tests: Enzyme linked immunosorbent assay (ELISA) is the most commonly performed test to detect HIV antibodies. There are various kind of ELISA based on the principle of test:

Indirect ELISA
Competitive ELISA
Antigen sandwich ELISA
Antigen and antibody capture ELISA

ELISA takes up to three hours to yield results. It has a major advantage of being economical although rapid tests give result within minutes. Commercial kits are available for ELISA and rapid tests. Rapid tests include:

Dot blot assays
Particle agglutination
HIV spot and comb tests
Fluorometric microparticle technologies

In addition supplemental tests are done for the confirmation.

Western blot

Indirect Immunofluorescence

Radio Immuno Precipitation Assay

Virus culture

Virus culture is another method for identifying HIV infection. Positive culture rates up to 98% are reported in confirmed seropositive individuals. The culture method is however, expensive, labour intensive, can take weeks for complete results and potentially exposes laboratory workers to high concentrations of HIV. Virus culture is used for research (Drug sensitivity, vaccine studies etc).

Polymerase chain reaction (PCR)

PCR can detect proviral DNA during window period, can differentiate latent HIV infection from active viral transcription and can quantitate the copy number of HIV DNA when used with external standards. PCR can successfully differentiate between HIV-1 and HIV-2 infections. Proviral DNA can be detected in peripheral blood mononuclear cells before seroconversion. Limitation to the Diagnostic use of PCR are rare false negatives, some of which can be avoided by the use of multiple primer pairs from conserved regions of the genome and false positive due to cross contamination of the PCR reaction mixture.

HIV-1 can be detected by PCR in the CSF of HIV infected patients independently of disease stage; spread of HIV-1 to the brain represents an early event during infection, which occurs in most asymptomatic individuals. PCR can also be used to detect HIV infection in neonates borne to HIV infected mothers.

Viral load assay

Quantitation of HIV RNA in plasma is useful for determining free viral load, assessing the efficacy of antiviral therapy and predicting progression and clinical outcome. Because baseline HIV viral load is predictive of survival at 10 years in patients with

nearly identical CD4 counts, assessment of baseline viremia prior to initiation of therapy is useful in patient management.

Indirect predictors of HIV infection

Decreased CD4 cells

Increased β_2 microglobulin

Increased IL2 receptors

Indicator diseases for AIDS

3.1.8 Treatment

HIV infection is a chronic infectious disease that can be treated, but not yet cured. There are effective means of preventing complications and delaying, but not preventing, progression to AIDS. At the present time, not all persons infected with HIV have progressed to AIDS, but it is generally believed that the majority will. People with HIV infection need to receive education about the disease and treatment so that they can be active partners in decision making with their health care provider.

A combination of several antiretroviral agents, termed Highly Active Anti-Retroviral Therapy HAART, has been highly effective in reducing the number of HIV particles in the blood stream (as measured by a blood test called the viral load). This can improve T-cell counts. This is not a cure for HIV, and people on HAART with suppressed levels of HIV can still transmit the virus to others through sex or sharing of needles. There is good evidence that if the levels of HIV remain suppressed and the CD4 count remains greater than 200, then the quality and length of life can be significantly improved and prolonged. Improved antiretroviral inhibitors against proteins such as Reverse transcriptase, Integrase and Tat are being researched and developed. One of the most promising new therapies is a new class of drugs called fusion or entry inhibitors..

As yet, no vaccine has been developed to prevent HIV infection or disease in people who are not yet infected with HIV, but the potential worldwide public health benefits of

such a preventive vaccine are vast. Researchers in many countries are seeking to produce such a vaccine, including through the International AIDS vaccine initiative.

3.1.9 Prevention of HIV infection

There is no cure for HIV infection. The only way to prevent HIV infection is through total avoidance of sources of transmission. AIDS prevention and control broadly includes:

- informing the general public about HIV transmission and risky behaviours.

- ensuring the safety of blood and blood products.

- counseling HIV infected persons, and

- taking action to reduce HIV transmission among injecting drug users.

3.2 Fungal pathogens frequently isolated from HIV/AIDS individuals

The Acquired Immune Deficiency Syndrome (AIDS) due to retrovirus HIV is characterized by the gradual loss of immune system functions. The hallmark of this process is a marked depression of cellular immunity. This often leads to several opportunistic infections including fungal infections. Studies on AIDS in the USA and Africa show that at least fifty to ninety percent of all patients contract a fungal infection at some time during the course of the illness and ten to twenty percent die as a direct consequence of these infections. The role of fungal infections became more important since *P. carinii*, which previously thought to be a protozoan, has been classified as a fungus on the basis of genetic studies.

The range of mycoses seen in AIDS patients is very wide. Almost all of the well-recognized fungal pathogens have been described in AIDS from different parts of the world, in addition to a number of various rare types.

The most common and some of the uncommon mycoses seen in patients of AIDS are listed below:

	Mycoses	Organisms	Main tissues involved
Common	Candidiasis	*C. albicans*	Oral mucosa, Oesophageal mucosa
	Cryptococcosis	*C. neoformans*	Brain, Meninges, Lung
	Histoplasmosis	*H. capsulatum*	Reticulo-endothelial system
	Coccidioidomycoses	*C. immitis*	Lungs
Uncommon	Aspergillosis	*Aspergillus* spp	Lungs
	Blastomycosis	*B. dermatitidis*	Lungs
	Penicilliiosis	*P. marneffei*	Reticulo-endothelial system
	Sporotrichosis	*S. schenckii*	Brain, Skin
	Dermatophytosis	*Trichophyton* spp *E. floccosum*	Nail, skin
	Pneumocystis infections	*P. carinii*	Lungs, Eyes and other organs

Coccidioidomycosis is a major mycosis in AIDS patients, but it is geographically limited to endemic areas (South Western States of United States and elsewhere in the Western hemisphere).

3.2.1 Candidiasis

Candidiasis is an acute or chronic, superficial or disseminated fungal infection caused by species of *Candida*. The disease manifests in a variety of forms. Since the pathogenic species of *Candida* forms the commensal flora of most individuals, the diagnosis of candidiasis should be carefully established. Up to 90% of HIV-positive individuals develop candidiasis at sometime during the illness. *Candida* infections in AIDS are usually limited to superficial candidiasis of varying degrees of severity in he oral cavity, throat and oesophagus. Recurrent often chronic vulvo-vaginal candidiasis is well described in female patients with AIDS. But, in general, whether male or female, oral thrush is the most common form and appears when the CD4 lymphocyte count is

about 200 cells/mm^3. Pulmonary and central nervous system *Candida* infections have also been reported in a small number of AIDS patients (Brooks *et al*, 2002).

The clinical specimens can be cultured on Sabouraud dextrose agar and incubated at 25^0c and 37^0c. The colonies appear in 3-4 days as cream colored, smooth and pasty. Sometimes the growth may be observed within an overnight incubation.

The culture of *Candida* species is treated with sheep or normal human serum and incubated at 37^0c for 2 to 4 hours. A drop of suspension is examined on the slide under the microscope. The germ tubes are seen as long tube like projections extending from the yeast cells. There is no constriction at the point of attachment to the yeast cell (Wheat, 1995).

Tests for the detection of antibodies against *Candida* are least helpful in severely immunosuppressed patients including patients of AIDS. Consistently rising titres are suggestive of deep infection. Kits are available commercially.

3.2.2 Cryptococcosis

Cryptococcosis is an, subacute or chronic fungal disease caused by an encapsulated yeast belonging to the genus *Cryptococcus*. There are many species of this genus prevalent ubiquitously in environment but only *C. neoformans* is significantly pathogenic to man and animals. This fungi involves opportunistic infection, primarily involves the lungs and disseminates to extrapulmonary sites through hematogenous route to different body organs particularly CNS, causing meningoencephalitis. The disease is one of the life threatening infections and responsible for high morbidity and mortality among the immunocompromised patients particularly associated with pandemic of acquired immunodeficiency syndrome (Wheat, 1995).

Cryptococcosis is the second most common fungal infection after candidiasis in HIV infected individuals and potentially the most serious infection. The initial infection most likely occurs through inhalation of basidiospores. The inflammatory response to be inhaled cryptococci produces a primary lung lymph node complex that limits spread of

the yeast. The fungus may remain dormant in the lungs until the immune system weakens and then can reactivate and disseminate to the CNS and other body sites. The disease usually occurs between the ages of 30 and 60 years and is uncommon in childhood. The incubation period of Cryptoccosis is usually 2-4 weeks. The disease is presumed to be resulting from the environment (Brooks *et al*, 2002).

Cryptococcosis is now the predominant cause of fatal fungal infection in patients with AIDS and ranks fourth among the CNS infections. It may occur at any point of time during the course of AIDS but is most common when the CD4 count falls below 200cells/mm^3. The extra-pulmonary cryptococcosis is one of the AIDS defining diseases. The frequent use of corticosteroids is the second most important risk factor for cryptococcal meningitis.

The prevalence of cryptococcosis in patients of AIDS varies from country to country: 3-6% in Europe, 6-10 % in the United States, and in up to 30% in parts of Africa. In AIDS, cryptococcosis is often insidious in onset, with few meningococcal symptoms or signs. Often the sole presenting symptom is mild headache. Less than 20% are somnolent, confused or obtunded. In some AIDS patients, however, the neurological signs are rare.

The clinical specimen can be observed directly under microscope by India ink preparation method. It can be isolated on Sabouraud dextrose agar.

The most effective test is the latex test for capsular antigen on CSF or blood. The antigen test is positive in over 90% of patients with untreated meningeal infection. Latex kits are available.

3.2.3 Histoplasmosis

Histoplasmosis is a systemic granulomatous disease caused by a thermally dimorphic fungus *Histoplasma capsulatum. H. capsulatum* is a dimorphic fungus and grows at 25-30^0con Sabouraud dextrose agar as a white to buff colony with in 6-8 weeks. When the mold is incubated at 37^0c on enriched media such as brain-heart infusion agar with

blood and cysteine, conidia germinate and converted into the yeast cells. The fungus is present as yeast in tissues and as filamentous form in the environment. The soil is the reservoir of this fungus. Adult men are affected more commonly than women but in children there is no sex difference (Wheat, 1994).

The infection with *H. capsulatum* develops when conidia or mycelial fragments are inhaled and subsequently converted into yeasts in alveolar macrophages in the lungs. The oval yeast cells of *H. capsulatum* parasitize macrophages, which are modulated by T-lymphocytes, resulting in localized granulomatous inflammation, which may assist in dissemination to reticulo-endothelial system.

It has been seen that individuals infected with HIV are at greater risk of developing disseminated histoplasmosis. The disseminated disease has also been reported to be involving the organ transplantation patients also (Brooks *et al*, 2002).

In 1985, the patients suffering from disseminated histoplasmosis and positive serology for HIV were defined as having AIDS according to the criteria of the centers for disease control and prevention, USA. It is a serious problem in AIDS patients and in the USA it is reported in as high as 6% patients.

Infection of thyroid gland, sinuses, prostate and epididymis with *H. capsulatum* is also rarely reported as a part of disseminated disease, even in geographical areas in which histoplasmosis is endemic.

Detection of circulating *Histoplasma* antigen is found to be a sensitive method for diagnosis of disseminated histoplasmosis in AIDS. Antigen levels fall in patient receiving antifungal treatment and rise in those who have relapsed. Kits are available commercially.

3.2.4 Coccidioidomycosis

Coccidioidomycosis is primarily an infection of the respiratory system caused by a dimorphic fungus *Coccidioides immitis*, found in soil of arid and semiarid areas. The infection occurs following inhalation of arthroconidia of the mycelial form of *C. immitis*

or reactivation of the latent infection in the immunocompromised patients. In the host, the arthroconidia swell and form spherules with a thick wall. The spherules enlarge and develop endospores. These mature spherules mechanically rupture, releasing endospores that spread locally and disseminate to the extra-pulmonary sites. The clinical features of disease can be described as;

Pulmonary Coccidioidomycosis

Disseminated Coccidioidomycosis

Chronic coccidioidal meningitis

Coccidioidomycosis in AIDS patients

Coccidioidomycosis is one of the frequent opportunistic infection among the HIV infected patients in the Southwestern USA. The disease in AIDS patients is nearly always a severe infection and was included in the surveillance case definition for AIDS in 1987. There is a high incidence of disseminated infection in AIDS patients living in the endemic regions as compared to the general population, which may be explained in part by reactivation of latent infection. The patients, who are immuno-compromised due to the solid organ transplantation, Hodgkin's disease, diabetes mellitus, 3^{rd} trimester of pregnancy or long-term corticosteroid therapy also, have an increased risk of developing disseminated disease (Wheat, 1995).

The colonies at 25^{0}c appear with in 3-5 days and are white to tan cottony which develop moderately rapid as a moist membranous culture at first and later on develop abundant aerial mycelia within 3-5 days but arthroconidia may not be apparent by 10 days (Brooks *et al*, 2002).

3.2.5 Aspergillosis

Aspergillosis is a systemic fungal infection found in immuno-compromised individuals. This is primarily a pulmonary infection with involvement of other body sites like paranasal sinuses and cutaneous tissues. It is caused by several species of genus *Aspergillu***s**, which are ubiquitously found in the environment worldwide in the

decaying organic matter. The disease process generally follows inhalation of the spores but the fungus may also gain directly entry to the body tissues through wounds or during surgery.

Aspergillosis is considered as the second most common fungal infection requiring hospitalization inn the United States. The frequency and related importance of these infections is on the rise in the developed countries, which is possibly related to increased number of immuno-compromised patients, owing to improved survival from AIDS malignancies and more intensive cytotoxic therapy, transplantation for organ dysfunctions and better therapy and prophylaxis for other fungal infections like candidiasis. *Aspergillus* infections in individuals with AIDS have been reported worldwide. *Aspergillus fumigatus* has been commonly encountered as the etiological agent of systemic aspergillosis in man and animals (Wheat, 1995).

Invasive aspergillosis is an increasingly common fungal infection and an important cause of morbidity and mortality in the immuno-compromised hosts, especially in patients with acute leukemia, bone marrow, solid organ transplantation or AIDS (Singh *et al*, 1991).

Invasive aspergillosis is well known to occur in various immuno-compromised populations, such as neutropenic patients with hematologic malignancies, patients with late stage of HIV infection and children with chronic granulomatous diseases (Holding *et al*, 2000).

This is common in severely immuno-compromised individuals who are on immunosuppressive drugs, corticosteroids or broad-spectrum antibiotics. There is widespread growth of the fungus in the lung tissue that may disseminate to involve other organs particularly kidneys and brain (Brooks *et al*, 2002).

The aspergillosis of CNS is serious fungal infection and majority of cases occur by hematogenous dissemination from pulmonary or gastrointestinal focus, more often in immuno-compromised individuals. Aspergillosis infection accounts for about 5% of the CNS fungal infections.

The clinical specimen can be cultured on Sabouraud dextrose agar with chloramphenicol. The species of Aspergilli are identified on the basis of their colony colour and morphologic features.

3.2.6 Blastomycosis

Blastomycosis is an acute or chronic pyogranulomatous fungal infection of respiratory system disseminating to the extrapulmonary sites like skin, bones and genitourinary system. This disease is also caused by one of the thermally dimorphic fungi, *Blastomyces dermatitidis*, a soil dwelling fungus. It is a broad-based yeast cells of 2-10 um diameter in tissue phase.

The infections with *B. dermatitidis* begin with inhalation of spores into the lungs. If the organism evades non-specific host defense mechanisms, the fungus undergoes a phase transition to yeast cells. These yeast cells increase in number in the parenchyma of the lung and spread to the other organs via bloodstream.

Blastomycosis is a T-cell opportunistic infection with a highly aggressive course in patients who have AIDS. The primary infection occurs after inhalation of aerosolized conidia into the lungs. In general, there is weight loss, fever, malaise, fatigue and other non-specific complaints. The men are affected six times more as compared to the women. Adults are more frequently affected than the children.

The infection with *B. dermatitidis* may infrequently be an opportunistic, notably in patients who are in the late stages of AIDS, transplants recipients and patients treated with immunosuppressive or cytotoxic chemotherapy (Wheat, 1995).

The most sensitive method for detecting small number of organisms is a histopathological section with Gomori's methamine silver staining which shows broad based yeasts with granulomatous inflammatory response (Brooks *et al*, 2002).

The culture of clinical specimen can be done on blood agar, Sabouraud dextrose agar, brain-heart infusion agar and blood-glucose-cysteine agar at 25^0c and 37^0c. The growth is very slow, taking about 2-4 week's time.

3.2.7 Penicilliosis

There are more than two hundred and fifty known species of the genus *Penicillium* which are widely distributed in the nature and found in soil as well as decomposing organic debris. They are non-pathogenic and used in manufacturing antibiotics like *Penicillium notatum* for penicillin, an antibacterial antibiotic and *Penicillium griseofulvin*, another effective antifungal antibiotic used in the treatment of dermatophytosis.

Penicillium marneffei is the only dimorphic fungus of this genus causing penicilliosis, an emerging systemic fungal disease of its restricted endemicity to the Southeast Asia.

P. marneffei causes an emerging clinical entity, penicilliosis marneffei, which is a progressive, disseminated and potentially fatal fungal infection in the later stages of the HIV infection.

There is dissemination of this fungus in immunocompromised as well as immunocompetent patients. Significantly, with spread of HIV pandemic throughout the world, *P. marneffei* infection has emerged as a new potential indicator disease of the acquired immunodeficiency syndrome in Southeast Asia and now constitutes the third most common AIDS-related opportunistic infection in a substantial proportion of cases in these endemic areas after tuberculosis and cryptococcosis.

The infection occurs via inhalation of airborne conidia. The primary site of infection in penicilliosis marneffei is the reticuloendothelial system. The granulomatous reaction occurs most often in reticuloendothelial system like bone marrow, liver, spleen and lymph nodes of immunocompetent patients. The yeast cells invade macrophages, survive and multiply intracellulary. The cell- mediated immune response to fungi leads to granuloma formation. The granulomas enlarge and become necrotic in the center and the yeast cells are released (Sirisanthana *et al*, 1994).

The clinical features includes low-grade fever, chills, malaise, marked weight loss, anemia, cough, generalized popular skin lesions with central umbilication, lymphadenopathy and hepatosplenomegaly.

The clinical signs in both HIV- positive and HIV- negative patients are almost similar except the former have a more acute onset, high fever and severe respiratory signs. Penicilliosis marneffei has also been included in the list of AIDS defining diseases in endemic areas. Infection of *P. marneffei* among the AIDS patients presents as a disseminated illness with fever, weight loss, skin lesions and pancytopenia and is invariably fatal if not diagnosed and treated in time (Wheat, 1995).

Moreover, the disease is most commonly seen in young adults infected with HIV but has also been reported from children. *P. marneffei* endophthalmitis has also been reported from a patient of AIDS in Thailand (Sirisanthana *et al*, 1994).

On Sabouraud dextrose agar and blood agar at 25^0c, greyish-white colonies grow within two days and subsequently become woolly-downey to granular in texture and yellow-orange, yellow-green, grey or green in centre. The periphery of colonies is white, grey-orange or purple orange and has radial folds. They are wrinkled, folded and have red-stained mycelia and sporulate poorly with greenish conidia (Brooks *et al*, 2002). The reverse is characteristically bright rose color due to diffusion of a water-soluble brick red pigment into the surrounding agar medium. The fungal culture media should not have cycloheximide as it is inhibitory to *P. marneffei*.

3.2.8 Sporotrichosis

Sporotrichosis is a chronic, pyogranulomatous fungal infection of cutaneous and subcutaneous tissues, which may remain localized or may show lymphatic spread with occasional dissemination to other parts of body. This is caused by *Sporothrix schenckii*, a thermally dimorphic saprobic fungus distributed worldwide.

S. schenckii is one of the six important thermodimorphic fungi of medical significance and causes a chronic lymphatic and subcutaneous infection. Sporotrichosis is broadly categorized into five clinical types. The first four are outcome of traumatic implantation and fifth one is due to inhalation of conidia. These are as follows:

Lymphocutaneous sporotrichosis

Fixed cutaneous sporotrichosis

Mucocutaneous sporotrichosis

Disseminated sporotrichosis

Pulmonary sporotrichosis

Disseminated sporotrichosis has been seldom reported in patients of AIDS (Kauffman *et al*, 2000).

3.2.9 Dermatophytosis

Cutaneous mycoses are caused by fungi that infect only the superficial keratinized tissue (skin, hair and nails). The most important of these are the dermatophytes, a group of about 40 related fungi that belong to three genera; *Microsporum, Trichophyton*, and *Epidermophyton*. Dermatophytes are probably restricted to the nonviable skin because most are unable to grow at 37^0c or in the presence of serum. Dermatophytoses are among the most prevalent infections in the world. Although they can be persistent and troublesome, they are not debilitating or life-threatening- yet millions of dollars are expended annually in their treatment. Dermatophytes are classified as geophilic, zoophilic, or anthropophilic depending on whether their usual habitat is soil, animals, or humans. Dermatophytes are acquired by contact with contaminated soil or with infected animals or humans (Weitzman *et al*, 1995).

Dermatophytes are identified by their colonial appearance and microscopic morphology after growth for 2 weeks at 250c on Sabouraud's dextrose agar. *Trichophyton* species, which may infect hair, skin, or nails, develop cylindric, smooth-walled macroconidia and characteristic microconidia. Depending on the variety, colonies of *T. mentagrophytes* may be cottony to granular; both types display abundant grape like clusters of spherical microconidia on terminal branches. Coiled or spiral hyphae are commonly found in primary isolates. The typical colony of *T. rubrum* has a white, cottony surface and a deep red, non diffusible pigment when seen from reverse. The microconidia are small and piriform, or pear-shaped. *T. tonsurans* produces a flat,

powdery to velvety colony that becomes reddish- brown on reverse; the microconidia are mostly elongate.

Epidermophyton floccosum, which is the only pathogen in this genus, produces only macroconidia, which are smooth-walled, clavate, two-to four-celled, and formed in groups of two or three. The colonies are usually flat and velvety with a tan to olive-green tinge. *E. floccosum* infects skin and nails but not hair.

The most common geophilic species causing human infections is *Microsporum gypseum*.

3.2.10 Pneumocystis infections

Pneumocystis carinii causes pneumonia in immunocompromised patients; dissemination is rare. Until recently, *P. carinii* was thought to be a protozoan, but molecular biologic studies have proved that it is a fungus with a close relationship to ascomycetes. *P. carinii* is present in the lungs of many mammals (rats, mice, dogs, cats, ferrets, rabbits) but rarely causes disease unless the host is immunosuppressed. Until the AIDS epidemic, human disease was confined to interstitial plasma cell pneumonitis in malnourished infants and immunosuppressed patients (corticosteroid therapy, antineoplastic therapy, and transplant recipients). Prior to the introduction of effective chemoprophylactic regimens, it was a major cause of death among AIDS patients. Chemoprophylaxis has resulted in a dramatic decrease in the incidence of pneumonia, but infections are increasing in other organs, primarily the spleen, lymph nodes, bone marrow (Su and Martin, 1994).

P. carinii has morphologically distinct forms: thin walled trophozoites and cysts, which are thick-walled, spherical to elliptical (4-6 um), and contain four to eight nuclei. Cysts can be stained with silver stain, toluidine blue, and calcofluor white. In most clinical specimens, the trophozoites and cysts are present in a tight mass that probably reflects their mode of growth in the host. *P. carinii* contains a surface glycoprotein that can be detected in sera from acutely ill or normal individuals.

P. carinii is an extracellular pathogen. Growth in the lung is limited to the surfactant layer above alveolar epithelium. In non-AIDS patients, infiltration of the alveolar spaces with plasma cells leads to interstitial AIDS-related *P. carinii* pneumonia. Blockage of the oxygen exchange interface results in cyanosis.

To establish the diagnosis of *P. carinii* pneumonia, specimens of bronchoalveolar lavage, lung biopsy, or induced sputum are stained and examined for the presence of cysts or trophozoites. Appropriate stains include Giemsa, toluidine blue, methenamine silver, and calcofluor white. A specific monoclonal antibody is available for direct fluorescent examination of specimens. *P. carinii* cannot be cultured. While not clinically useful, serology has been used to establish the prevalence of infection (Masur, 1992).

In the absence of immunosuppression, *P. carinii* does not cause disease. Cell mediated immunity presumably plays a dominant role in the resistance to disease, as AIDS patients often have significant antibody titers and *P. carinii* pneumonia is not usually seen until the CD4 lymphocyte count drops below 400/mm^3.

3.3 Antifungal Chemotherapy

There are a limited but increasing number of antibiotics that can be used to treat mycotic infections. Most have one or more limitations, such as profound side effects, a narrow antifungal spectrum, poor penetration of certain tissues, and the selection of resistant fungi. Promising new drugs are currently under development, and others are being evaluated in clinical trials. Finding suitable fungal targets is difficult because fungi, like humans, are eukaryotes. Many of the cellular and molecular processes are similar, and there is often extensive homology among the genes and proteins.

The classes of currently available drugs include the polyenes (amphotericin B and nystatin), which bind to ergosterol in the cell membrane; flucytosine, a pyrimidine analog; the azoles and other inhibitors of ergosterol synthesis; and griseofulvin, which interferes with microtubule assembly. Currently under investigation are inhibitors of

cell wall synthesis such as nikkomycin, which inhibits the synthesis of chitin, and pneumocandin, which inhibits the synthesis of ɣglucan.

3.3.1 Amphotericin B

The major polyene antibiotic is amphotericin B, a metabolite of streptomyces. Amphotericin B is the most effective drug for severe systemic mycoses. It has a broad spectrum, and the development of resistance is rare. The mechanism of action of polyenes involves the formation of complexes with ergosterol in fungal cell membranes, resulting in membrane damage and leakage. Amphotericin B has greater affinity or ergosterol than cholesterol, the predominant sterol in mammalian cell membranes. (Viscoli *et al*, 1999).

Amphotericin B is given intravenously as micelles with sodium deoxycholate dissolved in a dextrose solution. Though the drug is widely distributed in tissues, it penetrates poorly to the cerebrospinal fluid. Amphotericin B firmly binds to ergosterol in the cell membrane.

Amphotericin B has a broad spectrum with demonstrated efficacy against most of the major systemic mycoses, including coccidioidomycosis, blastomycosis, histoplasmosis, sporotrichosis, cryptococcosis, aspergillosis, mucormycosis, and candidiasis. The response to amphotericin B is influenced by the dose and rate of administration, the site of the mycotic infection, the immune status of the patient, and the inherent susceptibility of the pathogen.

3.3.2 Flucytosine

Flucytosine is a fluorinated derivative of cytosine. It is an oral antifungal compound used primarily in conjunction with amphotericin B to treat cryptococcosis or candidiasis. It is effective also against many dematiaceous fungal infections. It penetrates well into all tissues, including cerebrospinal fluid.

Flucytosine is actively transported into fungal cells by a permease. It is converted by the fungal enzyme cytosine deaminase to 5-flurouracil and incorporated into 5-flurodexyuridylic acid monophosphate, which interferes with the activity of thymidylate

synthetase and DNA synthesis. Mammalian cells lack cytosine deaminase and are therefore protected from the toxic effects of fluorouracil. Unfortunately, resistant mutants emerge rapidly, limiting the utility of flucytosine (Lortholary *et al*, 1999).

By itself, flucytosine is effective against chromoblastomycosis and other dematiaceous fungal infections.

3.3.3 Azoles

The antifungal imidazoles (eg, ketoconazole) and the triazoles (fluconazole and itraconazole) are oral drugs used to treat a wide range of systemic and localized fungal infections. The indications for their use are still being evaluated, but they have already supplanted amphotericin B in many less severe mycoses because they can be administered orally and less toxic. Other imidazoles, miconazole and clotrimazole, are used topically and are discussed below.

The azoles interfere with the synthesis of ergosterol. They block the cytochrome P450-dependent 14-ℑ-demethylation of lanosterol, which is a precursor of ergosterol in fungi and cholesterol in mammalian cells. However, the fungal cytochrome P450s are approximately 100-1000 times more sensitive to the azoles than mammalian systems. The various azoles are designed to improve their efficacy, availability, and pharmacokinetics and reduce their side effects. These are fungistatic drugs.

Ketoconazole is useful in the treatment of chromic mucocutaneous candidiasis, dermatophytosis, and nonmeningeal blastomycosis, coccidioidomycosis, paracoccidioidomycosis, and histoplasmosis. Of the various azoles, fluconazole offers the best penetration of the central nervous system. It is used as maintenance therapy for cryptococcal and coccidioidal meningitis. Oropharyngeal candidiasis in AIDS patients and candidemia in immunocompetent patients can also be treated with fluconazole. Itraconazole has demonstrated efficacy in the primary and maintenance therapy of histoplasmosis in AIDS patients, non-meningeal coccidioidomycosis, sporotrichosis, blastomycosis, chromoblastomycosis, aspergillosis, and dermatophytosis (Sheehan *et al*, 1999).

3.3.4 Griseofulvin

Griseofulvin is an orally administered antibiotic derived from a species of penicillium. It is used to treat dermatophytoses and must be given for long periods. Griseofulvin is poorly absorbed and concentrated in the stratum corneum, where it inhibits hyphal growth. It has no effect on other fungi.

Griseofulvin is clinically useful for the treatment of dermatophyte infection of the skin, hair, and nails. Oral therapy for weeks to months is usually required. Griseofulvin is generally well tolerated. The most common side effect is headache, which usually resolves without discontinuation of the drug. Less frequently observed are gastrointestinal disturbances, drowsiness, and hepatotoxicity (Sheehan *et al*, 1999).

3.3.5 Terbinafine

Terbinafine is an allylamine drug; it blocks ergosterol synthesis by inhibiting squalene epoxidase. Terbinafine is given orally to treat dermatophyte infections. It has proved quite effective in treating nail infections as well as other dermatophytoses. Side effects are not common but include gastrointestinal distress, headaches, skin reactions and loss of sense of taste. For the long-term treatment of tinea unguium, terbinafine-as well as itraconazole and fluconazole- may be given intermittently, using a pulse treatment protocol (Sheehan *et al*, 1999).

3.3.6 TOPICAL ANTIFUNGAL AGENTS

3.3.6.1 Nystatin

Nystatin is a polyene antibiotic, structurally related to amphotericin B and having a similar mode of action. It can be used to treat local candidal infections of the mouth and vagina. Nystatin may also suppress subclinical esophageal candidiasis and gastrointestinal overgrowth of candidia. No systemic absorption occurs, and there are no side effects. However, nystatin is too toxic for parenteral administration (Andriole, 1998).

3.3.6.2 Clotrimazole, Miconazole, & Other Azoles

A variety of antifungal azoles too toxic for systemic use are available for topical administration. Clotrimazole and miconazole are available in several formulations. Ecoconazole, butoconazole, tioconazole, and terconazole are also available. All of these compounds seem to have comparable efficacy.

Topical azoles have a broad spectrum of activity. Tinea pedis, Tinea corporis, Tinea cruris, Tinea versicolor, and cutaneous candidiasis respond well to local application of creams or powders. Vulvovaginal candidiasis can be treated with vaginal suppositories or creams. Clotrimazole is also available as an oral troche for treatment of oral and esophageal thrush in immunocompetent patients.

3.3.6.3 Other Topical Antifungal Agents

Tolnaftate and naftifine are topical antifungal agents used in the treatment of many dermatophyte infections and Tinea versicolor. Formulations are available as creams, powders, and sprays. Undecylenic acid is available in several formulations for the treatment of Tinea pedis and Tinea cruris. Although it is effective and well tolerated, antifungal azoles, naftifine, and tolnaftate are more effective. Haloprogin and ciclopirox are other topical agents commonly used in dermatophyte infections (Andriole, 1998).

3.4 Bacterial pathogens frequently recovered from HIV/AIDS individuals

3.4.1 *Streptococcus pneumoniae*

Introduction

The genera Streptococcus consist of chain-forming Gram-positive cocci that are facultative anaerobes. The pneumococcus is part of the normal naso and oropharyngeal flora of many healthy persons. Pneumococci can also be a primary cause of acute lobar pneumonia in previously healthy persons.

History/Origin

The pneumococcus was first identified in France and was found in United States in 1881. Subsequently, later on its recovery from all five continents was reported in 1939, indicating a wide geographic distribution. Despite intensive research, wide spread antibiotic use and mortality of pneumococcal pneumonia infections remains significant. *S. pneumoniae* remains the most common cause of bacterial pneumonia and otitis media worldwide.

Structure/Morphology

They are Gram-positive cocci occurring in pairs (diplococci) or, usually short, chains. The cocci are about 1⇑m in diameter; in diplococci they are ovoid or lanceolate in shape, with their distal ends narrowed. They are non-motile and non-sporing and all freshly isolated strains are capsulate.

Pathogenesis

HIV infections are responsible for immunologic defects not only in T-lymphocytes, but also in ℘lymphocytes, macrophages, polymorph nuclear cells and cytokine production. Although the primary immunologic defect in cell-mediated immunity depends in the humoral immune system also occurs and are easily immunologic squeal of HIV infection (Terpstra *et al*, 1989). The numerous abnormalities in the humoral immune system and photolytic cells increase the susceptibility of the HIV-1 infected person to bacterial infections particularly more caused by encapsulated bacteria.

In setting of HIV infection the predeposition to pneumococcal infection is probably due to dysfunctional host defenses rather than to increased colonization. Defect in mucus immunity could play a role in the susceptibility to pneumococcal infections because mucosal IgA may prevent bacterial adherence to mucosal surfaces and colonization with *S. pneumoniae* (Janoff *et al*, 1992)

The capsule of *S. pneumoniae* renders its resistant to phagocytosis. The ability to invade this important host defense mechanism allows *S. pneumoniae* to survive, multiply and

spread to various organs. The cell wall of *S. pneumoniae* consists of teichoic acid and the inflammatory response induced by Gram-positive cell wall differ from that induced by the endotoxin of Gram-negative organism, but does include recruitment of PMN neutrophils, changes in permeability and perfusion, cytokine release and stimulation of platelet activation factor. Other *S. pneumoniae* moieties in virulence is less clear, protein A, pneumolysis and peptide permease pleural effusion is the most common and empysemal pus in pleural space, one of the most serious complications of *S. pneumoniae*.

Clinical manifestations

Clinical presentation of pneumococcal pneumonia in patient with HIV infection is similar to that in normal host, most patients with pneumococcal pneumonia experience the acute onset of fever and productive cough (Forbes *et al*, 2002). In most patients with HIV disease and pneumonia, the complete blood cell count shows a relative leukocytosis.

Chest radiograph in-patient with pneumococcal pneumonia typically is abnormal and may be helpful in differentiating pyogenic pneumonia from other cause of lungs disease. Most patients present with segmental lobar or multilobar homogenous consolidation, (Polsky, *et al* 1986) although interstitial infiltrates have also been reported. Cavitation and pleural effusions is relatively uncommon in HIV infected patient with pneumococcal pneumonia.

Diagnosis

The approach to the diagnosis of pneumococcal pneumonia is same regardless of HIV serostatus. *S. pneumoniae* is usually isolated from sputum or blood. Precise streptococcal identification is based on the Gram stain and on biochemical properties, as well as on serologic characteristics when group antigens are present.

Identification

Hemolysis should not be used as stringent identification criterion. *S. pneumoniae* can be separated from other ℊhemolytic streptococci on the basis of sensitivity to surfactants, such as bile or optochin (ethyl hydrocupreine hydrochloride).

S. pneumoniae, which lacks a demonstrable group antigen by the Lancefield test, is *conventionally* identified by the quelling or capsular swelling test that employs type specific anticapsular antibody.

Epidemiology

S. pneumoniae is a human pathogen and have an environmental or zoonotic reservoir. It normally colonizes the nasopharynx, and prevalence studies have shown that it can be isolated from 5% to 10% of healthy adults and 20% to 40% of healthy children. The rate of colonization appears to be seasonal, with an increased prevalence seen during the winter.

Pneumococci are transmitted person-to-person by close contact. Transmission in day care centers has been demonstrated among infants and toddlers. In adults, transmission has been shown to be facilitated by crowded living conditions such as those found in prisons, military camps, homeless shelters, and nursing homes. Epidemics in schools, hospitals, or in the workplace are rare.

Population-based studies also have demonstrated that the incidence of pneumococcal disease in persons with HIV infection is extremely high. The respiratory tract is the most common site of invasive pneumococcal infection in HIV infected persons. Pneumococcal disease can occur at any time during the course of HIV infection.

S. pneumoniae continues to be a challenging organism to treat successfully in the 21st century. Surveillance data continue to reveal increasing resistance of *S. pneumoniae* to a variety of antimicrobial agents including penicillins, cephalosporins, macrolides, and quinolones. Currently, up to 40% of clinical infections are caused by a pneumococcal strain resistant to atleast one drug and 15% are due to a strain resistant to three or more drugs.

Treatment

As in the general population, the treatment of HIV related pneumococcal disease depends on the site of infection and whether or not the organism is susceptible to penicillin. HIV seropositive patients with moderate to severe pneumococcal pneumonia should be hospitalized and treated with parenteral antimicrobial agent. For hospitalized patient with documented penicillin sensitive pneumococcal pneumonia, penicillin (500,000 to 2,000,000 units intravenously every 4 to 6 hours) as the drugs of choice. Alternative antibiotic would include ampicillin first generation cephalosporin, macrolides and clindamycin. Milder cases of community acquired pneumoccal pneumonia may be treated with an oral regime as outpatient oral penicillin, first or second-generation cephalosporins, or macrolides would be appropriate. Most patients should be treated for approximately 10 days.

3.4.2 *Haemophilus* species

Members of the genus Haemophilus are obligatory parasites that colonize human and animal mucosae. The type species, *Haemophilus influenzae*, is associated with a wide range of respiratory and invasive infections, the latter occurring predominantly inchildhood.

H. influenzae is separated from most other *Haemophilus* species by its requirement for both X and V factors.

Haemophilus influenzae

It forms part of the normal commensal flora of the throat and naso pharynx of between 25 and 75% of healthy persons and acts opportunistically as a secondary invader in a variety of respiratory tract infections. It is frequently responsible for acute otitis media, sinusitis and infections involving the lower respiratory tract in patients with pre-existing pulmonary disease, such as chronic obstructive airways disease, cystic fibrosis or bronchiectasis. *H. influenzae* is the commonest bacterial pathogen associated with acute exacerbations of chronic obstructive airways disease.

Clinical features

Clinical presentation of *H. influenzae* infection does not differ from that in HIV negative patient. The presenting symptoms of fever and productive cough in cases of pneumonia are usually present. Out of 34 cases of *H. influenzae* pneumonia fever and productive cough is found in 100%cases, chest pain in 53% and dyspnea in 47%. Most patients had an elevated white blood cell count (Schlamn *et al*, 1989).

Diagnosis

Because history, physical examination and radiologic studies do not distinguish *H. influenzae* pneumonia from that caused by other bacteria, diagnosis require a positive culture. A culture of sputum confirms the diagnosis. A positive sputum culture alone should be interpreted with caution as *H. influenzae* can colonize the pharynx. A sputum Gram-stain with polymorphonuclear leucocyte, Gram-negative coccobacilli, and few epithelial cells are very suggestive in appropriate clinical settings (Collee *et al*, 1999).

Epidemiology

H. influenzae is one of the most common bacterial infections occurring in persons infected with HIV. *H. influenzae* is a well-known pathogen responsible for meningitis, epiglotitis, septic arthritis, cellulitis and bacteremia. It can also cause pneumonia, otitis and sinusitis as well as genital infections. Infections in adults are less common and tend to occur in elderly with chronic illness, or in younger adults with underlying medical condition associated with impaired immunity. Current reports suggest that infection increase the risk of acquiring *H. influenzae* infection (Musher *et al*, 1983; Moxon, 1995).

Prevention and Control

The species is naturally sensitive to a wide range of antibiotics, including ampicillin or amoxycillin, many of the newer cephalosporins, tetracyclines, sulphonamides, trimethoprim, fluoroquinolones and chloramphenicol. It has lesser intrinsic sensitivity to penicillin, erythromycin and ‘first generation’ cephalosporins (Campos *et al*, 1986).

3.4.3 Staphylococcal infections

Staphylococci are non-motile, non-spore-forming, and occasionally capsulate. Most are catalase positive. Their cell walls contain peptidoglycan (mucopeptide) and teichoic acids, important cell-adherence factors. Their peptidoglycan chains are linked by pentaglycine bridges (attacked by lysostaphin).

The most important human pathogen *S. aureus*, contains protein A, an antiphagocytic virulence factor, covalently incorporated into its cell wall. Most strains also contain 'clumping factor' (bound coagulase) on their outer surface, which binds to fibrinogen, thus causing the organisms to aggregate in plasma. Another (free coagulase) causes clotting of plasma in a tube test, and distinguishes this species from other human staphylococci (Gemmell, 1985).

Morphhology and Cultural Characters

S. aureus is approximately 1⇑m in diameter, and divides to form the clusters characteristic of the genus. On blood or nutrient agar, incubated in air for 18-24h at the optimal growth temperature of 37^0c, it forms colonies 1-3mm in diameter, although dwarf colonial forms are not uncommon. Colonies are smooth, low convex, glistening, densely opaque and of a butyrous consistency, sometimes surrounded by a narrow zone of haemolysis on blood agar, depending on the strain. Older colonies become translucent and sticky. Occasional strains are capsulated; their colonies are large, convex and glistening, becoming so slimy that run the surface of a tilted agar plate.
Pigmentation is characteristic of this species when grown aerobically, and ranges from cream through buff to gold.

S. aureus is tolerant of concentrations of sodium chloride that inhibit most other bacteria, and on mannitol salt agar it forms 1mm diameter yellow colonies surrounded by yellow medium due to acid formation.

Enzymes and toxins

Structural virulence factors of *S. aureus* include protein A, Capsule (some strains), and peptidoglycan. Enzymes include bound and free coagulases, nucleases, hyaluronidase,

proteinase, phosphatase, and fibrinolysin (staphylokinase). Recently, phosphatidyl inositol-specific phosphiliopase C has been proposed as a new virulence factor (Collee *et al*, 1999). Toxins include enterotoxins A-E, toxic shock syndrome toxin (TSST-1), epidermolytic toxins A and B, haemolysins ℑ, ℘↑, Ω and leucocidin.

S. aureus infections

Common infections caused by *S. aureus* include the following.

Pyogenic infections: folliculitis, impetigo, furuncles, carbuncles, breast abscess, post-operative wound infections, cellulites (skin and soft tissue); pyomyositis, psoas abscess (muscle); osteomyelitils, septic arthritis (bone); bronchopneumonia (especially post-influenzal), lung abscess, empyema (pulmonary); acute endocarditis of aortic or mitral valves, and of the tricuspid valve in intravenous drug users (cardic).

Disseminated infections: septicaemia, often with consequent metastatic secondary foci.

Toxin mediated illness: toxic shock syndrome, staphylococcal scalded skin syndrome, and staphylococcal food poisoning.

Diagnosis

The diagnosis is suspected in patients who have *S. aureus* in expectorated sputum and is established by recovery of *S. aureus* in blood cultures, empyema fluid, or transtracheal or transthoracic aspirates. False negative cultures for staphylococcus, unlike pneumococcus, are unusal.

Prognosis and treatment

The mortality rate is generally 30-40%, in part due to the serious associated conditions most patients have. Yet, a fulminant course with a lethal outcome sometimes occurs in previously healthy adults who develop this infection after influenza. Response to antibiotics tends to be slow, and convalescence is prolonged.

Most clinical isolates of *S. aureus* are resistant to benzylpenicillin, due to the production of a ℘lactamase that binds to the antibiotic and destroy its activity by opening it at the

℘lactam ring. Broad-spectrum penicillins such as ampicillin, amoxycillin and the ureidopenicillins are equally susceptible to staphylococcal ℘lactamase. Clavulanic acid inactivates staphylococcal ℘lactamase, and a combination of amoxycillin plus calvulanic acid has a place in therapy. Most of the cephalosporin antibiotics in current use are moderately resistant to staphylococcal ℘lactamases, though the newest of them are not as intrinsically active against staphylococci as the older ones.

Isolates of *S. aureus* resistant to penicillin and one or two other antibiotics such as tetracycline or erythromycin are not uncommon, and the injudicious use of topical antibiotics such as fusidic acid or mupirocin rapidly selects emerging resistant strains.

Of particular interest are *S. aureus* strains resistant to the ℘lactamase-resistant penicillins, methicillin, oxacillin, cloxacillin, flucloxacillin and others. Epidemic strains of these methicillin-resistant *S. aureus* (MRSA) are usually also resistant to several other antibiotics. During the past 15 years or so the appearance and spread world-wide of many such clones has caused major therapeutic problems in many hospitals, as well as diverting considerable resources to attempts at controlling their spread (Duckworth, 1989; Marples and Reith, 1992).

Methicillin resistance is a complex property, and more than one mechanism is involved (Fung-Tomc *et al*, 1991; Smith, 1992; Varaldo, 1993). Strains of MRSA that are ℘ lactamase-negatave can appear ‘penicillin-sensitive, methicillin-resistant’ on testing (Richardson and Quoraishi, 1990).

The major alternative is a cephalosporin, preferably cephalothin or cefamandole, cefazolin, or cefuroxime. Third-generation cephalosporins are somewhat less active than 1st or 2nd generation cephalosporins. Clindamycin is active against 90-95%.

Resistance to other antibiotics is achieved by a number of different mechanisms, depending on the class of antibiotic; these include membrane impermeability, alteration of the target site, and enzymic degradation of the antibiotic.

Multiple antibiotic resistances have profound implications for the treatment of staphylococcal infections. So far, all *S. aureus* strains remain sensitive to glycopeptide antibiotics vancomycin and teicoplanin, which are the mainstay of treatment of serious MRSA infections.

3.4.4 Gram-negative rods frequently isolated from HIV infected individuals

Gram-negative bacilli account for <2% of community-acquired pneumonias but for most nosocomial pneumonias, including fatal ones. The most important pathogen is *K. pneumoniae*, which causes Friedlander's pneumonia. Other usual pathogens are *P. aeruginosa*, *E. coli*, *Enterbacter* spp, *Proteus* spp, *Serratia marcescens*, and *Acinetobacter* spp. *P. aeruginosa* is a common pathogen in patients with cystic fibrosis, neutreopenia, AIDS, bronchiectasis, and pneumornias acquired in intensive care. Gram-negative bacillary pneumonias are rare in healthy hosts and usually occur in infants, the elderly, alcoholics, and debilitated or immunocompromised hosts, especially those with neutropenia (Brooks *et al*, 2002).

Cultural characteristics

E. coli can grow on simple media containing glucose as the sole carbon source. The optimal growth temperature is 36-37^{0}c. Most strains recovered in the clinical laboratory ferment lactose and thus grow as smooth, glossy, pink colonies on MacConkey's agar. At least 80% of strains are motile. All strains are methyl-red positive; they give negative H_2S in triple-sugar-iron (TSI) agar. Most strains form gas from glucose.

Klebsiella are non-motile. They are usually capsulate and can be recognized by their mucoid colonies on laboratory medium. The most frequently isolated klebsiellae ferment lactose.

P. aeruginosa is Gram-negative, non-sporing, motile rods. *P. aeruginosa* cultured from the respiratory tracts of patients with cystic fibrosis or other obstructive airways diseases. Six distinct colonial types of *P. aeruginosa* may be observed. Demonstration of the presence of the blue phenazine pigment pyocyanin is absolute confirmation of a strain as *P. aeruginosa* and thus the major diagnostic test. The yellow/green pigment

pyoverdin (flurescein) is also produces by most strains, giving the characteistic blue-green appearance of infected pus or cultures. Pyocyanin, pyoverdin and pyorubin are easily identified on nutrient or sensitivity test agars (Brooks *et al*, 2002).

Sign and Symptoms

E. coli often present in the lower respiratory tract, especially in surgical or otherwise debilitated patients who are being treated with antibiotics to which they are resistant. *K. pneumoniae* is responsible for Friedlander's pneumonia.

P. aeruginosa is the major cause of malignant otitis media, otitis externa. Panophthalmitis can result in partial blindness or loss of an eye. Endocarditis and septicaemia caused by *P. aeruginosa* is relatively rare but catties a mortality rate exceeding 70% in patients compromised by severe burns, cancer or drug addiction. Arguably, the most significant pathogenic role for *P. aeruginosa* at present is in the chronic debilitating pulmonary infections due to mucoid variants that are now the major cause of death in patients with cystic fibrosis.

Diagnosis

Gram-negative bacilli should be suspected in a patient with pneumonia who is in one of the risk categories noted above, especially with neutropenia or nosocomial pneumonia. Gram stain of sputum usually shows numerous gram-negative bacilli; however distinguishing the various species and genera on the basis of morphologic characteristics is impossible. Sputum cultures usually yield the pathogen. Positive cultures from blood, pleural fluid, or a transtracheal aspirate obtained before treatment are considered diagnostic.

Prognosis and treatment

The mortality for gram-negative bacillary pneumonia is about 25 to 50% despite the availability of effective antibiotics.

Antibiotics likely to be most effective for *P. aeruginosa* are the aminoglycosides tobramycin and gentamycin in combination with anti-pseudomonal penicillin such as

ticarcillin, or the ureido-penicillins, azlocillin and piperacillin. Newer agents with good activity include the carbapenems imipenem and meropenem and the monobactam aztreonam. Of the cephalosporins, ceftazidime has proved to be a useful non-toxic alternative to the aminoglycosides. Monotherapy with either ceftazidime or imipenem/cilastatin has been shown to be a safe and effective alternative to combination therapy for the treatment of serious hospital-acquired infections due to *P. aeruginosa* (Norrby *et al*, 1993). Quinolones, in particular ciprofloxacin, have provided a major advance as the ciprofloxacin, have provided a major advance as the first highly active anti-pseudomonal agents effective by oral administration.

For *Klebsiella*, a cephalosporin is the drug of first choice. TMP-SMZ, aminoglycoside, imipenem or meropenem, a fluoroquinolone, piperacillin, mezlocillin, aztreonam are the alternative drugs. Cephalosporins are also effective against *E. coli*.

3.4.5 Tuberculosis

Tuberculosis (TB) is a contagious disease that kills around 2 million people each year. One-third of the world's population is currently infected with TB and someone is newly infected every few seconds.

Relationship between TB and HIV

TB is the leading cause of death among HIV infected people; the WHO estimates that TB accounts for up to a third of AIDS deaths worldwide. When someone is infected with TB, the likelihood of them becoming sick with the disease is increased many times if they are also HIV positive.

Etiological agent of TB

TB is caused by a germ called *Mycobacterium tuberculosis*. A person can have active or inactive TB. Active TB or TB disease means the bacteria are active in the body and the immune system is unable to stop them from causing illness. People with active TB can pass the bacteria on to anyone they come into close contact with. When a person with active TB coughs, sneezes or spits, people nearby may breathe in the TB bacteria and

become infected. Left untreated, each person with active TB will infect on average between 10 and 15 people every year (Forbes *et al*, 2002).

People can also be infected with TB that is not active in the body. Inactive TB infection is also called latent TB. If a person has inactive TB, it means their body has been able to successfully fight the bacteria and stop them from causing illness. People who have inactive TB do not feel sick, do not have symptoms and cannot spread TB. In some people TB bacteria remain inactive for a lifetime without becoming active. But in some other people the inactive TB may become active TB if their immune system becomes weakened - for example by HIV. People with inactive TB are also called TB carriers (Brooks *et al*, 2002).

TB and HIV positive people

Because TB can spread through the air, the increase in active TB among people infected with both TB and HIV results in:

more transmission of the TB bacteria

more people with latent TB

more TB disease in the whole population.

People with latent TB are increasingly becoming infected with HIV, and many more are developing active TB because HIV is weakening their immune system. People who are co-infected with both HIV and latent TB have an up to 800 times greater risk of developing active TB disease and becoming infectious compared to people not infected with HIV.

People with advanced HIV infection are vulnerable to a wide range of infections and malignancies that are called 'opportunistic infections' because they take advantage of the opportunity offered by a weakened immune system. TB is an HIV related opportunistic infection. A person that has both HIV and active TB has an AIDS-defining illness.

There are several important associations between epidemics of HIV and TB:

TB is harder to diagnose in HIV positive people

TB progresses faster in HIV-infected people

TB in HIV positive people is more likely to be fatal if undiagnosed or left untreated

TB occurs earlier in the course of HIV infection than other opportunistic infections

TB is the only major AIDS-related opportunistic infection that poses a risk to HIV-negative people (TB in SAARC region, an uptate 2005).

Symptoms of TB

Symptoms of TB depend on where in the body the TB bacteria are growing. TB bacteria usually grow in the lungs. TB in the lungs may cause a bad cough that lasts longer than 2 weeks, pain in the chest and coughing up of blood or sputum. Other symptoms of TB disease include weakness or fatigue, weight loss, no appetite, chills, fever and night sweats (Brooks *et al*, 2002). Inactive TB has no symptoms.

Diagnosis

TB can be diagnosed by injecting a protein found in TB bacteria into the skin of an arm. If the skin reacts by swelling then the person is probably infected with TB. However, this method is not wholly reliable at detecting TB infections among HIV-infected people who have very weak immune systems.

Diagnosis of TB in the lungs may be made using an X-ray or sputum test. In cases of extrapulmonary TB (where the disease is affecting organs other than the lungs), fluid or tissue samples may be tested. But if the necessary facilities are not available then the TB diagnosis is often based on symptoms (Forbes *et al*, 2002).

Treatment

Active TB disease can almost always be cured with a combination of antibiotics. A proper combination of anti-TB drugs provides both prevention and cure. Achieving a cure takes about six months of daily treatment.

Several drugs are needed to treat active TB. Taking several drugs does a better job of killing all of the bacteria and is more likely to prevent them from becoming resistant to the drugs. To ensure thorough treatment, it is often recommended that the patient takes his or her pills in the presence of someone who can supervise the therapy. This approach is called DOTS (directly observed treatment, short course). DOTS cures TB in 95% of cases, and a six-month supply of DOTS costs as little as $10 per person in some parts of the world (WHO, 2005).

Prevention

A drug called isoniazid (INH) can be used as a preventative therapy for those who are at high risk of becoming infected with TB or for those who have inactive TB. People who have inactive TB but are not yet sick can take a course of isoniazid for several months to stop them developing active TB.

The WHO recommends that HIV positive people who have latent TB (but definitely not active TB) should be offered isoniazid preventive therapy as needed.

Multi-drug resistant TB (MDR-TB)

When a strain of TB is resistant to more than one antibiotic drug it is called multi-drug resistant TB or MDR-TB. These TB bacteria cannot be killed by the drugs usually used in DOTS. Drug resistance can arise when TB-patients do not take their medicine as prescribed. People can also catch MDR-TB from others.

MDR-TB is a serious problem and is very difficult to treat. The treatment of MDR-TB is much more expensive that DOTS and takes much longer. Being HIV positive does not of itself increase the chance of drug resistance (Telzak *et al*, 1999).

TB and HIV around the world

The importance of TB to the global HIV epidemic is enormous. Tuberculosis is a serious health problem in its own right but it is also the most likely cause of death for HIV positive people. Like HIV, TB has had an uneven impact around the world. In some parts of the world, TB is increasing after almost 40 years of decline. Escalating

TB rates over the past decade in many countries in sub-Saharan Africa and in parts of South-East Asia are mainly due to the HIV epidemic.

Between 1990 and 2005, TB incidence rates tripled in African countries with high HIV prevalence. Rates of TB are now rising across Africa by 3-4% annually. In 2004 an estimated 14 million people worldwide were living with dual HIV and TB infections, of which 70% were African.

The largest number of TB cases occurs in the South-East Asia Region, which in 2003 accounted for more than 3 million cases (more than a third of the global total). However, the estimated incidence per capita in sub-Saharan Africa is nearly twice that of the South-East Asia, at 345 cases per 100,000 populations in 2003. Also, the countries of Eastern Europe are facing a growing epidemic; there were over 143,000 estimated cases in Russia alone in 2003 (WHO, 2005).

TB is not only a problem in low and middle income countries. For example, there were 15,055 cases reported in the U.S. in 2002. TB in the UK is a disease that has never gone away, with 6,889 new cases reported in 2002. Although the UK's national rate is very low in comparison with most of the world, London has become one of the world's TB hotspots. In parts of London, TB rates are ten times the national rate - higher than in some countries of the former Soviet Union. About 10 per cent of people with TB in London are likely to be co-infected with HIV.

Worldwide TB control

Since DOTS was introduced on a global scale in 1991, more than 17 million people have received DOTS treatment. By the end of 2002, all 22 of the countries with the highest number of TB cases, which together have 80% of the world's estimated incident cases, had adopted the DOTS strategy. In total, 182 countries were implementing the DOTS strategy by the end of 2003, and 77% of the global population was living in parts of countries where the DOTS strategy was in place. Around 37% of all infectious TB cases were detected and then cured by high quality DOTS treatment services in 2003.

The emergence of multi-drug resistant TB threatens TB control efforts across the world, including in well resourced countries. In recent years, drug-resistant TB, including multidrug-resistant TB, was found in all regions of the world. The prevalence of MDR-TB was exceptionally high in almost all former Soviet Union countries surveyed, with drug resistance in new patients as high as 14%. High prevalence of MDR-TB was also found among new cases in China, Ecuador and Israel. Central Europe and Africa, in contrast, reported the lowest median levels of drug resistance.

WHO's leading infectious disease experts estimate there are 300,000 new cases per year of MDR-TB worldwide. There is also new evidence proving drug resistant strains are becoming more resistant, and unresponsive to current treatments. 79% of MDR-TB cases are now "super strains", resistant to at least three of the four main drugs used to cure TB (WHO, 2005).

3.4.6 Moraxellas infections

The moraxellas occur as components of the normal flora of the upper respiratory tract, the conjunctiva, the skin and the genital tract. The moraxellas are stout gram-negative cocci. They are strict aerobes, non-capsulate, non-motile (Brooks *et al*, 2002).

Morphology and staining

They are oval gram-negative cocci about 0.8⇑m in diameter. Sometimes organisms are single, but more often in pairs with adjacent sides flattened, occasionally found in groups of four as a result of characteristic division in two successive planes at right angles to one another. On occasion they may be found inside polymorphonuclear leucocytes.

Cultural characteristic

Aerobe with optimum temperature about 36^0c but growth of many strains occurs at 22^0c. Although CO_2 may enhance growth there is no absolute requirement. Most strains grow on nutrient agar. After incubation for 24h, colonies on blood or heated blood agar are 1-2 mm in diameter, non-haemolytic, often friable, white or greyish, convex with an

entire margin later becoming irregular. After 48h colonies are larger, more elevated with a raised opaque centre.

Laboratory diagnosis of *Moraxella catarrhalis*

M. catarrhalis is normally considered to be a harmless commensal of upper respiratory tract and is most often encountered when examining throat swabs and specimens of sputum. The finding of a few colonies of *M. catarrhalis* in a mixed culture containing other upper respiratory tract commensal organisms is probably of little or no significance. However in patients with compromised lung function, *M. catarrhalis* may be a pathogen of lower respiratory tract. In these patient a relatively pure growth of *M. catarrhalis* is often obtained from sputum and other specimens such as transtracheal aspirates.

Treatment

They are susceptible to a wide range of antibiotics but many strains produce β lactamase and are resistant to penicillin and ampicillin.

CHAPTER-IV

4. MATERIALS AND METHODS

4.1 Patient selection

This study has included HIV/AIDS individuals from Sparsa Nepal, Nabakiran plus, Vision plus, Maiti Nepal, Karuna Bhavan, Snehi samaj, Deep Jyoti, Shakti Milan Kendra and Nepal medical college. All confirmed HIV/AIDS individuals with or without respiratory tract infections were selected for the study.

4.2 Patient- information

Patient's name/code, demographic data, clinical symptoms, secondary infections, chronicity and other information were recorded from their case files, by interviewing with them or their care-takers, or by laboratory testing in a questionnaire (Appendix-1).

4.3 Specimen collection

Sputum samples were collected in a wide mouthed, sterile bottle and brought directly to the laboratory. As the sputum sample may contain some fastidious pathogens like *H. influenzae, S. pneumoniae,* it was processed soon after its collection. Patients were advised to collect 2ml of early morning sputum sample. Single specimen was collected from each individual.

4.4 Processing of sample

4.4.1 Macroscopic examination

This included the visual examination of the sputum specimens for

Consistency: Sputum may be purulent, mucopurulent, mucoid or salivary

Purulent : Green-looking, mostly pus

Mucopurulent: Green-looking with pus and mucus

Mucoid: Mostly mucus

Mucosalivary: Mucus with a small amount of saliva

When the sputum sample received contained mostly saliva, it was reported as "unsuitable for microscopic investigation" and requested for another appropriate sample.

Color

Red sputum: It indicates that the sample is contaminated with blood.

Green sputum: Green colored sputum usually contains *P. aeruginosa* as possible pathogens.

Brown sputum: It indicates the air is polluted with CO_2 and CO

4.4.2 Microscopic examination

Microcopic examination was performed for the detection and identification of pathogens. Specimens were examined as soon as possible after collection.

Microscopic examination was done by

Gram's staining

Ziehl-Neelsen (Z-N) staining

Culture of sample

Following culture media were used for the culture of sputum sample

Chocolate agar

Blood agar

MacConkey agar

Nutrient agar

Sabouraud's dextrose agar

4.4.3 Identification of isolated organisms

For the identification of isolated organisms, following microbiological techniques were performed.

Study of colony morphology/cultural characteristics

Study of colony morphology involved the observation of following characteristics:

Shape

Size

Elevation

Margin

Pigmentation

Haemolysis

Opacity

Consistency

Biochemical tests

Following biochemical tests were performed for the identification of the isolates as given by

Catalase test
Oxidase test
Coagulase test
Free coagulase
Bound coagulase
Indole test
Methyl-red test
Voges-Proskauer (VP) test
Citrate-utilization test
Triple-sugar iron test
Urea-hydrolysis test
Oxidation- fermentation
Nitrate reduction test
Motility test
Capsule staining

Some other specific tests

Satellitism test

Optochin sensitivity test

Bacitracin sensitivity test

Bile solubility test

Some tests for the identification of yeast-like fungi

Following methods were applied for the identification of yeast like fungi and filamentous fungi:

Germ tube production test

Lactophenol cotton blue mount

4.5 Antibiotic susceptibility test (Kirby-Bauer disc diffusion method)

After the isolation and identification of pathogenic organism from patients with disease, it is essential to determine the proper therapy. In present study, we performed the susceptibility test was performed by Kirby-Bauer method.

In this method, the test strain was uniformly inoculated on Mueller Hinton agar (MHA) plate and antibiotic discs are placed at the centre and periphery of plate (distance from centre to centre of the discs should be 2.4cm). Usually, 6 discs were placed on a 100 mm plate. Zone of inhibition was noted after overnight incubation at 37^0c.

Flowchart of the Methodology

CHAPTER-V

5. RESULTS

A total of 120 patients clinically diagnosed as having HIV/AIDS were included in the study. Sputum samples were collected from Sparsa Nepal, Nava Kiran, Maiti Nepal, Karuna Bhavan, Vision Plus, Deep Jyoti, Sneha Samaj, Shakti Milan Kendra and Nepal Medical College.

The study was conducted among 120 individuals; 69 males and 51 females. **Table-1** shows the pattern of microbial distribution in direct microscopic examinations (Gram's staining and AFB staining): Gram-positive bacteria-10, Gram-negative bacteria-22, Acid-fast bacilli (AFB)-4 and fungal isolates –16 among male patients whereas among female patients: Gram-positive bacteria-9, Gram-negative bacteria-15, AFB-2 and fungal isolates-20. Among 69 male patients, 45 (65.2%) samples showed positive growth on different cultural media whereas among 51 female patients, 33 (64.7%) gave positive growth on different cultural media **Table-2**.

Table-1: Findings of direct microscopic examination

Sex	Total	Bacteria (%)			Fungal elements (%)	AFB (%)
		Gram positive	Gram negative	Total		
Male	69	10 (14.4%)	22 (31.8%)	32 (46.3%)*	16 (23.1%)**	4 (5.7%)***
Female	51	9 (17.6%)	15 (29.4%)	24 (47.0%)*	20 (39.2%)**	2 (3.9%)***
Total	**120**	**19 (15.8%)**	**37 (30.8%)**	**56 (46.6%)**	**36 (30.0%)**	**6 (5.0%)**

* (P>0.05); ** (P>0.05); *** (P>0.05)

Table 2: Gender-wise culture positive rate

Sex	Total	Bacterial/Fungal growth	%	P-value
Male	69	45	65.2	P>0.05
Female	51	33	64.7	
Total	**120**	**78**	**65.0**	

K. pneumoniae was the predominant (28.6%) bacterial pathogens, followed by *E. coli* (17.9%) and *S. pneumoniae* (17.9%) (**Table-3**). Other bacterial isolates were *S. aureus* (16.1%), *P. aeruginosa* (8.9%), *M. catarrhalis* (7.0%) and *H. influenzae* (3.6%).

Table-4 shows that among the fungal pathogens, *C. albicans* was found to be predominant (44.4%) followed by *Aspergillus* spp. (30.6%), germ tube negative *Candida* spp (16.7%) and *Penicillium* spp. (8.3%).

Table 3: Types and frequency of bacterial isolates

Bacterial pathogens isolated	N	%
K. pneumoniae	16	28.6
E. coli	10	17.9
S. pneumoniae	10	17.9
S. aureus	9	16.1
P. aeruginosa	5	8.9
M. catarrhalis	4	7.0
H. influenzae	2	3.6
Total	**56**	**100**

Table-4: Types and frequency of fungal isolates

Fungi isolated	**N**	**%**
C. albicans	16	44.4
Aspergillus spp	11	30.6
Candida spp	6	16.7
Penicillium spp	3	8.3
Total	**36**	**100**

Prevalence of pathogens was shown in **Table-6**. It was high (65.5%) among age group above 40 whereas it was quite less (63.3%) among patients of age group 20-40. There was no growth among age group below 20. However, this difference was found to be statistically insignificant (P> 0.05).

Table-5: Polymicrobial isolation from HIV infected individuals

Pathogens isolated	**N**	**%**
C. albicans, E. coli	6	31.6
Aspergillus spp, *K. pneumoniae*	3	15.8
AFB, *E. coli*	2	10.5
E. coli, K. pneumoniae	2	10.5
Aspergillus spp, *P. aeruginosa*	2	10.5
AFB, *S. pneumoniae*	2	10.5
C. albicans, P. aeruginosa	2	10.5
Total	**19**	**100**

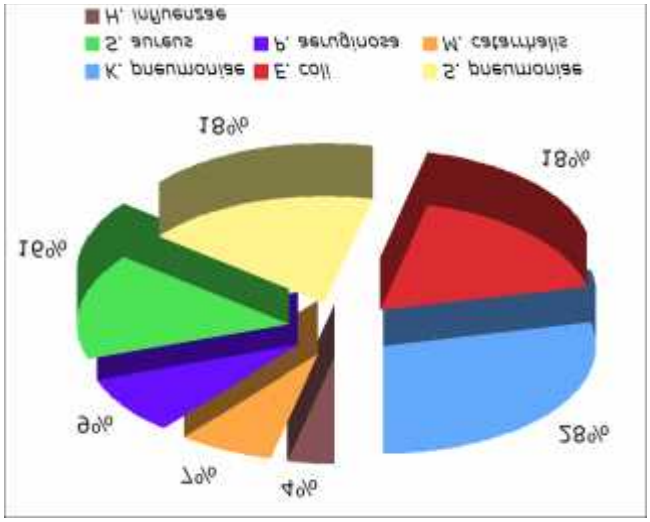

Fig-3 Pattern of bacterial infection among HIV seropositive individuals

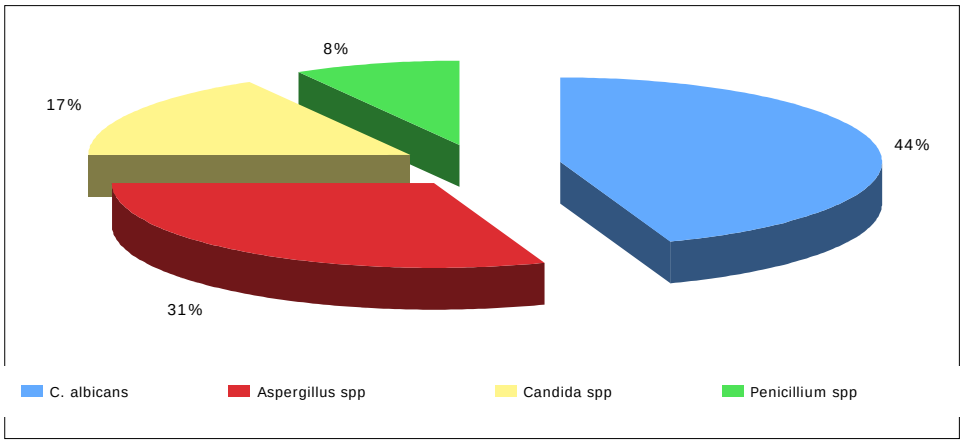

Fig-4 Pattern of fungal infection among HIV seropositive individuals

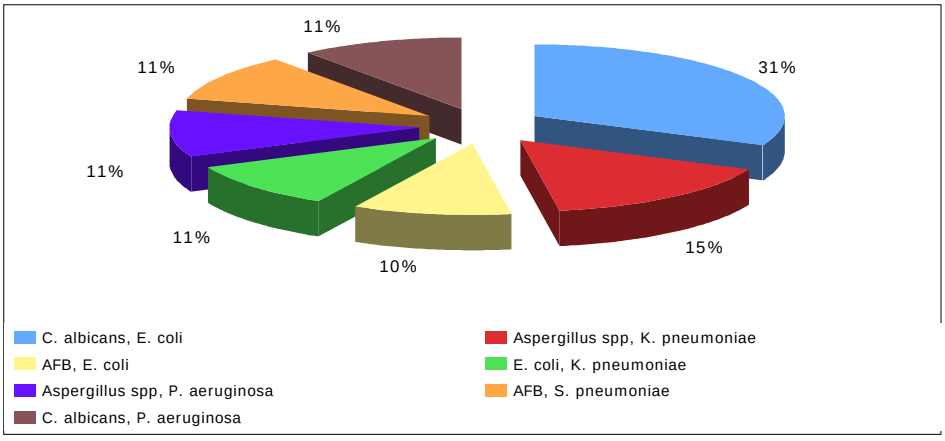

Fig-5 Pattern of polymicrobial infection among HIV seropositive individuals

Table-6: Prevalence of pathogens among different age groups

Age (Yrs)	Total	Growth positive	%	P-value
<20 Yrs	1	0	0.0	P>0.05
20-30 Yrs	40	26	65	
30-40 Yrs	50	32	64	
40-50 Yrs	29	20	65.5	
Total	**120**	**78**	64.8	

Among different ethnic groups, as shown in **Table-7**, *Dalit* was the group with high (70.0%) pathogen prevalence rate followed by *Tibeto-Burman* (66.7%) and *Indo-Aryan* (57.7%) groups. Here this difference in pathogen prevalence rates was not statistically significant (P>0.05).

Table-7: Prevalence of pathogens among different ethnic groups

Ethnic groups	Total	Growth positive	%	P-value
Dalit	10	7	70.0	P>0.05
Indo-Aryan	26	15	57.7	
Tibeto-Burman	84	56	66.7	
Total	**120**	**78**	**64.8**	

In this study, 65 individuals were studied who were living in different rehabilitation centers in Kathmandu and 55 individuals not living in the rehabilitation centers. Among

rehabilitation residents 35 (53.8%) were found to be infected with different bacterial and fungal pathogens and among those who were living in the community, 43 (78.1%) were found to be infected with different bacterial and fungal pathogens. There was association of LRTI with the resindential status of the study population (**Table-8**).

Table-8: Residential status and LRTI among HIV seropositive individuals

Residential status	**Total**	**Growth positive**	**%**	**P-value**
Living in rehabilitation center	65	35	53.8	P<0.05
Not Living in rehabilitation center	55	43	78.1	
Total	**120**	**78**	**65.9**	

Table-9: Antibiotic susceptibility pattern of the bacterial isolates

Antibiotics used / Bacterial Isolates	Chloramphenicol			Co-trimoxazole			Ampicillin			Gentamicin			Tetracycline		
	S	**R**	**% Sensitivity**	**S**	**R**	**% Sensitivity**	**S**	**R**	**% Sensitivity**	**S**	**R**	**% Sensitivity**	**S**	**R**	**% Sensitivity**
K. pneumoniae	10	6	62.5	6	10	37.5	8	8	50.0	9	7	56.2	7	9	43.7
E. coli	6	4	60.0	4	6	40.0	2	8	20.0	6	4	60.0	4	6	40.0
S. pneumoniae	5	5	50.0	3	7	30.0	2	8	20.0	5	5	50.0	5	5	50.0
S. aureus	5	4	55.5	3	6	33.3	2	7	22.2	5	4	55.5	5	4	55.5
P. aeruginosa	0	5	0	0	5	0	1	4	20.0	3	2	60.0	2	3	40.0
M. catarrhalis	2	2	50.0	2	2	50.0	1	3	25.0	2	2	50.0	1	3	25.0
H. influenzae	1	1	50.0	0	2	0	1	1	50.0	1	1	50.0	1	1	50.0
Total	**29**	**27**	**51.8**	**18**	**38**	**32.1**	**17**	**39**	**30.3**	**31**	**25**	**55.3**	**25**	**31**	**44.6**

CHAPTER-VI

6. DISCUSSION AND CONCLUSION

6.1 Discussion

Infections with opportunistic pathogens have been one of the hallmarks of AIDS since the beginning of the epidemic. An abundance of research and literature has been dedicated to these opportunistic fungi, viruses, and parasites. Less attention has been given to the bacterial infections complicating the course of persons infected with HIV. Even before HIV was found to be the causative agent of the syndrome, however, case reports appeared describing fulminant bacterial infections in these immunocompromised patients. It is now recognized that bacterial pneumonia and bacteremia occur at a higher frequency among HIV-infected patients compared to age-matched controls (Hirschtick *et al*, 1995; Meyer *et al*, 1994). Diseases caused by bacteria are responsible for a significant proportion of the morbidity and mortality seen in this population. Bacterial infections are the leading cause of death in HIV-infected patients (Nichols *et al*, 1989; Stein *et al*, 1992).

Among the opportunistic infections associated with HIV, diseases like pneumonia of bacterial origin occur at a rate many times higher in HIV infected patients than in the general population (Shailaja *et al*, 2004). In the present study, the bacterial isolates from the HIV infected patients were higher and also polymicrobial etiology in some of these patients is a significant finding, indicating the severity of the infection in this group.

As of June 30, 2006, 6990 HIV seropositive cases have been detected, with 1085 cases of AIDS (NCASC, 2006). As per the figures from National AIDS control organization (NACO), bacterial infection constituted 7% of opportunistic infections and the common organisms encountered in pulmonary infections were *S. pneumoniae*, *H. influenzae* and *S. aureus*. Tamang *et al* (2005) from Manipal Teaching Hospital, Pokhara has reported *H. influenzae* in 26.86%, *S. pneumoniae* in 21.16% and *M. catarrhalis* in 6.90%. In the present study, out of all the pathogens isolated, bacterial isolates constituted 51.4%

among which *K. pneumoniae* was isolated in 28.6% of cases and *E. coli* in 17.9% and in *S. pneumoniae* 17.9 %. Tchamran in his study on the lung diseases due to common bacteria in HIV infected individuals in African adults, noted 81% of infections due to *S. pneumoniae* and reported it to be the most offending pathogen in HIV reactive patients (Tchamran, 1997).

Concerning *S. aureus*, Levine *et al* (1990) recovered this pathogen in 23% of respiratory tract cultures performed in 129 consecutive HIV infected patients with an episode of respiratory disease. According to them, presence of *S. aureus* was found to be community acquired pneumonia in 28% of cases, of indeterminate significance in 62% and colonization in 10%. None of the patients with pneumonia were neutropenic or on corticosteroids. *S. aureus* was isolated in 16.1% in present study. *E. coli* is an uncommon cause of LRTI.

Steninhard *et al* (1992) determined the incidence of invasive *H. influenzae* disease in man with AIDS or HIV infection. According to them the cumulative incidence of invasive *H. influenzae* diseases in man 20-40 years of age with AIDS and in HIV infected men 20-49 years of age without AIDS were 79.2 and 14.6 per 100,000 respectively. Casadevalla *et al* (1992) reported 10 of 15 cases of adult *H. influenzae* type b bacteremia occurred in-patient with AIDS or who were at risk for AIDS. In present study, *H. influenzae* was reported in 3.6%.

P. aeruginosa infection in patients with HIV is often community acquired and is associated with substantial mortality. Dropulic in his study on the clinical manifestations of *P. aeruginosa* infection among patients with AIDS found that of the 73 episodes of *P. aeruginosa* infections, 13 were that of pneumonia (Dropulic *et al*, 1995). In the present study, 5 (8.9 %) of the isolates were *P. aeruginosa.*

M. catarrahalis is generally considered a commensal in the upper respiratory tract of adults, and its isolation from sputum is often reported as normal flora of the oropharynx. This appears to be a misconception as Sehgal and Shaimy (1994) reported this organism as the second most common isolate from patients suffering with lower respiratory tract

infections. In the present study *M. catarrhalis* was repeatedly isolated in 4 (7.0%) cases and was considered a pathogen. In all these cases the sputum was heavily loaded with the organisms. This organism needs to be looked for in HIV reactive patients when LRTI is suspected.

As of the end of December 2002 (UNAIDS update), the estimated number of PLWHA globally was 42 million (36.1 million at the end of 2000 and 40.1 million at the end of 2001), of which approximately 92% were adults and 8% children. During the year 2002, an estimated 5 million persons became newly infected with HIV (4.2 million adults and 0.8 million children) and 3.1 million (2.5 million adults and 0.6 million child) died. Since the beginning of documenting the burden of HIV/AIDS globally, there have been 25.4 million deaths due to HIV/AIDS. The trends in global HIV burden suggest that women and children are gradually facing more risk (UNAIDS, 2000; UN UNAIDS, 2001; AIDS 2002; UNAIDS, 2002).

Approximately one-third of the 36.1 million PLWH globally at the end of 2000, were co-infected with MTB, with 22% of these co-infected persons living with South-east Asia; the SAARC region countries comprising 17% of this co-infected total. With in Thailand, 60% of AIDS sufferers presenting at a Bangkok hospital between 1985 and 1991 had pulmonary TB suggesting that TB is most commonly reported cause of hospitalization and death among patients with AIDS in Thailand. In the Infectious Disease Hospital in Myanmar, 82% of the first 63 patients with AIDS had TB (WHO, 2002). Similarly, surveillance data from the SAARC region countries, India and Nepal revealed that 83% and 56% of patients with AIDS had TB of any organ. Percentage of adult (15-49y) TB/HIV co-infection is 2.9 in Nepal (Estimates, 2003)

About 55-89% of AIDS cases in India, were found to be suffering from extensive pulmonary TB. In the present study, *M. tuberculosis* was isolated in 6 HIV patients, out of 120 HIV seropositive individuals.

Selwyn *et al (*1989) report that in HIV-infected injection drug users, pyrogenic bacterial infections are both a substantial cause of pre-AIDS morbidity and mortality and a

significant predictor of progression to AIDS. In this prospective study of a cohort of patients from a methadone clinic, 13/318 HIV-infected patients without a diagnosis of AIDS died of bacterial infections, while1/411 HIV-negative patients died of bacterial infections.

Infections with non-typhoidal *Salmonella* have been described in patients with impaired host defenses, such as those with neoplastic disease, transplantation, cirrhosis, collagen vascular disease, renal failure requiring hemodialysis, and need for immunosuppressive drugs. An increased incidence of nontyphoidal salmonellosis in HIV-infected persons was originally noted in the early 1980s and nontyphoidal *Salmonella* septicemia became an AIDS-defining illness in 1987. Bacteremia, relapses, and severe disease are unusual in the immunocompetent host but characteristic of *Salmonella* infection in the HIV-infected population. Salmonellosis and bacteremia occur at an increased rate in persons with HIV (Jacobs *et al* 1985; Levine *et al*, 1991; Profeta *et al*, 1985; Smith *et al*, 1985; Sperber *et al*, 1987). A characteristic feature of salmonellosis in AIDS is the relapses that occur despite appropriate antibiotic therapy (Fischl *et al*, 1986; Glaser *et al*, 1985; Jacobs *et al*, 1985; Levine *et al*, 1991; Nadelman *et al*, 1985; Sperber *et al*, 1987). *S. typhimurium* and *S. enteritidis* are the two most common serotypes isolated from the blood of patients with AIDS in the United States.

Salmonella can cause focal infections in both the immunocompetent and immunocompromised host. Cases of endovascular infection, lung abscess, peritonitis, septic arthritis, osteomyelitis, brain abscess, subdural empyema, and meningitis have all been reported in persons with AIDS (Aliaga *et al*, 1997; Fernandezet *et al*, 1997).

Although less common than bacterial infections, serious fungal infections occur in the immunocompromised patient both as new infection and as reactivation of latent disease. Fungal pulmonary infections often precede the appearance of other opportunistics, but frequently co-exist with other pathogens (Rosen, 1994). Though pulmonary candidiasis is documented to be a very rare disease, occurring in late stages of AIDS, oral and oesophageal candidiasis is reported as the second most common (58%) opportunistic infection among HIV patients, from India. In the present study, 36 fungal isolates were

recovered from the sputum. *C. albicans* has been identified in 16/36 fungal isolates. To rule out the possibility of oropharyngeal colonization, a common feature among the HIV reactives, those cases with plenty of pseudohyphae on smear examination were considered as significant pathogens.

Meyohas, in a 7-year study on AIDS patients, reported isolated cases of aspergillosis from patients with a predisposing neutropenia due to HIV or steroid therapy (Meyohas *et al*, 1994). Shivananda (1992) in his study in 1192 on 825 patients with pulmonary infections found 15.39% of isolates to be *Aspergillus* species. Of these, *A. fumigatus* were 11.15%, *A. niger* were 3.2% and *A. flavus* was 0.96%. In her study of repeated sputum samples, Geetalakshmi (1999) from Chennai, documented pulmonary aspergillosis in 36 samples. The isolates from this study were *A. fumigatus, A. niger* and *Candida* species. Punkajalakshmi *et al* (1997*)* from Chennai, in her review, emphasized the emergence of candidiasis, aspergillosis, penicillosis and other pheohyphomycosis in patients infected with HIV. In present study *Aspergillus* spp were found to be 30.6%.

There is a high incidence of penicilliosis in AIDS patients in SEA; 10% of patients in Hong Kong get penicillosis as an AIDS-related illness. Cases of *P. marneffei* human infections (penicillosis) have also been reported in HIV-positive patients in Australia, Europe, Japan, the UK and the U.S. All the patients had visited Southeast Asia previously (Sirisanthana *et al*, 1998).

Although both the immunocompetent and the immunocompromised can be infected, it is extremely rare to find systemic infections in HIV-negative patients. The incidence of *P. marneffei* is increasing as HIV spreads throughout Asia. Infection rarely was documented before the AIDS epidemic. The first report of natural infection with *P. marneffei* was in a person with Hodgkin lymphoma who had lived in Southeast Asia (Disalvo *et al*, 1973). Only 8 cases of infection with *P. marneffei* were reported between 1964 and 1983 (Deng *et al*, 1985). The prevalence of infection has increased substantially, especially in persons who are infected with HIV (Supparatpinya *et al*, 1994). There were 92 cases diagnosed from 1987 to 1992 in Chiang Mai University Hospital, involving 86 patients who also were infected with HIV. Currently, this

infection is the third most common opportunistic pathogen in patients with AIDS in Thailand, after tuberculosis and cryptococcosis, despite the fact that it is endemic only to the northern part of Thailand (Sirisanthana *et al*, 1998). In this present study, *Penicillium* spp was found to be 8.3 %.

Shailaja *et al* has reported 44.28% of bacterial isolates, among which *K. pneumoniae* was isolated in 32.26%, *S. pneumoniae* in 25.81%, *S. aureus* in 12.9% *P. aeruginosa* in 9.68%, *M. catarrhalis* in 9.68% and *M. tuberculosis* in 42.89% in her study of LRTIs in patients with HIV infections. Similar organisms were also observed in present study.

K. pneumoniae was reported in 28.6%, *E. coli* in 17.9%, *S. pneumoniae* in 17.9%, *S. aureus* in 16.1%, *P. aeruginosa* in 8.9 %, *M. catarrhalis* in 7.0%, *H. influenzae* in 3.6 % and AFB in 5.0%. *C. albicans, Aspergillus* spp, *Penicillium* spp were also reported in 44.4%, 30.6% and 8.3%. *C. neoformans, H. capsulatum, C. immitis, B. dermatitidis* and *P. carinii* infections were not reported in present study. *Nocardia* spp, *Rhodococcus equi,* and *Legionella* spp were also not reported.

Polymicrobial etiology was also reported in present study. Their frequency of isolation were found to be: *E. coli* and *C. albicans* in 6 (31.6%), AFB and *E. coli* in 2 (10.5%), *K. pneumoniae* and *Aspergillus* spp in 3 (15.8%), *K. pneumoniae* and *E. coli* in 2 (10.5%), *P. aeruginosa* and *Aspergillus* spp in 2 (10.5%), AFB and *S. pneumoniae* in 2 (1.0.5%) and *P. aeruginosa* and *C. albicans* in 2 (10.5%).

6.2 Conclusion

Lower respiratory illness is a major clinical problem for patient infected with HIV/AIDS. *M. tuberculosis* is the major pathogen resulting in the morbity and mortality of these populations and second being the pneumonia. *C. albicans* is the major fungal pathogen isolated from patient infected with HIV/AIDS. Emergences of antibiotic resistant bacteria are threat for the patient with HIV.

CHAPTER-VII

7. SUMMARY AND RECOMMENDATION

7.1 Summary

Altogether 120 sputum's were collected from Nava Kiran, Vision plus, Deep Jyoti, Sneha Samaj, Shakti Milan Kendra, Maiti Nepal, Karuna Bhavan and Nepal Medical College.

- Presence of bacterial and fungal pathogens in sputum of HIV infected individuals were examined.
- The sputum samples were first preceded for direct microscopic examination and then subjected for culture.
- Z-N staining was performed to observe Acid-Fast Bacilli.
- Out of 120 HIV infected individuals, 78 (65%) gave positive growth on different cultural media. Among the bacterial pathogens *K. pneumoniae* (28.6%) was found to be most predominant followed by *E. coli* (17.9%), *S. pneumoniae* (17.9%), *S. aureus* (16.1%) *P. aeruginosa* (8.9%), *M. catarrhalis* (7.0%), *H. influenzae* (3.6%), and AFB (5.0 %).
- Among the fungal pathogens, *Candida albicans* was most predominant (44.4%) followed by *Aspergillus* spp (30.6%) and *Penicillium* spp (8.3%).
- Fungi like *C. neoformans, H. capsulatum, C. immitis, B. dermatitidis, P. carinii* and bacteria like *Nocardia* spp, *Rhodococcus equi*, *Legionella* spp were also not reported in present study.
- Among three ethnic groups, *Dalit* group was found to be more susceptible (70%) for infection.
- The prevalence of pathogens was found to be higher (65.5%) among HIV seropositive subjects of age 40-50 years.

- The prevalence of pathogens was found to be higher (78.1%) among HIV seropositive subjects not living in rehabilitation center.
- Polymicrobial isolation was also observed. Their frequency of isolation were found to be: *E. coli* and *C. albicans* in 6 (31.6%), AFB and *E. coli* in 2 (10.5%), *K. pneumoniae* and *Aspergillus* spp in 3 (15.8%), *K. pneumoniae* and *E. coli* in 2 (10.5%), *P. aeruginosa* and *Aspergillus* spp in 2 (10.5%), AFB and *S. pneumoniae* in 2 (1.0.5%) and *P. aeruginosa* and *C. albicans* in 2 (10.5%).
- Antibiotic susceptibility pattern was examined for bacterial isolates.
- Among Gram-negative bacteria, *K. pneumoniae* was most sensitive towards chloramphenicol (62.5%) whereas least sensitive towards co-trimoxazole (37.5%). *E. coli* showed 60% sensitivity towards chloramphenicol and gentamicin whereas 20% sensitivity towards ampicillin. *P. aeruginosa* was most sensitive towards gentamicin (60%) and least sensitive towards chloramphenicol. *M. catarrhalis* showed 50% sensitivity towards chloramphenicol, co-trimoxazole and gentamicin whereas 25% sensitivity towards ampicillin and tetracycline. *H. influenzae* showed 50% sensitivity towards chloramphenicol, ampicillin, gentamicin and tetracycline whereas least sensitive towards co-trimoxazole.
- Among Gram-positive bacteria, *S. pneumoniae* showed 50% sensitivity towards chloramphenicol, gentamicin and tetracycline whereas 20%sensitivity towards ampicillin. *S. aureus* showed 55.5% sensitivity towards chloramphenicol, gentamicin and tetracycline whereas 22.2% sensitivity towards ampicillin.

7.2 Recommendation

- As present study has shown a high rate of pathogen prevalence, physician should prescribe every HIV infected individuals to do sputum culture.
- Further research should be focused on viral infection that may trigger the bacterial/fungal infections.
- Serotyping should be done in order to specify the pathogens.
- This type of study can be carried among intravenous drug user groups.
- Incidence of infection can be studied throughout the year to obtain the seasonal frequency of the opportunistic infections.
- *S. aureus*, *E. coli*, *P. aeruginosa* are the frequently isolated pathogens in present study which are the most common agents involved in nosocomial infections as well. To prevent this, antibiotic and disinfectant policy should be set up in each hospital so as to prevent the infection.
- If the patient is found to be infected with fungal pathogens, broad-spectrum antibiotics should be withdrawn if possible.
- Further study can be conducted including equal number of non HIV seropositive individuals.

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APPENDIX-1

Questionnaire

Name of the institution:

Address:

Patient's name/code:

Sex:

Marital status:

Height:

Weight:

Complaints (symptoms if any)

First diagnosis of HIV:

Stage of HIV related syndrome:

Any secondary/opportunistic infection diagnosed:

Medication:

Report of the study:

Results of sputum examination

Macroscopic examination:

Color:

Blood:

Microscopic examination:

Gram staining:

Negative staining:

Giemsa staining:

LCB staining:

AFB staining:

Bacterial culture report:

Choice of drug based on antibacterial susceptibility testing:

Suggestion based on study:

APPENDIX-II

COMPOSITION AND METHOD OF PREPARATION OF DIFFERENT CULTURE MEDIA, BIOCHEMICAL MEDIA AND REAGENT USED FOR ISOLATION AND SENSITIVITY TESTING OF BACTERIA ISOLATED FROM SPUTUM SAMPLE

I. CULTURE MEDIA

Different types of culture media such as enrichment media, selective media and differential media were used. Composition and preparation of different types of culture media are given below. All the media used are supplied by Hi media company and final pH of all the medias at 25^0c is 7.4 ± 0.2.

1. NUTRIENT AGAR (Hi Media) M001

Ingredients	**gm/litre**
Peptone	5.0
Sodium chloride	5.0
Beef extract	1.5
Yeast extract	1.5
Agar	15.0

28 gm powder was suspended in 1000 ml distilled water and then boiled to dissolve completely. Then the medium was sterilized by autoclaving at 121^0c (15 lbs pressure) for 15 minutes.

2. BLOOD AGAR

The sterilized nutrient agar medium is cooled to 50^0c and 5-10% blood was added aseptically and mixed well before pouring.

3. CHOCOLATE AGAR

The sterilized blood agar plate was heated at 75^0c in hot air oven for 30 min until it gave chocolate colour.

4. MACCONKEY AGAR

Ingredients	**gm/litre**
Peptic digest of animal tissue	17.0
Protease peptone	3.0
Lactose	10.0
Bile salt	1.50
Sodium chloride	5.0
Neutral red	0.03
Agar	15.0

51.53 grams powder was suspended in 1000 ml distilled water and then boiled to dissolve completely. Then the medium was sterilized by autoclaving at 121^0c (15 lbs pressure) for 15 minutes.

5. SABOUROUD DEXTROSE AGAR

Ingredients	**gm/litre**
Sucrose	30
Sodium nitrate	2
Dipotassium phosphate	1
Magnesium sulphate	0.5
Potasium chloride	0.5
Ferrous sulphate	0.01
Agar	15
Final pH at 25^0c	7.3 ≀ 0.1

6. NUTRIENTS BROTH MOO2

Ingredients	gm/litre
Peptone	5.0
Sodium chloride	5.0
Beef extract	1.5
Yeast extract	1.5

13 grams powder was dissolved in 1000 ml distilled water and sterilized by autoclaving at 121^0c (15 lbs pressure) for 15 minutes.

ANTIBIOTIC SENSITIVITY TESTING MEDIUM

MULLER HINTON AGAR M173

Ingredients	gm/litre
Beef infusion form	300.0
Casein acid hydrolysate	17.5
Starch	17.0
Agar	17.0

38 grams powder was suspended in 1000 ml distilled water and then boiled to dissolve completely and the medium was sterilized by autoclaving at 121^0c (15 lbs pressure) for 15 minutes.

I. BIOCHEMICAL MEDIA

1. HUGH AND LEIFSON'S MEDIUM M826

Ingredients	gm/litre
Peptic digest of animal tissue	2.0
Sodium chloride	5.0
Dipotassium Phosphate	0.3
Glucose	10
Bromothymol blue	0.05
Agar	2.0

19.4 grams powder was suspended in 1000 ml distilled water. The medium was boiled to dissolve the medium completely. Dispensed in 5 ml amounts tubes and cotton plugged. Then the tubes were sterilized by autoclaving at 121^0c (15 lbs pressure) for 15 minutes.

SULFIDE INDOLE MOTILITY (SIM) MEDIA M181

Ingredients	**gm/litre**
Peptic digest of animal tissue	30.0
Beef extract	3.0
Peptonized iron	0.2
Sodium thiosulphate	0.025
Agar	3.0

36.23 grams powder was suspended in 1000 ml distilled water. The medium was boiled to dissolve the medium completely. Dispensed in 5ml amounts tubes and cotton plugged. Then the tubes were sterilized by autoclaving at 121^0c (15 lbs pressure) for 15 minutes.

MR-VP MEDIUM M070

Ingredients	**gm/litre**
Buffered peptone	7.0
Dextrose	5.0
Dipotassium phosphate	5.0

17.0 grams powder was suspended in 1000 ml distilled water. The medium was boiled to dissolve the medium completely. Dispensed in 5ml tubes and cotton plugged. Then the tubes were sterilized by autoclaving at 121^0c (15 lbs pressure) for 15 minutes.

SIMMONS CITRATE AGAR

Ingredients	**gm/litre**
Magnesium sulphate	0.2
Ammonium dihydrogen phosphate	1.0
Dipotassium phosphate	1.0
Sodium citrate	5.0
Sodium chloride	1.5
Agar	15
Bromothymol blue	0.08

24.2 gram was dissolved in 1000 ml distilled water and boiled to dissolve the medium completely. Medium was distributed about 3ml in test tubes and sterilized by autoclaving at 121^0c (15 lbs pressure) for 15 minutes. After autoclaving tubes containing medium were titled to form slant.

CHRISTENSEN UREA AGAR MEDIUM M112

Ingredients	**gm/litre**
Peptic digest of animal tissue	1.0
Dextrose	1.0
Sodium chloride	5.0
Disodium phosphate	1.20
Monopotassium phosphate	0.8
Phenol red	0.012
Agar	15.0

24 grams powder was suspended in 950 ml distilled water and sterilized at autoclaving at 115^0c (15 lbs pressure) for 20 minutes. After cooling to about 55^0c, 50ml of 40% urea was added and mixed well. Then 5ml was dispensed in test and set at slope position to make agar slant.

6. TRIPLE SUGAR IRON (TSI) AGAR M021

Ingredients	**gm/litre**
Peptic digest of animal tissue	10.0
Casein hydrolysate	10.0
Yeast extract	3.0
Beef extract	3.0
Lactose	10.0
Sucrose	10.0
Dextrose	1.0
Sodium chloride	5.0
Ferrous sulphate	0.20
Sodium thiosulphate	0.30
Phenol red	0.024

65 grams powder was dissolved in 1000 ml distilled water and boiled to dissolve the medium completely. Medium was distributed about 5ml in test tubes and sterilized by autoclaving at 121^0c (15 lbs pressure) for 15 minutes. After autoclaving tubes containing medium were tilted to form slant with a butt about 1 inch of long.

7. MANNITOL SALT AGAR M118

Ingredients	**gm/litre**
Proteose peptone	10.0
Beef extract	1.0
Sodium chloride	75.0
D-Manitol	10
Phenol red	0.025
Agar	15.0

111 grams powder was suspended in 1000 ml distilled water and then boiled to dissolve completely. Then the medium was sterilized by autoclaving at 121^0c (15 lbs pressure) for 15 minutes.

REAGENT

1. GRAM'S STAINING REAGENT

Crystal Violet Stain

Solution A

2.0 gm of Crystal Violet was dissolved in 20 ml of 95% ethyl alcohol.

Solution B

0.8 gm of ammonium oxalate was dissolved in 80.0 ml of distilled water.

Then the solution A and B were mixed.

Gram's Iodine

20 gm of potassium iodide was dissolved in 300 ml of distilled water and then 10 gm of iodine crystal was added to it. The volume was adjusted up to 1000 ml by adding distilled water.

Decolorizer

70% Acetone

Safranin (Counter Stain)

2.5gram safranin was dissolved in 1000 ml of 95% ethanol. Then the solution was diluted 1:10 with distilled water.

2. ZIEHL-NEELSON REAGENT

i) Carbol fuchsin stain

Composition

Basic fuchsin	10 gm
Absolute alcohol	100 ml
Solution of phenol 5% in water	1 litre

Preparation: 10 gm of basic fuchsin dye was dissolved in 100 ml of alcohol. Then the whole content was added to the phenol solution.

ii) Acid-Alcohol Decolourizer

Composition

Concentrated HCl	3 ml
95% alcohol	97 ml

Preparation: To 97 ml of 95% alcohol, 3 ml of concentrated HCl was added, mixed and transferred into a clean bottle.

iii) Malachite green counterstain (1%w/v)

Composition

Malachite green	1 gm
Distilled water	100 ml

Preparation: A stock solution of 1% malachite green was made in distilled water and for use; a small quantity was diluted with distilled water and kept in a clean bottle.

3. LACTOPHENOL COTTON BLUE

Composition

Phenol	10.0 gm
Cotton blue	0.04 gm
Lactic acid	10 ml
Glycerol	20 ml
Distilled water	10 ml

Preparation: Cotton blue was dissolved in distilled water by gently warming the solution. Then phenol was added in the solution and stirred for complete dissolution.

4. TEST REAGENTS

i) Catalase Reagent (3% H_2O_2)

Hydrogen peroxide	3 ml
Distilled water	97 ml

ii) Oxidase Reagent

Tetraethyl paraphenylene diamine dihydrochloride	1 gm
Distilled water	100 ml

iii) Methyl Red Indicator Solution

Methyl red	0.1 gm
Ethyl alcohol (95%)	300 ml
Distilled water	200 ml

iv) Barritt's Reagent

Solution A

ℑ-naphol	5 gm
Ethyl alcohol	100 ml

Solution B

Potassium hydroxide	40 gm
Distilled water	100 ml

1 ml of solution B and 3 ml of solution A was added to test suspension.

v) Kovac's reagent

Pure amyl or isoamyl alcohol	150 ml
Para dimethyl aminobenzaldehyde	10 gm
Concentrated hydrochloric acid	50 ml

vi) Bile Salt solution

Composition

Commercially available Sodium deoxycholate	10 gm
Distilled water	100 ml

Preparation: A 10% solution of Sodium Deoxycholate was prepared by adding 10 gm Sodium Deoxycholate powder in 100ml distilled water and transferred into a clean bottle.

APPENDIX-III

Procedure

1. **Gram's Staining**
   - A dried, heat fixed smear was prepared and the slide was covered completely with crystal violet solution for at least 1 min.
   - Then the stain was washed off with tap water and covered completely with Lugol's iodine solution for at least 20s.
   - Again the iodine solution was washed off with tap water. Then the slide was covered completely with acetone-alcohol decolouriser and left for about 10s.
   - Next, The slide was washed off with tap water and covered with counter stain solution (Safranin) for about 1 min.
   - Lastly, the counter stain was washed off with tap water, blotted and observed under microscope.

2. **Ziehl-Neelsen Staining**
   - A dried, oval shaped smear was prepared by means of a wooden stick.
   - The smear was then covered with carbol fuchsin solution. The smear was then heated until vapour just starts to evaporate. The slide was then left for about 5 min.
   - The smear was then washed off with distilled water and decolorized with 20% sulphuric acid until the initial red stain of carbol fuchsin disappeared.
   - Now, the smear was counterstained with 1% methylene blue for 1 min.
   - The smear was again washed off with distilled water, kept in the rack for air dry and observed under oil-immersion.

Reporting of sputum smears

If any definitive red bacilli are seen, report the smear as 'AFB positive', and give an indication of the number of bacteria present as follows:

More than	10 AFB/field,	Report +++
	1-10 AFB/field,	Report ++
	1-100 AFB/100 fields,	Report +
	1-9 AFB/100 fields,	Report the exact number

3. Antibiotic susceptibility testing

Kirby-Bauer method was used for antibiotic susceptibility test with following procedures:

1. Preparation of plates:

The agar plates were prepared in a way to make the thickness of medium of about 4 mm.

2. Preparation of inoculum:

For inoculum preparation, 3-4 pure culture colonies were transferred into a test-tube containing 2-3 ml of nutrient broth and was incubated at 37^0c for 2-4 hrs. to obtain turbidity.

3.Inoculation:

A sterile cotton swab was dipped into the turbid solution and was streaked (by means of swabbing) on the agar surface of MHA plate. The plate was then left 10 min at room temperature to dry the inoculum.

4.Application of the discs:

Using a sterile forcep, antibiotic discs were carefully placed on the agar surface of the plate with certain distance in between the discs so that the zones of inhibition do not get overlapped. Finally, the discs were pressed gently in order to make contact with media surface and the plate was left in the room temperature for 30 min. (Prediffusion time)

5.Incubation:

The plates were then incubated at 37^0c for overnight.

6.Reading of zone and its interpretation:

After incubation, the inhibition zones formed were measured and the result was interpreted on the basis of standered interpretative table given by difco laboratory (based on NCCLS guidelines).