CHAPTER-I

1. INTRODUCTION

The first reported case of death due to AIDS was in the year 1959 A.D. Since that time till 1981 death due to AIDS was limited to only the minority of population. The epidemic of HIV/AIDS was believed to occur only in the year 1981 A.D, since then the diseases spread so widely that now in the year 2006 A.D the disease have swept all the portion of world and now is one of the pandemic diseases. In many developing countries like Nepal, HIV/AIDS is the major public health problem, which is most likely to steep up in the near future. To quote the world development report 1993 A.D "Historians will look back on the later half of this century as having had one great medical triumph, the eradication of smallpox, and one great medical tragedy AIDS" (Cook, 1996).

After one decade; in 1993 A.D over 600,000 cases of HIV/AIDS had been reported to the WHO, which estimates that 2.5 million cases had actually occurred in that period (Virella, 1997). Due to the lack of effective treatment the death increases as the year passes by. Just after the two decades in the year 2003 A.D, 42 million people were infected. Till this year the total reported death due to HIV/AIDS tolls to 20 million. 80% to 90% of cases occur in third world countries with large foci in South America, India, Sub-Saharan, Africa and South East Asia.

This disease was first defined by CDC in United States of America in 1981. Since then the disease had infected tremendously large population including all races (except few white population in Europe) sex, and even age. Now HIV/AIDS has killed large number of individual around the world including the developing country like Nepal.

The first documented case of death due to HIV/AIDS in Nepal was reported in 1988. Since then the country is facing this deadliest disease of the recent century. Nepal is

constantly fighting for HIV/AIDS for more than two decades. In order to combat HIV/AIDS, the government of Nepal has established a separate body under the Ministry of Health. From the time of establishment the center is continuously combating HIV/AIDS in planning, policy making and implementing level. According to the recent report of NCASC around 6650 are infected with HIV and among which 322 died of HIV/AIDS and 362 people are PLWHA. To quote the recent report of UNAIDS the total cases of HIV/AIDS in Nepal in the year 2003 is around 10 thousand. .

Acquired Immune Deficiency Syndrome is a clinical representation of secondary immune deficiency diseases. Most of the cases of AIDS are due to the Human Immunodeficiency Virus. AIDS represent the late clinical stage of HIV infection. In the absence of effective treatment the HIV/AIDS case fatality rate is very high (80%-90%). Patient in developing countries like Nepal die within 3-5 years after the diagnosis of AIDS (Ministry of Health, 2003).

Initial HIV infection presents with mild fever, headache, diarrhea, rash and even lymphadenopathy. As the time passes, this infection with HIV leads to a condition known as AIDS. When a patient suffers from decrease in T-lymphocyte with CD4 marker cell count (<200 cells/µl) along with presence of various opportunistic infection and HIV infection, he/she acquires HIV/AIDS. The infection with HIV is responsible for the depletion of T-lymphocytes cells and other cells having CD4 protein on their surface. As HIV reproduce highly within the T-lymphocytes, their number decreases as the viral load increases. T-lymphocytes are that arm of immune system which helps to combat the infection with intracellular as well as extra cellular pathogens. Due to the decrease in number of these cells, body suffers from different types of opportunistic infection. It is clear that death in case of HIV/AIDS is not because of HIV infection but because of opportunistic infection.

HIV is transmitted from one human being to other in four different ways. Among them transmission due to contaminated needle holds the first place. Infection with HIV among Intravenous Drug (IVD) users is very high in developing countries like Nepal and most of Asia. Man having sex with man are among the high risk group of HIV infection. In the recent trend, Sexual transmission from female commercial sex worker to male is also high, in country like Nepal. Transmission due to blood transfusion and organ transplantation has been reduced in recent years by total screening of blood and blood products. Mother to child transmission is the only cause of pediatric HIV infection which can be reduced, to large extent, by the ARV therapy before delivery.

Demonstration of HIV antigen or antiserum in the blood is sufficient evidence of HIV infection in particular individual. Determination of P_{24} Protein in the blood by western blot or antibody detection by ELISA is the frequently used techniques done to identify individuals with HIV infection. The cases of HIV/AIDS are further conformed by CD4 count. Nucleic acid analysis is the essential tool for the identification of HIV infection even in the window period or in children below two years of age. RT-PCR is done as the more confirmatory test which is highly specific and sensitive too. In the developing country like Nepal, such tests are not feasible due to high cost. Microbiological, Parasitological and Histopathological examination for the identification of certain pathogens like *Pneumocystis carinii*, *Mycobacterium avium* and Kaposi's sarcoma virus can also considered as the counter indication of AIDS.

Death in HIV/AIDS is because of secondary infection. So the treatment of HIV infection should not be focused only on killing virus but also in treating secondary infections. There are basically three different stages of treating HIV infection.

1. Antiretroviral therapy used in order to reduce the viral load.
2. Immune-based therapy for the enhancement of immune system.
3. Treating secondary infection.

The combination therapy of these three will help the individual to prolong their life for up to or more than 20 years and also increase the time period of developing AIDS. In absence of this therapy the individual with HIV infection develops AIDS within 2 to 3 years and die within 5 years.

Antiretroviral therapy is highly expensive treatment and once it is started it must be continued throughout lifetime. ARV therapy is basically concerned with reducing the total viral load in the serum. Once the viral load is reduced, the CD4 count will automatically increase. Now a days more advance technique has been developed to reduce viral load in serum and it is called HAART (Highly Active Antiretroviral Treatment). It is mostly practiced in developed countries rather than in developing countries like Nepal, where ARV itself is not feasible. Immune based therapy depends upon the type of secondary infection that develops. It is not only tough, but also very expensive treatment, since individual organisms require separate treatment. When an individual suffers from more than one infection this treatment is more complicated.

For developing country like Nepal, the most feasible treatment is treating the secondary infection as soon as it develops. In doing so, we can also increase the time of HIV progressing to AIDS in a particular individual. Treating secondary infection is one of the cheapest, but not easy methods. Proper identification of an organism is the primary step and is of vital importance. Information regarding these organisms must be kept in proper way, in order to find out the local isolates. Antibiotic sensitivities of these local isolates must be performed on the regular basis in order to reduce the development of drug resistant strain.

Properly planned treatment of the secondary infection in HIV cases along with ARV therapy helps to prolong the life of PLWHA in this country. Keeping this thing in mind, this small work in the field of HIV/AIDS was done in collaboration with Maiti Nepal and Central Department of Microbiology in order to set the local isolates among the PLWHA. Different samples have been analyzed for the detection of various organisms

causing secondary infection. Cerebral spinal fluid, bronchial aspirates and other body secretions has not been included in this study.

CHAPTER-II

2. OBJECTIVES

2.1 General Objectives

The general objective of this study is to set the correlation of decrease level of T lymphocytes with CD4 marker count along with the secondary infection among HIV infected individuals.

2.2 Specific objectives

1. To determine the blood serum level of T lymphocytes with CD4 marker in HIV positive cases.
2. To determine the different type of opportunistic infection present in the patient with HIV infection.
3. To correlate the T lymphocyte count with different type of opportunistic infection present in the HIV/AIDS patients.
4. To examine serum for the presence of antigen against the HIV1/HIV2 and Hepatitis B virus.
5. Microbiological and Hematological examination of Blood.
6. Microbiological examination of Sputum, Throat swab and Urine.
7. Parasitological and Microbiological examination of Stool.

CHAPTER-III

3. LITERATURE REVIEW

3.1 Immunodeficiency disease

The immune system is like the proverbial two-edged sword. On the one hand, humans are dependent on immune system for survival; on the other hand, they are vulnerable to disorder in its function ranging from immunodeficiency states to hypersensitivity disorders. Put more succinctly, the disorder range from those caused by “too little” to those caused by “too much or inappropriate” immunoreactivity. To encompass this spectrum, the various disorder of immune function is considered. Immunodeficiency disease is one among them (Robbins, 2001).

Immunodeficiency disease may develop because of inherited defect in the development of the immune system, or they may be secondary to the effects of disease that affect the normal immune system. Clinically, they present with increased susceptibility to infection and, sometimes, cancer. The type of infections in a given patient depends largely on the component of immune system that is deficient. Immunodeficiency diseases are broadly classified into two types.

1. Primary immunodeficiency disease
2. Secondary immunodeficiency disease

Primary immunodeficiency occurs as a genetic defect. Due to the mutation in certain genes the production of immune cells or immune components are greatly reduced. They usually come to attention early in life because of the vulnerability of the child to recurrent infections. Although these immune disorders are relatively uncommon, they are often devastating because many affected patients develop fetal infection. Primary immunodeficiency states are experiments of nature that have greatly helped our understanding of the ontogeny and regulation of the immune system.

Secondary immune-deficiency is the condition in which there is depletion in cells or component of immune system due to any means or agent acquired during life time. Many of these secondary states can be accounted for by the loss of immunoglobulins, inadequate synthesis of immunoglobulins, or loss of lymphocytes (as may occur due to drugs and systemic infection), but other mechanisms may also be operative. AIDS is one example of secondary immunodeficiency in which there is depletion of T-lymphocytes with CD4 marker.

3.2 Cells of immune system

The immune system comprises of the molecules, cells and organs which are involved in host defense against infection. Immunoglobulin, compliment protein, interferon and interleukins are some of the molecules involved in immune system. Cells involved in the immune system can be classified into granulocytes and agranulocytes. Among agranulocytes, lymphocytes are considered to be the most specific arms of the immune system. These cells are specific to the target, can recognized between self and non-self and do posses memory. Lymphocytes are broadly classified into two classes according to their nature of combating infection. B lymphocytes produce immunoglobulin that react with antigen and neutralize it. On the other hand, T lymphocytes act upon intracellular pathogen and destroy it.

3.2.1 B Lymphocyte

B cells constitute 10% to 20% of the circulating peripheral lymphocytes population. The defining feature of cells in the B-cell lineage is their ability to synthesize protein called immunoglobulins. No other cells express these proteins. Upon antigenic stimulation, B cells form plasma and memory cells, plasma cells secrete immunoglobulins. Each immunoglobulin binds specifically and with high affinity to its own particular small molecular ligand, which may be any of the vast number of chemical determinants found in proteins, carbohydrates, lipids, or other macromolecules. The molecular determinants bound by various immunoglobulin proteins can be referred to collectively as antigens. Decrease in B cells level either by

genetic defect or by acquiring infection posses a great threat to the host. Once the humoral immunity is decreases large number of pathogenic organism infect the host.

3.2.2 T Lymphocyte

T cells do not express immunoglobulin but, instead, detect the presence of foreign substances by way of surface proteins called T-cell receptors (TCR). T cells are the mediators of cellular immunity and are essential for induction of humoral immunity to most naturally encountered antigens. 60% to 70% of the peripheral lymphocytes are T lymphocytes. According to the function T cells are classified as helper T cell and cytotoxic T cell. Besides TCR all T cell contains a marker protein called as CD3 (Cluster of Differentiation). In addition to CD3 proteins, T cells express a variety of other nonpolymorphic function associated molecules, including CD4 and CD8. These two molecules serve as co receptors and are express on two mutually exclusive subsets of T cells. CD4 is expressed in approximately 60% of mature CD3+T cells, whereas CD8 is expressed on about 30% of T cells. Thus in normal individual the ratio of CD4:CD8 is 2:1. The CD4+ and CD8+ T cells perform distinct but somewhat overlapping functions. T cells with CD4 marker are often called “helper” T cells because the soluble molecules secreted by them influence virtually all other cells of the immune system, including B cells, natural killer cells, and macrophages. The T cells with CD8 marker, like CD4 cells, can secrete cytokines, but they are best known for their ability to kill virus-infected or tumor cells by direct cytotoxicity. Decrease level of T cells not only impairs its function but also slow down the differentiation of other cells of immune system. Decrease concentration of T cells with CD4 marker by HIV leads to a chronic condition known as AIDS, where secondary infection due to opportunistic infection occurs and our body cannot fight against it, the result of which is death.

3.3 Acquired immunodeficiency syndrome

The acquired immunodeficiency syndrome (AIDS) is characterized by the progressive loss of the CD4+helper/inducer subset of T lymphocytes, leading to severe immunosuppression and constitutional disease, neurological complications, and

opportunistic infection and neoplasms that rarely occur in person with intact immune function. (CDC, 2000). The term AIDS first appeared in the Morbidity and Mortality Weekly Report of the CDC in 1982 to describe "…. A disease, at least moderately predictive of a defect in cell-mediated immunity, occurring with no known cause for diminished resistant to that disease" (CDC, 1982).

For surveillance purpose, the CDC currently defines AIDS in an adult or adolescent age 13 years or older as the presence of one of 25 AIDS-indicator conditions, such as KS, PCP or disseminated MAC. In children younger than 13 years, the definition of AIDS is similar to that in adolescents and adults, except that lymphoid interstitial pneumonitis and recurrent bacterial infection are included in the list of AIDS-defining conditions (CDC, 1987). The AIDS-defining condition can be summarized as below.

- Candidiasis of bronchi, trachea, or lungs
- Candidiasis, esophageal
- Cervical cancer, invasive
- Coccidioidomycosis, disseminated or extrapulmonary
- Cryptococcosis, extrapulmonary
- Cryptosporidiosis, chronic intestinal (greater than 1 month's duration)
- Cytomegalovirus disease (other than liver, spleen, or nodes)
- Cytomegalovirus retinitis (with loss of vision
- Encephalopathy, HIV-related
- Herpes simplex: chronic ulcer(s) (greater than 1 month's duration); or bronchitis, pneumonitis, or esophagitis
- Histoplasmosis, disseminated or extrapulmonary
- Isosporiasis, chronic intestinal (greater than 1 month's duration)
- Kaposi's sarcoma
- Lymphoma, Burkitt's (or equivalent term)
- Lymphoma, immunoblastic (or equivalent term)
- Lymphoma, primary, of brain

- Mycobacterium avium complex or M. kansasii, disseminated or extrapulmonary
- Mycobacterium tuberculosis, any site (pulmonary or extrapulmonary) Mycobacterium, other species or unidentified species, disseminated or extrapulmonary
- Pneumocystis carinii pneumonia
- Pneumonia, recurrent
- Progressive multifocal leukoencephalopathy
- Salmonella septicemia, recurrent
- Toxoplasmosis of brain
- Wasting syndrome due to HIV

On the basis of laboratory finding AIDS can be define as the condition where there is depletion in the level of peripheral T lymphocytes with CD4 marker less than 200 cells per cubic millimeter of blood. Normally the ratio of T cell with CD4 marker to CD8 is 2:1 and if this ratio alters such that it is 1:2 than such condition also indicates AIDS.

In order to over-come these complicate definition for many developing countries like Nepal, where diagnostic facilities may be minimal WHO clinical case definition of AIDS was introduced. This definition was developed at WHO workshop on AIDS at Bangui, Central Africa Republic. According to WHO, AIDS in an adults is define by the existence of at least two of the major signs associated with at least one minor sign, in the absence of known cause of immunosuppression such as cancer or severe malnutrition or other recognized aetiologies (Cook, 1996).

1. Major signs
    a) Weight loss >10%of body weight
    b) Chronic diarrhea > 1 month
    c) Prolonged fever > 1 month (intermittent or constant)
2. Minor signs
    a) Persistent cough for>1 month

b) Generalized pruritic dermatitis
c) Recurrent varicella zoster
d) Oropharyngeal candidiasis
e) Chronic progressive and disseminated herpes simplex infection
f) Generalized lymphadenopathy

Paediatric AIDS is suspected in an infant of child presenting with at least two major and two minor signs in the absence of known causes of immunosuppression.

1. Major signs
   a) Weight loss >10%of body weight
   b) Chronic diarrhea > 1 month
   c) Prolonged fever > 1 month (intermittent or constant)
2. Minor signs
   a) Generalized lymphadenopathy
   b) Oropharyngeal candidiasis
   c) Repeated common infection(s) (otitis pharyngitis)
   d) Persistent cough for>1 month
   e) Generalized dermatitis
   f) Confirmed material HIV infection

Above all these definitions, the presence of generalized Kaposi's sarcoma or *Pneumocystis carinii* pneumonia or *Mycobacterium avium* complex infection are sufficient by themselves for the diagnosis of AIDS.

3.4 HIV and immunodeficiency

The evidence for HIV's primary role in the pathogenesis of immunodeficiency disease like AIDS is reviewed by several scientists. In addition, many scientists have responded to specific arguments from individuals who assert that AIDS is not caused by HIV. The present discussion reviews the AIDS epidemic and summarizes the evidence supporting

HIV as the cause of AIDS. When the epidemiological studies show that only 80-85% of the death due to AIDS is associated with the HIV infection the things become more complicated. By 1983, several research groups had focused on retroviruses for clues to the cause of AIDS. In May 1983, the first report providing experimental evidence for an association between a retrovirus and AIDS was published. They first gave the name lymphadenopathy associated virus (LAV) that causes AIDS. The French group of scientist led by Dr. Luc Montagnier subsequently reported that LAV was tropic for T-helper cells, in which it grew to substantial titer and caused cell death. On the other side of world, researcher in San Francisco subsequently reported the isolation of a retrovirus they name the AIDS associated retrovirus (AARV) from AIDS patients in different risk group, as well as from different asymptomatic people from AIDS risk group. They found that AARV like that of LAV and HTLV-III isolated by Dr. Robert Gallo grew substantially in peripheral blood mononuclear cells and killed CD4+ T cells

During the same period, HTLV-III and AARV were isolated from the brain of children and adults with AIDS-associated encephalopathy, which suggested a role for these viruses in the central nervous system disorders seen in many patients with AIDS. By 1985, analysis of the nucleotide sequences of HTLV-III, LAV, and AARV demonstrated that the three viruses belonged to the same retroviral family and were strikingly similar. Taking these data in consideration in the year 1986, the International Committee of Viral Taxonomy renamed the viruses the human immunodeficiency virus (HIV) (Coffin, 1986).

After the year 1986, it was concluded that, one of the cause of AIDS is HIV and the fact is accepted by all the people around the world. Once this retroviral hypothesis of HIV is set, LAV, AARV and HTLV-III were no more used to formulate the cause of AIDS.

3.5 Clinical manifestations of HIV infection

There is a wide spectrum of clinical features following HIV infection, varying from asymptomatic infection to AIDS. During the first few years of infection most patients

have no symptoms or only minor illness. However within a 6-years period of infection approximately 30% will have developed AIDS and at least a further 30% will have suffered some form of illness. There are progressive immunological defects apparent in most individual who have been infected for more than 5 years, and latent infection in the central nervous system is also common. It is likely that most patients who are relatively well in the first 5 years will develop major HIV-related illness in the later life (Shanson, 1999).

Because patients may have HIV infection but not manifest the actual syndrome of AIDS, a reclassification of HIV infection into four stages is used by the Central for Disease Control and Prevention. The first stage is acute infection by HIV; patient with acute disease may experience fever, diarrhea, rash, lymphadenopathy, night-sweats, and a general fatigue similar to that in acute infection mononucleosis. These symptoms may last a few weeks or up to six months.

After the acute infection, most patients will be asymptomatic. The asymptomatic stage is designated stage II disease. At this time, the patients may have few or no symptomatic complaints. There is antibody evidence of HIV infection, but it may be difficult to culture the virus from T-lymphocytes. The patients may have minor laboratory abnormalities but immune deficiency related infections or cancers are not present (Alcamo, 1994).

The third stage of disease is termed persistent generalized lymphadenopathy (PGL). Patient in this stage have generalize lymphadenopathy for greater than 6 months in two or more areas of their bodies (excluding the inguinal area) and have antibody evidence for HIV (positive ELISA and western blot tests). PGL is not an end stage of HIV infection but an early manifestation of AIDS. Studies reveal that there is probably a 6 to 10 percent chances per year that patients with stage II and III disease will develop symptomatic HIV infection.

Stage IV disease can also be viewed as “symptomatic HIV infection.” At this stage, patients begin experiencing symptoms from their immunodeficiency due to gradual loss of helper T-lymphocytes. Stage IV is further subdivided into five different section in relation to the specific symptoms of immunodeficiency. The CDC classification of HIV infection is summarized in the table given below.

Table 1: CDC classification of HIV infections

Stages	New Classification	Common Name
Group I	Acute HIV infection (mononucleosis-like, aseptic meningitis)	Acute infection
Group II	Asymptomatic	Healthy carrier
Group III	Persistent generalized lymphadenopathy (PGL)	AIDS related complex (ARC)
Group IV	Other diseases A) Constitutional disease (fever, weight loss, diarrhea) B) Neurological disease (dementia, myelopathy, peripheral neuropathy) C) Secondary infections 1. CDC defined AIDS-associated 2. Other specific infections D) Secondary cancers E) Other conditions attributed to HIV infection or immunosuppression	 AIDS related complex (ARC) AIDS related complex (ARC) AIDS ARC AIDS ARC

From the manifestation of HIV infection it is now clear that AIDS can be cause by HIV but HIV infection in itself is not the supportive evidence of AIDS.

3.6 HIV and its pathogenicity

Human immunodeficiency virus (HIV), previously known as HTLV III, LAV, and AARV belong to the family Retroviridae and the genus Lentivirus.

3.6.1 General properties of retroviridae

The family Retroviridae includes a large number of disease producing animal viruses, several of which are of clinical importance to humans. Retroviridae are distinguished from all other RNA viruses by the presence of an unusual enzyme, reverse transcriptase, which converts a single stranded RNA viral genome into double-stranded viral DNA. Because these viruses reverse the order of information they are termed retroviruses. Retroviridae are divided into several genera on the basis of nucleotide sequences similarity and genome structure. Among them one is Lentivirus, which includes the human immunodeficiency viruses 1 and 2. The lentiviruses cause neurological and immunological diseases, but do not have the oncogenic properties. The discovery that AIDS is also caused by HIV greatly increased the attention to this family of viruses (Strohl, 2001).

Despite their wide range of diseases manifestations, all retroviruses are similar in structure, genomic organization, and mode of replication. Retroviruses are enveloped particles. The viral envelope, formed from the host cell membrane, contains 72 spiked knobs. These consists of a transmembrane protein, TM (fusion protein), which is linked to a surface protein, SU (attachment protein), that binds to a cell receptor during infection. Host cell proteins, including the major histocompatibility complex class II proteins, are also found in the envelope.

3.6.2 Morphology of HIV

As we know that, HIV belongs to the family Retroviridae and the genus is Lentivirus it cause neurological and immunological diseases but not oncogenic in nature recent studies have shown that HIV also infect the kidney cells. The shape of the virus is spherical. Its matrix is icosahedral and the capsid or core is cylindrical. The size of the

virus is 80-100 nm in diameter. The HIV is enveloped virus, envelop consists of lipid membrane derived from the host and viral protein in it. About 72 copies of envelop protein are present. It consists of 3 or 4 molecules of a cap like head known as glycoprotein 120 (gp 120) and 3 or 4 mol of glycoprotein 41 (gp 41) anchor of viral envelope. Inner to the envelope is the viral capsid often known as matrix it is made up of viral protein (P) 17/18. Inner to it is viral core bullet-shaped consists of 2000 copies of P24. Inner to this core is P7 known as nucleocapsid which encloses the genetic copy of RNA. There are three viral proteins inside the P24 core they are Reverse transcriptase, Integrase, and Protease. Regarding its genomic materials, it contains two single stranded positive senses RNA. Its genome is 9.2 Kb and consists of 9 Genes, classified as structural and functional gene. Structural gene comprises gag gene, pol gene, and env gene. Besides this there are six functional genes namely vif gene, vpr gene, vpu gene, nef gene, tat gene, and rev gene.

3.6.3 Genomic organization of HIV

As already mention above, the complete sequence of HIV-I contain a 9.2 kb genome. Long Terminal Repeats (LTRs) flank the genome for gag, pol, and env which lie in the stander order for retroviruses. The gag gene (55 kDa), which code for a polyprotein that is cleaved post synthetically into three immunogenic structural proteins, which are P17/18 (matrix protein), P24 (core protein), and P7 (nucleocapsid protein). Next to gag is pol gene (66 kDa) which codes for reverse transcriptase, proteases, integrases, and ribonucleases. The third structural gene of HIV, the env gene (160 kDa) codes for transmembrane and surface proteins. The products of this gene are expressed as envelope glycol-proteins gp120 and gp41.

There are additional frames on either side of the env gene coding for at list 6 proteins, of which 5 are involved in the regulation or accessory function of HIV expression (Collier, 1998). These genes include: tat (transactivation gene) which encodes a peptide of 14 kDa, which acts in a positive feedback loop by binding to the stem-loop region of LTR-RNA in association with cellular proteins at a defined target sequence. Binding of

tat results in as much as a 1000-fold increases in expression of all viral genes. Regulatory of virus gene or the rev gene encode a protein of 19 kDa. Rev has an important function in regulating virus expression by allowing the transport of full-length, spliced mRNA from the nucleus to the cytoplasm. In the absence of rev, viral mRNA is either degraded or rapidly spliced into RNAs that can be fully transcribed. These resultant products are non-structural regulatory proteins. Nef (negative factor gene) codes for a protein of 27 kDa which actual function remains unclear. Recently it has been shown to down-modulate surface CD4 expression by lysosomal degradation. Additionally, nef has been identified as having considerable kinase activity and contributing to viral expression in the absence of cellular activation. The gene product of vif gene (viral infectivity factor gene) is incorporated into virus particles in a way that confers infectivity on the virion, possibly influencing uncoating early in the infection. Cell-to-cell transmission is not impaired in HIV defective for vif, but transmission of free virus to cell is impaired. It seems likely that vif acts late in the viral life cycle, during viral assembly. The vpu (in HIV-1:16 kDa) and vpx (HIV-2:12-16 kDa) are gene that code for viral proteins of less well defined function. Because the 2 proteins differ from each other but are both antigenic, they can be exploited in serological tests to distinguish between infections by either virus. By contrast, the detection of unique type-specific sequences vpu for HIV-1 and vpx for HIV-2 has proved most useful in distinguishing between infections by these viruses. Vpr gene (viral protein R) codes for a protein which is 14 kDa that imparts a rapid growth advantage to HIV-1. It is assemble in virion and may be associated with gag during transport. Vpr seems to play a role with respect to nuclear import of the preintegration complex in unactivated cells. Another function attributed to this gene has been its ability to block cells in the replication cycle. The purpose of this function is not understood.

3.6.4 Replication strategy of HIV

Although the genomes of HIV is both single-stranded, positive-sense mRNA and are capable of coding for protein synthesis in translation assays *in vitro,* linear translation of

the sequence is unlikely to lead to comprehensive expression of viral proteins. The functionality of the proteins produced relies on splicing of their mRNAs. RT enzymatic action and reverse transcription seem to be required to produce a DNA intermediate. Once reverse transcription occurs, retrovirus DNA can be found in the nucleus, either as closed circular, supercoiled copies, or as linear provirus copies, both unintegrated and integrated. Because HIV use the normal mechanisms of cellular protein synthesis for expression, it seems likely that, in addition to the regulatory proteins already described, the internal and external environment of the cell influence virus expression, possible through interaction of certain normal intracellular proteins with the virus LRT. Such mechanisms may explain the cellular activation requirements for virus replication in HIV-infected T cells stimulated externally by antigens, superantigens or cytokines. As another example, the HIV LRT is known to have a sequence to which the lymphoid-specific cellular enhancer, NF-kB, binds. These interactions also leave open the possibility for co-factors, such as infections and non-specific cellular activators, in the progression of disease in infected individuals.

HIV expression and replication are not continuous but are likely to be stepwise. In a resting cell, there is little transcription of virus. Cell activation leads to the transcription of HIV mRNA in general, but the expression of structural viral proteins is repressed by the inhibitory sequences, RRE, in the mRNA, so that the low levels of prematurely truncated short transcripts are synthesized. However, the mRNA species that code for the regulatory proteins, including tat and rev, are transcribed. As the expression of tat and rev increases, a threshold is achieved when the positive feedback loop becomes active and transactivation is mediated by tat. At the same time, the action of rev allows the transport of functional mRNA transcripts into the cytoplasm where they are translated into proteins. As these are expressed, viral structural proteins are abundantly synthesized. Intense expression of virions leads to programmed cell death, usually with the accumulation of episomal poxviruses. Cell cycle blockade in the G2/M phase macrophages seems to be more likely to sustain low levels of persistent virus release without cell death, as do lymphocytes infected with the HTLVs.

3.6.5 Pathogenesis of HIV

As the AIDS pandemic pass its second decade, concepts regarding the mechanisms of HIV pathogenesis have made important contribution to our understanding of the disease. Many of these advances have revolved around correlation of disease progression and viral body burden. However, several other areas related to disease progression have emerged as important. For instance, changes in CD4 T cell subtypes have been observed that may provide an understanding of the mechanisms of cell biology of AIDS. These findings which show a steady decline in Th1 cells whereas Th2 cells seems to remain at steady levels, provide insight into lymphoid subset profiles. Likewise, the finding that native CD4 T cells are lost during disease progression at a higher rate than CD8 or memory cells may imply a more certain immunological defect. This effect may be further exemplified by an infected individual's loss of immune responsiveness first to specific recall antigens, than to allogeneic antigens and finally to mitogen stimulation.

The mechanism explaining the actual loss of CD4 T cells in still unresolved. Specifically, it remains to be determined whether the virus can directly account for CD4 cell loss via infection followed by programmed cell death or whether indirect mechanisms, such as gp120-CD4 antibody cross-linking, viral protein-induced cellular dysregulation or antiviral bystander effects are involved.

Finally, it should be mention that, as a prerequisite for HIV or HTLV replication, cellular activation and proliferation are important factors. The role of cellular activation, whether induced by antigens via the specific T cell receptor or cytokines, remains a new and unexplained phenomenon of the cell's control over virus expression. Cellular models have been developed that permit synchronization of replicating virus for studies of potential therapeutic targets that may be sought to interrupt the viral life cycle. Concepts regarding intervening illnesses and infections as non-specific activators of T cells from HIV-infected individuals are currently being investigated to explain

differences observed between those who progress to disease rapidly compared with those who progress more slowly.

Serum from infected patient are able to inhibit both functions of the envelop proteins, i.e. fusion and virus attachment in internalization. As a result some sera from infected patients neutralize virus infection *in vitro*. Variability in antigenic expression by the virus could influence the degree of neutralization, although some neutralizing activity for all HIV-1 strain is present albeit at low levels, in the sera from infected humans. Furthermore, recent studies demonstrate that many HIV isolates in culture show passive shedding of free gp120 into the tissue culture fluid from infected cells. This in turn leads to blocking of antibody neutralization.

3.7 HIV/AIDS and opportunistic infection

Once we found that majority of AIDS is due to late HIV infection, now we focus our literature totally on HIV/AIDS. Death in HIV/AIDS is not due to HIV virus but of secondary infection. How HIV leads to opportunistic infections is the major concern of this section. The term ‘opportunist’ is not an exact one. ‘Opportunist’ organisms have three main characteristics (Shanson, 1999):

1. They are usually organisms of low pathogenicity: e.g. Pseudomonas aeruginosa, Cytomegalovirus, Pneumocystis carinii etc.
2. They cause serious infections mainly when the host defense mechanisms are impaired.
3. They can behave as ‘conventional pathogens’ but under opportunistic conditions may cause atypical clinical presentations or disseminated lesions.

Thus, opportunistic organisms cause diseases only when the immune system of the host is weak and is harmless in the case of normal individual. Infection of such organisms in normal individuals are asymptomatic, or they experience symptoms so mild they do not seek medical attention and may not even realize that their illness was because of opportunistic organisms.

The timing and development of opportunistic infection in people with HIV is a product of the host and the microbial environment within which we all live. Although virtually all aspect of the immune system are affected by HIV, defect in cellular immunity (involving T cells and macrophages) predominate. Most opportunistic infections are with pathogens against which the host is normally defended through cellular immunity. The organisms that take advantage of HIV-related defect in macrophage and T cell function can be listed as follows (Stewart, 1997):

Bacteria

Mycobacterium tuberculosis

Mycobacterium avium complex

Salmonella species

Viruses

Cytomegalovirus

Herpes simplex

Varicella zoster

Epstein-Barr virus

Fungi

Candida species

Cryptococcus neoformans

Aspergillus species

Histoplasma capsulatum

Coccidioiodes immitis

Protozoa

Pneumocystis carinii

Toxoplasma gondii

Microsporidia

Cryptosopridia
Leishmania

3.7.1 Abnormalities in cell mediated immunity

Cell-mediated immunity is required to attack organism within cells, where they are protected from antibody-mediated killing. It functions by two main effectors mechanisms: delayed-type hypersensitive and cytotoxic T lymphocytes (CTLs). In delayed-type hypersensitivity, intracellular organisms (particularly fungi, protozoa, and the few intracellular bacteria) are killed by macrophages which have been activitated by cytokines released by CD4+ T cells. CTLs are also activitated by cytokines from CD4+ cells, and act (in particular against viruses) by killing infected cells. Both mechanisms are damaged by HIV infection. Loss of delayed-type hypersensitivity at skin and mucosal surfaces accounts for the predominance of infection at these sites in the early stage of symptomatic HIV disease (CD4 cell count 500-200 µL). A third arm of cell-mediated immunity is provided by natural killer cells. This non-T, non-B (null) matures as large granular lymphocytes and is characterized by the cell surface markers CD16. They functions through direct non-specific killing of virus-infected or malignant cells and specific killing of antibody-coated cells (antibody-depended cell-mediated cytotoxicity).

3.7.2 Abnormalities humoral immunity

Antibodies are produced by plasma cells, which are formed by proliferation and differentiation of B lymphocytes under the control of cytokines produced by helper T cells. This process induces a switch from producing IgM to IgA and IgG antibodies under T cell guidance. Antibodies eradicate organism mostly by enhancing phagocytosis by neutrophils and macrophages (opsonization). HIV causes a non-specific, polyclonal stimulation of B cells, leading to their activation and, ultimately, to hypergammaglobulinaemia (predominantly IgG and IgA). Auto antibodies may be produced that are directed against several host proteins, including platelets, lymphocytes, neutrophils, and myelin. Certain allergic reactions, especially to drugs, are

more frequently in HIV infected people, and are likely to be antibody-mediated. Despite producing more immunoglobulins, B cells in HIV-infected people fail to respond appropriately. Intracellular bacterial infection becomes a major problem in the late disease, and reactivation of infections with pathogens such as cytomegalovirus and *Toxoplasma gondii* produce no IgM response (Stewart, 1997).

3.7.3 Source of opportunistic pathogens

The usual opportunistic pathogens are those that are ubiquitous in soil or water or commonly latent within the body after infection at an early age.

Table 2: Source of opportunistic pathogens

Organism	Source	Route of Infection
Pneumocystis carinii	Endogenous reactivation, infected humans	Inhalation
Toxoplasma gondii	Kitty litter, uncooked meet, endogenous reactivation	Ingestion
Cryptosopridia	Water, infected animals and humans	Ingestion
Microsporidia	Water, infected animals and humans	Ingestion
Cryptococcus neoformans	Soil, bird droppings, animals	Inhalation
Aspergillus species	Soil	Inhalation
Cytomegalovirus	Endogenous reactivation, infected humans	Sexual or close blood contacts
Herpes simplex	Endogenous reactivation, infected humans	Sexual contacts
Varicella zoster	Endogenous reactivation, infected humans	Uncertain
Mycobacterium avium	Soil, water	Inhalation, ingestion

Some pathogens commonly infect people with HIV that are rare in other immunocompromised patients. For some this is due to the severe, broad and unrelenting nature of advance HIV immune depletion, but latent infection with some pathogens may also be higher in people with HIV. Some of the common source of pathogens is shown in table 2.

3.7.4 Common opportunistic pathogens

Though all opportunistic disease may occur in AIDS patients throughout the world, the relative importance of specific diseases may be different. For instance, major opportunistic diseases in Europe, the Americas and Asia, such as *Pneumocystis carinii* pneumonia, atypical mycobacteriosis and Kaposi's sarcoma, less frequent in African AIDS patients than elsewhere. In contrast, tuberculosis, chronic diarrhea, cerebral toxoplasmosis and bacteraemia due to non-opportunistic pathogens, such as *Salmonella typhimurium* and *Streptococcus pneumoniae*, are all very common. In South-East Asia systemic infection with *Penicillium marneffei* is a common opportunistic mycosis in patients with HIV infection (Cook, 2001).

Besides this, tuberculosis is also the leading cause of death among the AIDS patients in these regions. Additional consequences of dual HIV/*M. tuberculosis* infection include a high mortality among tuberculosis patients with HIV infection (mostly due to HIV related illness), a growing rate of multidrug resistance of *M. tuberculosis,* and an up to tenfold increase in sever skin reactions-including Stevens-Johnson syndrome, during antituberculous therapy in HIV-positive patients. Kaposi's sarcoma, an angioproliferative disorder of endothelial origin, is found in some of the patients with AIDS.

Some of the common organisms that are generally encounter in the patients with HIV infection are presented here.

3.7.4.1 Bacteria

a) Campylobacter jejuni

Campylobacter jejuni is the one of the cause of bacterial diarrhoea in the patient with low immunity. The organism is responsible for the large bowel infection. *Campylobacter jejuni* is gram-negative slender, curved rods, 0.2-0.5 × 1.5-5 µm, appearing in comma, S-shaped or spiral ("gull-wing") forms (Collee, 1996).

Campylobacter can cause either gastrointestinal or extra intestinal infections. Extra intestinal disease, including meningitis, endocarditic, and septic arthritis, is being recognized increasingly, particularly in patients with AIDS and other immunocompromised individuals (Forbes, 2002).

There are no special requirements for the collection, transport, and processing of clinical specimens for the detection of *campylobacter*; the two most common specimens submitted to the laboratory are feces and blood. The identification is made on the basis of its morphological and cultural characteristic. The organism is microaerophilic, i.e. for growth they required oxygen at tension lower than in air. The optimum gaseous composition for growth is 5% oxygen, 10% carbon dioxide, and 85% nitrogen. This bacterium grows well but slowly on good quality nutrient agars; growth may take 2-5 days to develop fully on suitable media. On freshly poured solid media, the colonies are flat, moist, translucent, and when young, are like water droplets. They commonly range from 0.5-3 mm in diameter and tend to become confluent along the streaks made by the inoculating wire.

Campylobacter jejune is oxidase positive, nitrate positive, urease negative, Catalase positive, and usually do not form H_2S on triple-sugar iron agar. The ability to hydrolyse hippurate is considered to be the diagnostic for the strain of *C. jejune* (Collee, 1996).

b) Haemophilus influenzae

Haemophilus influenzae is a small, non-motile Gram-negative, fastidious, facultative anaerobic bacterium in the family Pasteurellaceae. *H. influenzae* is highly adapted to its human host. It is present in the nasopharynx of approximately 75 percent of healthy children and adults. It is usually the non encapsulated strains that are harbored as normal flora, but a minority of healthy individuals (3-7 percent) intermittently harbor *H. influenzae* type b (Hib) encapsulated strains in the upper respiratory tract.

Pharyngeal carriage of Hib is important in the transmission of the bacterium. *Haemophilus* "loves heme", more specifically requires a precursor of heme in order to grow. Nutritionally, *Haemophilus influenzae* prefers a complex medium and requires preformed growth factors that are present in blood, specifically X factor (i.e., hemin) and V factor (NAD or NADP). In the laboratory it is usually grown on chocolate blood agar which is prepared by adding blood to an agar base at 80 degrees. The heat releases X and V factors from the RBCs and turns the medium a chocolate brown color. The bacterium grows best at 35-37°C and has an optimal pH of 7.6.

Haemophilus influenzae is generally grown in the laboratory under aerobic conditions or under slight CO_2 tension (5% CO_2), although it is capable of glycolytic growth and of respiratory growth using nitrate as a final electron acceptor.

The pathogenesis of *H. influenzae* infections is not completely understood, although the presence of the type b polysaccharide capsule is known to be the major factor in virulence. Encapsulated organisms can penetrate the epithelium of the nasopharynx and invade the blood capillaries directly. Their capsule allows them to resist phagocytosis and complement-mediated lyses in the non-immune host such as patients with AIDS.

c) Streptococcus pneumoniae

Streptococcus pneumoniae is gram positive lancet-shaped arranged in pairs or in short chain, alpha-haemolytic cocci. The organism produces pneumolysin that degrades red blood cells under anaerobic conditions (observed as α haemolysis). *Streptococcus pneumoniae*, commonly known as pneumococci, is the primary etiological agent in pneumonia of all types it is particularly responsible for lobar pneumonia. Patients with HIV/AIDS, congestive heart failure, chest trauma, multiple myeloma, sickle cell anemia and, hypogammaglobulinemia are most susceptible to pneumococcal infections. Pneumococci are identified by solubility in bile. An autolysin (peptidoglycan-degrading enzyme) is released by bile from the cell membrane and binds to a choline-containing teichoic acid attached to the peptidoglycan. The autolysin then digests the bacterial cell wall resulting in lysis of the cell. If the cells are grown in ethanolamine instead of choline, ethanolamine is incorporated into the teichoic acid. The autolysin then cannot lyse the cell wall.

Capsule is highly prominent in virulent strains and its carbohydrate antigens vary greatly in structure among strains. The capsule is anti-phagocytic and immunization is primarily against the capsule. Capsular vaccines are available for susceptible individuals; immunity is serotype-specific. Using appropriate type-specific antisera, the capsule on isolated bacteria can be "fixed" and becomes visible microscopically (the Quellung reaction) which is useful in microbial identification. Complement activation by teichoic acid may explain the attraction of large numbers of inflammatory cells to the focal site of infection

The presence of a capsule allows an escape from phagocytosis, resulting in an intense inflammatory response in hosts who are immunologically naive. Colonization of the oropharynx by bacterial adherence to human pharyngeal cells is the usual first step.

The alternative pathway of the complement is first activated. Anticapsular antibodies are effective in providing protection against pneumococcal infection. They appear 5-8

days after the onset of infection. By this time, fever usually disappears in the absence of treatment. Natural immunity follows infections as well as colonization. Studies on the prevalence of measurable levels of antibody in the US suggest that the adult population is susceptible to most *S. pneumoniae* that commonly cause infection.

d) Staphylococcus aureus

The *staphylococci* are gram-positive spherical cells, usually arranged in grape-like irregular clusters, facultative anaerobes and are Catalase positive they grow readily on many types of media and active metabolically, fermenting carbohydrates and producing pigments that vary from white to deep yellow. Most of them are normal flora of skin and nose. It is one of the commonest causes of opportunistic infections in the hospital and community; including pneumonia (Jawetz, 1980*). Staphylococcus aureus* is beta-hemolytic on sheep blood agar, can ferment Manitol, Golden pigmented, Coagulase-positive. It contains an IgG-binding protein A in the cell wall, produces a clumping factor (different from coagulase) that binds fibrinogen. Free protein a binds to immunoglobulin and complement, blocking Fc and complement receptors and is thus anti-phagocytic.

S. aureus causes a number of different disease entities associated with production of certain exotoxins. In addition to these "disease-specific" exotoxins, other cell lytic exotoxins (alpha, beta [sphingomyelinase C], gamma and delta toxins and leucocidins) may be produced. In addition, some tissue-degrading enzymes may be involved in spreading (e.g. lipase and hyaluronidase).

e) Klebsiella pneumoniae

Klebsiella pneumoniae is Gram negative, non-motile and nonflagellated, beta-lactamase positive, lysine positive, citrate positive and produces gas. This bacterium produces large sticky colonies when plated on nutrient media. Its pathogenicity can be attributed to its production of a heat-stable enterotoxin. *K. pneumoniae* infections are common

among HIV/AIDS patients, where they cause pneumonia (characterized by emission of bloody sputum). It is facultative anaerobic bacilli, Oxidase negative, reduce nitrates to nitrites; ferment lactose, urease positive. It contains thick capsule, which is a major virulence factor and is antiphagocytic. Its outer membrane contains lipopolysaccharide (LPS) in which lipid A portion is endotoxic, O (somatic) antigen is serotype specific. The bacteria overcome innate host immunity through several means. Alcoholics and people with compromised immune systems are at increased risk of infection.

K. pneumoniae, which is normally found in the respiratory tract of approximately 10% of the population, can cause pneumonia in immunocompromised individuals and diabetics. Lung abscesses may form. A significant clinical clue is the production of thick, blood tinged, viscous sputum. Pneumonia because of *Klebsiella spp* infection carries a fatality rate of 50%. If bacteraemia develops, the prognosis is even worse.

f) Pseudomonas aeruginosa

Pseudomonas spp are obligate aerobic gram-negative rods that do not ferment glucose for energy. These organisms are called non-fermenter. They derive their energy by oxidation of sugars rather than fermentation, so they are Oxidase positive. *Pseudomonas aeruginosa* is one of the major species in the pseudomonas genus and derives its name from the production of various pigments, including pyoverdin, a yellow-green pigment, and pyocyanin, a blue or green pigment. *Pseudomonas aeruginosa* is motile, nutritionally versatile, and can grow at a variety of temperatures (optimally at 37°C, but can also grow at 42°C). It can also compose part of the normal microbial flora of humans, with rates of colonization enhanced in hospitalized and immunocompromised patients. Although *Pseudomonas aeruginosa* is ubiquitous in the environment, pseudomonal infections are mainly opportunistic and effect compromised hosts. *Pseudomonas aeruginosa* pneumonia occurs almost exclusively in patients with compromised lung function or systemic immunocompromised. Exposure to the hospital

environment especially in ICUs, endotracheal intubations, and prior use of antibiotics increases the likelihood of *Pseudomonas aeruginosa* pneumonia.

P. aeruginosa is Oxidase positive, Positive for growth at 42°C, Positive for hydrolysis of acetamide, Positive for growth on cetrimide agar, Positive for nitrate reduction, smells like grape or corn-tortilla and gives Sticky non-lactose fermenting colony in MacConkey agar. *P. aeruginosa* is capable of producing pyocyanin, pyoverdin, pyorubin, and pyomelanin pigments.

Furthermore, damage of the mucosal epithelium by proteases, including a protease released by *Pseudomonas* sps and proteases released by inflammatory cells, leads to exposure of cellular cytoskeleton with strong binding affinity for *Pseudomonas* sps. The virulence of the strains of *Pseudomonas spp* is enhanced by the production of high levels of exotoxins A.

g) *Salmonella* sps

Salmonella is gram-negative rods belonging to Enterobacteriaceae family. They are motile with peritrichous flagella. In aerobic and anaerobic conditions, they grow readily on ordinary media producing colonies indistinguishable from those of other. Mostly they are prototrophic and grow in minimal-salts medium with a suitable carbon sources. Usually the organism is glucose, maltose, mannitol and sorbitol fermenter with production of acid and gas. They cannot ferment sucrose, salicin and adonitol. They lacks tryptophanase enzyme so they cannot utilize tryptophan to produce Indole. They are urease negative so cannot utilize urea. They give a positive methyl red reaction and negative Voges-Proskauer reaction. *Salmonella* grows well with citrate as sole source of carbon. Most salmonella gives a positive reaction for H_2S in triple sugar iron agar but the exception includes strains of Paratyphi A. Nearly all *Salmonellae* with the exception of *typhi* and *paratyphi* A produce arginine dihydrolase, lysine and ornithine decarboxylase but not glutamic acid decarboxylase.

h) Mycobacterium avium

Organisms of the *Mycobacterium avium* complex (MAC) are ubiquitous in the environment. *M. avium* is the etiologic agent in >95% of patients with AIDS who develop disseminated MAC disease. An estimated 7%--12% of adults have been previously infected with MAC, although rates of disease vary in different geographic locations. Although certain epidemiologic associations have been identified, no environmental exposure or behavior has been consistently associated with the subsequent development of MAC disease in susceptible persons.

The mode of transmission for MAC infection is thought to be through inhalation, ingestion, or inoculation through respiratory or gastrointestinal tract portals of entry. Household or close contacts of those with MAC disease do not appear to be at increased risk for experiencing disease, and person-to-person transmission is unlikely. In the absence of effective combination ART or chemoprophylaxis in those with advanced immunosuppression, the incidence of disseminated MAC disease among persons with AIDS ranges from 20%--40%. For those with a $CD4^+$ T lymphocyte count <100 cells/μL who are receiving effective prophylaxis or those who have responded to ART with a sustained increase in $CD4^+$ T lymphocyte count to levels >100--200 cells/μL, the overall incidence rate has been estimated at 2 cases per 100 person-years. Most cases of MAC disease occur among persons with $CD4^+$ T lymphocyte counts <50 cells/μL. Other factors that are associated with increased susceptibility to MAC disease are high plasma HIV-1 RNA levels (>100,000 copies/mL), previous opportunistic infections (particularly CMV disease), previous colonization of the respiratory or gastrointestinal tract with MAC, and reduced in vitro lymphoproliferative immune responses to *M. avium* antigens, possibly reflecting defects in T-cell repertoire.

i) Mycobacterium tuberculosis

Tuberculosis is an important pulmonary complication of HIV/AIDS for many regions. HIV infection represents the greatest risk factor for the development of active tuberculosis. Instead of a life time risk of developing disease of 10%, those with HIV infection have a risk of 8%-10% per year. If immunosuppressed, progression from infection can be rapid, taking months rather than years. It is contagious via the respiratory rout to other HIV-infected persons and those not infected with the virus. It is thought to hasten the progression of HIV disease, but it is treatable. Tuberculosis can be present at different stages of HIV infection. The clinical pattern changes with the degree of underlying immunosuppression, being similar to adult post-primary infection early immune deficiency and similar to primary infection in advance immune deficiency. In the later situation, the patients may present with non-specific features such as fever, weight loss, and fatigue and lymph-node enlargement.

Extrapulmonary tuberculosis is much more common in HIV-infected individuals than in general population, occurring in up to 70% of the patients with HIV and tuberculosis. The commonest forms of extrapulmonary tuberculosis are lymphadenitis and military disease, but other sites such as the central nervous system, bone marrow and genitourinary tract can be involved (Stewart, 1997).

Mycobacterium tuberculosis is acid-fast bacilli, which shares the property of acid fastness. It is resistant to decolourization by week mineral acid after staining with one of the arylmethane dyes. *M. tuberculosis* is complex unicellular organism with a wide range of antigenic determinants. They are bacillary or cocco-bacillary and vary from 0.5 to 10 µm in length. *M. tuberculosis* gives eugonic (rough, tough, and buff growth on glycerol and pyruvate egg. The niacin test and urease test are positive. It is an aerobe that grows only at 37^0C and is usually sensitive to standard antimycobacterial therapy.

j) Treponema pallidum

Treponema pallidum is the causative agent of syphilis. The organism is spirochetes and is not stained by normal gram stain technique. It is approximately 0.1-0.5 µm wide and

5-15 µm long with 6-14 coils. The organism is microaerophilic in its oxygen requirements and can only be grown in tissue culture (Virella, 1997).

Treponema pallidum is transmitted by direct contact and transplacentally. Clinical laboratory workers, hospital personal, and blood transfusion recipients may contact the disease accidentally. Thus the incidence of this disease is high among the patients with HIV/AIDS, who have encountered the disease via sexual or direct contact with blood.

3.7.4.2 Fungi

a) Candida albicans

Candida albicans is often present in the skin, mouth, vagina, and intestinal tract of healthy persons and animals, where it lives without causing disease. The organism is small yeast that forms filaments called pseudohyphae when cultivated in laboratory media. As body defense is compromised (as in the case of HIV infection) or when changes occurs in the microbial population it flourishes and causes many number of form of candidiasis (Alcamo, 1994).

Candidiasis always develops at some stage of HIV illness, first appearing, on average; at a CD4 cell count of about 275/µL. Presentation include mucosal involvement, flexural involvement, paronychia, balanitis, angular cheilitis, and onychomycosis. The most common form of this disease present as candidiasis in vagina often called as vulvovaginitis of vaginal thrust and oral candidiasis of oral thrust.

b) Cryptococcus neoformans

Cryptococcus neoformans is the commonest cause of meningitis in HIV infection and can also cause pulmonary disease (Stewart, 1997). The organism is found in the soil of urban environment and grows actively in the dropping of pigeons but within the pigeons itself. Cryptococcus may become airborne with gusts of wind and subsequently enters the respiratory passageways of humans.

Cryptococcus neoformans cell have a diameter of about 5 to 6 µm and are embedded in a gelatinous capsule that provide resistant to phagocytosis. The cell penetrates to the air sacs of the lungs, but the symptoms of infection are generally rare. However, if the organism pass to the blood stream and localize in the meninges and brain, the patients may experience piercing headaches, stiffness in the neck, and paralysis (Alcamo, 1994).

3.7.4.3 Protozoa

a) Pneumocystis carinii

Pneumocystis carinii is an opportunistic pathogen, causing life threatening pneumonia in immunocompromised patients, particularly in AIDS patients. Infection probably occurs by inhaling the organism in aerosols. Some infections may also occur from reactivation of a latent infection. HIV-associated pneumocystic pneumonia is found in developing country but it is not a major cause of death in AIDS patients as in USA, Europe, and elsewhere (Cheesbrough 2, 2000).

The cysts of the organism are 10-12 µm in diameter. Trophozoites are smaller (1-5 µm), pleomorphic and is not seen by silver staining. Giemsa is necessary to demonstrate trophozoites and the internal structure of the cyst which encloses two to eight sporozoites. The organism may be demonstrated in the sputum obtained by inhalation of saline (induced sputum), bronchial washing and is impression smear from lungs tissue. Open lungs biopsy is traumatic and only considered if transbronchoscopic biopsy fails (Collee, 1996).

b) Cryptosporidium parvum

Cryptosporidium parvum is thought to have world wide distribution but is particularly prevalent in tropical and developing countries. The disease is transmitted by the faecal-oral route with infective oocysts being ingested. The oocysts is small, round to oval, measuring 4-6 µm. *Cryptosporidium parvum* infection is higher in the warm wet season, and lower in the drier cool months.

In persons with abnormal immune responses, particularly with AIDS, infection with *Cryptosporidium parvum* can cause acute and often fetal diarrhoeal disease and also respiratory disease. Autoinfection can occur with infective oocysts sporulating in the intestine (Cheesbrough 1, 2000).

c) Toxoplasma gondii

Toxoplasma gondii is an animal coccidian parasite which causes toxoplasmosis, with congenital toxoplasmosis being the most serious form of human infection. It has a world wide distribution. The disease is transmitted by the ingestion of oocysts in food, water, or from hands contained with faeces from an infected cat. Transmission can also occurs by transplant transmission (as in HIV/AIDS), by blood transfusion, or by ingesting the parasites in uncooked meet of an infected animal intermediated host (Cheesbrough 1, 2000).

In the early stages of infection, the parasite invades phagocytic cells and mononuclear leucocytes. In the chronic stages of infection, the parasites multiply intracellular in the tissues, forming cysts. The commonest cause of focal cerebral lesions in HIV/AIDS is toxoplasmic encephalitis, which is almost always due to reactivation of past infection. This infection is followed by fever, headache, mental deterioration and seizures.

d) Giardia lambia

Giardia lambia has a worldwide distribution and is particularly common in the tropic and sub-tropics, in areas where water supplies and the environmental becomes faecally contaminated. The disease is transmitted by faecal-oral rout. The organism occurs in two different forms, trophozoite and cysts. Trophozoite is small pear-shaped flagellate with a rapid tumbling and spinning motility, often likened to a falling leaf, measuring 12-15 × 5-9 µm. Cysts are small and oval measuring 8-12 × about 6 µm. Internal structure includes four nuclei grouped at one end, azonemes, median bodies, and remains of flagella.

In HIV/AIDS it causes abdominal pain, severe diarrhoea, flatulence, vomiting, weight loss, malabsorption with lactose intolerance. Symptoms can be severing in the children under the age of 3, and in the undernourished. Those with gastrointestinal disorder or bacterial infection of the intestine tend to be more susceptible to this infection.

3.7.4.4 Virus

a) Human herpes virus type 8 (HHV8)

Human herpes virus type 8 causes a type of malignancy among the HIV/AIDS people especially homosexually male. This malignancy, called as Kaposi's sarcoma, which in days before HIV was seen only in elderly men, is now a sign of HIV infection, especially in healthy men. Kaposi's sarcoma is most commonly manifested as skin lesions usually red, purplish or brown colored. Sometime these follows skin creases and can occurs quite symmetrically like those of pityriasis rosea. These lesions need to be differentiated from other pigmented lesions, including melanocytic naevi, dermatofibromas, bruises, pyogenic granulomas, haemangiomas and bacillary angiomatosis.

Any part of body surface can be affected, but the lesions commonly occur inside the mouth on the hard palate. A skin biopsy should always be obtained to confirm diagnosis, as even Kaposi's sarcoma can have atypical presentation (Stewart, 1997).

b) Cytomegalovirus

Cytomegalovirus (CMV) is a prevalent virus that causes minimal symptoms or asymptomatic infection in otherwise healthy adults, but may cause severe disease when the immune system is not well developed or compromised. Polymorphs, Monocytes, B-lymphocytes, and epithelial cells may all be latently infected. CMV can be recovered from more than 90% of immunocompromised patients (particularly in HIV/AIDS). CMV infection can present as fever, pneumonia, esophagitis, enteritis, hepatitis, retinitis, uveitis- virtually any type of infection.

c) Hepatitis B virus

Hepatitis B virus (HBV) is a non-cytopathic virus and contains a partially double-stranded DNA genome. This virus predominantly infects hepatocytes and belongs to the hepadnavirus family. HBV, by contrast, is almost exclusively an immune-mediate disease. The outcome of infection is largely determined by the age at infection, which relates to the maturity of the immune respond (Dore, 2001).

This virus infection becomes more severe in the patients with suppress immune system such as HIV infection. The prevalence of HBV and HIV infection is high among the individual using intravenous drugs and practicing multi-partner sex.

d) Varicella-zoster virus

In patients with HIV/AIDS varicella-zoster is the etiologic agents of herpes zoster. This disease is believed to result from the reactivation of virus, which remains latent for life in the sensory ganglia of patients who had chickenpox. The vesicular rash occurs in a dermatomal distribution and is most often seen on the chest, but it may be located anywhere. The vesicles have a sharply demarcated border that is not seen in other type of herpes lesions. The rash is very painful; in some cases, precedes the rash, making diagnosis difficult.

e) Herpes-simplex virus

Herpes-simplex is the most important viral infection because it can cause chronic ulceration and pain. If there is no other region for immunosuppression, infection that persists for than a month indicates progression to AIDS. Herpes-simplex may present as vesicles or ulcer on the palate, gingival margins, lips of face. Besides these it may rarely lead to radiculopathy, sometimes with myelopathy, usually in association with a recent history of active genital herpes.

3.8 HIV/AIDS related systematic diseases

The infections with HIV destroy the immune system in such a way that people who loss their life because of HIV/AIDS suffers from various or almost all systematic disease. The week immune system cannot overcome the large majority of opportunistic organisms invading the body. The most common systematic disease encountered in the developing country like Nepal is discussed below.

3.8.1 HIV/AIDS and respiratory disease

Up to two-third of people with HIV will have a respiratory illness associated with their infection. Many of these illnesses are treatable and some are preventable. Aggressive investigation of pulmonary symptoms and signs is essential (Mitchell, 1995). Most of the pulmonary diseases are due to bacterial and unusual organisms. The lungs is one of the most common sites of problem, usually late in the chronology of HIV induced disease. Accurate diagnosis and confirmation of unusual infection or disease processes in the lungs will be the trigger for asking could it be HIV (Stewart, 1994).

Pneumocystic carinii pneumonia is the most frequently identified serious opportunistic infection in HIV disease. It is also one the most common AIDS defining illness in the undiagnosed HIV infected patient. The most frequently occurring infection in the developing country like Nepal is tuberculosis. It occurs relatively early, with CD4 cell count in the range 200-400 µL. Besides these, pyogenic bacterial infections occur more frequently in HIV-infected persons. The most common organisms include *Streptococcus pneumoniae, Haemophilus influenzae*, and *Branhamella catarrhalis*. Infection with *Staphylococcus aureus, Legionella pneumophila* and gram negative organism may also occur.

MAC infection is an extremely common infectious complication of advance HIV infections, although it is not considered as major pulmonary pathogens. Chronic

suppurative lung and sinus disease are being reported with increasing frequency. Sinus disease can be more chronic, bilateral and more severe than in HIV-negative individuals. Pulmonary disease in HIV infection can be listed below.

Table 3: Pulmonary disease in HIV infection.

Condition	CD4 cell count (perμL)
Infection	
Pneumocystic carinii pneumonia	<200
Bacterial pneumonia	<250
Suppurative lungs and sinus disease	<100
Mycobacterium tuberculosis	<400
Mycobacterium avium complex	<100
Cytomegalovirus	<100
Malignancy	
Kaposi's sarcoma	<150
Non-Hodgkin's lymphoma	<100
Lungs carcinoma	
Mechanical	
Airways diseases/emphysema	<250
Pueumothorax	-
Vascular	
Pulmonary hypertension	-
Interstitial lung disease	
Lymphoid interstitial pneumonitis	>500

Besides bacterial infections there are some viral infections that are common as respiratory pathogens in HIV patients. The most predominant being the Cytomegalovirus, occurring when the CD4 cell count falls below 100/μL. HIV may

itself causes direct pulmonary damage. Viral upper respiratory tract infections are more common in HIV infection.

Among the fungal infection, *Cryptococcus neoformans* and disseminated candidiasis are common in HIV infections. Disseminated candidiasis may also involve the lungs.

Two common malignancies associated with HIV can involve the lungs, Kaposi's sarcoma and non-Hodgkin's lymphoma. An increased incident of malignancies in immune suppressed patients have been reported, but there is nothing unusual about feature of bronchial carcinoma that points to underlying HIV infection in smokers.

The lungs in HIV can be affected by a number of functional or mechanical problems. Pueumothorax, cystic pulmonary disease, and an emphysema-like process are seen in advance disease in patients without previous infective lungs problems. HIV infection has also been associated with pulmonary hypertension.

In conclusion, we can say that the lungs are a primary target for the opportunistic infections and malignancies affecting those with HIV infection. In the patients whose HIV infection is undiagnosed, PCP is the commonest clue to its presence. Early diagnosis prevents morbidity and mortality. Less commonly, interstitial lungs disease and tuberculosis, often "primary" or clinically atypical, will be the clue to underlying HIV infection. Other pulmonary complications are usually a late manifestation of HIV infection, which has usually (but not always) already been diagnosed.

3.8.2 HIV/AIDS and gastrointestinal disease

Gastrointestinal disease is common in patients infected with HIV and can represent the first significant clinical illness. Diarrhoea, dysphagia, abdominal pain, jaundice or gastrointestinal bleeding may be the result of opportunist infection, AIDS-related neoplasia, or infection with HIV alone. The spectrum of gastrointestinal tract and liver involvement in HIV infection is broad and has been well reviewed recently. While

significant gastrointestinal disease tends to occur late in the natural course of HIV infection, clinical symptoms or signs occurs at all stages in the chronology of HIV disease.

About 15% of patients with HIV infection and AIDS present with acute, severe abdominal pain requiring urgent evaluation. The pain is often an intermittent or recurrent problem, which may be associated with chronic diarrhoea. The differential diagnosis is broad and includes problems unique to HIV disease as well as those seen in the general population.

Oesophageal disorders occur in about 40% of AIDS patients. The main symptoms are difficulty in swallowing and pain on swallowing. The vast majority of patients with HIV infection and dysphagia will have *Candida* oesophagitis, which can occur during any stage of HIV infection, including seroconversion illness. Other causes include oesophagitis due to CMV, herpes simplex virus or MAC, idiopathic ulcer and Kaposi's sarcoma.

Diarrhoea is the most common gastrointestinal symptom in HIV infection, affecting up to 90% of patients and becoming more frequently as immune deficiency progresses (Johansen, 1990). Most patients have some degree of malabsorption and many are malnourished; diarrhoea and weight loss are independent predictors of mortality.

Anorectal disease is common in homosexual men and may include anal fistulas, anal ulcers secondary to infection with herpes simplex virus and CMV, non-specific idiopathic ulceration, lymphoma and squamous-cell carcinoma. Anal intraepithelial neoplasia appears to be associated with human papilomavirus infection and accelerated by HIV-induced immune deficiency.

Although the incidence of gastrointestinal haemorrhage in patient with HIV is unknown, serious gastrointestinal blood loss occurs in only a small percentage, but is

associated with high mortality (up to 30%). Common causes of bleeding, such as peptic ulcer disease, should be considered as well as HIV-related infection and neoplasia. CMV is the opportunistic infection most likely to cause gastrointestinal bleeding, as it infects vascular endothelium, leading to ichaemia and mucosal ulceration. It can affect the oesophagus and stomach but more often is a cause of colonic ulceration or diffuse colitis. Neoplasms of the GI tract occasionally cause bleeding. Common pathogens involved can be listed as follows.

Table 4: Common pathogens in HIV-related diarrhoea

Organism	Small bowel (duodenum/jejunum)	Large bowel (colon/terminal ileum)
Bacteria	*Mycobacterium avium* complex *Salmonella* species	*Campylobacter* species *Shigella* species *Yersinia* species *Aeromonas* species *Clostridium* species
Protozoa	*Cryptosporidium* species *Microsporidia* *Cyclospora* species *Giardia lambia*	*Entamoeba histolytica*
Viruses	Rotavirus Astrovirus Calicivirus Picornavirus HIV	Cytomegalovirus Adenovirus Herpes virus

3.8.3 HIV/AIDS and hematological disease

Patients with HIV infection may present with symptoms and/or signs of anaemia, neutropenia or thrombocytopenia at all stages, including seroconversion. Although the principle defect of HIV infection is in the production of CD4 lymphocytes, multilineage hematopoietic cell defects are common. The severity of anaemia and neutropenia, thought not thrombocytopenia, is related to fail in the CD4cell count and progress of HIV disease (Stewart, 1997).

Thrombocytopenia occurs in all patient groups infected with HIV and has several causes. The recent studies showed that thrombocytopenia occurs more frequently in patients with AIDS (up to 21%) or CD4 cell counts below 200/µL (30%), than in patients without AIDS (9%) or with CD4 cell counts between 200 and 500/µL (8%). Thrombotic thrombocytopenic purpura is a rare disorder associated with HIV infection, which also may present before other symptoms of disease progress.

Normochromic normocystic anaemia (haemoglobin level below 115 g/L) with an inappropriately low reticulocyte count is seen in 70% to 95% of patients on progression to AIDS. Although this is the late stage of HIV infection, clinical presentation and/or diagnosis may unfortunately be delayed until this time. Symptoms of anaemia are likely to dominate the clinical picture. The causes include infection, particularly with *Mycobacterium avium* complex or cytomegalovirus, tumor such as Kaposi's sarcoma or lymphoma, and AIDS induced suppression of progenitor cells.

It is most important to review the patient's differential white cell count, as a normal total white cell may mask important diagnostic clues to HIV infection such as neutropenia, lymphopenia or lymphocytosis. Neutropenia (neutrophil count below 1500/µL) may occur rarely with seroconversion or more commonly (in up to 40% of patients) with progression to AIDS. Neutrophil counts below 1000/µL are manifested clinically in mouth ulceration, skin infection, fever or septicaemia. Patients may present

with peripheral blood features of myelodysplasia, such as anaemia and neutropenia with hypogranular neutrophils, although the diagnosis of AIDS is usually apparent at this time. Other blood film abnormalities include vacuolated monocytes and, occasionally, phagocytosed pathogens such as *Candida*.

Generalized lymphadenopathy and mild to moderate splenomegaly may occur with the initial seroconversion reaction, with ongoing asymptomatic infection or with AIDS. Any person who present with generalized lymphadenopathy that persist for more than three months and who has been sexually active should be advised to the need for HIV testing.

3.8.4 HIV/AIDS and urinary tract infection

Urinary tract infection in HIV/AIDS includes fluid and electrolyte disorders, glomerular disease, tubulointerstitial disease, and parenchymal disease. Renal parenchymal and fluid and electrolyte abnormalities may complicate hospitalization in 20% of patients. HIV-specific renal abnormalities are being defined, but most follow opportunistic infection or drug nephrotoxicity. Principles of treatment are largely the same as for patients without HIV.

Volume depletion is the most common cause of renal insufficiency in patients with HIV/AIDS, often a result of vomiting and diarrhoea. Hyponatraemia is common in advance HIV disease, with volume depletion being a factor in about 40 % of cases. It is associated with increase mortality in patients with AIDS.

HIV-associated nephropathy is a distinctive clinicopathological entity, with focal and segmental glomerulosclerosis, proteinuria, nephritic syndrome and frequently progression to renal failure over weeks to months. More common in intravenous drug users, it may occur at any stage of HIV infection. Hypertension is rare, oedema often not prominent, and haematouria infrequently. Aetiology may be related to direct

infection by HIV or penetration of renal parenchyma by CD4 lymphocytes and macrophages.

3.9 Mode of HIV transmission

HIV is transmitted by any of the four routes. These include sexual contacts (Heterosexual or homosexual), administration of infected blood or blood products, sharing of contaminated needles, and perinatal transmissions. In contrast to North America and Europe, where the overwhelming majority of AIDS patients acquired the disease by homosexual contacts or where intravenous drugs addicts, bidirectional heterosexual transmission is the major mode of infection in the developing country like Nepal.

In the absence of amplifying factors, the efficiency of penile-vaginal intercourse is low for HIV transmission (well below 1%). Factors which enhance the risk of heterosexual transmission of HIV include higher viraemia (during acute infection and more advance stage of disease) in the infecting partner, sex during menstruation, receptive anal intercourse, the presence of other sexually transmitted diseases, and possible lack of circumcision in men, cervical ectopy and the use of desiccating vaginal agents. In particular, genital ulcers such as chancroid, syphilis, and genital herpes, and to a lesser extent, gonorrhoea and chlamydial infection, enhance the risk of sexual transmission of HIV (Cook, 1995).

HIV has been transmitted by transfusions with whole blood, plasma, clotting factors, or cellular fractions of blood (Strohl, 2001). Blood transfusions continue to be a source of HIV infection in many parts of the developing world. This clearly demonstrates that the existent of a fairly simple technology-HIV antibody tests-is not enough for assuring safe blood transfusions.

Transmission can occur by inoculation with HIV-contaminated needles-ether accidentally or through the use of shared needles or syringes by drugs users.

Nosocomial transmission of HIV by syringe and needles undoubtedly occurs, but its role in the spread of HIV is poorly documented-although it is probably very low. However, outbreak of infection-associated nosocomial infection in Eastern Europe demonstrated that infections for medicals during medical purpose can be a source of HIV infection in the community (Cook, 1995).

Mother-to-child transmission during pregnancy, during delivery or through breast feeding is the second most common mode of spread of HIV-1 in the developing world. It is strikingly that the observed rates of mother-to-child transmission of HIV-1 have been consistently higher in Asia and Africa than in Europe and North America. Factor playing a role in this higher risk of transmission include transmission through breast feeding, more frequent advance immunodeficiency (associated with higher HIV viraemia) and more common occurrence of chorioamnionitis as a result of genital infection. In several, but not all, studies maternal HIV-1 infection was associated with adverse pregnancy outcome, including stillbirth, premature delivery and low birth weight.

3.10 Laboratory diagnosis of HIV infection

Demonstration of HIV-1/2, their proteins, or their nucleic acids in the serum of a particular individual is an effective diagnosis of HIV infection. Besides this monitoring of CD4 cells in plasma, determining the condition of ratio of CD4 to CD8 cells in the peripheral blood also serve as a diagnostic tool for HIV/AIDS.

3.10.1 WHO testing strategy for HIV infection

There are large numbers of test that determines the presence of HIV infections, WHO recommends three testing strategies to maximize accuracy while minimizing cost (WHO, 1992). Which strategy is most appropriate will depend upon the objective of test and the prevalence of HIV in the population as shown in table 4.

Table 5: WHO recommendation for HIV testing strategies according to test objectives and prevalence of infection in the population.

Objective of testing	Prevalence of infection	Testing strategy
Transfusion/donation safety	All prevalence	I
Surveillance	>10%	I
	≤10%	II
Diagnosis		
Clinical signs/symptoms of HIV infection/AIDS	All prevalences	II
Asymptomatic patients	>10%	II
	≤10%	III

Strategy I: All samples are tested with one ELISA or rapid/simple test (hereafter referred to as test).

Strategy II: All samples are first tested with one test. Any reactive samples are subjected to a second test based on a different principle and/or a different antigen preparation.

Strategy III: All samples are first tested with one test. Any reactive samples are tested with a different test. Samples found reactive by the second test are subjected to a third and different test.

3.10.2 Microbiological investigations of HIV infection

HIV can be isolated from the lymphocytes of heparinized blood samples of a high proportion of patients with asymptomatic as well as symptomatic infections but the co-

cultivation procedures require with ‘permissive’ cell lines, and the techniques used to detect infection of the tissue culture, are currently only available as research procedures.

Screening serum HIV antibody tests usually depends on either direct or indirect ELISA assays involving plastic microtiter trays or beads coated with HIV antigens or antibody. Both HIV-1 and HIV-2 should be tested for by initial screening antibody tests. Blood is the usual sample, but recently screening test with saliva and urine has also been introduced. Two more reliable first generation commercial screening tests had good specificity, but occasional false positives occurred as well as false negatives. Most recent commercial ELISA and other antibody tests have been introduced using specific monoclonal antibodies or more specific p24 core protein or various envelope antigens. These tests allow infection to be detected serologically earlier than first-generation tests and the sensitivity is also improved for detecting HIV antibodies in a patients with advance AIDS. More than 97% of patients with AIDS and at least 99% of patients with PGL or ARC have serum antibodies to HIV detected with these tests. Greater than 93% of patients accruing HIV infection appear to seroconvert during the first 3 months of infection (Shanson, 1999).

Commercial PCR test for HIV RNA in EDTA plasma samples are valuable for detecting HIV infection in infants when the results of antibody tests may be difficult to interpret. Molecular biology tests can also be useful for investigating suspected outbreaks of HIV in exceptional circumstances in the community and rarely in hospitals following surgery or dental practices. Nucleic acid quantification tests are useful for prognosis assessments and decisions about starting and monitoring antiretroviral drug therapy.

3.11 Treating HIV/AIDS

Because of the progressive nature of the disease, it is the HIV infection that is treated as the clinical problem, rather than focusing on the end stage-AIDS-alone. Thus, treatments of HIV/AIDS can be broadly classified into three categories. Antiretroviral therapy targeting each step in the HIV viral replications, but in recent trends only those

drug directed against the reverse transcriptase and viral protease have been mostly used. Immune based therapy used to enhance the immune system as far as practical. Finally, treating secondary infection as soon as they emerge. The final category of treatment posses the greatest threat once it is not properly managed. Existence of MDR strain of bacteria, because of treatment, causes the death earlier rather than prolong the life span of PLWHA (Strohl, 2001).

3.11.1 Antiretroviral therapy for HIV/AIDS

There is no clear evidence that combination therapy is more effective at suppressing the virus and delaying disease progression than monotherapy. With more than ten agents available, the management challenge is tailoring combination therapy to the needs of individual patients (Stewart, 1997).

Currently available antiretroviral drugs are classified broadly into three categories. Among them the first antiviral agents to be used were the nucleoside analogues. Besides this, nonnucleoside inhibitor and protease inhibitor were also used. Some of the antiviral drugs are mention below (Strohl, 2001).

Unlike DNA polymerase, which makes very few mistakes in the replication of DNA because of its proofreading activity, reverse transcriptase has no ability to proofread. Therefore DNA synthesis by the viral reverse transcriptase produces many errors. This results in mutations in all of the HIV gene, and accumulation of a pool of mutant viruses in any individual patient. In the presence of an antiviral drug, there is a strong selection for mutation that confers resistant to that drug, and the high mutation rate ensures that such mutations will occur. The answer to this therapeutic dilemma has been to use multi drugs that act on different steps in viral replication cycle, because the probability of several different mutations occurring simultaneously in the appropriate gene in the same genome is very low. In addition, certain of the drug combinations are synergistic, that effects on reducing viral load being considerably greater than merely the sum of the individual drug effects. The use of potent combination regimens can

limit viral replication and dissemination to lymphoid tissue, thus creating a similar reservoir of chronically infected cells, and limiting the potential for viral mutations that could lead to acquisition of drug resistance. However, even this approach is not entirely effective, because HIV also exhibits a relatively high rate of recombination, enabling mutations from two different genomes to be combined into one virus particle (Strohl, 2001).

Table 6: Currently available antiretroviral drugs

Drugs type	Drugs
Nucleoside reverse transcriptase inhibitors (NRTI)	1) Abacavir sulfate 2) Didanosine (ddl) 3) Lamivudine (3TC) 4) Stavudine (d4T) 5) Zalcitabine (ddc) 6) Zidovudine (AZT)
Nonnucleoside reverse transcriptase inhibitors (NNRTL)	1) Delavridine 2) Efavirenz 3) Nevirapine
Protease inhibitors	1) Amprenavir 2) Indianvir sulfate 3) Lopinavir 4) Nelfinavir mesylate 5) Ritonavir 6) Saquinavir

Currently, combinations of various drugs described above that are given three or sometime four at a time are being evaluated for efficacy, both in short-term reduction of viral load and/or increase in CD4+ cell count, and in long term survival. The most commonly used combinations of the drugs are:

1. Two nucleoside reverse transcriptase inhibitor with one protease inhibitor
2. Two nucleoside reverse transcriptase inhibitor with one nonnucleoside reverse transcriptase inhibitor.
3. Two nucleoside reverse transcriptase inhibitor with two protease inhibitors.

Choice of the drugs regimen is individualized based on criteria such as tolerability, drug-drug interactions, convenience/adherence, and possible baseline resistance. All such multi-drugs therapies are commonly referred to as "highly active antiretroviral therapy" or HAART. Unfortunately, whereas such multi drugs therapy can lower the viral load to undetectable levels, virus reemerges if HAART is stopped, indicating that the HIV has not been eradicated. Therefore with current drugs therapy, HIV continues to exist in sanctuaries, such as the central nervous system, semen, or nonreplicating T lymphocyte reservoirs. Thus HIV infection is currently both chronic and incurable with this therapy.

3.11.2 Immune-based therapy for HIV/AIDS

The immune system raises vigorous responses to HIV infection that is more effective in reducing viral replication than many of the antiviral agents used to date. In a few patients this immune response is maintained, effectively suppressing viral replication and delaying diseases progression. However, in most cases, the efficacy of the immune response wanes over time. The aims of immune-based therapy are (Stewart, 1997):

1. To augment the immune response to HIV so that viral replication is suppressed and diseases progression is delayed.
2. To augment immune responses broadly so that patients can resist opportunistic infections and the development of malignancy.
3. To suppress those aspects of the immune response to HIV this may facilitate viral replication or causes disease in their own right.

The immune-based therapy is basically targeted against the HIV infection, autoimmunity diseases due to HIV, Immune deficiency, malignancy due to HIV,

opportunistic infection in HIV/AIDS, and cachexia. Immune-based therapy is in its infancy and to date no therapies are licensed. This therapy is given basically for enhancing HIV-specific immunity by therapeutic vaccines, hyperimmune plasma and immunoglobulin therapy, and cellular therapy. General immune potentiators are also used as immune-based therapy. These include cytokines therapy such as interleukin-2, interleukin-12, and interferon gamma; immunomodulators such as thymic hormones, dialyzable leukocyte extract, Isoprinosine, and N-Acetylcysteine. Immunoglobulin therapy and therapy that is used for enhancing specific immune response to opportunistic pathogens are the most popular immune based therapy.

3.11.3 Treating secondary infections in HIV/AIDS

The bulk of the morbidity and mortality associated with HIV infection is due to conditions (most commonly secondary infections) that arise in the setting of the resultant immune defects. Early in the history of HIV epidemic, most physicians waited for opportunistic disease to arise and than attempted to treat them as best as possible. It was later demonstrated that prophylaxis was possible for a number of opportunistic infections- extending both the symptom-free and overall survival of HIV infected individuals.

Specific opportunistic diseases are encountered at particular degree of immune suppression. If the CD4 count is used as a marker for immune status, certain conditions are rarely encountered until the CD4 counts has fallen below a given level. Calculating this condition the pre-prophylaxis treatment is possible. Treating secondary infection may be the cheapest way of treating patients with HIV/AIDS but negligence may produce the devastating results by evolving multi drug resistant organisms.

In conclusion we can say that there is no hard and fast rule for treating PLWHA but the combination of the three therapy mention above, under the guideline of experts will prolong the life span of PLWHA to great extent.

Conclusion

HIV and AIDS have been repeatedly linked in time, place and population group; the appearance of HIV in blood supply has preceded or coincides with the occurrence of AIDS cases in every country and region where AIDS has been noted. Among individuals without HIV, AIDS-like symptoms are extraordinary rare, even in population with many AIDS cases. Individuals as different as homosexual men and infants have all developed AIDS with only one common denominator: infection with HIV (CDC, 2000). Laboratory workers accidentally exposed to highly concentrated HIV and health care workers exposed to HIV-infected blood have developed immunosuppression and AIDS with no other risk factor for immune dysfunction. Scientist have now used PCR to find HIV in virtually every patients with AIDS and to show that HIV is present in larger and increasing amounts even in the pre-AIDS stages of HIV disease. Researchers also have demonstrated a correlation between the amount of HIV in the body and progression of the aberrant immunologic processes seen in people with AIDS.

Despite this plethora of evidence, the notation that HIV dose not cause AIDS continues to find a wide audience in this decade, with potential negative impact on HIV-infected individuals and on public health efforts to control the epidemic. HIV-infected individuals may be convinced to forego anti-HIV treatment that can forestall the onset of serious infections and malignancies of AIDS (Edelman, 1991). Pregnant HIV-infected women may dismiss the option of taking AZT, which can reduced the likelihood of transmission of HIV from mother to infant (Connor, 1994).

People may be dissuaded from being tested for HIV, there by missing the opportunity, early in the course of disease, for counseling as well as for treatment with drugs to prevent AIDS-related infections such as PCP. Such prophylactic measures prolong survival and improve the quality of life of HIV-infected individuals.

Most troubling is the prospect that individuals will discount the threat of HIV and continue to engage in risk sexual behavior and needle sharing. If public health message on AIDS prevention are diluted by misconception that HIV is not responsible for AIDS, otherwise preventable cases of HIV infection and AIDS may occur, adding to the global tragedy of the epidemic.

CHAPTER-IV

4. MATERIALS AND METHODS

This study is performed in Sonja Jeevan Kendra of "Maiti Nepal", Tribhuvan University department of Microbiology, and Siddhi polyclinic. Maiti Nepal is one of the best rehabilitation centers for HIV/AIDS victim which is located in central of Kathmandu valley.

4.1 Materials

Different equipments, glassware, chemicals, reagents and, media have been used during the study. Since the study was carried in three different laboratories the equipments varied accordingly. Details of chemicals, reagents and media are provided in Appendices II, III, IV, and V.

4.1.1 Equipments and glassware

Some of the equipments frequently used during the study and their manufacturer are given below.

Table 7: Equipments used during study

Equipments	**Manufacturer**
Biological safety cabinet	Dalton (USA)
Incubator	Sanyo (Japan)
Autoclave	Sakura (Japan)
Hot air oven	Sakura (Japan)
Microscope	Olympus20X (Japan)
Weighing Machine	Chyo MP 300 (Japan)
Refrigerator	Toshiba (Japan)
Colorimeter	Santos (India)
WBC Counter	Santos (India)

Most of the glass wares used during the study were made up of borosil and were manufactured in India. Petri plates used were both disposable and reusable. The reusables were manufactured by Hi-Media cooperation India. Other glassware includes measuring cylinders, conical flask, beakers, ESR tubes, biochemical test tubes and other test tube, frosted slides, and Hemocytometer.

4.1.2 Chemicals and reagents

All the chemicals and reagents used in this study are prepared according to "District Laboratory Practice in Tropical Countries Part 1 and 2" by Monica Cheesbrough (2000). The chemicals and reagents used in this study are supplied by Hi-Media Pvt. Ltd India. Procedure for preparing these chemicals and regents are listed in Appendices II and III

4.1.3 Media

Culture media as well as biochemical media used are all supplied by Hi-Media. Except Ogawa Egg Medium all other media were prepared according to the instruction indicated in "Preparation of different media" published by Hi-Media Limited. Ogawa Egg Medium was prepared according to the guideline provided by National Tuberculosis Center. Preparation procedures of different media are mention in Appendices II.

4.2 Methodology

Methodology used during this study is explained by different attributes, they are defined as follows.

4.2.1 Site selection

This study was solely performed in Sonja Jeevan Kendra of Maiti Nepal. Maiti Nepal is one of the best rehabilitation centers for HIV/AIDS victim. It is located in Pingalasthen, Gaushala the central of Kathmandu valley. This social organization is holding around 100 individual both male and female and even children infected with HIV. All PLWHA

are regularly examined and their CD4 count is monitored every six month. Those whose CD4 count is below 200 cell/mm^3 are given antiretroviral therapy. Among total PLWHA majority of the population comprises of women of sexually active group, followed by children below 10 years of age, and very few male.

4.2.2 Study population

Study population comprises those individuals who are HIV positive and whose CD4 cell count was known. These people are rehabilitated in Maiti Nepal and frequently available for the study. The study population has been selected irrespective of their mode of transmission of HIV, age and sex. Since the study principally focused on the CD4 cell count, those individual who recently visited the Sonja Jeevan Kendra and whose HIV infection is determined and CD4 count has not been done are not included in this study. Children less then five years of age and whose consent cannot be taken were also not included in this study.

4.2.3 Sample population

Fifty one individuals with HIV positive and CD4 cell count were included in the study. Among them forty nine were female and only two were male. Verbal as well as written consent were taken from all the sample population and only those who were ready to participate for whole period of time were included in the study. Voluntary involvement of the individual was highly encouraged and once the individual reject for participation, they are then not included in study, in any period of time.

4.2.4 Sample size

Only twenty one individual were ready to voluntary for the study. These individual gave the written consent for the participation in the project. Since the study was done taking different clinical samples verbal consent among the study sample were taken during the processing of each samples. Death of subject did occur during the study, further processing of their samples was not possible, but were included in study with all the result obtained before their death. Some individual in the sample population who

previously volunteer along with the written consent for the participation refuse for some clinical samples were also included with the result of all previous study except the one that they reject.

4.2.5 Time frame

The study was started from August 2004 A.D. and ends at the end of January 2007. During the first three month of study literature review, proposal writing, and site selection process was done. It took nearly two month for the Maiti Nepal to give the acceptance letter. Laboratory work was started from 7th November 2004 till last of December 2005.

4.2.6 Methods

Both qualitative as well as quantitative method has been used in this study. CD4 cell count, total blood count, differential blood count, Hemoglobin concentration, and erythrocytes sedimentation rate are some of the quantitative attribute observed during the study. Qualitative attributes includes microbiological and parasitological examination of different clinical samples. Semi-quantitative analysis of urine for the detection of enteric bacterial pathogens was also done.

4.2.7 Study type

The study was completely hospital based cross-sectional study. All the clinical samples were collected and processed in clinic laboratory, and University laboratory. The study was conducted with the limited people in a particular time frame with out control group.

4.2.8 Data analysis

The results obtained were all analyzed using standard statistical technique. Most of the results obtained were analyzed by comparing with the CD4 cell concentration and some were checked for correlation using different tools and technique including SPSS verson10 software.

4.3 Collection and processing

Five different types of clinical samples were taken for the determination of secondary infection among the PLWHA. These include blood, urine, stool, sputum, and throat swab. Collection and processing of these samples varies accordingly.

Blood samples were collected aseptically for various purposes. Nearly 10 ml of blood were drawn from each individual. 2-3 ml of which were collected in a vial containing anticoagulant for the determination of CD4 cell count. The processing was done in Siddhi polyclinic. 5 ml was poured in culture bottle for the detection of bacterial pathogens along with the culture media (BHI broth) in the ratio 1:10. These bottles were incubated in 37^0C for 21 days. In a regular interval of 72 hours they were streaked on Blood agar, MacConkey agar and Chocolate agar. With nearly 1 ml, ESR test was done in ESR tube. Total hemoglobin concentration, and HIV test by kit method were also performed. From the remaining, thick and thin smear were made for Gram stain, Acid fast stain, and Wright's stain.

With the help of sterile cotton swab, sample from throat were processed. Swabbing and inoculation in different media were done in sterile zone. Throat swab collected was first streaked in chocolate agar followed by blood agar and Manitol Salt agar. All the plates were then incubated at 37^0C for 24 hours. After inoculation thin smear for Gram stain and thick smear for Acid Fast stain were prepared.

A sterile container was given to each patient for subsequently three days for the collection and processing of sputum sample. Patients were requested to give the deep induced and saliva free sputum as far as practical. Sample was inoculated in Blood agar, MacConkey agar, Chocolate agar, and Ogawa Egg-Medium. They were then incubated at 37^0C for 48 hours except Ogawa medium which was incubated for six weeks at 37^0C. Macroscopic and microscopic observation was done for the detection of presence of blood, pus, epithelial cell and bacteria as well as fungi. Gram stain, Acid Fast stain, and Giemsa stain was done for the detection of pathogens in sputum.

Stool samples were collected aseptically for the detection of both bacterial as well as parasites. The samples were collected early in the morning and processing was done within two hours. When the specimens is formed or semi formed a thick suspension were made in sterile peptone water and loop full of it was streak in Blood agar, MacConkey agar, and SS agar. Before inoculating in a SS agar the sample was enriched in a Selenite F broth at 37^0C for 6 hours. For unformed stool sample was inoculated directly. The stool sample was then examined physically and under microscope. For microscopic observation slides were prepared for Gram stain, Acid Fast stain, Modified Acid Fast stain, Basic Fuchsin stain, wet mount preparation with normal saline and iodine.

Collection of urine was done in a sterile container by following mid-stream urine collection method, in the early morning. They were then transfer aseptically in the laboratory, and the processing was done immediately for the culture, and routine examination within two hours. One loop full of properly mixed sample was inoculated in each plate of Blood agar, MacConkey agar, and Potato Dextrose agar. Plates were then incubated at 37^0C for 24 hours. Glucose, Protein, and P^H test were done by dipping the tri-stick in the remaining of the sample. From the remaining 8-10 ml of urine routine examination were done by pouring it into test tube and centrifuging it at 3,000 rpm for 10 minutes. From the sediments, Gram stain, Acid Fast stain, and wet preparation were done.

4.3.1 Blood sample

Venous blood was collected from a vein in the arm with a plastic syringe of 10 ml capacity. Thus collected blood was then processed for determining various specific and non specific parameters. The result obtained was then correlated with the concentration of T cells with CD4 marker in the peripheral blood. The various parameters studied in our study include:

4.3.1.1 Differential white blood cell count

Thin blood film was made from well-mixed EDTA ant coagulated blood. To prevent EDTA associated blood changes, blood film was made as little delay as possible. The prepared blood film was then stained with Wright stain for 5 minutes, after which it was air dried and observed under the microscope for counting purpose. The counting was done to all the cells till its number toll to 100 and they are then expressed in percentage. Thus obtained result was then correlated with the concentration of T cell with CD4 marker in peripheral blood.

4.3.1.2 Haemoglobin concentration

Haemoglobin was measured by haemoglobin cyanide photometric method. In this study 20 µl of patient blood was diluted in 5 ml Drabkin diluting fluid. It was mixed properly and left for 5 minutes. The colorimeter was set zero using Drabkin Solution at 540 nm and the absorbance of patient's diluted blood was taken against the blank. The concentration was then determined with the help of standard graph obtained after plotting the value of absorbance against the concentration of standard haemoglobin solution whose concentration was known. Thus obtained result was then correlated with the concentration of T cell with CD4 marker in peripheral blood.

4.3.1.3 Erythrocyte sedimentation rate

In present study, aseptically collected anticoagulated blood was drawn into the Westergren tube up to the 0-mm mark with the help of syringe. These tubes were then placed in the Westergren Stand in a completely upright position. After 1 hour the height of the column of plasma in mm graduation starting from the 0-mm at the top of the tube was noted. Thus obtained result was then correlated with the concentration of T cell with CD4 marker in peripheral blood.

4.3.1.4 Staining

Staining of direct blood specimen was done in order to determine the blood born pathogens. In this study three different stains were used for the detection of blood pathogen.

a) Gram stain

A thin smear (coagulated portion of blood) was prepared at the center of well labeled, clean, and greezfree glass slide. It was air dried and heat fixed, after which the smear was flooded with Crystal violet for 1 minutes, it was washed with water, followed by gram's iodine for 1 minutes. The smear was washed with water and then decolorized with acetone-alcohol for 10 seconds till the purple color disappears; it was again washed with water and counter stain with Safranin for 1 minute. Thus prepared stained smear was air dried and observed under microscope in 100x objective with oil immersion. The result obtain were expressed as Gram positive/negative, rod/cocci, chain/bunch or any other if observed. The result of Gram stain directs the processing of biochemical test and aids in the identification of particular organism. Further details of Gram Stain are also mention in appendix IV.

b) Acid-Fast stain (Ziehl-Neelsen method)

As Gram stains this technique is also routinely performed in our study for different clinical samples and all the procedure are similar unless mention. A thick smear was prepared from the given sample (coagulated blood) at the center of clean, greezefree, frosted slide which is marked properly. This smear is air dried, heat fixed, and flooded with carbol fuchsin as a primary stain. The slide was then heated till the stain evaporates and left for 5 minutes after which it was washed with water and decolorized with 3% acid-alcohol for 5 minutes till the red color disappears. The slides were cleaned with water and then counter stain with malachite green for 3 minutes. It was then air dried and observed under the oil immersion power of microscope. This stain basically focused in the identification of Mycobacterium tuberculosis, M. avium, and other

related organism that is generally encountered in the immune compromised patients. Further details of Gram Stain are also mention in appendix IV.

4.3.1.5 Serological test

For the detection of Hepatitis B virus and *Treponema pallidum* serological test were done. Besides this the further conformation of the HIV infection was also done serologically in the lab. HIV 1/2 test and HBsAg test were done by kit method provided by Chembio Diagnostic Systems and Standard Diagnostics respectively. The procedure was followed according to the instructions provided along with the kit. For the syphilis Rapid Plasma Regain (RPR) test was done. 20 µl of serum sample was placed in RPR plate and mixed with one drop of reagent in a circular manner, after proper mixing it was then shake for about 8 minutes and the result were interpreted along with the positive control. The entire serological tests were reported as reactive or no reactive. Thus obtained result was then correlated with the concentration of T cell with CD4 marker in peripheral blood.

4.3.1.6 Culture

The culture of blood was done in order to determine the bacteria present in the blood. In this study we culture blood first in Brain Heart Infusion (BHI) broth, after incubation at 37^{o}C for 48 hours we sub-culture it in different agar medium like Blood agar, MacConkey agar, and chocolate agar. The blind subculture was done every 48 hour for up to 21 days. If the BHI bottle shows turbidity and there was growth on any agar medium then the sample was consider as positive and further biochemical test was done in order to identify the organism. The different biochemical tests used during our study are presented in the Appendices portion at the end of this report.

Aseptically collected 5 ml of blood was dispense in a bottle containing 50 ml of BHI broth, the lid was tight properly and incubated at 37^{o}C. After 48 hours all the bottles were checked for the turbidity and all the samples were incubated in BA, CA, and MA. The chocolate agar was incubated at 5-10% CO_2 concentration and rests were incubated

at 37oC in incubator. Colonies obtained from the blood agar were Gram stained; sub-culture on Nutrient agar and broth. The NA and NB were incubated at 37^{o}C for 24 hour and after which all the biochemical tests were done for the identification. BA was checked for haemolysis, MA for the lactose fermenter and non lactose fermenter, and CA for watery as well as pin head colony. Gram stain was done for all the isolates on these agar plates. Thus obtained different organism was then correlated with the concentration of T cell with CD4 marker in peripheral blood.

4.3.2 Throat swab sample

Throat sample was taken for this purpose; with the help of sterile cotton swab moisten in sterile normal saline. The sample was collected in sterile zone and processed immediately. Sample collected was first swab into the chocolate agar followed by BA, MA, and PDA after which a thin and thick smear was prepared in two different glass slides.

4.3.2.1 Staining

Gram staining and Acid Fast staining was done to the prepared thin and thick smear respectively. Process involve in these staining are mention in unit 4.3.1.4 and appendix IV.

4.3.2.2 Culture

Sample inoculated in BA, MA, and PDA is incubated at 37oC for 24 hour. CA was incubated in 5-10 % CO2 jar at 37^{o}C for 24 hour. After 24 hour the plate showing growth were gram stained, sub-culture in NA and NB. Colony morphology on CA, BA, and MA and biochemical test were done as mention in section 4.3.1.6. For PDA colony producing fruity smell was observed. Thus obtained different organism was then correlated with the concentration of T cell with CD4 marker in peripheral blood.

4.3.3 Sputum sample

Sputum was processed in order to correlate the secondary infection related to lower respiratory tract infection with CD 4 cell count. From the first day sample physical examination, microscopic, and bacteriological examination was done. Acid-Fast staining was done in all three days.

4.3.3.1 Physical examination

Sample from all three days were processed for physical examination. We observed for consistency of samples, whether it is mixed with saliva, and presence of blood. The samples mixed with saliva were rejected for culture.

4.3.3.2 Staining

A thick smear was prepared for the Acid-Fast staining from all three days sample and was processed as mention in section 4.3.1.4 (b) and appendix IV. Gram stain was done on only first day sample as mention in 4.3.1.4 (a) and appendix IV.

a) Giemsa stain

This staining was done in order to detect *Pneumocystis carinii* in the sputum. Sputum sample was first fixed with double volume of normal saline and centrifuge at 5000 rpm for 15 minutes; the sediment was then made a thin smear. The smear was air dried, fixed with methanol for 2 minutes, and flooded with diluted (1:25) Giemsa stain for 15 minutes. It was air dried and observed under microscope for the presence of cyst of pathogens. Further details of Giemsa Stain are also mention in appendix IV.

4.3.3.3 Culture

The culture for the sputum is same as that of throat swab mention in section 4.3.2.2 except for the Ogawa Egg Medium. The sputum from first day was inoculated in a slant of Ogawa Egg Medium and incubated at 37^{o}C for six weeks. After six weeks rough, buff, and tough colony was observed. Thus obtained different organism was then correlated with the concentration of T cell with CD4 marker in peripheral blood.

4.3.4 Stool sample

Stool sample was studied in order to determine the GI tract infection in PLWHA. Bacteriological as well as parasitological examination was done with reference to following parameters.

4.3.4.1 Physical examination

Aseptically collected stool was observed for its color, appearance, and presence and absence of blood, mucus, and pus.

4.3.4.2 Microscopy

In microscopy, Gram stain and Acid-Fast stain was done as mentioned in 4.3.1.4 (a, b). Wet mount preparation was done in order to detect parasites. Further details of Wet preparation, Acid-Fast Stain, and Gram Stain are also mention in appendix IV.

a) Modified Acid-Fast stain

It was done to detect *cryptosporidium* from the stool sample. The concentration of decolorizer used is 1 % instead of 3% acid alcohol, and rest of the process is same as AF stain.

b) Basic fuchsin stain

This staining was done in order to detect *Campylobacter* species. Thin smear of the stool sample was made, air dried, gently heat fixed, and covered with 1% basic fuchsin for 30 seconds. Washed well with water, air dried, and examined using 100X oil immersion objective. Smear was looked for abundant small delicate, spiral curved bacteria, S-shaped, and short spirocheatal forms.

c) Wet mount

One drop of normal saline was placed at the centre of the clean, well labeled slide. With the help of stick sample was taken touching multiple site in the stool. It was mixed

properly and covered with cover slip. The over flooded sample was shocked with the blotting paper and observed under 45X objective for the identification of different parasites. The parasite identified was then correlated with the CD4 count.

4.3.4.3 Culture

Three different types of media were used for the stool sample. The processing on BA and Ma was similar as mention above. For the detection of Salmonella the sample was first inoculated in Selenite F Broth and incubated at 37^{o}C for 6 hours. After which one loop of sample from broth was aseptically transferred into the SS agar. All the plates were incubated at 37^{o}C for 24 hours. SS agar was observed for black colored colony. Typical colonies from SS agar and MA were transferred into NA and NB for the biochemical tests. Thus obtained different organism was then correlated with the concentration of T cell with CD4 marker in peripheral blood.

4.3.5 Urine sample

Urinary tract infection is common problem in PLWHA which etiology can be detected by examining the urine sample. Various parameters are observed in urine.

4.3.5.1 Physical examination

Urine collected in a sterile container was first observed for its color, turbidity, and presence or absence of blood.

4.3.5.2 Biochemical

Biochemical examination of urine was done in order to determine the pH, Glucose, and protein concentration using commercially available tri-stick. The results were drawn by comparing the colors on tri-stick obtain after dipping into the urine sample for few seconds.

4.3.5.3 Microscopic

Urine sample was centrifuged and its sediments was microscopically examine by Gram stain, AF stain, and wet preparation. Procedure for Gram and AF stain is mentioned in section 4.3.1.4 (a, b). Further details of Microscopic procedure are also mentioned in appendix IV.

a) Wet preparation

One drop of sediments was placed in the slide, covered with cover slip and observed under microscope immediately before drying. This preparation was examined for the presence of pus cells, epithelial cells, erythrocytes, cast, crystals, and fungi.

4.3.5.4 Culture

With the help of standard loop whose diameter was 4 mm sample was streaked on BA, MA, and PDA. MA was used in order to quantify the lactose fermenting colonies besides other parameter. Biochemical test was done from NA and NB inoculated from BA and MA. PDA was checked for growth of Fungi. Thus obtained different organism was then correlated with the concentration of T cell with CD4 marker in peripheral blood.

4.4 Quality control

It was of utmost importance to perform quality throughout the study to obtain results that were both reliable and desirables. Therefore, quality control was applied at different levels during the study.

a) During sample collection care was taken to check for any contamination and only the samples collected in leak proof container were selected.
b) Samples were processed on the day of collection as soon as possible.
c) All the processing of samples was performed aseptically wearing masks and gloves within the biological safety cabinet.

d) Freshly prepared media were used every time and each batch of media was put into quality check. For that a freshly made plate was incubated with control bacterial strains and the other incubated uninoculated. Growth obtained on the inoculated plate helped to make out the type of growth to look for where as growth on the uninoculated plate clearly indicated that the media was contaminated.
e) Each new batch of strains and reagents were tested for their effectiveness by making a comparison with the control stained smear slides.

CHAPTER-V

5. RESULTS

Correlation of secondary infection with varying concentration of T cells with CD4 marker in peripheral blood was determined by examine different clinical samples. These include blood, throat swab, sputum, stool, and urine. The results obtained are then categorized into three categories, first group includes the individual with CD4 cells count above 500, second group with the individual whose CD4 cells count was 500 to 200, and finally to third where CD4 cells count was below 200 cells per mm^3. Among 21 individuals studied for 5 different samples different organisms were obtained from different samples and single individual for multiple infection were also obtained. The results of the study can be summarized as follows:

5.1 Total cases of HIV infection with relation to T-cell with CD4 marker count

Total case was categorized into three categories; among 3 categories, first category comprises 33.33% (7) of total population, followed by second which comprises 57.14% (12), and last with 9.53% (2). The grouping of the cases was done irrespective of their age, sex, mode of transmission of the disease, and treatment with antiretroviral drugs.

Table 8: Total cases of HIV infection with relation to T-cell with CD4 marker count

CD4 count	Above 500 cells/mm^3	500 – 200 cells/mm^3	Below 200 cells/mm^3
Total cases	**7**	**12**	**2**
Percentage (%)	**33.33**	**57.17**	**9.53**

5.2 Body mass index with relation to T-cell with CD4 marker count

Among total cases 33.33% had low BMI, 61.9% BMI in normal range, and only 4.77% were obese. The result is summaries in table 9.

Table 9: Body mass index with reference to CD4 cells count.

CD4 count/ Body Mass Index (BMI) (Kg/m^2)	Above 500 cells/mm^3		500 – 200 cells/mm^3		Below 200 cells/mm^3		Total	
	No	%	**No**	%	**No**	%	**No**	%
Below 20 (loss weight)	**1**	14.29	**5**	41.67	**1**	50	**7**	33.33
20-30 (Healthy)	**5**	71.42	**7**	58.33	**1**	50	**13**	61.9
Above 30 (Obese)	**1**	14.29	0	0.0	0	0.0	**1**	4.77
Total cases	**7**		**12**		**2**		**21**	

5.3 Differential white blood cell count

Different types of white blood cell were count and expressed in percentage. The result obtain was tabulated along with age and CD4 cell count.

Table 10: Differential WBC count

L No	Age	CD4	**Lymphocyte**	**Neutrophil**	**Eosinophil**	**Monocytes**	**Basophil**
9	22	869	**35**	**63**	**2**	**0**	**0**
2	26	833	**31**	**66**	**2**	**1**	**0**
7	21	792	**38**	**41**	**14**	**7**	**0**
19	30	570	**40**	**58**	**2**	**0**	**0**
20	20	565	**32**	**59**	**5**	**4**	**0**
17	20	556	**45**	**58**	**3**	**4**	**0**
14	40	519	**29**	**63**	**3**	**5**	**0**
21	35	492	**40**	**53**	**4**	**5**	**0**
6	24	487	**33**	**46**	**12**	**9**	**0**
16	28	462	**38**	**59**	**3**	**0**	**0**
3	23	437	**26**	**69**	**2**	**3**	**0**
12	26	417	**39**	**53**	**3**	**5**	**0**
4	23	405	**39**	**56**	**4**	**11**	**0**
8	22	365	**32**	**60**	**4**	**4**	**0**
5	25	363	**23**	**62**	**9**	**6**	**0**
11	23	361	**18**	**72**	**8**	**2**	**0**
18	30	353	**36**	**54**	**6**	**4**	**0**
1	24	330	**42**	**50**	**3**	**5**	**0**
13	35	239	**37**	**50**	**6**	**7**	**0**
15	26	169	**14**	**76**	**6**	**4**	**0**
10	30	139	**40**	**58**	**2**	**0**	**0**

5.4 Haemoglobin concentration and ESR

Haemoglobin concentration (g/dl) and ESR of blood was obtained and is presented along with the CD4 cell count and age of the given patient.

Table 11: Haemoglobin concentration and ESR

Lab No	Age	CD4	**Hb**	**ESR**
9	22	869	**11.3**	**22**
2	26	833	**10.4**	**25**
7	21	792	**12.1**	**11**
19	30	570	**11.5**	**20**
20	20	565	**10**	**15**
17	20	556	**13**	**16**
14	40	519	**12**	**10**
21	35	492	**8.9**	**24**
6	24	487	**8.7**	**10**
16	28	462	**10**	**12**
3	23	437	**11.8**	**22**
12	26	417	**8.7**	**27**
4	23	405	**11.6**	**54**
8	22	365	**10.8**	**15**
5	25	363	**9.4**	**10**
11	23	361	**12.6**	**27**
18	30	353	**8.9**	**13**
1	24	330	**8.7**	**6**
13	35	239	**12**	**10**
15	26	169	**6.5**	**67**
10	30	139	**5.7**	**37**

5.5 Organism isolated from blood with relation to CD4 cell count

From Gram stain, AF stain, and culture from different media along with the biochemical test performed. Most of the sample showed no growth in the BHI. From the first category where the CD4 cell count was between 1000-500 there was no growth on

BHI, but 14.29% of the patient in that group were positive for *Treponema pallidum* which was detected through RPR test. Similarly 14.29% of the same population was suffering from Hepatitis B virus infection. 83.33% of sample from second group where CD4 count was 500-200 showed no growth. 25% were positive for syphilis and 16.67% for *Salmonella* infection. Where as in the last group whose CD4 count was below 200, 50% of them were growth negative in BHI and 50% were positive for *Staphylococcus aureus.* As a whole 85.71% was growth negative in BHI, 19.05% was syphilis positive, 9.52% was *Salmonella* positive, 4.76% of the total population was positive for both *Staphylococcus aureus* and Hepatitis B virus.

Table 12: Organism isolated from blood with relation to CD4 cell count

CD4 count/ Organism isolate	Above 500 cells/mm^3		500 – 200 cells/mm^3		Below 200 cells/mm^3		Total	
	No	**%**	**No**	**%**	**No**	**%**	**No**	**%**
Bacteria								
Salmonella sps	0		**2**	**16.67**	0		**2**	**9.52**
Staphylococcus aureus	0		0		**1**	**50**	**1**	**4.76**
Treponema pallidum (RPR method)	**1**	**14.29**	**3**	**25**	0		**4**	**19.05**
Virus								
Hepatitis B (Kit Method)	**1**	**14.29**	0		0		**1**	**4.76**
No growth (BHI)	**7**	**100**	**10**	**83.33**	**1**	**50**	**18**	**85.71**
Not Participated	0		0		0		0	
Death of Subjects	0		0		0		0	
Total cases	**7**		**12**		**2**		**21**	

5.6 Organism isolated from throat swab with relation to CD4 cell count

Among the total population 9.52% of cases did not participate during the processing of throat swab. 33.33% of the sample was no growth on BA, CA, and MA. Remaining 57.15% of the cases were positive for different types of organism. We were able to isolate and identify four different types of organism. *Staphylococcus aureus* comprises the 23.81% of total population followed by *Streptococcus pneumoniae* 19.05%, *Moraxella catarrhalis* 9.52%, and *Klebsiella pneumoniae* 4.76%. According to the CD4 cell count in first group most of the sample that is 42.86% were free from bacteria that were cultural able in the media used, and all four group of organisms infected the group. 14.29% were infected with *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Moraxella catarrhalis*, and *Klebsiella pneumoniae*. In second group, 8.33% did not participated, 33.33% were no growth sample, and 25% of each was infected with *Staphylococcus aureus* and *Streptococcus pneumoniae*. Remaining 8.33 was infected with *Moraxella catarrhalis* and non with *Klebsiella pneumoniae*. Similarly in the third group 50% did not take part and remaining was infected with *Staphylococcus aureus*.

Table 13: Organism isolated from throat swab

CD4 count/ Organism isolate	Above 500 cells/mm^3		500 – 200 cells/mm^3		Below 200 cells/mm^3		Total	
	No	**%**	**No**	**%**	**No**	**%**	**No**	**%**
Staphylococcus aureus	**1**	**14.29**	**3**	**25**	**1**	**50**	**5**	**23.81**
Moraxella catarrhalis	**1**	**14.29**	**1**	**8.33**	0		**2**	**9.52**
Klebsiella pneumoniae	**1**	**14.29**	0		0		**1**	**4.76**
Streptococcus pneumoniae	**1**	**14.29**	**3**	**25**	0		**4**	**19.05**
No growth	**3**	**42.86**	**4**	**33.33**	0		**7**	**33.33**
Not Participated	0		**1**	**8.33**	**1**	**50**	**2**	**9.52**
Death of Subjects	0		0		0		0	

Total cases	**7**	**12**	**2**	**21**

5.7 Organism isolated from sputum with relation to CD4 cell count

In sputum 14.29% of the total studied sample showed no growth. *Streptococcus pneumoniae* was the most common organism, 23.81% of population suffer from this infection. Other organism isolated were *Haemophilus influenzae*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* comprising 14.29% of the patients. *Staphylococcus aureus* and *Mycobacterium tuberculosis* occurs in 9.52% of the studied population. Majority of the infection due to *Streptococcus pneumoniae* occurs in the second category where the CD4 cell count was 500-200. *Mycobacterium tuberculosis* infection in our studied did not occur in the first category but in remaining two, it occurs in equal proportion infecting each individual in those groups. Similar is the case of Staphylococcus *aureus* but the pattern of distribution other organism occurred randomly among three groups.

Table 14: Organism isolated from sputum

CD4 count/ Organism isolate	Above 500 cells/mm^3		500 – 200 cells/mm^3		Below 200 cells/mm^3		Total	
	No	**%**	**No**	**%**	**No**	**%**	**No**	**%**
Staphylococcus aureus	0		**1**	**8.33**	**1**	**50**	**2**	**9.52**
Haemophilus influenzae	**1**	**14.29**	**2**	**16.66**	0		**3**	**14.29**
Klebsiella pneumoniae	**1**	**14.29**	**2**	**16.66**	0		**3**	**14.29**
Streptococcus pneumoniae	**2**	**28.58**	**3**	**25**	0		**5**	**23.81**
Mycobacterium tuberculosis	0		**1**	**8.33**	**1**	**50**	**2**	**9.52**
Pseudomonas aeruginosa	**2**	**28.58**	**1**	**8.33**			**3**	**14.29**
No growth	**1**	**14.29**	**2**	**16.66**	0		**3**	**14.29**
Not Participated	0		0				0	
Death of Subjects	0		0		0		0	
Total cases	**7**		**12**		**2**		**21**	

5.8 Organism isolated from stool with relation to CD4 cell count

Stool sample were classified into two separate classes, first the isolated protozoan parasites were studied by wet mount preparation. In protozoa, three different types of organism were identified among which the most predominant was *Cryptosporidium parvum* comprising 19.05% of total population. Among total cases only 9.52% of population was infected with *Giardia lambia*, followed by *Entamoeba histolytica* which was found in the first group population comprises 4.76% of the total population. Death of subject occurs during the study and the patient was from third category, not only that we were unable to isolate any protozoa from that group. 38.10% of total population shows no parasites and 23.81% from total did not participated.

Table 15: Protozoa isolated from stool sample

CD4 count/ Organism isolate	Above 500 cells/mm^3		500 – 200 cells/mm^3		Below 200 cells/mm^3		Total	
	No	**%**	**No**	**%**	**No**	**%**	**No**	**%**
Giardia lambia	0		**2**	**16.66**	0		**2**	**9.52**
Cryptosporidium parvum	**2**	**28.58**	**2**	**16.66**	0		**4**	**19.05**
Entamoeba histolytica	**1**	**14.29**	0		0		**1**	**4.76**
No Parasites	**2**	**28.58**	**5**	**41.67**	**1**	**50**	**8**	**38.10**
Not Participated	**2**	**28.58**	**3**	**25**	0		**5**	**23.81**
Death of Subjects	0		0		**1**	**50**	**1**	**4.76**
Total cases	**7**		**12**		**2**		**21**	

Microbiological examination shows the infection of four different organism including *Mycobacterium avium* and *Campylobacter Jejuni* which infected 9.52% of total population. The most common infection was of *Salmonella* sps. infecting 28.57% of

total population, and *Salmonella paratyphi* 4.76% of the total population. 4.76% of total population died, 23.81% did not participated, and 19.05% samples were no growth. *Mycobacterium avium* infection only occurs in second and third category and *Salmonella paratyphi* in second. Major infection of the first category was due to *Salmonella* sps which is 42.86% of that group population.

Table 16: Bacteriological examination of stool

CD4 count/ Organism isolate	Above 500 cells/mm^3		500 – 200 cells/mm^3		Below 200 cells/mm^3		Total	
	No	**%**	**No**	**%**	**No**	**%**	**No**	**%**
Mycobacterium avium	0		**1**	**8.33**	**1**	**50**	**2**	**9.52**
Salmonella sps.	**3**	**42.86**	**3**	**25**	0		**6**	**28.57**
Campylobacter Jejuni	0		**2**	**16.66**	0		**2**	**9.52**
Salmonella paratyphi	0		**1**	**8.33**	0		**1**	**4.76**
No growth	**2**	**28.58**	**2**	**16.66**	0		**4**	**19.05**
Not Participated	**2**	**28.58**	**3**	**25**	0		**5**	**23.81**
Death of Subjects	0		0		**1**	**50**	**1**	**4.76**
Total cases	**7**		**12**		**2**		**21**	

5.9 Organism isolated from urine with relation to CD4 cell count

Including *Candia albicans* a fungi and *Nocardia sps.*, we were able to isolate six different type of organism from urine. *Escherichia coli* 14.29% and *Proteus mirabilis* 14.29% were among the most predominant isolates followed by *Candia albicans* 9.52%, *Klebsiella pneumoniae* 9.52%, *and Nocardia sps.* 4.76%, and *Staphylococcus*

aureus 4.76%. 9.52% of total population died, 14.29% did not take part, and 19.05% of sample we were unable to isolate any pathogenic organisms.

Table 17: Organism isolated from urine

CD4 count/ Organism isolate	Above 500 cells/mm^3		500 – 200 cells/mm^3		Below 200 cells/mm^3		Total	
	No	**%**	**No**	**%**	**No**	**%**	**No**	**%**
Candia albicans	**2**	**28.58**	0		0		**2**	**9.52**
Escherichia coli	0		**3**	**25**	0		**3**	**14.29**
Klebsiella pneumoniae	**1**	**14.29**	**1**	**8.33**	0		**2**	**9.52**
Nocardia sps.	**1**	**14.29**	0		0		**1**	**4.76**
Proteus mirabilis	**1**	**14.29**	**1**	**8.33**	**1**	**50**	**3**	**14.29**
Staphylococcus aureus	**1**	**14.29**	0		0		**1**	**4.76**
No growth	**1**	**14.29**	**3**	**25**	0		**4**	**19.05**
Not Participated	0		**3**	**25**	0		**3**	**14.29**
Death of Subjects	0		**1**	**8.33**	**1**	**50**	**2**	**9.52**
Total cases	**7**		**12**		**2**		**21**	

5.10 Correlation of secondary infection with the concentration of peripheral T cell with CD4 marker

We isolate and identify nearly 20 different types of organisms from five different samples. The result obtain thus indicate that nearly all of the case is suffering from some type of infection not only that many individuals were suffering from multiple infection. Death of 9.52% of total population did occur those who died suffer from multiple infections. Most of the patient who suffers from multiple infections belongs to the second category. The predominant organism that our study found was *Staphylococcus aureus*, and *Streptococcus pneumonia*.

Table 18: Total organisms isolated in correlation with CD4 cell count

CD4 count/ Organism isolate	Above 500 cells/mm^3		500 – 200 cells/mm^3		Below 200 cells/mm^3		Total
	No	**%**	**No**	**%**	**No**	**%**	**No**
Bacteria							
Campylobacter Jejuni	0		**2**	**100**	0		**2**
Escherichia coli	**1**	**16.67**	**5**	**83.33**	0		**6**
Haemophilus influenzae	**1**	**33.33**	**2**	**66.67**	0		**3**
Klebsiella pneumoniae	**3**	**50**	**3**	**50**	0		**6**
Moraxella catarrhalis	**1**	**50**	**1**	**50**	0		**2**
Mycobacterium avium	0		**1**	**50**	**1**	**50**	**2**
Mycobacterium tuberculosis	0		**1**	**50**	**1**	**50**	**2**
Proteus mirabilis	**1**	**33.33**	**1**	**33.33**	**1**	**33.33**	**3**
Pseudomonas aeruginosa	**2**	**67.67**	**1**	**33.33**	**0**		**3**
Salmonella paratyphi	0		**1**	**100**	0		**1**
Salmonella sps.	**3**	**37.50**	**5**	**62.50**	0		**8**
Staphylococcus aureus	**2**	**22.22**	**4**	**44.44**	**3**	**33.33**	**9**
Streptococcus pneumoniae	**3**	**33.33**	**6**	**67.67**	0		**9**
Treponema pallidum	**1**	**25**	**3**	**75**	0		**4**
Protozoa							
Cryptosporidium parvum	**2**	**50**	**2**	**50**	0		**4**
Entamoeba histolytica	**1**	**100**	0		0		**1**
Giardia lambia	0		**2**	**100**	0		**2**
Fungi							
Candia albicans	**2**	**100**	0		0		**2**
Nocardia sps.	**1**	**100**	0		0		**1**

Virus							
Hepatitis B	**1**	**100**	0		0		**1**

Other organism that was frequently encountered includes *Salmonella* sps. *Escherichia coli* and *Klebsiella pneumoniae*. *Campylobacter Jejuni*, *Haemophilus influenzae*, *Treponema pallidum*, and *Cryptosporidium parvum* were also isolated. Organism such as *Salmonella paratyphi*, *Entamoeba histolytica*, *Nocardia* sps, and Hepatitis B were isolated in very few numbers.

Among the third group, whose CD4 cell count was below 200, *Mycobacterium avium*, *Mycobacterium tuberculosis*, *Proteus mirabilis*, and *Staphylococcus aureus* were most common isolates. 50% of the total infection due to *Mycobacterium avium* and *Mycobacterium tuberculosis* occurs in this group. Where as 33.33% of Proteus *mirabilis*, and *Staphylococcus aureus* population were also present in this category. Infection due to other organism did not occur in this group.

Campylobacter Jejuni, *Salmonella paratyphi*, and *Giardia lambia* were only present in second category where CD4 cell count was 500-200. *Treponema pallidum* (75%) also occurs in this group. Other predominant in this group are *Escherichia coli* (83.33%), *Streptococcus pneumoniae* (67.67%), *Haemophilus influenzae* (66.67%), and *Salmonella Sps* (62.50%). 50% of total isolates of *Klebsiella pneumoniae*, *Moraxella catarrhalis*, *Mycobacterium avium*, *Mycobacterium tuberculosis*, and *Cryptosporidium parvum* occurred in this category.

Entamoeba histolytica, *Candia albicans*, *Nocardia Sps.*, and Hepatitis B infection occurred only in this category. Other predominant organism includes *Pseudomonas aeruginosa* (67.67%), followed by *Cryptosporidium parvum* (50%), *Moraxella catarrhalis* (50%), and *Klebsiella pneumoniae* (50%). *Campylobacter Jejuni*,

Mycobacterium avium, *Mycobacterium tuberculosis*, *Salmonella paratyphi*, and *Giardia lambia* infection did not occurred in this group.

5.11 Organism according to CD4 count category

The most predominant organism that infected the given category of population can be list as follows. Those organisms which are not listed in this list infect the minor population of all three categories.

Table 19: Organism according to CD4 count category

above 500 cells/mm^3	500 – 200 cells/mm^3	below 200 cells/mm^3
Entamoeba histolytica	***Campylobacter Jejuni***	***Mycobacterium avium***
Candia albicans	***Giardia lambia***	**(50%)**
***Nocardia* sps.**	***Salmonella paratyphi***	***Mycobacterium***
Hepatitis B		***tuberculosis***
		(50%)
Pseudomonas aeruginosa	***Escherichia coli***	
(67.67%)	**(83.33%)**	
	Treponema pallidum	
	(75%)	
	Streptococcus pneumoniae	
	(67.67%)	
	Haemophilus influenzae	
	(66.67%)	
	***Salmonella* sps.**	

	(62.50%)	

From the above table we can see that most of the organism isolated was from the people whose CD4 cell count is in the verse of declining. Unusual organisms such as MAC were isolated from individual whose CD4 count is below 500. Most of other organisms were opportunistic pathogens.

CHAPTER-VI

6. DISCUSSION

Correlation of secondary infection with peripheral level T Lymphocytes with CD4 marker (CD4) count in HIV/AIDS patients was done taking five different samples. Most of the infections were due to opportunistic organisms indicating the depletion of immune components in HIV infection.

Multiple recurrent bouts of infections with fungi, bacteria, and viruses occur as the CD4+ cell counts declines. For example, the nervous system can be the site of opportunistic infections with *Toxoplasma*, *Cryptococcus*, and *Mycobacteria*. Since our study did not include the samples from brain we were unable to generalize these infections in context to our patients. The lungs are also primarily affected by opportunistic infections, PCP being one of the most common. Mycobacterium infections are also common problem in the lungs. Our study prevails that the most common infection in lungs is because of *Streptococcus pneumoniae*. Serious gastrointestinal tract illnesses are due to opportunistic pathogens, but these may be in concern with HIV infection. CMV colitis is a common problem, but HIV is often present as well. Protozoal parasitic disease, as well as infections with gram-negative enteric bacteria was the sources of gastrointestinal disorders. Our study was also true in this context. Infections in the urinary systems and even in circulatory system are common in HIV/AIDS. Hematological disorders were often encountered in our study.

Besides this, BMI was calculated to determine the progressive loss of body weight. We found that those patients whose CD4 count was below 500 were all under weight. This figure indicates that they were progressing to AIDS.

6.1 HIV/AIDS and hematological diseases

The most common HIV related hematological diseases include anaemia, neutropenia, thrombocytopenia or lymphoid disorders. Normochromic normocystic anaemia (Hb level below 11.5 g/dl) with an inappropriate low reticulocyte count is seen in 70% to 95% of patients on progression to AIDS (Street, 1994). In our finding (Table 11) 80% of patients whose CD4 count is below 500 have Hb level below this mark (11.5 g/dl), indicating that they are on the progression of AIDS. Although this is the late sign of HIV infection, clinical presentation and/or diagnosis may unfortunately be delayed until this time. Symptoms of anaemia are likely to dominate the clinical picture.

Differential WBC count can also serve as an indicator for the progression of AIDS. The normal percentage of lymphocytes in the adults is 25-40% (Cella, 2000). We found that in most of the cases there was no remarkable depletion in the total percent count since there is great depletion in the T-lymphocytes with CD4 marker count. Since the body maintain the homeostasis there is no remarkable change in lymphocytes unless the dropdown is very severe. Neutropenia was not prominent in our study. Normally in the healthy adult the percent of neutrophil is 54-75%, most of the cases lies in this value except few exception. The basic cause might be the opportunist infection basically the bacterial infections. ESR was also calculated, all of the patients showed elevated level of ESR indicating they are suffering from some short of infections.

6.2 HIV/AIDS and blood pathogens

The HIV is targeted towards the blood cells; besides this it can be isolated from brain and kidney cells. Other pathogens that are encountered in blood include HBV, *Mycobacterium*, *Staphylococcus*, and other organisms which are systematic in nature but transmitted via blood. We were able to isolate *Treponema pallidum* among 19.05% of the total cases. Syphilis is one of the common problems among the HIV patients where the mode of disease transmission is due to sexual contacts or IV drug users. Most of our cases include the CSW so the result is not so alarming. Besides this 40.76% were suffering from HBV. Among all patients one of them was found to be suffering from

both HBV and syphilis. Other isolates that occurred were *Staphylococcus aureus* and *Salmonella* sps. Both of the organisms are not common in healthy individual. Due to the suppress immune system the organism might have habituated themselves in the blood. In case of HIV/AIDS *Staphylococcus aureus* might be responsible for causing the high temperature (fever). The toxin secreted by the bacteria is highly pyogenic in nature and one of the prominent clinical diagnoses in HIV is elevated temperature. *Salmonella* might be responsible for the fever but with different pathogenesis. Both of the organisms are common micro flora of the healthy human, explaining the endogenous source of opportunistic infections.

6.3 HIV/AIDS and organism from respiratory tract

The respiratory tract is one of the commonest sites of infection associated with HIV. Among all, infection due to *Mycobacterium tuberculosis* is most predominant in contest of Nepal. Due to immunocompromised host, person with latent tuberculosis infection who is also infected with HIV develops clinical tuberculosis at an increased rate. Instead of a 10% lifetime risk of developing TB, 60%-80% of adults with dual infections may develop TB. This interaction has resulted in a parallel pandemic of TB: even in the country like Nepal where around 10%-15% of the adult populations have dual infections, annual TB rate increased 5-10 folds during the half of the 1990s. No conclusive data indicates that any infection, including *Mycobacterium tuberculosis* infection, accelerates progression to AIDS in HIV infected persons. Our finding suggested that only minority of the population (9.52%) were infected with active form of disease.

Other respiratory tract infections include infection due to PCP, MCA infections, bacterial infections, viral infections, and infections due to fungi. Infections such as PCP and MAC were only common in the patients with HIV/AIDS whose CD4 cell count is below 200. From our finding, we were unable to isolate these organisms in any of the cases from sputum sample. From the sputum we were able to isolate many opportunistic organisms (Table 14). Among them the most predominant was *Streptococcus*

pneumoniae (23.81%), followed by *Haemophilus influenzae*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* (14.29% each). All of these bacteria are pyogenic and normal flora of human body. Most of these infections were predominant among the patients whose CD4 cell count was below 500 cells. Infections due to *Staphylococcus aureus* and *Legionella pneumophila* also occurs among the HIV infected cases. In our study only 9.52% of total cases presented with *Staphylococcus aureus* where as patients with *Legionella pneumophila* infections was absent in our survey.

HIV may itself cause direct pulmonary damage. Viral upper respiratory tract infections are more common in HIV infection. Other viral infection includes Cytomegalovirus, which occurs when the CD4 cell count drop below 100. Due to the limited resources our study was not focused on isolating the respiratory virus so though they might be present in our cases we might have overcome them.

Cryptococcus neoformans is also a common cause of pulmonary infections in the persons with compromised immune system. Other fungal infections include PCP and disseminated candidiasis. The PCP is the most common presentation of AIDS where CD4 cell counts drops below 200. Induced sputum with the direst guidelines of physician is required for the isolation of this fungus from sputum. Our study was also unable to detect this organism from any of the cases due to the lack of expert's guideline. Candidiasis was also not recorded from our finding as a respiratory tract infection.

From upper respiratory tract infection where we took throat swab as an sample we encountered *Branhamella catarrhalis* (9.52%). Other organisms were common to that of sputum. The most dramatic results obtained was the isolation of 23.81% of *Staphylococcus aureus* which was comparably very high to that of sputum (9.52%). This result might be due to the nature of organism who loves places where salt concentration is high.

6.4 HIV/AIDS and intestinal pathogens

Diarrhoea is the most prominent gastrointestinal symptom in HIV infection. Almost every individual with HIV infection develops this condition once in their life time. The causative agent of diarrhoea in case of HIV/AIDS varies broadly. Some time it is so difficult to isolate a particular organism from sever cases. Most of the cases in the late stage suggest that HIV itself is responsible for the sever diarrhoea. Other unusual organisms responsible may be MAC, *Cryptosporidium*, *Cyclospora*, and Rotavirus. Diarrhoea may become sever and even can be the cause of death if not treated in case of HIV infection. Management has three basic aims: detection of treatable causes; relief of symptoms; and prevention of malnutrition. Most patients have some degree of malabsorption and many are malnourished if not managed well. The most important thing that should be taken in mind is diarrhoea and weight loss are two different predictors of mortality in AIDS.

The causative agents of diarrhoea vary according to the level of CD4 cell in the peripheral blood. Certain pathogens such as CMV, MAC, and other unusual pathogens as mention are likely to cause disease in the setting of low CD4 cell counts (<100). Other pathogenic organisms that are frequently encountered include *Campylobacter Jejuni*, *Salmonella* sps, *Giardia lambia*, and *Entamoeba histolytica*. No pathogen is isolated in some AIDS patients with diarrhoea despite extensive evaluation. Although there may be mucosal damage from bacterial outgrowth or unidentified pathogens, primary HIV infection of the gut may be the cause. HIV has been found within mucosal epithelium in up to 40% of patients with advance disease, epithelial cell damage has not been directly demonstrated; small bowel biopsy in this setting often shown an increase in crypt:villus ratio without inflammatory cell infiltrate. ART may be associated with resolution of diarrhoea.

Non-infecting causes of diarrhoea are uncommon but include; neoplastic involvement of the gut, particularly by Kaposi's sarcoma and non-Hodgkin's lymphoma, idiopathic ulcerative colitis in response to conventional therapy, autonomic neuropathy,

Didanosine therapy, pancreatic insufficiency secondary to pentamidine or Didanosine therapy or opportunistic pancreatic infection, and lactose intolerance, especially in patients receiving lactose-containing placebo formulation in clinical trail.

6.5 HIV/AIDS and urinary tract infections

Infection of urinary tract is not so much severing compare to that of other systematic infections. Since most of the cases of HIV/AIDS are seen among the people of sexually active group genital organs may serve as the port of infection of many sexually transmitted diseases. Besides this, HIV serves as a major pathogen in some of the complication of urinogenital system. Due to the suppress immune system extra-pulmonary tuberculosis is one of the top most disease occurring among HIV/AIDS patients. Among which renal tuberculosis is one of them. Both *Mycobacterium tuberculosis* and *M. avium* are responsible for causing renal tuberculosis among PLWHA.

UTI monitored with the urine as a sample may includes some of the cutaneous diseases occurring among the patients with HIV. Some of the diseases are as such that they occur at some stage of HIV infection. The skin is the largest and most visible organ of the body, and a perception of good health depends on its appearance as well as its function. As about 90% of HIV-infected patients develop cutaneous signs and symptoms, diagnosis and management are vital in recognizing progression of HIV infection.

Candidiasis always develops at some stage of HIV illness, first appearing, on average, at a CD4 cell count of about 275. Most yeast infections are superficial minor infections, affecting the skin or mucous membrane of mouth and vagina. Vaginal thrust might be the source of *Candia* in the urine of HIV patients. In our finding, 9.52% of total cases were found to have vaginal thrust, as we were able to isolate the *Candia* from them. Sometime the yeast cell might be isolated from urine from the female. Due to the presence of *Lactobacillus* near the vaginal region reduced the pH of the surrounding favoring the growth of fungi, like yeast. Before concluding the remarks that the yeast isolated from our sample are due to vaginal thrust clinical examination is necessary.

In this study we were also able to isolate *Nocardia* Sps. from urine sample. Only 4.76% of the total populations showed the organism. It is not the usual finding in the urine sample as such. A *Nocardia* infection tends to occur in immunocompromised hosts, and some of the species causes self-limited skin infections where as other are responsible to cause progressive cutaneous and lymphocutaneous disease. Other complications that occur among the HIV infected individual due to these organisms include respiratory infections and neurological infections. Since we were only able to isolate this organism from only one sample the result cannot be generalized.

Other common organisms that were isolated were among the common pathogens of UTI. These include *Proteus, Klebsiella,* and *Staphylococcus aureus*. We were also able to isolate significant amount of *E. coli* from the urine indicating that the organism is one of the source of UTI. Infections with such organism might be of exogenous source.

6.6 HIV/AIDS and opportunistic infection as a whole

As we now know that there are three basic stage of treating HIV infections as soon as it emerges. ART, which is very expensive treatment in contest of Nepal, will not provide a significant result, if given without monitoring. So the total cost regarding the ARV is high, if we provide in proper guideline. Secondly, immune-based therapy, which is not only, very expensive but requires attention of individual organism as they infect the patients. Most organisms do not have immune-based treatment, so for them the treatment procedure will be in dilemma, despites of its high specificity and reliability. Thirdly, treating secondary infection as it immerges, sounds simple and cost effective is the best way of increasing the life span of individual with HIV infection in the contest of Nepal. Treating secondary infections on the other hand is one of the challenging jobs for all of us, who are working in this field. Treating secondary infection as soon as emerges or getting ready before it emerges is the right way of treatment. For this purpose we need hand to hand support from clinician as well as biomedics.

The first job in this sector is to isolate and identify the local organisms that are infecting the PLWHA in contest of Nepal. These identified organisms must be categorized with certain parameter that is most predominant among these individuals. The parameter must be chosen in such a way that it specify certain period of infectivity. In our opinion we think that, T-cell with CD4 marker in peripheral blood count or viral load in peripheral blood or both might be the best indicators of categorizing secondary infections among the HIV patients. We choose CD4 count as our indicators and try to categorize the isolated organism from PLWHA.

In this study, we isolated nearly 20 different type of organism from the patients with different level of CD4 count. Most of the organisms were common isolates and some are found only among the individual with certain level of CD4 count. Since we followed the traditional way of culturing and identifying the organism based upon bio-chemical test we might have escaped other organism which cannot be isolated from the technique that we implicated. In order to generalize this treatment more and more sample should be tested and large number of population must be included for the study.

In this study, we categorized the organism into three different classes, according to the CD4 cell count. First categorize comprises of those individuals whose CD4 cell count is above 500; *Entamoeba histolytica, Candia albicans, Nocardia* sps, and Hepatitis B were present only among them. On the basis of our study, we can say that, these organisms infect the individuals only in the initial period of HIV infection where the viral load is comparable very low and CD4 count is high. *Candia albicans* infection in vagina and oral cavity itself is the suggestive parameter for testing the presence of HIV infections. Hepatitis B is such an infection which is common among the sexually active group. Since HIV is transmitted via sexual contact it has a direct relationship with STD such as Hepatitis B. The period of death after infection with this virus is comparative very low compare to HIV infection even if the Hepatitis B infection occurs alone. Reports even say that, if a person is infected with both viruses i.e. HIV and HBV, the death of such subject occur early because of HBV even before he develops advance

HIV infections. Thus, it is very common that we encounter such viral infections early in the period of patients. Besides these, *Nocardia* infection is also among the immunocompromised individuals. This organism infects in such conditions where immune system is very week, but in our cases where sample size is comparably very low, and we were able to isolate only among one individual, the data cannot be generalized on this basis.

In the second category, where the CD4 cell count lies between 500 to 200; *Campylobacter jejuni*, *Giardia lambia*, and *Salmonella paratyphi* were isolated. These organisms do not have such remarkable significant relation with reduced CD4 cell counts. They are encounter generally among the individuals without HIV as well. The most upcoming result was obtained in this class is that, 75% of total syphilis cases fall in this category. This again supports the fact that HIV and STD can occur as counterpart. The late developments of syphilis suggest that the disease is slow progressing type rather than HBV infection. Most of the organisms that were isolated among the individual with CD4 count between 500-200 were normal flora of healthy humans. Besides this, many individuals were infected in this category compare to other two. It came to our knowledge that, most of the secondary infections develops in this period and the infections is due to opportunistic organisms.

In last category where CD4 cell count was below 200, we were unable to isolate any specific organisms among the group populations. The region might be due to low sample population or classical method followed for isolating and identifying. *Mycobacterium avium* and *Mycobacterium tuberculosis* were also isolated from this population. *M. avium* develops in the late stage of HIV infections. In many parts of world, along with KS and PCP MAC is also considered as one of the indicators of HIV/AIDS. 50% of the population in this category died during the study period, which makes this population non-referencing type. Multiple infections were one of the common scenarios among the individual in this category followed by unusual organism from not common sources. For example in this category, we isolated *Staphylococcus*

aureus from blood. This picture clearly indicates that once individual reaches the period where the drop down of CD4 cell is very low the site of infection by opportunistic pathogen is not fixed. Most of the death occurs in this period, so is consider being the most critical period among all three classes.

Isolating, identifying, and categorizing the pathogens that infect during various stage of HIV infection is only minor job done in term of treating secondary infections. The difficult task arises after this, i.e. choosing the best drugs for that particular organism. The drugs must id cleverly chosen in such a way that, it must be focused on treating the infection rather than creating multi-drug resistant organisms. Multi-drug resistant organism is the greatest threat for PLWHA than any other thing. In order to prevent this careful monitoring of the drugs must be done. In order to bring the effective treatment, we should set the drug profile level for all the common isolated in every individual. After extensive research we will be able to set the national guideline for treatment of secondary infections (as of ART) in contest of Nepal.

The obtain result must be informed to the patient so as he/she can discuss the matter along with the physician in obtaining right drug for treatments. The right drug for the right organism will act as a magic bullet in treating secondary infections that occurs during the period of HIV infections, which in turn will prolong the time of developing AIDS.

At the end, if the three therapy as mentioned earlier goes parallel for treating HIV infection, we can make people live longer than they have expected even being a carrier of HIV. The conclusion of this study showed that, treating secondary infection is not the solution of treating HIV infection but is the major tools for the developing nations like ours to generate hope for extending long life to those who have suffered from this tragic epidemic disease of twenty-first century.

CHAPTER-VII

7. SUMMARY AND RECOMMENDATION

7.1 Summary

The summary of our study on correlation of secondary infection with peripheral T-cell with CD4 cell count can be summarized as follows:

1 Out of 126 samples from 5 different regions of 21 patients 71 were positive for different organisms including virus. 56.35% were total positive samples, out of which 84.51% were bacterial infection, 9.86% were infection due to protozoa, 4.23% due to fungi, and 1.41% was viral infection.

2 Total patients were classified into three categories according to their level of T-cell with CD4 marker in the peripheral blood. The first category comprises 33.33% of total population whose CD4 cell count was above 500 cells per mm^3, followed by second where 57.17% of total population with CD4 cell count was between 500 to 200 cells per mm^3 lies, and third which comprises only 9.53% of total population where CD4 count was below 200 cells per mm^3.

3 Most of the positive samples were from second category, where 56.34% of total positive samples occurred. In first category 35.21% of total positive cases occurred, followed by third where 8.45% of total positive samples were present.

4 Out of 42 samples from first category 59.52% were positive. From positive cases we were able to isolate *Entamoeba histolytica*, *Candia albicans*,

Nocardia Sps., and Hepatitis B. These organisms were not found in remaining two categories. Among other isolates *Pseudomonas aeruginosa* was most predominant in this group.

5 *Campylobacter Jijuni*, *Giardia lambia*, and *Salmonella paratyphi* occurred in second category only. Among total population in this category 56.34% were infected. Most of the organism isolated falls in this category, among which *Treponema pallidum, Escherichia coli*, *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Salmonella Sps* were most predominant.

6 In third category, out of 9 samples studied, 66.67% of the group populations were positive for different organisms. Death of one subject occurred during the processing of stool and urine reducing the sample size. None of the isolate occurs only in this category but *Mycobacterium avium*, *Mycobacterium tuberculosis, Proteus mirabilis*, and *Staphylococcus aureus* were isolated in this category.

7 Body Mass Index was calculated in order to determine the loss of weight. 33.33% of total population were under weight where as 4.77% were over weight. Most of the under weight population lies on the third category, where 50% of total group population were under weight, followed by second group 41.67% and first 14.29%.

8 Haemoglobin concentration and erythrocyte sedimentation rate shows no significant correlation with CD4 count.

9 There was no relationship between the CD4 count and concentration of different type of leucocytes.

10 As the CD4 cell count goes on decreasing individual start suffering from various and multiple infections. Most of the organisms infecting are the normal flora of the healthy human. Infection due to multiple and unusual organisms are common as individual CD4 count decreases.

7.2 Recommendation

From our study following recommendation can be made for the further research in this field:

1 For this type of study, more time and sample are strongly required. Since our study was done in limited time and with small group of individual the inference of the study cannot be drawn on this basis.

2 Further extensive research should be carried out to know the likely causative pathogens encountering in PLWHA which includes Molecular Biology technique such as Polymerase Chain Reaction (PCR) and DNA probe that can identify organism which are difficult to culture in normal laboratory condition.

3 Techniques for identifying different virus such Kaposi's sarcoma, Cytomegalovirus, Herpes zoster, and Herpes simplex must be implemented. Besides this organism such as *Pneumocystis carinii*, *Toxoplasma*, *Histoplasma*, *Isospora*, and other rear organism should be detected using modern advance technique.

4 Large variety of culture media specific for particular organisms should be implemented in this study. Culturing the anaerobes must be encouraged along with the procedure identifying different fungi and protozoa.

5 Malignancies such as Burkett's Lymphoma, Immunoblastic Lymphoma, and cervical cancer should be included in the study along with HIV related Encephalopathy.

6 The correlation of secondary infection should not be done along with the CD4 cell count; assay of HIV load should be included in the study. RT-PCR or bound DNA PCR should be carried out side by side with CD4 cell count and secondary infection.

7 People with ARV and HAART should be monitored separately from those who are not receiving treatment.

8 Treating the secondary infection as it occurs in the PLWHA is a crucial point of treatment. Proper isolation and identification of a local isolate causing secondary infection in given place to the given person can be of vital importance in prolonging the life of infected people. After identification antibiotic sensitive test to that local isolate must be done in regular basis. The misuse of antibiotic should be strongly discouraged.

9 This study was conducted in a limited time frame, it should be done in all seasons through out the year and with people in different stage if HIV infection. People having very high as well as very low level of both CD4 cells and viral load should be included in the study.

10 Besides monitoring the secondary infection, immune component such as interleukins and interferon in blood must be monitored. Comparison between the individual receiving treatment for secondary infection and untreated should be done.

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APPENDIX I

Questionnaire

A) Examination of Blood specimen

Date:-

Clinical Profile

Name: - Age/Sex: - Lab No.:-

Hematological Examination:-

Total count (WBC):- Hemoglobin Concentration:-

Differential Count: - Erythrocytes Sedimentation Rate:-

Neutrophils:-

Eosinophils:-

Basophiles:-

Lymphocytes:-

Monocytes:-

Immunological and Serological Examination:-

HIV ½ Test (Kit Method):-

Hepatitis B surface antigen Test (Kit Method):-

Syphilis Test (RPR Method):-

Lymphocytes Count:-

Serum CD4 T-Cell Count: - Serum CD4 T-Cell %:-

Microscopic Examination:-

Gram's Stain:-

Acid Fast Stain:-

Giemsa Stain:-

Bacteriological Examination:-

Culture Media / Days of Incubation	2nd	7th	14th	21st
BHI Broth				
Mac-Conkey Agar				
Blood Agar				
Chocolate Agar				

Biochemical Test:-

Oxidase: -	Catalase: -	Coagulase: -
Urease: -	Citrate: -	MR/VP:-
TSI: -	SIM: -	O/F:-

Other Media If Used:-

Remarks:-

Performed By: -

Checked By:-

Date:

B) Examination of Throat Swab

Date:-

Clinical Profile

Name: - Age/Sex: - Lab No.:-

Microscopic Examination:-

Gram's Stain:-

Acid Fast Stain:-

Bacteriological Examination:-

Culture on Blood Agar:-

Chocolate Agar:-

Mac-Conkey Agar:-

Biochemical Test:-

Oxidase:-	Catalase:-	Coagulase:-
Urease:-	Citrate:-	MR/VP:-
TSI:-	SIM:-	O/F:-

Other Media If Used:-

Remarks:-

Performed By:- Checked By:-

C) Examination of Sputum

Date:-

Clinical Profile

Name: - Age/Sex: - Lab No.:-

Physical Examination:-

Purulent Mucopurulent Mucoid Mucosalivary

Blood:- Present / Absent

Microscopic Examination:-

Gram's Stain:-

Acid Fast Stain:-

Giemsa Stain:-

Bacteriological Examination:-

Culture on Blood Agar:-

Chocolate Agar:-

Mac-Conkey Agar:-

Biochemical Test:-

Oxidase: -	Catalase: -	Coagulase: -
Urease:-	Citrate:-	MR/VP:-
TSI: -	SIM: -	O/F:-

Remarks:-

Performed By: - Checked By:-

D) Examination of Urine Specimen

Date:

Clinical profile

Name: Lab No. :

Age/sex: Date:

Physical examination

Appearance

1) Color: Colorless Pale Yellow Deep Yellow Brown
2) Turbidity: Clear Cloudy
3) Blood: Present Absence

Biochemical Examination

1) Glucose Protein pH

Microscopic Examination

1) Gram's Stain
2) Acid-Fast Stain
3) Wet preparation: Erythrocytes Leucocytes Epithelial cell
   Casts Crystals Fungi
   Parasite eggs and larvae

Reading of Culture Plates

1) Blood agar:
2) MacConkey Agar: LF NLF

Biochemical Tests

Oxidase: - Catalase: - Coagulase: -

Urease: - Citrate: - MR/VP:-

TSI: - SIM: - O/F:-

Performed By: - Checked By:-

E) Examination of Stool

Date:-

Clinical Profile

Name: - Age/Sex: - Lab No.:-

Physical Examination:-

Color:-

Appearance:-

Presence of: - Blood Mucus Pus

Microscopic Examination:-

Gram's Stain:-

Acid Fast Stain:-

Modified Acid Fast Stain:-

Basic Fuschin Stain:-

Wet Mount (Saline):-

Bacteriological Examination:-

Culture on Blood Agar:-

Mac-Conkey Agar:-

SS Agar:-

Biochemical Test:-

Oxidase: -	Catalase: -	Coagulase:
Urease: -	Citrate: -	MR/VP:-
TSI: -	SIM: -	O/F:-

Other Media If Used:-

Remarks:-

Performed By: - Checked By:

APPENDIX I I

A. Composition and Preparation of different Culture Media

The culture media used were from Hi-media Laboratories Pvt. Limited, Bombay, India

1. **Blood Agar Base**

   Blood Agar Base (infusion agar) with 5% sheep blood.

Composition	**gram/liter**
Protease peptone	15.0
Liver digest	2.5
Yeast extract	5.0
Sodium chloride	5.0
Agar	15.0

   Final pH at 25^{o}C 7.4± 0.2

   Directions: To redhydrate, 40gm of Blood Agar Base was suspended in 1000ml of distilled water and soaked for 20 minutes. It was sterilized by autoclaving for 15 minutes at 121^{o}C., it was allowed to cool to 50^{o}C and 5% defibrinated sheep blood was added. The media was mixed gently and poured into sterile Petri-plates.

2. **Chocolate Agar**

   For the preparation of chocolate agar, blood agar was prepared as mentioned above and it was heated at 70^{o}C in water bath until the color change from bright red to dark brown and poured into sterile Petri-plates.

3. **MacConkey Agar**

Composition	**gram/liter**
Peptone	20.0
Lactose	10.0
Bile-salts	5.0
Sodium chloride	5.0
Neutral red	0.075
Agar	12.0

Final pH at 25^{o}C 7.2±0.2

Directions: 52 grams of the medium was suspended in 1000 ml distilled water dissolved by boiling and sterilized by autoclaving for 15 minutes at 121^{o}C (15 lbs pressure).

4. **Nutrient Broth**

Composition	**gram/liter**
Peptone	5.0
Sodium chloride	5.0
Beef extract	1.5
Yeast extract	1.5

Final pH at 25^{o}C 7.2±0.2

Directions:

13 grams of the medium is suspended in 1000ml distilled water. The medium is autoclaved at 15 lbs. pressure (121^{o}C) for 15 minutes.

5. **Mannitol Salt Agar**

Composition	**gram/liter**
Beef extract	1.0
Protease peptone	10.0
Sodium chloride	75.0
D-Mannitol	10.0

Phenol red	0.025
Agar	

Final pH at 25^{o}C 7.4±0.2

Directions: 111 grams of the medium was suspended in 1000 ml distilled water and boiled to dissolve it completely. It was sterilized by autoclaving at 121^{o}C for 15 minutes in 15lbs. pressure.

6. Nutrient Agar

Composition	**gram/liter**
Peptone	5.0
Sodium chloride	5.0
Beef extract	1.5
Yeast extract	1.5
Agar	15.0

Final pH at 25^{o}C 7.4±0.2

Directions: 37 grams of media was suspended in 1000ml distilled water and boiled to dissolve completely. The medium was autoclaved at 121^{o}C for 15 minutes in 15lbs. pressure and dispensed in Petri plates.

B. Composition and Preparation of Bio-chemical Media

1. Sulphate Indole Motility (SIM)

Composition	**gm/liter**
Peptic digest of animal tissue	30.00
Beef extract	3.00
Peptonized iron	0.20
Sodium thiosulphate	0.025
Agar	3.00

Final pH at 25^{o}C 7.3±0.2

Directions: 36.23 grams of the medium was suspended in 1000ml of distilled water and was boiled to dissolve completely. It was distributed in tubes and then sterilized by autoclaving at 121^{o}C for 15 minutes in 15lbs. pressure.

2. **Simmons's Citrate Agar**

Composition	**gm/liter**
Magnesium Sulphate	0.20
Ammonium Dihydrogen phosphate	1.00
Dipotassium phosphate	1.00
Sodium citrate	2.00
Sodium chloride	5.00
Agar	15.0
Bromothymol blue	0.08

Final pH at 25^{o}C 6.8± 0.2

Directions: 24.2 grams of medium was dissolved in 1000 ml of distilled water, distributed in tubes and then sterilized by autoclaving at 121^{o}C for 15 minutes in 15lbs. pressure. Then the tubes were allowed to cool in slanted position.

3. **MR-VP media**

Composition	**gm/liter**
Buffered Peptone	7.00
Dextrose	5.00
Dipotassium phosphate	5.00

Final pH (at 25^{o}C) 6.9 ±0.2

Directions: 17 grams of MR-VP powder was dissolved in 1000ml of distilled water. It was distributed in tubes and sterilized by autoclaving at 121^{o}C for 15 minutes in 15lbs. pressure.

4. Triple Sugar Iron Medium

Composition	**gm/liter**
Peptic digest of animal tissue	10.00
Casein enzymic hydrolysate	10.00
Yeast extract	3.00
Beef extract	3.00
Lactose	10.00
Sucrose	10.00
Dextrose	1.00
Ferrous Sulphate	0.20
Sodium chloride	5.00
Phenol red	0.024
Agar	12.00

Final pH at 25^{o}C 7.4±0.2

Directions: 65 grams of the medium was dissolved in 1000ml of distilled water, and distributed in tubes. It was sterilized by autoclaving at 121^{o}C for 15 minutes in 15lbs. pressure. Then the tubes were allowed to cool in slanted position.

5. Christensen urea Agar

Composition	**gm/liter**
Peptone	1.0
Dextrose	1.0
Sodium Chloride	5.0
Dipotassium Phosphate	1.2
Monopotassium Phosphate	0.8
Phenol Red	0.012
Agar	15.0

Final pH at 25^{o}C 7.4 ± 0.2

Directions: 24 grams of the medium was suspended in 950 ml distilled water and sterilized by autoclaving at 121^{o}C for 15 minutes. After cooling to about 45^{o}C, 50 ml of 40 % urea was added and mixed well. Then 5 ml was dispensed in test tube and was allowed to set in sloped form.

6. Oxidation /Fermentation(O/F) medium

Composition	**gm/liter**
Peptone	2.0
Sodium chloride	5.0
Dipotassium phosphate	0.3
Bromothymol blue	0.008
Agar	2.0

Final pH at 25^{o}C 6.8 ± 0.2

Directions:9.4 grams of the medium was rehydrated in 1000ml cold distilled water and then heated to boiling and dissolve completely. The medium was distributed in 1000ml amounts and sterilized in the autoclave for 15 minutes at 15 lbs pressure (121^{o}C). To 100ml sterile medium aseptically added 10 ml of sterile Dextrose and mixed thoroughly and dispensed in 5 ml quantities into sterile culture tubes.

APPENDIX III

A) Composition and Preparation of Stains and Reagents

1. Gram's stain

a. Crystal violet stain

Composition	gram/liter
Crystal violet	20
Ammonium oxalate	9
Ethanol (absolute)	95 ml
Distilled water	876 ml

Directions: 20 gm crystal violet was dissolved in 95 ml of absolute alcohol to which 9 gm ammonium oxalate suspended in about 200ml distilled water was added. Finally, the total volume was adjusted to 1000 ml by adding distilled water.

b. Lugol's iodine solution

Composition	gram/liter
Potassium iodide	20
Iodine	10
Distilled water	1 liter

Directions: 20 grams of potassium iodide was dissolved in quarter of distilled water and mixed well until potassium iodine was dissolved completely. To it 10 grams of iodine was added and mixed until the iodine was dissolved and the volume was adjusted to 1000 ml by adding distilled water.

c. Acetone alcohol decolorizer

Composition	**gram/liter**
Acetone	500 ml
Ethanol (absolute)	475 ml
Distilled water	25 ml

Directions: Distilled water and absolute ethanol was mixed and then measured volume of acetone was added.

d. **Safranin stain**

Composition	**gram/liter**
Safranine, 99% dye content	10
Distilled water	1000ml

Directions: The dye was added to the distilled water in a bottle and shaked to dissolve.1, % stock solution was diluted to make the working solution.

1% stock solution	40 ml
Distilled water	360 ml

2. Acid Fast Stain

a) **Carbol fuchsin**

Composition	**gram/liter**
Basic fuchsin	10
Ethanol absolute	100 ml
Phenol	50
Distilled water	1000 ml

Directions: Weight the basic fuchsin on a piece of clean paper and transfer to a container. Measure the ethanol and add to the bottle. Mix at the intervals until the basic fuchsin is completely dissolved. With great care, weight the

phenol in a beaker. Measure the water, and add some of it to the beaker to dissolve the phenol. Transfer to the bottle of stain, and mix well. Add the reminder of the water, and mix well.

b) **Acid Alcohol (3% v/v)**

Composition	**Volume**
Ethanol absolute	680 ml
Distilled water	290 ml
Hydrochloric acid, concentrated	30 ml

Directions: Measure the ethanol and transfer to leak-proof container. Measure the water and add to the alcohol, and mix well. Measure the hydrochloric acid add to the solution, mix well.

c) **Malachite Green**

Composition	**gram/liter**
Malachite green	5
Distilled water	1000 ml

Directions: Weight malachite green in a paper, and transfer to a bottle. Measure the water and add about a quarter of it to the bottle. Mix until the dye is completely dissolved. Add the remainder of water and mix well.

3. Modified Acid-Fast Stain

The composition and preparation of stain is similar to that of Acid-Fast stain except the concentration of acid alcohol, which is 1% in this stain. 1% acid alcohol is prepared by dilution from the 3% acid alcohol.

4. Giemsa Stain

Composition	**gram/liter**
Giemsa powder	7.6
Glycerol	500 ml
Methanol	500 ml

Directions: Weigh the Giemsa on a piece of clean paper, and transfer it to a dry brown bottle which contains a few dry glass beads. Measure the methanol and add to the stain. Mix well. Measure the glycerol and add it to the stain. Place the bottle of stain in a water bath at 50-60^{0}C, for up to 2 hours to help the stain to dissolve. Mix well at intervals.

5. Wright's Stain

Composition	**gram/liter**
Wright stain powder	2.5
Methanol	1000 ml

Directions: Weight the Wright powder and transfer it to a dry bottle. Add a few glass beads. Measure the methanol and add this to the stain. Mix well at intervals until the powder is completely dissolved. Warm the solution at 37^{0}C to dissolve the dye.

6. Normal saline (0.8% w/v)

Composition	**gram/liter**
Sodium chloride	8.5
Distilled water	1 liter

Directions: 8.5 gram of sodium chloride was dissolved completely in 1 liter distilled water. The bottle was labeled and stored at room temperature.

B) Composition and Preparation of Test Reagents

1. Catalase reagent

To make 100 ml

Composition

Hydrogen peroxide solution	3 ml
Distilled water	97 ml

Directions: 3 ml of hydrogen peroxide was added to 97 ml distilled water, mixed well and stored away from light.

2. Oxidase reagent

To make 10 ml

Tetramethyl-p-phenylene diamine dihydrochloride	0.1 ml
Distilled water	10 ml

Directions: 0.1 ml Tetramethyl-p-phenylene diamine Dihydrochloride was dissolved in 10 ml distilled water and mixed well. Strips of Whatman's number 1 filter paper was soaked in the Oxidase reagent and dried before storing in a dark bottle tightly.

3. Kovac's reagent

To make 40 ml

4-dimethylaminobenzaldehyde	2 gm
Isoamyl alcohol	30 ml
Hydrochloric acid (concentrated)	10 ml

Directions: 2 grams 4-dimethylaminobenzaldehyde was dissolved in 30 ml Isoamyl alcohol and to it 10 ml concentrated hydrochloric acid was added and mixed well.

4. Methyl-red solution

To make 50 ml	
Methyl-red	0.05gm
Absolute ethanol	28 ml
Distilled water	22 ml

Directions: 0.05 grams of methyl red was dissolved in 28 ml ethanol. To it distilled water was added and mixed well.

5. Voges-Proskauer reagent

a. **alpha-napthol ,5%, color intensifier**

To make 100 ml	
Alpha-Napthol	5 gm
Ethyl alcohol, absolute	100 ml

Directions: 5 grams Alpha-Napthol was added to 100 ml of ethyl alcohol.

b. **Potassium hydroxide ,40% ,oxidizing agent**

To make 100 ml	
Potassium hydroxide	40 gm
Distilled water	100 ml

Directions: 40 grams of potassium hydroxide was added to 100 ml distilled water and mixed till dissolved.

APPENDIX IV

Methodology of staining

1. Gram's stain

This procedure developed by Hans Christian Gram for differential staining of bacteria is still the most widely used method for differentiating bacteria into two broad categories Gram positive Gram negative , on the basis of the ability of the organism to retain the primary stain crystal violet

Method

For Gram staining smear made on clean, grease-free glass slide was flooded with crystal violet for 30 seconds .it was washed with distilled water and flooded with Lugol's iodine solution for 1 minute .again washed with water and flooded with ethyl alcohol for 10 seconds ,air dried and observed under low , high and oil immersion objective.

Interpretation

Gram-positive bacteria that retained the crystal violet staining after alcohol treatment appeared dark purple in color where as the Gram-negative bacteria that were decolorized after alcohol treatment and took the color of counter stain Safranin thus appearing pink.

Reporting of Gram stain

1. Number of bacteria present, whether many, moderate, few or scanty.
2. Gram reaction of bacteria , whether Gram positive or Gram negative
3. Morphology of the bacteria.
4. Presence of pus cells and their number
5. Presence of yeasts cells and epithelial cells.

2. Acid Fast Stain

While the majority of bacteria are stainable by either simple or Gram staining procedures, a few genera, particularly the members of the genus *Mycobacterium tuberculosis* and *Mycobacterium leprae* represent bacteria that are pathogenic to humans, the stain is of diagnostic value in identifying these organisms.

The characteristic difference between mycobacteria and other organisms is the presence of a thick waxy (lipoidal) wall that makes penetration by stains extremely difficult. Once the stain has penetrated, however, it cannot be readily removed even with the vigorous use of acid alcohol as a decolorizing agent. Because of this property, these organisms are called acid-fast, while all other microrganisms, which are easily decolorized by acid alcohol, are non-acid fast. The acid fast stain uses three different reagents namely carbol Fuchsin, acid-alcohol and methylene blue.

Method

For Acid Fast staining smear made on clean, grease-free glass slide, after air dry and heat fixed was flooded with Carbol Fuchsin. The stain was heated until the vapors just begin to rise (at about 60oC). Smear should not be over heated. The smear was left for 5 minutes. It was then washed with clean water and flooded with 3% v/v acid alcohol for 5 minutes or until the smear is sufficiently decolorized, i.e. pale pink. The smear was again wash with clean water. Malachite green was then added for 3 minutes and then washed with water. The back of the slide was wiped clean and placed in a draining rack for the smear to air dry. The smear was then examined microscopically using 100x oil immersion objective.

Interpretation

Acid Fast Bacilli appears red, straight or slightly curved rod, occurring singly or in small groups, many appear beaded. The cells and the background appear green. When any definite red bacilli are seen, the smear was report as “AFB Positive” and indication of number of bacteria present was reported as follows

Number of bacteria	Report
> 10 AFB/Field	+++
1 -10 AFB/Field	++
10 -100 AFB/100 field	+
1 – 9 AFB/100 field	Exact No.

3. Modified Acid Fast Stain

Modified acid fast stain was performed in order to detect *Cryptosporidium parvum* from the stool sample. Oocysts of *C. parvum* can be easily identified in smear stained by the modified acid fast stain method following concentration by the formol ether oocyst concentration technique.

Method

The smear was prepared from the sediment obtained by the formol ether oocyst concentration technique. It was air-dried and fixed with methanol for 2-3 minutes. The smear was then stained with unheated carbol fuchsin for 15 minutes and wash off with water. Decolorization was done with 1% acid-alcohol for 10-15 seconds and again washed with water. Counter stain was done with malachite green for 30 seconds, washed with water and slide was left in the draining rack for the smear to dry. The smear was then examined microscopically using 100x oil immersion objective.

Interpretation

The oocysts of *Cryptosporidium parvum* appears small, round to oval, pink red stained bodies measuring 4-6 µm. Some may contain a single deeply stained red dot.

4. Giemsa Stain

Giemsa is a Romanowsky stain that is widely used in Paracytology to stain blood parasites. It is also used to stain *Histoplasma* species, and the internal bodies of Pneumocystis *carinii* cysts, and occasionally bacterial capsules.

Method

The smear was fixed with covering it with methanol for 2-3 minutes and was allowed to air-dry. The slide was placed; smear downwards, in a Petri plate supported in each side with a thin piece of stick. The diluted stain (1:20 in buffered water) was poured in the dish and covered with lid. The stain was left for 30 minutes and washed with buffered water. The back side of slide was wrapped with a blotting paper and the slide was placed in a draining rack to air-dry. The smear was then examined microscopically using first 40x and then 100x oil immersion objective.

Interpretation

The organism appears Blue-mauve to dark purple, depending upon the stage of development. Nuclei of host cell appears dark purple, cytoplasm of host cell pale blue, and bacteria pale to dark blue.

5. Wet Preparation

Wet preparation is done in order to detect fecal parasites. They are useful for the detection of motile trophozoites, ova and cyst present in moderate numbers, and to detect erythrocytes, cellular debris or excess fat.

Method

In a clean, well labeled glass slide one drop of normal saline was placed. Using a piece of stick small amount of specimen was mixed with the saline. A smooth thin preparation was made and covered with cover slip. The entire preparation was observed for the presence of any form of parasites.

Interpretation

The type and number of organism were reported as follows.

Organism/preparation	Report
1-3	Scanty
4-10	Few
11-20	Moderate
21-40	Many
>40	Very Many

APPENDIX V

Methodology for Bio-Chemical tests used for identification of pathogens

1. Catalase test

This test is based on the ability of bacteria to produce Catalase an enzyme that catalyses the rapid release of oxygen from hydrogen peroxide.

$H_2O_2 \longrightarrow H_2 + O_2$

Method

With a sterile glass slide a small amount of pure growth of pure growth from NA was transferred to a clean, grease free slide. To it a drop of 3% hydrogen peroxide solution was added and observed for the evolution of gas.

Interpretation

Rapid evolution of gas bubbles indicated positive test.

2. Oxidase test

It is based on the presence in presence in bacteria of cytochrome Oxidase that oxidizes 1% Tetramethyl-p-phenylene diamine Dihydrochloride forming end product indophenols which is dark purple in color.

Method

A small amount of growth on agar was picked with a sterile glass rod and rubbed on moist filter paper impregnated with Oxidase reagent.

Interpretation

If positive, color of filter paper changed from light to dark purple within 10 seconds.

3. Coagulase test

It is of two types based on the detection of free or bound coagulase

a. Slide coagulase test

It demonstrated the presence of bound or cell surface associated coagulase also known as clumping factor which binds plasma fibrinogen, without the need for a coagulase reacting factor, causing agglutination of the organism by binding them together with aggregated fibrinogen.

Method

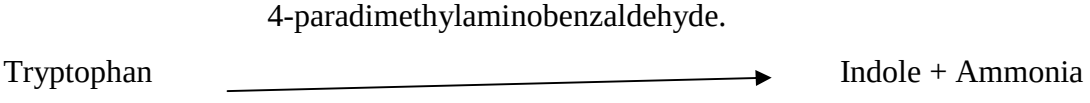
A drop of normal saline was placed near both the end of a clean glass slide. To both drops pure growth being tested was added and made into smooth suspension. In the absence of visible agglutination .A drop of plasma was added to the test drop and distilled water was added on the control.

Interpretation

Presence of visible agglutination in the coagulase plasma a drop and smooth, homogenous suspension in the control indicated positive slide coagulase test. Clumping in both drops occurred when the test organism auto agglutinated indicating negative slide coagulase test.

4. Indole test

This test is particularly helpful in identifying enterobacteria which break down the amino acid tryptophan present in medium and release Indole, as detected by a color reaction after treatment with kovac's reagent containing 4-paradimethylaminobenzalde-hyde.



Method

By using sterile wire SIM medium was inoculated with pure growth of the test organism. After overnight incubation at 37^{o}C few drops of Kovac's reagent was added to the tube and any color change was noted.

Interpretation

Appearance of red color indicated positive test for Indole production.

5. Methyl-red test

This test is used to differentiate enterobacteria which are capable of acid production by fermentation of glucose to give red color with the indicator methyl red.

Method

Pure growth of the test organism was inoculated into sterile MR-VP broth. After overnight incubation at 37^{o}C, a drop of methyl red was added.

Interpretation

Positive MR Test was indicated by the appearance of red coloration indicating acid production whereas formation of yellow or orange color was seen in negative test.

6. Voges-Proskauer test

This test is based on the ability of organisms to ferment glucose with the production of acetoin (acetylmethylcarbinol).

Method

MR-VP broth was inoculated with the test organism and after overnight incubation a few drops of VP reagent was added to it.

Interpretation

Pink color was observed in positive test indicating the formation of acetoin.

7. Triple-sugar Iron (TSI) test

This test is based on the ability of organism to utilize triple sugars (glucose, sucrose and lactose) in TSI medium. Lactose or sucrose fermenting organism will first utilize glucose producing acid end producing acid end products and then it will start fermenting sucrose or lactose thus causing both slant and butt of medium to remain yellow even after 18-24 hours incubation. However non lactose and non-sucrose fermenting organism will start fermenting peptone first after exhaustion of glucose thus producing ammonia which changes the slant to red.

Method

Test organism was inoculated to TSI medium by first stabbing the butt of the tube and then streaking the surface of the slant. After overnight incubation at 37^{o}C color of both the slant and butt, presence or absence of gas and H_2S was observed.

Interpretation

The result of the TSI was interpreted in 5 different ways as given below.

a) (Yellow) Acid / (Yellow) Acid, Gas, H_2S ⟶ Glucose, Lactose/Sucrose fermenter, H_2S producer.

b) (Red) Alkaline/ (Yellow) Acid, No Gas No H_2S only ⟶ Glucose not lactose/sucrose fermenter, not aerogenic, no H_2S production

c) (Red) Alkaline/No change ⟶ Glucose, Lactose and Sucrose non- fermenter

d) (Yellow) Acid /No change ⟶ Glucose- oxidizer

e) No change/ No change ⟶ Non fermenter

8. Urease test

This test is helpful in differentiating enterobacteria on the basis of their ability to produce Urease. Urease degrades urea present in the medium forming ammonia and Carbondioxide rendering the medium containing phenol red, alkaline as indicated by color change.

$$\text{Urea + water} \xrightarrow{\text{Urease}} \text{Ammonia + Carbondioxide}$$

Method

Pure growth of the test organism was streaked on the slant of the Urease medium and incubated at 37^{o}C for 24 hours. After incubation any color change in medium was observed.

Interpretation

Change of color from initial light pink to dark pink indicated positive result.

9. Oxidation-Fermentation test

This test is useful in differentiating carbohydrates oxidizing organisms from carbohydrates fermenting ones.

Method

The test organism was inoculated into two sets of the Hugh-Leifson's medium tubes containing bromothymol blue as indicator. Paraffin was added to one of the tubes to maintain anaerobic environment. The tubes were incubated at 37^{o}C for up to 4 days and examined daily for carbohydrate utilization as shown by acid production.

Interpretation

Change in color of the medium from green to yellow in both tubes showed that the organism was fermentative whereas acid production in open tube only indicated that the organism was oxidative type.

APPENDIX VI

1 Written consent for the active participation during the period of study

मेरो नाम शिवराम पन्त, म त्रिभुवन विश्वविद्यालयको सूक्ष्म जीव बिज्ञान विभागमा स्नातकोत्तर तहको पाठ्यक्रम अनुसार थेसिस लेख्नु पर्ने भएकोले मैले HIV/AIDS र यसमा देखा पर्ने सहायक रोगहरुको बारेमा अध्ययन, अनुसन्धान गर्ने निर्णय गरेको छु जसमा तपाईको पुर्ण सहयोगको अपेक्ष गर्दछ ।

अनुसन्धानको क्रममा तपाईको व्यक्तिगत बिवरणको पुर्ण रुपमा गोप्य राखिने छ । यो अध्ययन करीव १ (एक) बर्षको रहने छ । यस अनुसन्धानको सिलसिलामा तपाईको रगत, खकार, घांटीको स्वाव, दि्सा र पिसावको नमूना संकलन गर्न जरुरी हुने भएकोले ती नमुनाहरु क्रमैसंग उपलब्ध गराईदिनु हुने छ भन्ने मैले आशा राखेको छु । सो नमूना संकलन गर्दा प्रत्येक पल्ट तपाईको पुर्व स्वीकृति पछि मात्र लिइने छ भनि तपाईलाइ बिश्वस्त गराउंदछु । माथि उल्लेखित नमूना मध्ये कुनै नमूना दिन नचाहनु भएको खण्डमा तपाईको इच्छाको कदर गरिने छ ।

यस अनुसन्धानको क्रममा तपाईलाई HIV कहां कसरी सरेको हो सो कुरा उल्लेख गरिने छैन । साथ साथै तपाईलाइ यस अनुसन्धानबाट आफूलाई कुनै प्रकारको अप्ठ्यारो महशुस भई अनुसन्धानबाट अलग हुन चाहनु भएको खण्डमा रोकिने छैन र यसरी बिचमा नै अलग हुनु भएको सहयोगीहरुको व्यक्तिगत बिवरण पनि गोप्य राखिने छ ।

<u>नमूना संकलन गर्ने विधि</u>

१. रगत	:१० ml रगत सूइको माध्यामबाट तपाईको पाखुराबाट लिइने छ।
२. घांटीको स्वाब	:घांटीमा रहेको Tonsil भन्ने ग्रन्थिबाट कपासको माध्यामबाट लिइने छ ।
३. खकार	:तपाईको खकार ३ (तीन) दिन सम्म नियमित रुपमा लिइने छ । पहिलो र दोश्रो बिहान सवेरै र तेश्रो पटकको तेश्रो दिनको कुनै पनि समयमा लिइने छ ।
४. दिसा र पिसाब	:यी नमूनाहरु बिहान सबेरै एक पल्ट मात्र लिइने छ ।

मेरो यस अध्ययन अनुसन्धानबाट यसमा संलग्न रहनु भएकोमा तपाईलाई आर्थिक तथा भौतिक रुपमा कुनै प्रकारको लाभ नभए पनि यस अनुसन्धानबाट निस्केको निचोडबाट नेपालमा रहेका HIV/AIDS पिडित समुदायको उपचारमा केहि हदसम्म टेवा पुग्ने विश्वास मैले लिएको छु । यसर्थ यस अनुसन्धानको क्रममा आवश्यक पर्ने नमूनाहरु उपलब्ध गराई सहयोग गर्नं हुनेछ भन्ने अपेक्ष लिएको छु ।

Shiva Ram Panth

M. Sc. Microbiology

माथि उल्लेखित सबै बिवरण मैले राम्ररी पढेको छ’रमलाई अरुको सहयोगबाट प’र्ण रुपमा जानकारी गराइएको छ ।
यस अन’सन्धानको क्रममा आवश्यक भएको नम’नाहरु कसैको दवावमा नभइ आफ्नै स्वच्छाले उपलब्ध गराइ सहयोग गर्ने छ’।

क्र सं.	नम”ना	हस्ताक्षर	मिति
१	रगत		
२	घांटीको स्वाब		
३	खकार		
४	दिसा		
५	पिसाब		

APPENDIX VII

1 Internship/Volunteership agreement with Maiti Nepal

INTERNSHIP / VOLUNTEERSHIP AGREEMENT

Terms of Reference for Mr. / Mrs. / Ms. SHIVA RAM PANTH

Name: SHIVA RAM PANTH

Title: VOLUNTEER

Duration: 2004-12-7 → 2005-8-7

1. Reporting

Reports to: Dr. Mukta Bhattarai

Code of conduct:

1. You shall during the period of internship / volunteer ship:-
   a. Diligently and faithfully carry out all the instructions given to you from time to time.
   b. Maintain complete secrecy about the affairs and prevailing system of the organisation.
2. You will not be allowed to interfere in the private matters of the organisation.
3. Photography is strictly prohibited.
4. You will not be allowed to take personal interview with any of the children or girls of Maiti's shelter.
5. Smoking inside the premises is strictly prohibited.
6. Interns / Volunteers are not allowed to bring any visitors without prior notice to the organisation. In case, if the volunteer wants to bring any visitors they should inform the organisation at least one week before and get the permission from the concerned authority.
7. Permission from the concerned authority is a must, if interns / volunteers want to bring any kind of commodity inside Maiti's premises.
8. In case of complaint or suggestion, you are welcome to the office.
9. Work according to the rules and regulations of the organisation.
10. You will abide by the rules and regulations of the organisation, which may be enforced from time to time.

At all times, interns / volunteers are asked to abide by Nepali laws and ensure that conduct is consistent with accepted norms as well as good conduct. The internship / volunteer ship can be suspended or terminated due to breach of the above rules and regulations or due to behavior that compromises the reputation of Maiti Nepal.

Medical Insurances food, accommodation and transportation:

Medical, food, accommodation and transportation is the sole responsibility of the interns / volunteers. It is assumed that interns / volunteers will cover their own expenses and make her / his arrangements.

Status of the intern / volunteer:

The intern / volunteer is engaged as an independent and shall be treated as such for all purposes, including but not limited to, HMG taxation, withholding, unemployment insurance, and worker's compensation. The intern / volunteer will not be considered an employee of Maiti Nepal for any purpose.

Internship / Volunteer experience letter:

Letter of internship / volunteer will be given only to those interns / volunteers who will spend 6 months with the organisation.

Clearance of bills:

All bills that may be owed to Maiti Nepal (i.e. phone, food etc.) must be cleared prior to separation from Maiti Nepal.

Shiva Rani Panth
(Full Name)
Intern / Volunteer)

Date

Mr. Anoop Singh Gurung
Administrative Officer

07/12/04
Date

APPENDIX VIII

1 Volunteership letter from Maiti Nepal

SWC Aff. 1137
Regd. 413/049/50

माइती नेपाल • Maiti Nepal

Ref. No. 2005/12/0997

Date 18 November 2005

TO WHOM IT MAY CONCERN

This is to certify that Mr. Shiva Ram Panth completed his volunteer ship from 07 December 2004 till 07 August 2005. As a volunteer he helped us in the Pathology Laboratory of Maiti Nepal's Sonja Jeevan Kendra (Clinic), Kathmandu. He has been involved in performing different Microbiological and Hematological tests of HIV / AIDS patients under the supervision of Dr. Mukta Bhattarai during his period in Sonja Jeevan Kendra.

He has been very helpful and cooperative during his internship.

We wish him success in all his endeavors.

Thank you,

Sincerely,

Anoop Singh Gurung
Administrative Officer

83-Maiti Marg, Pingalsthan • P.O. Box 9599, Kathmandu, Nepal
Tel: 977-1-4494816, 4492904 • Fax: 977-1-4489978 • Email: program@maitinepal.org or maiti@ccsl.com.np • www.maitinepal.org
हाम्रो आवाज : चेलीबेटी बेचबिखनबाट मुक्त समाज । Our voice: A society free from girls and women trafficking