SERO-PREVALENCE OF SYPHILIS AMONG HIV POSITIVE SERUM SAMPLES OBTAINED FROM NATIONAL PUBLIC HEALTH LABORATORY, TEKU, KATHMANDU, NEPAL

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Master of Science in Microbiology (Medical)

By

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TO WHOM IT MAY CONCERN

This is to certify that **Mr. Sudeep Dahal** has completed this dissertation work entitled **"Sero-prevalence of syphilis among HIV positive serum samples obtained from National Public Health Laboratory"** from November 2008 to April 2009 as a partial fulfillment of M. Sc. Degree in Microbiology under the supervision of Prof. Dr. Sarala Malla, Ex-Director of NPHL.

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ABSTRACT

Sexually transmitted infections (STIs) have been prioritized not only because of their high incidence, complications and sequalae but also due to the socioeconomic impact and their role in transmission of HIV. HIV/syphilis co-infection presents a serious health problem. This study was carried out at National Public Health Laboratory, Nepal from November 2008 to April 2009, aiming at finding the prevalence of syphilis among HIV sero-reactive individuals. The serum specimens from HIV suspected individuals attending NPHL were tested by two rapid tests and ELISA using the WHO algorithm. The HIV positive individuals were further screened for syphilis by RPR followed by TPHA testing for those turned reactive with RPR. Reactive upon testing by both methods was set criterion for active syphilis determination. Data obtained from laboratory processing and the questionnaire was analyzed using Winpepi version 3.8. Out of 1094 samples tested, 30.6% were confirmed as HIV positives. Of the 303 samples, further screened for syphilis by RPR and TPHA tests confirmed a true syphilis sero-prevalence rate of 14.2%; 51.1% being confirmed as false positives. Majority of the HIV infected were males (61.5%) which was statistically significant ($p<0.05$). Similarly, the syphilis co-infection was also higher among males (62.8%) but was statistically insignificant ($p>0.05$). The highest prevalence of HIV was found in age group 25–34 years (46.9%), followed by 35–44 years (28.9%) which was statistically significant ($p<0.05$). Similarly, the highest prevalence of syphilis co-infection was also observed in age groups 25–34 years (60.4%) followed by 35–44 years (25.6%) but was statistically insignificant ($p>0.05$). The notable rate of syphilis among HIV positives is a public health concern. It indicates the need of introducing STIs screening in HIV positive individuals at national level.

Key words: HIV, Syphilis, HIV/syphilis co-infection, RPR, TPHA

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LIST OF ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome
ANC	Antenatal clinic
ART	Antiretroviral therapy
BFP	Biological false positive
CDC	Centers for Disease Control and Prevention
CNS	Central nervous system
CSF	Cerebrospinal fluid
DFA	Direct fluorescent antibody
DFA-TP	Direct fluorescent antibody *Treponema pallidum*
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme linked immunosorbent assay
EIA	Enzyme immunoassay
FSW	Female sex worker
FTA-ABS	Fluorescent treponemal antibody absorption
HIV	Human immunodeficiency virus
HTLV	Human T-cell lymphotropic virus
HAART	Highly active antiretroviral therapy
IFA	Indirect fluorescent antibody assay
IDU	Intravenous drug user
LIA	Line immunoassay

MSM	Men having sex with men
MSW	Male sex worker
MTCT	Mother to child transmission
NCASC	National Center for AIDS and STD control
NIAID	National Institute of Allergy and Infectious Diseases
NPHL	National Public Health Laboratory
NSBSW	Non-street based sex worker
OI	Opportunistic infection
PCR	Polymerase chain reaction
PLWHA	People living with HIV/AIDS
RIPA	Radio immuno-precipitation assay
RT	Reverse transcriptase
RNA	Ribonucleic acid
RIA	Radioimmunoassay
RPR	Rapid plasma reagin
SACTS	STD/AIDS Counseling and Training Services
SBSW	Street based sex worker
STD	Sexually transmitted disease
STI	Sexually transmitted infection
STC	SAARC Tuberculosis Center
TPHA	*Treponema pallidum* hemagglutination assay
VDRL	Veneral disease research laboratory
WHO	World Health Organization

CHAPTER – I

1. INTRODUCTION

Sexually transmitted infections (STIs) are among the first ten causes of unpleasant diseases in young adults in developing countries and the second major cause of unpleasant diseases in young adult women. Adolescents and young adults (15–24 years) make up only 25% of the sexually active population, but represent almost 50% of all new acquired STIs. STIs are epidemics and present an enormous health and economic consequences (Ros *et al.*, 2008). There has been a significant increase in the frequency and diversity of sexually transmitted diseases (STDs) for the last two decades. Leaving some fluctuations, their incidence remains very high (De Schryver *et al*., 1990).

Acquired immunodeficiency syndrome (AIDS) is a disease of the human immune system caused by the human immunodeficiency virus (HIV) (Sepkowitz, 2001; Weiss, 1993), a complex member of the *Lentivirus* genus of the Retroviridae family (Rivera *et al*., 2010). AIDS is a severe condition appeared after infection by HIV. Therefore, HIV infection leads to AIDS when at least one of the serious complications develops and the CD4+ T-lymphocytes count decreases substantially (McCutchan, 2008). HIV attacks and destroys certain populations of leucocytes essential to the body's immune system leading to weakened immune system. In fact when HIV infects a cell, it may remain latent for 7–10 years and usually without any symptoms or only minor illness. Gradually, the virus becomes activated destroys the defense mechanisms making it prone to other opportunistic infections (for e.g. TB) and other indicator conditions of AIDS, a late clinical stage of infection (STC, 2004). According to the staging system introduced by the World Health Organization (WHO), patients with AIDS are grouped under Stage IV which includes toxoplasmosis of the brain, candidiasis of the esophagus, trachea, bronchi or lungs and Kaposi's sarcoma (WHO, 1990). For more than two decades, HIV and AIDS has been a growing challenge worldwide. HIV/AIDS is

recognized as a global emergency demanding the attention on the international health agenda and one of the most important public health issues (Wasti *et al.*, 2009). The spread of HIV/AIDS has reached a pandemic form within a short span of time (Kallings, 2008). A total of 33.4 million people are estimated to be living with HIV across the globe, 2.7 million people became infected with the virus and 2 million people have lost their life due to AIDS (UNAIDS/WHO, 2009).

The first case of AIDS in Nepal was reported in July 1988 (Subedi *et al.*, 1992). Nepal has progressed from being a low-prevalence, low-risk population to one with a "concentrated" epidemic in the early 2000 (Neupane *et al.*, 2003), with prevalence exceeding 5% in at least two risk groups; namely sex workers and injecting drug users (IDUs). HIV prevalence in these groups appears to have been rising rapidly over recent years (Furber *et al.*, 2002). UNAIDS has estimated the adult (15–49 years) HIV prevalence rate of 0.5% by the end of 2007 in general population whereas the number of people living with HIV in the same time has been estimated to be 70,000 (UNAIDS/WHO, 2008b). According to the National Centre for AIDS and STD Control (NCASC), the cumulative number of HIV positive cases, as of August 2010, is 16,262. Among them, 65% were males and 35% were females. Most of the infected people are in the age group of 20–49 years old (NCASC, 2010).

Syphilis, a chronic and systemic sexually transmitted infection, is caused by the spirochaete *Treponema pallidum* subspecies *pallidum* (Laford *et al.*, 2006). Syphilis is a systemic disease with a wide variety of presentations in which symptomatic periods (primary, secondary and tertiary) alternate with periods of clinical latency (Zetola *et al.*, 2007a). Syphilis remains a major public health problem in sub-Saharan Africa and in the developing world (DeSchryver *et al.*, 1990; Gerbase *et al.*, 1998). Compared to syphilis in developed countries, the worldwide burden of syphilis is formidable (Lafond *et al.*, 2006). An estimated 12.22 million cases of syphilis occurred worldwide in 1999– slightly below the 1995 estimate (WHO, 2001). South and Southeast Asia (SEA), with an estimated 5.8 million new cases in 1997 (45% of global new cases), is the major

focus for STIs. Studies in South Asia indicate extremely high prevalence rate of STIs in groups such as sex workers (Thakor *et al.*, 2004). Out of 12 million new cases of syphilis estimated worldwide annually by WHO, 4 million occurs in Africa (Gerbase *et al.*, 1998). Syphilis prevalence rates range from 1% to 20% in some developing countries (Laga *et al.*, 1994).

In Nepal, prevalence of STDs is quite high and estimated to be around 3% of the population (Subedi *et al.*, 1996). The major hospitals of the country report higher prevalence of gonorrhoea followed by syphilis, chancroid and herpes genetalis (Dixit, 2005). In the context of Nepal, various studies among high risk individuals have reported that the seroprevalence of syphilis ranges from 3.5–28% in (SACTS, 2001; Poudel *et al.*, 2003; Bhatta *et al.*, 1994). A multi centre, cross-sectional study of healthy males from different development regions of Nepal showed the prevalence rate for syphilis to be 0.85%; the occurrence of syphilis being highest (1.39%) in mid-western development region and least (0.58%) in eastern development region (Shrestha, 2008). The active syphilis rate, a significant correlate of the risk for HIV transmission, had dropped to 9.5% in 2003 from 18.8% in 1999 in Nepal (USAID, 2005).

HIV infection and syphilis are linked. Syphilis increases the risk of transmitting as well as getting infected with HIV (NIAID, 2009). The interaction of syphilis and HIV infection is reportedly complex (Turbadkar *et al.*, 2007). In patients co-infected with HIV and *T. pallidum*, cutaneous lesions may be more severe, symptomatic neurosyphilis may be more likely to develop, the latency period before the development of meningovascular syphilis may be shorter, and the efficacy of standard therapy for early syphilis may be reduced (Hall *et al.*, 2006). HIV infection may cause more severe manifestations of early syphilis or more rapid progression to late syphilis (neuro- and gummatous syphilis) (Goh, 2005). Features of secondary syphilis may be particularly pronounced and more aggressive in HIV co-infected patients (Karumudi *et al.*, 2005). Diagnosis and treatment are more complicated in patients co-infected with HIV and syphilis (Kent *et al.*, 2008).

The frequency of HIV/syphilis co-infection depends on the prevalence of the two infections in a given patient group and on individual risk factors (Pialoux *et al.*, 2008). Studies have shown strong correlation between high rates of syphilis and increased rates of HIV infection within a sexually active population (Horn *et al.*, 2002). An estimated 16% of all patients and 28% of men with syphilis have co-infection with HIV in the United States (Zetola *et al.*, 2007a). A study carried out among patients attending a comprehensive adolescent health center in New York with a positive serologic test for syphilis revealed 15.3% being infected by HIV (McCabe *et al.*, 1993). Different studies conducted in India among HIV infected patients had revealed the syphilis co-infection rate to be in the range of 4.34–21.6% (Mahajan *et al.*, 2008; Dhanvijay, 2000). A study conducted among HIV infected sex trafficked women and girls in Nepal revealed those HIV positives were more likely to be infected with syphilis than HIV negatives (Silverman *et al.*, 2008). A study conducted by New ERA/SACTS in 1999 among sex workers and truckers in Nepal showed sex workers and truckers with syphilis had a ten-fold higher risk of getting HIV than those without syphilis (New ERA/SACTS, 2000).

There are no sufficient data available on HIV/syphilis co-infection in Nepalese context as very few researches have been carried out. The available few data are also not representative to the HIV positive populations. Lack of sufficient data on the co-infection adversely affects the disease management, control and prevention strategies of the government. So, this study aims to reveal some information on HIV-syphilis co-infection in our context which could be of importance for concerned authorities in evaluating control interventions and assisting in strategic plans to reduce the disease burden. The study has been designed to estimate preliminary burden of syphilis-HIV co-infection among the different groups. As HIV and STIs could easily spread from “high risk” groups to the general population, base line seroprevalence data may serve as an indicator for the emergence of HIV and STIs in such population. Co-existence of syphilis and HIV infection in the patients will indicate need for further public health efforts if spread of AIDS is to be controlled.

CHAPTER – II

2. OBJECTIVES

2.1 General Objective

To determine the sero-prevalence of syphilis among HIV positive serum samples obtained from National Public Health Laboratory (NPHL)

2.2 Specific Objectives

a) To screen the HIV infection among the suspected individuals visiting NPHL.
b) To detect syphilis among HIV positive individuals.
c) To estimate the rate of HIV/syphilis co-infection.
d) To correlate the serological findings with other variables.

CHAPTER – III

3. LITERATURE REVIEW

3.1 Human immunodeficiency virus (HIV)

HIV infection is caused by a retrovirus known as human immunodeficiency virus (HIV) (type1 and 2) which breaks down the body's immune system and leads to acquired immune deficiency syndrome (AIDS) leaving the person susceptible to opportunistic infections (OIs) and some malignancies (Joshi *et al.*, 2003). HIV progressively destroys some types of white blood cells called CD4+ T-lymphocytes. These lymphocytes help to protect the body against foreign cells, infectious organisms, and cancers. Many of the complications of HIV infection, including death, usually result from these OIs and not from HIV infection directly (McCutchan, 2008).

AIDS was first recognized by the U.S. Centers for Disease Control and Prevention (CDC) in 1981 and the causative agent of the disease was controversially discovered in 1983 (Gallo, 2006). It was called human T-lymphotropic virus-III (HTLV-III) in U.S., lymphadenopathy-associated virus (LAV) in France, and AIDS-associated retrovirus elsewhere (Coffin *et al.*, 1986). In 1986, the virus was named HIV (Sepkowitz, 2001).

3.1.1 Properties of HIV

HIV is a retrovirus, which, like many other viruses, stores its genetic information as RNA. When HIV enters a human cell, it releases its RNA, and an enzyme called reverse transcriptase (RT) makes a DNA copy of the viral RNA. The resulting DNA is integrated into the cellular DNA. Thus, HIV is called a retrovirus, referring to the reversed (backward) process (McCutchan, 2008).

There are two strains of HIV known to exist namely, HIV-1 and HIV-2. HIV-1 is the virus that was initially discovered and termed LAV. It is more virulent, relatively easily

transmitted, and is the cause of the majority of HIV infections globally. HIV-2 is less transmittable and is largely confined to West Africa (Reeves *et al.*, 2002). HIV-1 originated in West-Central Africa in the first half of the 20th century when a closely related chimpanzee virus first infected humans (McCutchan, 2008).

HIV is roughly spherical enveloped virus with the diameter of about 110 nm (90-120 nm). Viral membrane is composed of two lipid bilayers derived from the host cell membrane when virus buds out during replication. Embedded in the viral envelope are glycoprotein (gp) 120, and a stem consisting of gp41 molecules that anchor into the viral envelope. This glycoprotein complex enables the virus to attach to and fuse with target cells to initiate the infectious cycle. The glycoprotein gp120 determines viral tropism by binding to target-cell receptors, while gp41 mediates fusion between viral and cellular membranes (Chan *et al.*, 1997). The virion has an outer icosahedral shell composed of the viral protein p17 and an inner cone shaped core composed of 2000 copies of viral protein p24, enclosing the ribonucleoproteins. The genome is diploid, composed of two identical single stranded positive sense RNAs tightly bound to nucleocapsid proteins, p7 and enzymes needed for the development of the virion such as RT, proteases, ribonuclease and integrase (Simmonds, 2006; Butel, 2007). The HIV genome contains nine genes three major or structural genes (*gag*, *pol* and *env*) and six non-structural regulatory genes [*tat*, *rev*, *nef*, *vif*, *vpr* and *vpu* (or *vpx* in the case of HIV-2)] (Chakraborty, 2003). The structural genes are required for a replicating retrovirus i.e. they contain information needed to make the structural proteins for the progeny virus particles. The non-structural genes regulate viral expression and are important in disease pathogenesis in vivo i.e. they control the ability of HIV to infect cells, produce new copies of virus causing disease condition (Butel, 2007).

HIV is completely inactivated by treatment for 10 minutes at room temperature with any of the following 10% household bleach, 50% ethanol, 35% isopropanol, 1% Nonidet P40, 0.5% Lysol, 0.5% paraformaldehyde, or 0.3% hydrogen peroxide. The virus is also inactivated by extremes of pH (pH 1.0, pH 13.0). HIV is readily inactivated

in liquids or 10% serum by heating at 56°C for 10 minutes, but dried proteinaceous material affords marked protection (Butel, 2007).

3.1.2 Pathogenesis and clinical features

In HIV infection, there are three distinct phases: Acute infection (4–8 weeks), Asymptomatic infection or Clinical Latency (10–11 yrs) and, ultimately AIDS defining conditions and death (2–3 yrs). After the establishment of primary infection, the virus is disseminated to lymphoid organs where it persists with minimal expression for the time of clinical latency and finally a profound expression of HIV provirus occurs leading to immune suppression and onset of OIs as well as neoplasms and eventually, the death (Schupach, 2003).

3.1.2.1 Acute retroviral syndrome

Acute infection is a transient symptomatic illness associated with high-titer HIV replication and a robust and expansive immunologic response to the invading pathogen in which most individuals (80–90%) develop influenza or mononucleosis-like illnesses (Kahn *et al.*, 1998). In the acute phase (first several weeks following infection), the person may experience mild fatigue and fever as the virus copy number (viral load) in the blood increases approaching several million viruses per ml (Global Health Council, 2010; Piatak, 1993). The high viral load increases the person's ability to infect someone else as the person appears apparently well (Global Health Council, 2010). The most common signs and symptoms include fever (median maximal temperature 38.9°C), fatigue, maculopapular rash, headache, lymphadenopathy, pharyngitis, myalgia, arthralgia, aseptic meningitis, retro-orbital pain, weight loss, depression, gastrointestinal distress, night sweats, and oral or genital ulcers. A morbilliform rash (maculopapular) occurs in 40–80% of persons with symptomatic acute infection which may be difficult to detect in darkly pigmented people. The acute illness may last from a few days to >10 weeks, usually <14 days. The CD4+ cell count usually decreases during acute HIV infection but may remain in the normal range over the ensuing weeks; the CD8+ cell count increases, thus, inverting the CD4:CD8 ratio (Kahn *et al.*, 1998).

3.1.2.2 Asymptomatic infection or clinical latency

During the asymptomatic phase (months or years after infection), both the viral load and the risk of transmission are decreased. Within one to three months, antibodies become detectable in the blood. This phase may last for many years, though most people start to experience symptoms within 10 years (Global Health Council, 2010). There are individuals with long-term latency resulting in clinically and immunologically healthy for 10–15 years post-HIV seroconversion, with stable CD4+ counts (Buchbinder *et al.*, 1994). The incubation time may be as short as 2–3 years in 5–10% of patients (rapid progressors) (Schupbach, 2003).

3.1.2.3 AIDS

AIDS is the end stage clinical manifestation of HIV infection. Due to relentless production of HIV proteins, maintained by continuous viral replication in productively infected cells, and the ensuing elimination of host cells over many years finally lead to the destruction of immune system, which is clinically manifested by opportunistic infections and tumors. The infection in central nervous system (CNS) may lead to distinct HIV-associated disease, including the HIV associated dementia complex, vacuolar myelopathy, and sensory neuropathy (Schupbach, 2003). In this final phase, viral load increases due to virus release into the blood and CD4+ T-cells decrease as these cells are destroyed. At this point, most infected people develop symptoms of the disease, including weight loss and fever. The transition from "living with HIV" to a diagnosis of AIDS occurs when the CD4+ count drops from a normal range of 500–1500 cells/mm^3 to <200 cells/mm^3 or when the CD4+ cells are only 14% of total lymphocytes (Global Health Council, 2010; CDC, 1992).

3.1.3 Epidemiology

3.1.3.1 The global burden

HIV/AIDS causes debilitating illness and premature death in people during their prime years of life and has devastated families and communities. UNAIDS and the WHO estimate that AIDS has killed more than 25 million people since it was first recognized

in 1981, making it one of the most destructive pandemics in recorded history (UNAIDS/WHO, 2005). According to new estimates the total number of people living with HIV/AIDS (PLWHA) after 9 years of its first detection in 1981 became nearly 10 million. The number became doubled to 20 million after another 4 years and tripled in 1998. At the end of 2003, it reached 38 million. In 1990, the adult (15–49) rate of HIV infection was <0.5% and it has been increased to 1.1% as end of 2003 (STC, 2004). By the end of 2008, 33.4 million people were living with HIV, more than 90% of them from the developing world. The number of PLWHA has been increasing and the impact of HIV/AIDS on women and girls has been particularly devastating. Women now comprise almost 50% (15.7 million) of adults living with HIV (UNAIDS/WHO, 2009). In 1993, AIDS was the fourth leading cause of death for women in developing countries; it is now the leading cause of death among women aged 15–44 in developing countries (Global Health Council, 2010). The impact of HIV/AIDS on children and young people is a severe and growing problem. About 2.1 million children <15 years are living with HIV and millions others lost their parents due to AIDS. In 2008, 430,000 children <15 were infected with HIV and 280,000 died of AIDS (UNAIDS/WHO, 2009). The number of children living with HIV increased from 1.5 million in 2001 to 2.5 million in 2007 (UNAIDS/WHO, 2007). Among all PLWHA in the world, 22.4 million (two-thirds) live in sub-Saharan Africa. Adult prevalence is highest in this region, where 5.2% of adults aged 15–49 years are PLWHA (5.8% in 2001). Outside of sub-Saharan Africa, the region with the largest number of PLWHA is Asia**.** The number of PLWHA in this region accounts for 15% of the global burden. An estimated 4.7 million were living with HIV in this sub region in 2008. India accounts for roughly half of Asia's HIV prevalence (UNAIDS/WHO, 2009). India has an estimated 2.5 million infections and an estimated adult prevalence of 0.36% (UNAIDS/WHO, 2007). Prevalence rates of PLWHA are lowest in East Asia, with the majority of PLWHA in South and SEA (UNAIDS/WHO, 2008a). Everyday more than 7,400 men, women and children are infected with HIV. Since the late 1990s, the number of people newly infected with HIV has declined each year. In 2001, 3.2 million people were newly infected versus 2.7 million in 2008. This decline in new infections has contributed to

stabilizing the percentage of PLWHA. New infections continue to occur in young people. Nearly 16% of all new infections in 2008 occurred among children <15 and 45% of new infections occur in young people (15–24 years). Most new infections occur in sub-Saharan Africa with 1.9 million people were newly infected in 2008, about 20% less than in 2001. Of the 2 million people who died in 2008, 1.7 million were ≥15 years of age and 280,000 were <15 years. Nearly all deaths due to AIDS occur in the developing world. In 2008, 70% occurred in sub-Saharan Africa and over 16% in South, East and SEA. (UNAIDS/WHO, 2009).

HIV/AIDS in Asia was first detected in the early to mid-1980s. Thailand was the first Asian nation to report HIV infection followed by an explosive epidemic. Among the SAARC countries first AIDS cases were reported in 1986 by India and Pakistan and by 1993 all SAARC countries started reporting AIDS cases (STC, 2004). An estimate of global summary of HIV/AIDS has been shown below:

Table 1: Global Summary of HIV/AIDS, 2008 (UNAIDS/WHO, 2009)

Regions	Adults and children newly infected with HIV	Adults and children living with HIV	Adult and children deaths due to AIDS
Sub-Saharan Africa	1.9 million	22.4 million	1.4 million
South and Southeast Asia	280,000	3.8 million	270,000
Eastern Europe and Central Asia	110,000	1.5 million	87,000
Latin America	170,000	2.0 million	77,000
East Asia	75,000	850,000	59,000
Middle East and North Africa	35,000	310,000	20,000
North America	55,000	1.4 million	25,000
Caribbean	20,000	240,000	12,000
Western and Central Europe	30,000	850,000	13,000
Oceania	3,900	59,000	2,000
World	2.7 million	33.4 million	2 million

3.1.3.2 HIV/AIDS in Nepal

The first case of AIDS in Nepal was reported in July 1988 (Subedi *et al.*, 1992). According to the National Centre for AIDS and STD Control (NCASC), the cumulative number of HIV positive cases, as of August 2010, is 16,262. Among them, 65% were males and 35% were females. Most of the infected people are in the age group of 20–49 years old (NCASC, 2010). UNAIDS has estimated the adult (15–49 years) HIV prevalence rate of 0.5% by the end of 2007 in general population whereas the number of people living with HIV in the same time has been estimated to be 70,000 of which 17,000 were women (UNAIDS/WHO, 2008b). The distribution of HIV prevalence across the country is uneven. It shows that almost 50% of all HIV infection lies in the terrain highway epidemic region which constitutes from the east to the west of the country, followed by the hill region 19%, far western and Kathmandu valley 16% each respectively (Wasti *et al.*, 2009). According to the national HIV Sentinel Surveillance conducted among patients with sexually transmitted infections (STI) in six sentinel sites in Nepal, the prevalence of HIV was much higher in the Western sites and increases as we move from East to West and was almost double in the male patients (3.4%) as compared to 1.9% in the female patients (NCASC, 2001).

The HIV/AIDS situation in Nepal has been described as an impending crisis (Seddon, 1998). Over the last few years, HIV/AIDS epidemic in Nepal has gained ground and Nepal has progressed from a low prevalence country to one with so called concentrated epidemic in certain subgroups of the population (Neupane *et al.*, 2003). Until recently, Nepal possessed only scattered data regarding the prevalence of HIV. Nepal's HIV epidemic is largely concentrated in most at risk populations (MARPs), such as female sex workers (FSWs), intravenous drug users (IDUs), migrant labourers, men having sex with men (MSM) and transgender (The World Bank, 2008; Pokhrel *et al.*, 2000). A situation analysis study of HIV/AIDS conducted in 2000 has identified the young people, mobile populations, FSWs, MSM, IDUs and children as the most vulnerable to HIV/AIDS in Nepal (Pokhrel *et al.*, 2000). Most of the HIV infections in Nepal have been caused by HIV-1 though recently seroevidence of HIV-2 has been reported from

Bhairahava, Nepal (Chander *et al.*, 2004). The most recent behavioural surveys (in 2005) showed HIV prevalence among IDUs to be 52% in Kathmandu, 32% in the Eastern Terai districts, 22% in Pokhara and 12% in the Western Terai districts (UNAIDS/WHO, 2008a). IDUs appear to be extensive in Nepal and to overlap with commercial sex. In 2007 an estimated 6,557 IDUs were living with HIV or AIDS (about 10% of the total AIDS cases). The burden of HIV among IDUs is heavy in the Highway Districts and Kathmandu Valley, where 30% of all PLWHA are IDUs. HIV prevalence among IDUs in 2007 was 34.7%, significantly lower than 51% in 2003. This decline in prevalence is, to some extent, supported by improving behavioral indicators measured by three successive rounds of integrated biological and behavioral surveys (IBBS). There are between 25,000–34,000 FSWs in Nepal with an estimated HIV prevalence of 1.3–1.6%. HIV infection rates among street-based sex workers (SBSWs) in the Kathmandu Valley are between 15–17%. Nationally, clients of FSWs have an estimated HIV prevalence of 2%. High number of sex workers migrates or are trafficked to Mumbai, India, to work, thereby increasing HIV prevalence in the sex workers' network in Nepal more rapidly. It is estimated that 50% of Nepalese sex workers in Mumbai brothels are HIV positive (The World Bank, 2008). Recently, HIV prevalence of 38% has been found among repatriated Nepalese sex-trafficked females and one half of the women and girls trafficked to Mumbai were HIV-positive with increased risk among those trafficked prior to age 15 years (Silverman *et al.*, 2007). Similarly, another study among male migrant returnees from Indian cities in Doti districts has reported a prevalence rate of 8% (Poudel *et al*., 2003). Although accurate data on sex between men are not available, a recent report suggests that MSM activity in Nepal is similar to MSM activities in of the rest of South Asia. A national estimate of MSM, including MSW, is 64,000–193,000. HIV prevalence among MSM in the Kathmandu Valley is estimated to be about 3.3% (3.4% among non-MSWs and 2.9% among MSWs) (The World Bank, 2008). The overall seroprevalence of HIV among the total blood donors in nation wide and in Central Blood Transfusion Service (CBTS), Kathmandu through the six years of review (2001–2007) was 0.33% and 0.4% respectively, indicating a significant decreasing trend in HIV seroprevalence in Nepal (Tiwari *et al.*, 2008). Around 10 years

ago, Nepal was described as a country having comparatively lower prevalence of HIV/AIDS compared to other countries in SEA. Seasonal migration to Indian Cities for seeking job and sexual trafficking across a porous Indian border, fuelled by recent political insurgency, has raised Nepal's HIV prevalence second highest in the region after India (Seddon,1998; Singh *et al.*, 2005). A summary of HIV/AIDS in Nepal has been shown below:

Table 2: Estimated number of adults and children living with HIV*

Categories	2001	2007
Adults (15+) and children	56000	70000
Low estimate	41000	50000
High estimate	80000	99000
Adults (15+)	55000	68000
Low estimate	41000	49000
High estimate	78000	97000
Adult rate (15–49)%	0.5	0.5
Low estimate	0.3	0.4
High estimate	0.7	0.7
Women (15+)	12000	17000
Low estimate	8200	12000
High estimate	18000	25000

* Estimates include all people whether or not they have developed symptoms of AIDS

Source UNAIDS/WHO, (2008b)

3.1.4 Modes of transmission

AIDS is a blood-borne disease with sexual, parenteral, and perinatal modes of transmission (Guss, 1994). Though the overwhelming majority of infections occur through sexual contact, HIV continues to threaten children of HIV-positive mothers and IDUs (Global Health Council, 2010). The transmission of HIV requires contact with a body fluid that contains the virus or infected cells. HIV can appear in nearly any body

fluid, but transmission occurs mainly through blood, semen, vaginal secretions, and breast milk of an infected person. HIV is not transmitted by casual contact (such as touching, holding, or dry kissing) or by close, nonsexual contact at work, school, or home. No case of HIV transmission has been traced to the coughing or sneezing of an infected person or to a mosquito bite (McCutchan, 2008).

3.1.4.1 Sexual transmission

Globally, HIV is mostly transmitted through sexual contact. The exchange of body fluids during sexual intercourse is the apparent mode of transmission. Both the fluid and cellular components of semen has been found to contain HIV, as do endocervical secretions. Sexual practices, including the practice of anal intercourse and vaginal intercourse during menses, sexual mixing patterns and level of condom use, has all been recognized as factors affecting spread (Folks *et al*., 1998). Biological factors also seem to affect the efficiency of transmission; these factors include the level of viraemia, infectivity and virulence of a particular HIV strain, and presence of STDs such as genital ulcers (Plummer *et al*., 1991).

3.1.4.2 Transmission via blood and blood products

Recipients of unscreened blood and blood products from HIV infected donors are at high risk of HIV infection. The likelihood of HIV infection occurring in recipients of HIV positive blood is close to 100%. In countries where screening of blood for HIV has been instituted, the risk for HIV transmission through screened blood has been estimated to be 1/36000 to 1/225000 per unit transfused. This residual risk is due to antibody-negative infected donors in the 'window' period prior to seroconversion. Transmission of HIV by transplantation of vascular organs such as liver, heart, kidneys and bone marrow has occurred (Folks *et al*., 1998).

3.1.4.3 Transmission through injecting drug use

Among IDUs, HIV is transmitted by parenteral exposure to HIV-infected blood through the use of contaminated needles or other injection equipment. Factors associated with

HIV infection in IDUs include duration (years) of injection, frequency of needle sharing, number of needle-sharing partners and number of injections. In addition, unsafe sexual practices may contribute some infections among IDUs (Nelson *et al.*, 1991).

3.1.4.4 Perinatal (vertical) transmission

Mother-to-child transmission plays an important role in HIV infections in children. In prospective studies of infants born to HIV-infected women, transmission rates have ranged from 13–40%. The transmission of the virus from the mother to the child can occur *in utero* (during pregnancy), intrapartum (at childbirth) or postnatally (via breast feeding). Infection can occur during parturition as a result of transplacental bleeding or contact of abrasions with virus-containing fluids during passage along the birth canal (Folks *et al*., 1998). In about 30–50% of pregnancies involving women infected with HIV who are not treated, HIV is transmitted to the fetus through the placenta or at birth during passage through the birth canal (Ramachandran, 1988), although it may be less frequent with caesarian than vaginal delivery (Folks *et al*., 1998). In the absence of treatment, the transmission rate up to birth between the mother and child is around 25%. However, where combination antiretroviral drug treatment and Cesarian section are available, this risk can be reduced to as low as 1%. Of the estimated 700,000 children who were infected with HIV in 2003, about 315,000 were infected through breast-feeding (Coovadia, 2004). The risk from breastfeeding depends on the duration of breastfeeding but may be as high as 75% (McCutchan, 2008). Instances of HIV transmission via breast milk from mothers who were infected by blood transfusion after delivery have been described and virus has been isolated from milk (Folks *et al*., 1998).

3.1.4.5 Transmission in health care settings

Skin, mucous membranes and needle stick exposures to blood and other body fluids are frequent in the health care setting. Transmission of HIV after mucous membrane or cutaneous exposure seems to be much rarer (Folks *et al*., 1998). The average risk of seroconversion after a needlestick injury with HIV positive blood is about 0.3% (Tokars *et al*., 1993).

3.1.5 Laboratory diagnosis of HIV infection

The use of repeatedly reactive enzyme immunoassay (EIA) followed by confirmatory Western blot or immunofluorescent assay remains the standard method for diagnosing HIV infection (Chou *et al.*, 2005; Rivera *et al.*, 2010). A DNA polymerase chain reaction (PCR) and/or viral culturing are the standard detection methods in infants and young children (Rivera *et al.*, 2010). Other tests, such as tests to measure viral load or p24 antigen, detect HIV in the blood sooner after infection than tests that detect antibodies to HIV (McCutchan, 2008). Specific antibody to HIV is produced shortly after infection, but the exact time depends on several factors, including host and viral characteristics. Importantly, antibody may be present at low levels during early infection but not at the detection limit of some assays. Using the early-generation tests, antibody could be detected in most individuals by 6–12 weeks after infection. Newer-generation assays, including the third-generation antigen sandwich assays, can detect antibody at about 3–4 weeks after infection. This window period before the detection of antibody can be shortened by several days using antigen tests, and by several more days using nucleic acid detection methods. Therefore, in most individuals, the window period may be only 2–3 weeks if an all-inclusive testing strategy is used (Constantine, 2006).

Tests to detect antibody to HIV can be classified as: 1) screening assays, which are designed to detect all infected individuals, or 2) confirmatory (supplemental) assays, which are designed to identify individuals who are not infected but who have reactive screening test results. Accordingly, screening tests possess a high degree of sensitivity, whereas confirmatory assays have a high specificity. These classes of assays, performed in tandem, produce results that are highly accurate, reliable, and appropriate to protect the blood supply or assist in the diagnosis of HIV infection (Constantine, 2006).

3.1.5.1 Screening assays

Regardless of the particular screening test used, serum or plasma samples first are tested (screened) using a test with high sensitivity, most often an enzyme-linked immunosorbent assay (ELISA), “rapid test” or “simple method” (Constantine, 2006).

i. Enzyme-linked immunosorbent assays/Enzyme immunoassays (ELISA)

ELISA is the most commonly used test to screen for HIV infection because of its relatively simple methodology, inherent high sensitivity, and suitability for testing large numbers of samples, particularly in blood testing centres. A common feature of all varieties of ELISA is the use of enzyme conjugates that bind to specific HIV antibody and substrates/chromogens that produce colour in a reaction catalyzed by the bound enzyme conjugate (Constantine, 2006).

a. Indirect ELISA

The most popular ELISA involves an indirect method in which HIV antigen is attached to a well of a 96-well microtiter plate. After addition of serum suspected of containing antibodies to HIV in the well, other reagents are added which include enzyme conjugates that bind to specific HIV antibody and substrates/chromogens that produce colour in a reaction catalyzed by the bound enzyme conjugate (Constantine, 2006).

b. Competitive ELISA

Alternate ELISA methodologies include a competitive format in which specific HIV antibody in the sample competes with an enzyme-bound antibody reagent for antigen sites on the solid phase. The more antigens present in the sample, the less free antibody will be available to bind to the antigen-coated well. In this method, colour development is inversely proportional to specific HIV antibody concentration (Constantine, 2006; Goldsby *et al*., 2003).

c. Double antigen sandwich assay (DAGS)

A more recent addition to ELISA technology is the antigen sandwich method in which an enzyme (alkaline phosphatase or horseradish peroxidase) is conjugated to an HIV antigen (similar to the immobilized antigen on the solid phase). The antibody in the sample is “sandwiched” between two antigen molecules, one immobilized on the solid phase and one containing the enzyme. Subsequently, the addition of substrate results in colour development in proportion to antibody concentration. The antigen sandwich

ELISA is considered the most sensitive screening method, given its ability to detect all isotypes of antibody (including IgM). One disadvantage of this method is the relatively large volume (150µl) of sample required, which may make repeat testing and testing of samples from infants difficult (Constantine, 2006).

d. Fourth generation assays

The new generation of combination ELISAs that simultaneously detect both antigen and antibody has been developed and marketed, and offers advantages for decreasing the time, personnel, and costs necessary to perform each assay individually. These assays have demonstrated a high analytical sensitivity of detection that is most likely attributed to the combination of a third-generation format (antigen sandwich) for antibody detection and the ability to simultaneously detect HIV p24 antigen. Due to their ability to detect p24 antigen, the fourth-generation ELISAs will be of value in detecting early and established HIV infection (Constantine, 2006).

ii. Rapid tests

Rapid assays for detecting specific HIV antibody were developed in the late 1980s, and are defined as tests that can yield results in <30 minutes (Constantine, 2006). Their sensitivity is as high as 100%, but they must be followed with confirmatory Western blotting or immunofluorescence antibody testing, as with conventional HIV antibody tests (Rivera *et al.*, 2010). When performed correctly, rapid HIV assays are accurate and have wide utility in a number of testing situations. Technical errors are common with these assays because users become careless with these simple procedures. They are easy to perform and have utility in developing countries, where facilities may not be optimal, stable electricity may be unavailable, and formal education programs for laboratorians are absent (Constantine, 2006).

a. Dot blot/Immunoblot

They produce a well-circumscribed coloured dot on the solid phase surface if the test is positive. These rapid assays incorporate a built-in-control to indicate that the test was

performed correctly. This control is an anti-human immunoglobulin that binds any immunoglobulin in the sample and produces a separate indicator when all reagents are added appropriately. Some assays substitute an IgG binding dye (protein A gold reagent) for the anti-immunoglobulin conjugate, thereby decreasing the procedure by a step (Constantine, 2006).

b. Immunochromatographic (ICT) assays

The newer one-step rapid assays, also known as ICT assays, are convenient, self-contained tools for HIV serologic testing, consisting of a flat cartridge device, usually plastic or paper. Whole blood, oral fluid, or serum is placed at the tip of the device and allowed to diffuse along a strip that is impregnated with reagents (often protein A colloidal gold) that bind and permit visual detection of HIV antibodies; some use third-generation (antigen sandwich) technology. The test can be performed on whole blood, or blood collected via fingerstick (Constantine, 2006).

c. Dipsticks

Other rapid test formats include dipsticks, in which antigen is attached on the “teeth” of comb-like devices; several of these rapid tests have the ability to differentiate HIV-1 and HIV-2. Disadvantages include a subjective interpretation, difficulty in reading if the laboratorian is colour-blind, and a higher cost than that of the ELISA (Constantine, 2006).

d. Simple tests

This type of HIV test requires longer than 30 minutes for results, but consists of procedures that can be performed easily without instrumentation. Within this class of tests are agglutination assays in which antigen-coated particles (red blood cells, latex particles, or gelatin particles) are allowed to react with serum antibodies to form visible clumping (agglutination). If red blood cells are used, the technique is termed passive hemagglutination; with the use of latex particles, it is known as latex agglutination (Constantine, 2006).

3.1.5.2. Confirmatory (supplemental) assays

Most testing algorithms require the use of very specific assays, such as the Western blot, indirect fluorescent antibody (IFA) assay, or the radioimmunoprecipitation assay (RIPA), to verify reactive screening test results (Constantine, 2006).

i. Western blot test

The Western blot probably is the most widely accepted confirmatory assay (gold standard) for the detection of antibodies to the retroviruses. It is based on using an electrophoretic technique to separate HIV antigens derived from a lysate of virus grown in culture. This technique denatures the viral components, imparts a negative charge to the antigens, and separates them primarily on the basis of their molecular weights. The separation of antigens in the technique allows for the identification of specific antibodies to each of the viral antigens in a subsequent set of steps similar to the ELISA methodology (Constantine, 2006).

ii. Indirect immunofluorescent antibody (IFA) assay

In this technique, cells (usually lymphocytes) are infected with HIV and are fixed to a microscope slide. Serum containing HIV antibodies is added and reacts with the intracellular HIV. The slide is washed and allowed to react with anti-immunoglobulin antibodies with a covalently bound fluorescence label attached. The reaction is visualized using a fluorescent microscope. This technique has the advantage of providing definitive diagnosis of samples that have yielded indeterminate results by Western blot analysis (Constantine, 2006).

iii. Line immunoassay

In this assay, recombinant or synthetic peptide antigens are applied on a nitrocellulose strip, rather than electrophoresed as in the Western blot. This use of “artificial” antigens decreases the presence of contaminating substances derived from cell culture that can cause interference and sometimes false reactions. A number of reports have verified that the accuracy is equivalent to the Western blot (Constantine, 2006).

3.1.6 Treatment

Antiretroviral therapy (ART) is the mainstay in HIV treatment (Rivera *et al.*, 2010). Antiretroviral treatment has transformed AIDS from an inevitably fatal condition to a chronic, manageable disease in some settings (Simons *et al.*, 2006). Several classes of drugs are used to treat HIV infection. All of the drugs, called antiretroviral drugs, block the activity of one of the enzymes HIV needs to replicate inside human cells. These drugs include reverse transcriptase inhibitors, protease inhibitors, fusion inhibitors and integrase inhibitors. They prevent the virus from replicating (McCutchan, 2008). Antiretroviral drug combinations are more beneficial than monotherapy, and combination regimens (usually three-drug combinations) that include protease inhibitors are of greater benefit than combination regimens without these drugs (Palella *et al.*, 1998). These combinations of drugs are often referred to as highly active antiretroviral therapy (HAART). HAART can delay or prevent AIDS in HIV-infected people, thus extending their life (McCutchan, 2008). The use of HAART could decrease the spread of HIV from infected persons by decreasing viral loads (Quinn *et al.*, 2000). HAART neither cures the patient nor does it uniformly remove all symptoms but with ART many HIV-infected individuals have experienced remarkable improvements in their general health and quality of life, which has led to a large reduction in HIV-associated morbidity and mortality in the developed world (Palella *et al.*, 1998).

3.1.7 Prevention

No vaccine is currently available for preventing HIV infection. Many candidate vaccines are under development and are at different stages of testing. Vaccine development is difficult because HIV mutates rapidly, is not expressed in all cells that are infected, is not completely cleared by host immune response after primary infection and lack of an appropriate animal model. The only way to avoid epidemic spread of HIV is to avoid exposure to the virus by maintaining a healthy lifestyle that minimizes or eliminates the high risk factors (Brooks *et al.*, 2007; Hamlyn *et al.*, 2007). Abstaining from sex until marriage, being faithful to the partner and consistently and correctly using condoms are the three prevention interventions that eliminate the high risk factors

(Global Health Council, 2010). People who are likely to come into contact with blood or other body fluids at their job should wear protective latex gloves, masks, and eye shields. These universal precautions apply to body fluids from all people and not just those from people with HIV (McCutchan, 2008).

3.2 Syphilis

Syphilis is a sexually transmitted infectious disease caused by the bacterium *Treponema pallidum* (Chamberlain, 2002). The genus name, *Treponema*, is derived from the Greek term for "turning thread" (Waseem *et al.*, 2009). *T. pallidum* is a member of the order Spirochaetales, family Spirochaetaceae (spirochetes), and genus *Treponema*, which includes four human pathogens and at least six human non-pathogens. The pathogenic species are *T. pallidum subsp. pallidum* which causes venereal syphilis, *T. pallidum subsp. endemicum*, which causes endemic syphilis (bejel), *T. pallidum subsp. pertenue*, which causes yaws, and *T. carateum*, which is the etiologic agent of pinta (Larsen *et al.*, 1995). *T. pallidum subsp. endemicum* (bejel), *T. pallidum subsp. pertenue* (yaws), and *T. carateum* (pinta) can be differentiated from *T. pallidum* by the clinical manifestations of their respective diseases and, more recently, by genetic differences (Lafond *et al.*, 2006). Human is the only known natural host of *T. pallidum* (French, 2007). Syphilis is a chronic, multi-stage disease with diverse and wide-ranged manifestations and is acquired by direct contact, usually sexual, with active primary or secondary lesions. Infection also occurs when organisms cross the placenta to infect the fetus in a pregnant woman (Lafond *et al.*, 2006). Syphilis has the nickname of "great imitator" in the history of medicine because of its diverse clinical manifestations that occur in different stages of the disease (Ho, 2002). Understanding of disease pathogenesis and how host-pathogen interactions influence the course of disease have been compromised by the fact that the organism can not be grown *in vitro* (Peeling *et al.*, 2006).

In 1905, Fritz Schaudinn, Erich Hoffmann and Fred Neufeld, working in the women's ward of the Department of Dermatology at the Charite Hospital in Berlin, became the first group in the world to observe the causative agent of syphilis, *T. pallidum*. The

pathogen's etiological significance was subsequently demonstrated by Schaudinn and Hoffmann and other scientists. The detection of *T. pallidum* was the first decisive step towards the development of diagnostic and therapeutic procedures in subsequent years (Kohl *et al.*, 2005; Souza, 2005). In Europe, syphilis was epidemic in the late 15th century and its manifestations were quite severe and was called the 'great pox', 'lues venereum', and 'morbus gallicn' (Singh *et al.*, 1999).

3.2.1 Properties of *T. pallidum*

Although given various names following its discovery, the causative organism of syphilis was finally named *Treponema* because of its resemblance to a twisted thread and *pallidum* because of its pale color (Singh *et al.*, 1999). *T. pallidum* is 0.1–0.18μm in diameter and 6–20μm in length, making it invisible by light microscopy (Larsen *et al.*, 1995). It can not survive for long outside the human body (McCutchan, 2008a). It exhibits characteristic corkscrew motility due to endoflagella, with rapid rotation about the longitudinal axis and flexing, bending and snapping about the full length (Larsen *et al.*, 1995). Endoflagella are located in the periplasmic space. The spiral-shaped body of *T. pallidum* is surrounded by a cytoplasmic membrane, which is enclosed by a loosely associated outer membrane. A thin layer of peptidoglycan between the membranes provides structural stability (Lafond *et al.*, 2006).

Metabolic-pathway analysis shows that genes encoding all of the enzymes of the glycolytic pathway are present in *T. pallidum*, suggesting that it uses several carbohydrates as energy sources. The organism has previously been demonstrated to survive better in very low concentrations of oxygen and is therefore considered microaerophilic (Singh *et al.*, 1999). In proper suspending fluids and in the presence of reducing substrates, it may remain motile for 3–6 days at 25°C. In whole blood or plasma stored at 4°C, organisms remain viable for at least 24 hours, which is of potential importance in blood transfusions. Drying and elevation of the temperature to 42°C kills the spirochete rapidly. Treponemes are readily immobilized and killed by trivalent arsenical, mercury and bismuth (contained in drugs of historic interest in the treatment

of syphilis) (Brooks *et al*., 2007). *T. pallidum* is still one of the most penicillin-sensitive micro-organisms known (Lowhagen, 1990).

3.2.2 Pathogenesis and clinical features

Entry of *T. pallidum* probably occurs through areas of "microtrauma," usually in mucous membranes, and most sexual transmission of syphilis probably occurs from the genital and mucous membrane lesions of primary and secondary syphilis (French, 2007); and then there is a rapid systemic spread via the blood and lymphatics (Chamberlain, 2002). The natural history of syphilis is usually divided into early and late stages by an arbitrary time of one year. It is more infectious in the early stage of syphilis comparing with the late stage. Early syphilis is further divided into primary, secondary and early latent syphilis. Late syphilis includes late latent syphilis and different forms of tertiary syphilis namely neurosyphilis, cardiovascular syphilis and gummatous (Ho, 2002).

3.2.2.1 Primary syphilis

The lesion of primary syphilis occurs at the site of initial inoculation of *T. pallidum*. It is usually single and painless but can be multiple and painful. It tends to begin as a macule that becomes a papule, which then ulcerates. A 2–3 week incubation period usually occurs between the inoculation of T pallidum and development of the lesion (the range of incubation period is reported as being 9–90 days). Local, non-tender lymphadenopathy is often associated with this lesion. If left untreated, a lesion heals spontaneously 4 or 5 weeks later (range of healing 3–10 weeks). Because the ulcers are usually painless and can occur at sites where they are not visible (perianally or in the anal canal, vagina) or not recognized (mouth ulceration), many individuals with primary syphilis do not present to services or are not diagnosed at presentation (French, 2007)

3.2.2.2 Secondary syphilis

Four to eight weeks after primary syphilis, *T. pallidum* becomes a systemic infection with bacteraemia (French, 2007). In the general population, this stage overlaps the

primary syphilis stage in approximately one-third of patients (Singh *et al.*, 1999). This secondary stage of syphilis is characterised by a generalized and usually symmetrical macular papular rash, which is often widespread and may also involve the scalp, palms and soles in 50–80% of cases (Rompalo *et al.*, 2001; Hutchinson *et al.*, 1994). Occasionally this rash is predominantly papular, and rarely these papules ulcerate. This can be associated with generalized lymphadenopathy and mucosal ulceration. These ulcers may coalesce on the buccal mucosa, forming "snail track" ulcers, and in the genital regions (where there are opposing membranes) they can cause wart like lesions called *condyloma lata*. These features are often accompanied by constitutional symptoms such as fevers and malaise. The widespread vasculitis during secondary syphilis may lead to a broad range of syndromes such as hepatitis, iritis, nephritis, and involvement of the cranial nerves, particularly the VIII (auditory) nerve. These complications of secondary syphilis are relatively uncommon, occurring in less than 10% of individuals. Individuals with secondary syphilis, who do not have treatment, improve spontaneously over 3–6 weeks. About a quarter of patients have relapsing episodes of secondary syphilis, with recurrence of rash, mucosal ulceration, and fevers. These relapses are rare after one year and almost never occur after two years. The infection then becomes asymptomatic (latent) (French, 2007).

3.2.2.3 Latent syphilis

Latent syphilis is defined as the period after infection with *T. pallidum* in which patients are seroreactive but show no other evidence of disease (Baughn *et al.*, 2005; Ho, 2002). It is also defined as the period from disappearance of the secondary manifestations until therapeutic cure occurs or tertiary manifestations develop (Singh *et al.*, 1999; Lafond *et al.*, 2006). Latent syphilis is usually divided into early latent and late latent syphilis by an arbitrary time of one year (Ho, 2002). Early latent syphilis is the stage of disease that occurs up to one year after inoculation. A patient with early latent syphilis is considered to be infectious due to the 25% risk of relapse to secondary syphilis (Singh *et al.*, 1999; Lafond *et al.*, 2006). Late latent syphilis is also asymptomatic and is defined as infection for >1 year after inoculation. Serologic testing during the late latent stage is

positive, but sexual transmission is unlikely (Lafond *et al.*, 2006), due to lack of lesions (Zetola *et al.*, 2007a). However, organisms may seed the bloodstream intermittently during latent syphilis and can infect the developing foetus during pregnancy (Lafond *et al.*, 2006). This possibility of vertical transmission is the reason for routine syphilis screening of all pregnant women. Although cure without treatment is questioned, many patients will remain in this latent phase indefinitely (Zetola *et al.*, 2007a).

3.2.2.4 Tertiary/Late syphilis

Today, when coincidental antibiotic therapy is common, late manifestations of syphilis are rarely seen (Lafond *et al.*, 2006). Approximately 15–40% of individuals who are not treated or with late latent syphilis will develop the late manifestations of syphilis (tertiary syphilis) with men at increased risk compared with women (Singh *et al.*, 1999; CDC, 2007). The three main manifestations of late syphilis are: neurosyphilis, cardiovascular syphilis and gummatous syphilis (French, 2007).

i. Neurosyphilis

As well as being a manifestation of secondary syphilis, meningovascular syphilis can also occur in tertiary syphilis. The incubation period is usually 5–12 years, and its symptoms are similar to those of early meningo-vascular syphilis (French, 2007). Parenchymatous neurosyphilis results when chronic meningoencephalitis causes destruction of cortical parenchyma (McCutchan, 2008a). It is the involvement of spinal cord (dorsal columns) and brain by syphilis. The incubation period of this is usually 10–20 years. The spinal cord syndrome is called tabes dorsalis, and the brain syndrome is called general paresis of the insane (French, 2007). Initial symptoms may include headache, neck stiffness, dizziness, behavioral abnormalities, poor concentration, memory loss, lassitude, insomnia, and blurred vision (McCutchan, 2008a).

ii. Cardiovascular syphilis

Cardiovascular syphilis, usually seen as aortic insufficiency or aneurysm, is observed in 10% of untreated patients (Lafond *et al.*, 2006). Cardiovascular syphilis usually occurs

15–30 years after primary syphilis and may occur in any large vessel. It is characterised by an aortitis usually affecting the proximal aorta. It may cause aortic incompetence (which may be complicated by heart failure), coronary ostial stenosis (presenting as angina), and aortic medial necrosis causing aortic aneurysm (French, 2007). Symptoms include brassy cough, obstruction of breathing due to pressure on the trachea, hoarseness due to vocal cord paralysis resulting from compression of the left laryngeal nerve, and painful erosion of the sternum and ribs or spine (McCutchan, 2008a).

iii. Gummatous syphilis

Progressive inflammation caused gumma (late benign syphilis), a localized form of tissue and bone destruction, in 15% of patients with untreated syphilis (Lafond *et al.*, 2006). Gummatous syphilis is often referred to as benign late syphilis because these lesions rarely cause total physical incapacity or death, but when the lesions occur in organs such as the brain or heart, serious complications may occur; including ulcers of the skin, collapse of the palate or nasal septum, or organomegaly (Singh *et al.*, 1999; Lafond *et al.*, 2006). These granulomatous lesions usually occur 3–12 years after primary syphilis (French, 2007). Some have reported that gumma develops from 1–46 years after healing of secondary lesions, with the majority developing by the end of the 15th year (Singh *et al.*, 1999). They can occur in almost any tissue but most commonly present when they affect skin/bone (French, 2007). They may also occur in the liver, heart, brain, stomach, and upper respiratory tract (Lafond *et al.*, 2006).

3.2.2.4 Congenital syphilis

Pregnant women with syphilis can transmit the infection to the fetus. Transmission is usually transplacental and is particularly likely during the first two years of infection. About a third of babies born to mothers with early syphilis are born without infection and a third with congenital syphilis; a third of pregnancies will result in miscarriage or stillbirth. The prognosis is particularly poor if symptoms of syphilis are present in the first few weeks after birth (French, 2007). Congenital syphilis has been divided into two clinical syndromes early and late congenital syphilis. Early congenital syphilis refers to

those clinical manifestations that appear within two years of life. Those features that occur after two years, and usually manifest near puberty constitute late congenital syphilis (Genc *et al.*, 2000). Antibiotic treatment of the mother during the first two trimesters is usually sufficient to prevent negative outcomes, but later treatment or lack of treatment may result in complications and results in high risk pregnancy (Lafond, *et al.* 2006). Untreated early syphilis results in death of the fetus in up to 40% of those pregnancies (NIAID, 2009). "Snuffles" or persistent rhinitis is one of the earliest clinical manifestations, occurring in 4–22% of infants (up to 50%). The nasal discharge may be profuse and purulent or blood tinged and is highly infectious. Late manifestations of congenital syphilis include Hutchinson's triad of interstitial keratitis, peg-shaped upper incisors, and eighth-cranial-nerve deafness (Singh *et al.*, 1999).

3.2.3 Epidemiology

3.2.3.1 The global burden

STDs are hyper-endemic in many developing countries. In industrialized countries, the bacterial STD (syphilis, gonorrhoea, chancroid) declined from the peak during the second world war till up to late 50s, then increased during the 60s and early 70s, and they have been decreasing again from the late 70s till the present. Infection rates are similar in both women and men, but women and infants bear the major burden of complications and serious sequelae (DeSchryver *et al.*, 1990). Globally, there are more than 900,000 cases of pregnant women infected with syphilis every year, resulting in 360,000 fetal or perinatal deaths and in the birth of 270,000 infants with serious or permanent impairment (CDC, 1999). A WHO report published in 2001 provides estimates of the extent of the world's STD epidemics as they were in 1999 (previous reports were published in 1990 and 1995). As of early 2007, there are no more recent international estimates. The WHO estimates that 340 million new cases of syphilis, gonorrhea, chlamydia and trichomoniasis occurred throughout the world in 1999 in men and women aged 15–49 years. The largest number of new infections occurred in the region of South and SEA, followed by sub-Saharan Africa and Latin America, and the Caribbean. The highest rate of new cases per 1,000 population occurred in sub-Saharan

Africa. An estimated 11.76 million cases of syphilis occurred worldwide in 1999 - slightly below the 1995 estimate (12.22). The greatest number of cases was estimated to have occurred in South and SEA, with 5.8 million cases, while a further 3.5 million cases occurred in sub-Saharan Africa (WHO, 2001).

The burden of syphilis varies widely according to individual variations and the development status of nations (e.g., rates among sex workers in parts of Africa are as high as 47%). Seroprevalence in prenatal clinics in Africa is estimated at 4–15% (Singh *et al.*, 1999). Referring to sub-Saharan Africa in particular, prevalence varies from country to country. It is 2.3% in Zimbabwe, 6% in Tanzania, 9% in Ethiopia, 12% in Mozambique and 1.6% in South Africa (Patel *et al.*, 2008). With syphilis seroprevalence estimated at 8.3% in sub-Saharan Africa, approximately 1.6 million pregnant women with syphilis remain undiagnosed, including >1 million attending antenatal care (Gloyd *et al.*, 2001). Studies of pregnant women in Africa have revealed rates of 17.4% in Cameroon, 8.4% in South Africa, 6.7% in Central African Republic and 2.5% in Burkina Faso (WHO, 2001). Researches in four cities in sub-Saharan Africa showed the prevalence of syphilis to be >10% in Zambia; 6% in Cameroon, 3–4% in Kenya and 1–2% in Benin (Buve *et al.*, 2001). Prevalence of syphilis in rural Ugandan population varies from 11–13% (Wagner *et al.*, 1994; Kamali *et al.*, 1999).

Serological surveys in India have revealed high seroprevalence rates ranging from 9.07% among high risk STI patients in Himachal Pradesh to 21.9% in long distance truck drivers in central India (Reynolds *et al.*, 2006). Different studies conducted in India had revealed a syphilis seroprevalence rate of 0.5–4.5% in blood donors (Ekadashi, 2008; Bhattacharya *et al.*, 2007; Kothari *et al.*, 2002), 1–6% in IDUs (Baveja, 2003; Jindal *et al.*, 2007) and 22.7% in sex workers (Thakor *et al.*, 2004). Different studies conducted in China had revealed a syphilis rate of 19.8% in MSM (Ruan *et al.*, 2009), 15.7% in FSWs (Ruan *et al.*, 2006) and 5.4% among IDUs (Jia *et al.*, 2008). A study carried out among MSWs in Thailand revealed the syphilis prevalence to be 7.6% (Kunawararak *et al.*, 1995).

A study conducted in Central America showed syphilis prevalence in the range of 3–15% among MSM and 2–25% among FSWs (Nunez *et al.*, 2009). A study conducted among Jamaican population showed syphilis prevalence to be 2.7% in general adult population, 4.7% in pregnant women and 2.2% in children (Smikle *et al.*, 1990). A study conducted in Mexico revealed syphilis seroprevalence rate of 6.4% among FSWs (Hernandez *et al.*, 1998). Studies conducted in Brazil revealed syphilis rate of 24.4% among patients attending the AIDS Reference Center (Morimoto *et al.*, 2005) and 18% among the prisoners (Massad *et al.*, 1999).

The table below shows that increases in prevalence (1995–1999) was seen in Eastern Europe and Central Asia, sub-Saharan Africa, Latin America and the Caribbean.

Table 3: Estimated new cases of syphilis (in millions) among adults (WHO, 2001)

Region	1995			1999		
	Male	Female	Total	Male	Female	Total
North America	0.07	0.07	0.14	0.054	0.053	0.107
Western Europe	0.10	0.10	0.20	0.069	0.066	0.136
North Africa and Middle East	0.28	0.33	0.62	0.167	0.197	0.364
Eastern Europe and Central Asia	0.05	0.05	0.10	0.053	0.052	0.105
Sub-Saharan Africa	1.56	1.97	3.53	1.683	2.144	3.828
South and Southeast Asia	2.66	3.13	5.79	1.851	2.187	4.038
East Asia and Pacific	0.26	0.30	0.56	0.112	0.132	0.244
Australia and New Zealand	0.01	0.01	0.01	0.004	0.004	0.008
Latin America and Caribbean	0.56	0.70	1.26	1.294	1.634	2.928
Total	5.55	6.67	12.22	5.29	6.47	11.76

3.2.3.2 Prevalence in Nepal

Syphilis seroprevalence in Nepal has been studied mostly among the high risk groups as very scarce information has been published on the prevalence among the healthy general population. A retrospective data analysis for seroprevalence of antibodies to

syphilis was carried out during the period 2003/04 among healthy Nepalese males. The study showed the prevalence of syphilis to be 0.6%. The STD prevalence rate in women was 4.7% and its prevalence in Kathmandu was 1–5% in 1998 (Joshi *et al.*, 2003). A multi-centre, cross-sectional study of healthy males from different development regions of Nepal showed the prevalence rate for syphilis to be 0.85%; the occurrence of syphilis being highest (1.39%) in mid-western development region and least (0.58%) in eastern development region (Shrestha, 2008). A descriptive cross sectional study conducted among blood donors in Central Blood Transfusion Service (CBTS) in Nepal showed the overall seroprevalence syphilis to be 0.42%; 0.45% in male and 0.24% in female (Karki *et al.*, 2008). The seropositivity of syphilis among blood donors in Bhairahava, western Nepal, has been reported to be 0.39% (Chander *et al.*, 2003). Syphilis infection was documented 20.4% among sex trafficked women and girls in Nepal (Silverman *et al.*, 2008). A recent study conducted among MSM in Kathmandu city showed a prevalence rate of 2.1% for syphilis (Acharya *et al.*, 2006). A study conducted among 341 FSWs in Kathmandu Valley revealed syphilis seroprevalence of 28%. Gynecological examination based on symptoms revealed that 72% of the FSWs were infected with some type of STD. The prevalence of syphilis was the most common (Bhatta *et al.*, 1994). A study carried out among FSWs and truckers in the Terai region in March-July 1999 revealed nearly half of the sex workers (47.3%) had at least one STD. Nearly 20% had syphilis and 7% had a history of syphilis. About 10% of the truckers were infected with at least one STD, and 5% were infected with syphilis (New ERA/SACTS, 2000). A study conducted among FSWs in the Kathmandu Valley in March-August 2001 showed a prevalence of active syphilis to be 14.3% among SBSWs and 3.5% among NSBSWs which was almost four times higher in SBSWs as compared to NSBSWs (SACTS, 2001). 4% of FSWs in Pokhara were infected with syphilis in 2004 in a study conducted by New ERA and SACTS (New ERA/SACTS, 2005). A study done among sexually active persons in six sentinel sites in Nepal showed the prevalence of active syphilis to be 4.02% in male and 2.8% in female patients (NCASC, 2001). The active syphilis rate had dropped to 9.5% in 2003 from 18.8% in 1999 in Nepal (USAID, 2005).

3.2.4 Modes of transmission

Syphilis is a systemic disease which can be spread by sexual contact, via vertical transmission and blood transfusion (Murray *et al.*, 2002).

3.2.4.1 Sexual transmission

Syphilis is a sexually transmitted infection, and the more sexual partners that individuals have, the more likely they are to acquire syphilis. Syphilis is passed from person to person through direct contact with syphilis sore. Sores occur mainly on the external genitals, vagina, anus, or in the rectum. Sores also can occur on the lips and in the mouth. Transmission of the organism occurs during vaginal, anal, or oral sex (CDC, 2007). For a single, unprotected sexual contact, the risk of transmission of syphilis is 30–60% (WHO, 2007; Larsen *et al.*, 1995).

3.2.4.2 Vertical transmission

The syphilis bacterium can infect the baby of a woman during her pregnancy. Depending on how long a pregnant woman has been infected, she may have a high risk of having a stillbirth or of giving birth to a baby who dies shortly after birth (CDC, 2007). The mother can transmit the infection transplacentally to the fetus by contact of the newborn with a genital lesion. Breast feeding does not result in syphilis transmission, unless an infectious lesion is present on the breast (Genc *et al.*, 2000). Vertical transmission of early syphilis during pregnancy results in a congenital infection in at least 50–80% of exposed neonates (Waseem *et al.*, 2009). More than one million infants are born with congenital syphilis each year (French, 2007).

3.2.4.3 Blood transfusion

The transmission of syphilis can also occur by blood transfusion, for this reason the WHO and the Food and Drug Administration recommended the screening of the disease in all donations of blood (Salvador *et al.*, 2004). The risk of transmission through blood is though negligible due to improved donor selection, uniform serologic testing of all blood donors and a shift from transfusion of fresh blood to transfusion of refrigerated

blood components (Singh *et al.*, 1999). Transmission via blood products is theoretically possible since organisms may survive for at least 24 hours in whole blood/plasma stored at 4°C (Brooks *et al.*, 2007), but storing blood for 5 days assures safety (Richens, 1992).

3.2.5 Laboratory diagnosis of syphilis

The diagnostic tests of syphilis are broadly divided into three categories: i) direct microscopic examination, ii) indirect non-treponemal (screening) and treponemal (confirmatory) serologic tests, and iii) direct antigen detection tests (molecular biology-based method) currently used in research settings and as gold standards for test evaluation (Ho, 2002; Larsen *et al.*, 1995).

3.2.5.1 Direct microscopic examination

When lesions are present, the most specific and easiest means of diagnosing syphilis is by direct detection of the organism. A positive result on microscopic examination is definitive evidence of syphilis if infection with other pathogenic treponemes can be excluded (Larsen *et al.*, 1995). Dark-field examinations and direct fluorescent antibody (DFA) tests are the definitive methods for diagnosing early syphilis (CDC, 2006).

i. Dark-field microscopy

Since *T. pallidum* can not be cultured in the laboratory, dark-field microscopy is used to identify the treponemes (seen as a motile spirochaete in a saline solution) from samples taken from the lesions of primary and secondary syphilis which allows the immediate diagnosis of syphilis (Peeling *et al.*, 2004; French, 2007). Because the width of *T. pallidum* are too narrow and too slender to be seen under the ordinary microscopy, dark-field examination is the most productive during primary, secondary, infectious relapsing and early congenital syphilis when moist lesions containing large numbers of treponemes (e.g., chancres, condylomata latum, or mucous patches) are present (Larsen *et al.*, 1995; Ho, 2002). It requires special equipment and training, and is not suitable for oral or rectal samples because of the potential presence of non-pathogenic spirochetes in those sites (Zetola *et al.*, 2007a).

ii. Direct fluorescent antibody (DFA) test

In mid-1960s, the direct fluorescent antibody (DFA) test for *T. pallidum* had been developed. The results can be read under the fluorescence microscope equipped with a dark-field condenser (Ho, 2002).The test detects and differentiates pathogenic treponemes from non-pathogenic treponemes by an antigen-antibody reaction; thus, the organism is not required to be motile and viable in the DFA-TP. Because the conjugates used are specific for pathogenic strains of *Treponema* spp., the DFA-TP is applicable to samples collected from oral or rectal or intestinal lesions. However the test cannot distinguish between the pathogenic strains of *Treponema* spp (Larsen *et al.*, 1995).

3.2.5.2 Serological tests

Serological testing remains the mainstay of laboratory diagnosis for primary, secondary, latent, and tertiary syphilis (Larsen *et al.*, 1995; Nadarajah, 1990). The basic principle of serologic test for syphilis is to measure or demonstrate either the specific or non-specific anti-treponemal antibody, hence inferring that the examined patient has been exposed to *T. pallidum*. The first demonstrable humoral immunologic response is the production of specific anti-treponemal IgM at the end of second week, and IgG at about 4 weeks after exposure to *T. pallidum.* Serological tests for syphilis are divided into two categories: i) non-treponemal (anti-lipoid antibody detection) and ii) treponemal (anti-*T. pallidum* antibody detection) serologic tests. They are further divided into different tests based upon different methodology (Ho, 2002).

i. Non-treponemal tests

The standard status non-treponemal tests can be used as qualitative tests for initial screening or as quantitative testes to follow treatment (Peeling *et al.*, 2004). In non-treponemal tests, the antibodies to be measured are non-specific treponemal antibodies. It is based upon the reactivity, both IgM and IgG, of sera from patients with syphilis to non-specific cardiolipin-cholesterol-lecithin antigens (Ho, 2002). The non-treponemal tests measure IgM and IgG antibodies to lipoidal material released from damaged host cells as well as to lipoprotein-like material and possibly by cardiolipin released from the

treponemes. In the qualitative tests, undiluted patient's serum is used simply to measure the presence or absence of antibody. In the quantitative tests, serial two-fold dilutions are made, and the serum is diluted until an end point is reached. Quantitative reactions are reported in terms of the highest dilution in which the specimen is fully reactive. Non-treponemal tests used for screening have the advantage of being widely available, inexpensive, convenient to perform on large numbers of specimens, and necessary for determining the efficacy of treatment (Horn, 2002). Limitations of the non-treponemal serologic tests include their lack of sensitivity in early dark-field-positive primary cases and in late syphilis and the possibility of a prozone reaction or false-positive results. Without some other evidence for the diagnosis of syphilis, a reactive non-treponemal test does not confirm *T. pallidum* infection as they are not very specific for syphilis. Therefore, when the non-treponemal tests are used as screening tests, all reactive results should be confirmed with a treponemal test (Larsen *et al.*, 1995). The most commonly used non-treponemal tests are: a) Venereal disease research laboratory (VDRL) test and b) Rapid plasma reagin (RPR) test (Zetola *et al*., 2007a).

a. Venereal disease research laboratory (VDRL) test

The test introduced in the year 1946, is performed by mixing heat–inactivate patient's serum with a freshly prepared suspension of cardiolipin-lecithin-cholesterol antigen and reading the resulting flocculation microscopically with a low-power objective (Young, 2006). The VDRL test continues to be used in some settings, although it has no advantages over the RPR for diagnosis of syphilis (LaFond *et al.*, 2006). The test is reactive in 78% of patients with primary syphilis. In late syphilis, about one fourth of untreated patients have negative VDRL results. The sensitivity of the test is 100% during secondary syphilis (Larsen *et al.*, 1995).

b. Rapid plasma reagin (RPR) test

The test introduced in the year 1957, uses cardiolipin antigen with choline chloride (to block inhibitors in serum, eliminate the need for heat-inactivation and allow testing of plasma), EDTA (to stabilize the antigen and allow it to be used for up to 6 months when

stored at 4–10°C, and finely divided carbon particles (to enable the result to be read by eye) (Young, 2006). The test is reactive in 86% of patients with primary syphilis (Larsen *et al.*, 1995). Like VDRL, the test is most sensitive, almost 100%, during the middle stages of syphilis. It is less sensitive during the earlier and later stages of the disease. After treatment with antibiotics, the levels of syphilis antibodies should fall which can be monitored with another RPR test. Unchanged or rising levels can mean a persistent infection (Levin, 2007). It is more sensitive, simpler and less time consuming for screening purposes than VDRL test (Sharma *et al.*, 1992). Automation, antigen stability, the ability to use plasma rather than serum and macroscopic observation makes the test more conducive for use in clinical laboratories (Lafond *et al.*, 2006).

ii. Treponemal tests

Treponemal tests, which detect anti-treponemal antibodies qualitatively, are more complex and use *T. pallidum* as the antigen and are based on the detection of specific antibodies directed against treponemal components. Treponemal tests are used primarily to verify reactivity in the non-treponemal tests. The treponemal tests also may be used to confirm a clinical impression of syphilis in which the non-treponemal test is non-reactive but there is evidence of syphilis, as in late syphilis. Unfortunately, treponemal tests are technically more difficult and costly to perform than non-treponemal tests and cannot be used to monitor treatment (Larsen *et al.*, 1995). Treponemal tests are further divided by different methodology such as: a) Fluorescent treponemal antibody absorption (FTA-ABS), b) *T. pallidum* haemagglutination assay (TPHA), and c) Enzyme immunoassay (EIA) (Ho, 2002).

a. Fluorescent treponemal antibody absorption (FTA-ABS)

The FTA-ABS test is an observer dependent technique to detect indirectly the presence of anti-treponemal antibodies on microscopic slide pre-fixed with *T. pallidum* antigen by a fluorescence microscope. Because sorbent is added to absorb the non-specific antibodies to enhance the specificity, the word absorption is added after FTA test to differentiate this test from less specific fluorescent treponemal antibody test (Ho, 2002).

b. *T. pallidum* haemagglutination assay (TPHA)

The TPHA test, developed by Rathlev and Tomizawa in 1967, has high sensitivity and specificity, but is not sensitive enough for the diagnosis of primary syphilis (Matsumoto *et al.*, 1993). In TPHA, purified *T. pallidum* antigens are attached to Turkey RBCs in order to detect the specific anti-treponemal antibodies in patient's serum. A reactive result is defined as passive agglutination of red cells (Ho, 2002). The patient's serum is screened at an initial dilution of 1:80 (Gillespie, 1994). If particle is used instead of red cell, it is called *Treponema pallidum* Particle Agglutination (TPPA) test (Ho, 2002).

c. Enzyme immunoassay (EIA)

EIA uses reaction indicators that employ enzyme acting on an antigen-antibody complex to produce measurable chromogenic end products which can be read by a spectrophotometric device. The antigen used in EIA can either be cardiolipin, purified treponemal antigen or recombinant treponemal antigen. Purified treponemal antigen and recombinant treponemal antigen are used in most of the commercially available EIA tests. Advantages of EIA serologic test are more objective, less labour intensive and automated (Ho, 2002).

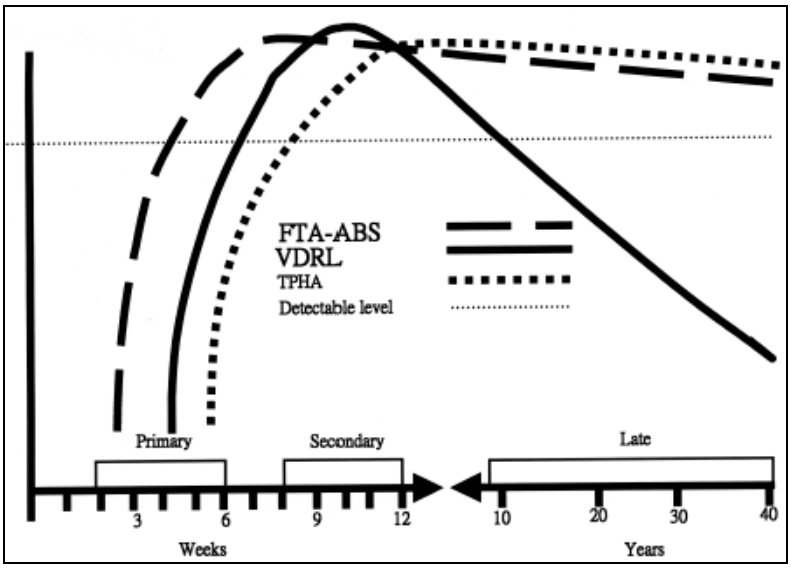

Figure 1: The relationship between levels of syphilis antibodies vs. time (Ho, 2002)

3.2.5.3 Molecular biology based method

The shortcomings of the standard tests for syphilis for the diagnosis of early primary, congenital, and neurosyphilis, as discussed above, have made techniques based on the detection of treponemal DNA or antigens very appealing. By far, DNA probes and PCR are the most commonly used molecular-based methods to detect treponemal DNA or antigen (Larsen *et al.*, 1995; Ho, 2002) but are not readily available for routine clinical use (Singh *et al.*, 1999). PCR could be extremely valuable in diagnosing infection in congenital syphilis (passively transferred antibodies now confuse the diagnosis), in diagnosing neurosyphilis (the only serologic test is only 50% sensitive), in diagnosing early primary syphilis (the only tests available currently are microscopic), and finally, in distinguishing new infections from old infections (now only a rise in titre can be used) (Larsen *et al*., 1995).

3.2.6 Treatment

Intramuscular Benzathine Penicillin G 2.4 mega-units either as a single dose or weekly in two to three doses is the mainstay of treatment of syphilis in developing countries (Goh, 2005), and has been used effectively for more than 50 years, with preserved sensitivity patterns and the best track record of efficacy (Kassuto *et al.*, 2003), to achieve clinical resolution, whether the resolution of symptoms or the prevention of sexual transmission, and to prevent late stage manifestations (Horn, 2002). The preparation(s) used, the dosage and the length of treatment depend on the stage and clinical manifestations of the disease (CDC, 2006). In patients allergic to penicillin (approximately 10%), oral doxycycline 100 mg twice daily for 2 weeks is given or tetracycline 500 mg four times daily for 2 weeks or azithromycin 500 mg daily for 1 week (Goh, 2005). Patients should be warned of the Jarisch-Herxheimer (JH) reaction occurring in 70–90% of patients that causes a flu-like illness characterized by low-grade fever (a rise in temperature to 102°F), myalgias, headache, tachycardia, hyperventilation, vasodilation with flushing, mild hypotension and malaise that begin within a few hours after initiation of therapy and last for 12–24 hours (Dylewski *et al.*, 2007; Waseem *et al.*, 2009; Singh *et al.*, 1999). JH reactions can be serious in patients

with neurosyphilis, oculosyphilis and cardiovascular syphilis and may be ameliorated by prednisolone 10–20 mg three times a day for 3 days starting 24 hours before giving anti-treponemal treatment (Goh, 2005). The cure of syphilis depends not only on the antibiotic effect but also on the integrity of the immune capacity of the host (Singh *et al.*, 1999). Once syphilis has been diagnosed the response to treatment is monitored by the decline of non-treponemal titre as clinical signs and symptoms may subside spontaneously even without treatment. It is important that a baseline non-treponemal serologic test must be done quantitatively before treatment and followed by the same testing method such as VDRL or RPR to monitor the treatment response (Ho, 2002).

3.2.7 Prevention

A five-point syphilis control plan including public education, screening, clinical treatment, partner notification, and prophylactic treatment has been defined. This framework still forms the basis of primary prevention of syphilis control today. Educating the general public about the consequences and prevention of syphilis and other STDs is paramount in the primary prevention of these diseases. Mass treatment of populations with a high prevalence of infection has also been effective (Singh *et al.*, 1999). Syphilis is likely to remain a common disease worldwide, and some awareness of its prevention, presentation, diagnosis, and treatment is important for all clinicians. Many of the tools for effective syphilis control (such as antenatal screening to prevent congenital syphilis) are already well established but have not been fully implemented in many parts of the world. Comprehensive sexual health promotion programs have been shown to reduce syphilis prevalence. Primary prevention, together with provision of easily accessible syphilis diagnostic and treatment services, will remain the cornerstone of syphilis control (French, 2007). Clinicians are key participants in syphilis control, because they must educate patients, counsel them in sexual risk reduction, and routinely and frequently screen those at increased risk (Zetola *et al.*, 2007b; Chamberlain, 2002). By identifying populations affected by syphilis, clinical services can be made more accessible, key determinants of continued transmission can be identified, and targeted interventions can be developed (Kilmarx *et al.*, 1995).

3.3 HIV/syphilis co-infection

People living with HIV and AIDS commonly have other infections. This is a result of the shared transmission mode of these infections with HIV or as a result of immuno-suppression. These co-infections not only complicate management of patients with HIV but also contribute to increased HIV transmission rates (WHO, 2007). Syphilis has been associated epidemiologically with acquisition and transmission of infection with HIV (Stamm *et al.*, 1988; Wasserheit, 1992; Greenblatt *et al.*, 1988). Syphilis has been estimated to increase HIV transmission 2–9 fold and HIV acquisition 2–4 fold (Chesson *et al.*, 2003). There is now good evidence that syphilis and HIV act synergistically with regard to both transmission and progression of both diseases (Kofoed *et al.*, 2006; Zetola *et al.*, 2007b, Lynn *et al.*, 2004). Syphilis can mimic HIV infection and vice versa chancre versus chronic mucocutaneous ano-genital herpes in AIDS, secondary syphilis versus primary HIV infection, neurosyphilis versus neurological complications of HIV infection (Rompalo *et al.*, 2001). In fact, HIV infection, like syphilis, is a protean disease that has a number of clinical presentations and they interact with each other in different ways from serology to clinical presentation (Ho, 2002). Previously having syphilis is a risk marker for HIV infection (Quinn *et al.*, 1990), laboratory tests for syphilis may be modified in persons with HIV infection (Hutchinson *et al.*, 1991; Rompalo *et al.*, 1992); currently recommended therapy for syphilis may be less effective for persons with HIV infection (Johns *et al.*, 1987; Hall *et al.*, 2006).

3.3.1 Pathogenesis in HIV/syphilis co-infected patients

There is strong epidemiological evidence suggesting that a history of past syphilis increases the risk of AIDS (Funnye *et al.*, 2003) and that HIV alters the natural history of syphilis as it does for some STDs (Fleming *et al.*, 1999; Kassuto *et al.*, 2003). While the strong correlation between syphilis and HIV infections may be largely due to their mutual association with high-risk behaviour, there is also the possibility that a syphilitic chancre may provide a superior opportunity for the spread of HIV (Quinn *et al.*, 1990). Both primary syphilis and other ulcerative genital lesions that increase the risk of HIV infection, disrupt the epithelial or mucosal surfaces, which provide a portal of entry for

the virus to enter the systemic circulation (Lafond *et al.*, 2006; Reynolds *et al.*, 2006). This may explain why anal and other traumatic sexual practices may enhance HIV acquisition. Disruption of the genital mucosa is associated with the recruitment of inflammatory cells such as CD4+ T-lymphocytes and macrophages (Blocker *et al.*, 2000; Wasserheit, 1992), the primary targets of HIV, which are found in abundance in syphilitic lesions (Lafond *et al.*, 2006). Presence of these cells can facilitate transmission of HIV virions from HIV infected persons to uninfected persons or provide additional targets for HIV entry in HIV negative persons who are being exposed to the virus (Blocker *et al.*, 2000). The chancres and ulcerative lesions, thus, increase the acquisition of HIV by providing target cells for potential infection; and increase HIV transmission by facilitating transfer of cell rich fluid or blood (Funnye *et al.*, 2003).

The severity of syphilis in HIV infected patients probably varies widely depending on host immunity (Kassuto *et al.*, 2003). The clinical presentation of syphilis in HIV positive patients is commonly the same as in HIV negative patients, however, unusual and atypical features may be seen. Accelerated progression through the syphilitic stages may occur. This progression may be related to the level of immuno-suppression. The primary stage of syphilis may consist of larger, deeper and multiple or more extensive chancres (up to 70% of patients) in the HIV patient that take longer to heal (Rompalo *et al.*, 2001; Schofer *et al.*, 1996). The course of syphilis may be more rapid in HIV infected patients and are more likely to present with secondary syphilis (Hall *et al.*, 2006) and those with secondary syphilis are more likely to have persistent chancres (Hutchinson *et al.*, 1994). The rash of secondary syphilis can mimic many dermatologic conditions, such as tinea versicolor, pityriasis rosea, scabies, fixed drug eruptions, and erythema multiforme; in HIV-infected individuals taking ART, it has been misdiagnosed as an antiretroviral drug reaction (Hall *et al.*, 2006). The signs and symptoms of secondary syphilis are all common findings in HIV infected patients with/without opportunistic infections. The altered immune responses of HIV infected patients may lead to atypical clinical presentations of secondary syphilis (Funnye *et al.*, 2003). Patients with HIV infection who acquire syphilis may be more likely to progress

to clinical neurosyphilis than those without HIV infection (Musher *et al.*, 1990; Hall *et al.*, 2006; Zellan *et al.*, 2004) that include early pathologies such as acute syphilitic meningitis and late pathologies such as CNS gummatous lesions (Kassuto *et al.*, 2003). Alteration in the natural course of syphilis by HIV is due to the profound defects in cell mediated immunity. In patients with early syphilis and HIV infection, a synergistic immunodeficiency state may be due to a transient treponemal induced immuno-suppression. Alternatively, meningeal inflammation induced by either pathogen could allow the other agent to penetrate further into the central nervous system (Hall *et al.*, 2006). Approximately one-third of patients with early syphilis have invasion of treponemes in the CSF, regardless of their HIV status (Schofer *et al.*, 2006; Rolfs *et al.*, 1997). It is important to recognize that central nervous system disease can occur during any stage of syphilis in HIV (Horn, 2002). The latency period before the development of meningo-vascular syphilis may be shorter among the co-infected patients (Hall *et al.*, 2006). HIV-positive immuno-suppressed patients progresses to tertiary manifestations more rapidly (Rolfs *et al.*, 1997) and there is an increased frequency of ophthalmic disease e.g., uveitis, keratitis, optic neuritis, conjunctivitis, optic atrophy, or chorioretinitis disease (Kassuto *et al.*, 2003; Lynn *et al.*, 2004; Karumudi *et al.*, 2005).

3.3.2 Epidemiology of HIV/syphilis co-infection

In the 1980s, studies showed that 70% of serum specimens from homosexual men with AIDS reacted in treponemal tests for syphilis (Rogers *et al.*, 1983). More studies in the heterosexual population indicate that approximately 60% of HIV-infected females had reactive syphilis serologic test results (Castro *et al.*, 1988). The studies revealed a high rate of HIV/syphilis co-infection. In general, 15% of adolescents and adults with syphilis are co-infected with HIV (Waseem *et al.*, 2009). In the U.S., surveillance data is available for syphilis and HIV separately, and few are available on the co-infection rate (Lopez-Zetina *et al.*, 2000). A survey conducted among patients attending STD clinics in U.S. revealed 24.3% of the patients with syphilis were positive for the AIDS virus, in contrast with 3.5% of the patients who did not have syphilis (Quinn *et al.*, 1990). An epidemic of HIV infections among college students who are primarily MSM

have been reported from North Carolina, a state with one of the highest syphilis rates in the south-eastern United States. During the 6-year period, (2000–2005) there were 1460 (20.8%) HIV positive men aged 18–30 years reported in North Carolina; 111 (7.6%) were co-infected with syphilis; 90 (6.2%) were co-infected with early syphilis. Data available for 551 (25.7%) HIV-positive women diagnosed from 2002–2005; only 13 (2.4%) were co-infected with syphilis; 6 (1.1%) were co-infected with early syphilis (Sena *et al.,* 2008). A literature review including 30 studies on HIV prevalence among patients with primary diagnosis of syphilis had shown a mean co-infection seroprevalence of 15.7% in the U.S. seroprevalence among men was 27.5% and 12.4% among women. Seroprevalence in MSM ranged from 64.3–90% and from 22.5–70.6% in IDUs (Blocker *et al.,* 2000). A study conducted in U.S. revealed 13.4% of the patients visiting public health clinics had positive STD test results. Among distinct STDs, syphilis was the least frequent (7.5%), but was reported in the highest proportion (10.1%) of all new HIV infections and conferred the greatest risk for newly diagnosed HIV (Huhn *et al.,* 2008). A study was conducted in a hospital in U.S. among patients with suspected sexually transmitted diseases. The study revealed syphilis seroprevalence in those who were HIV positive was 31.7%, as compared to 5.9% in those who were HIV negative (Ansell *et al.*, 1994). A similar study conducted among HIV positive individuals in U.S. revealed 8.3% in the cohort having syphilis (Malone *et al.*, 1995). A study conducted in Cuban HIV/AIDS female patients revealed a syphilis seroprevalence rate of 20.3%. In the study 67 women were used as controls (i.e. negative to the virus). Only 11.9% females showed reactivity to the reaginic antibodies. The results showed a close association between syphilis and HIV/AIDS and that both diseases may coexist in a same patient (Rodriguez *et al.*, 2004). An investigation carried out in 181 female prostitutes in Honduras revealed the prevalence of HIV/syphilis co-infection to be 2.2% (Venegas *et al.,* 1991). A study on the prevalence of HIV/syphilis co-infection among 830 HIV/AIDS patients at a hospital in Rio de Janeiro revealed 2.7% of the patients being co-infected by syphilis. The ratio between men and women with co-infection was approximately 4:1 (Signorini *et al.,* 2007). A cross-sectional study of STD outpatients conducted at four major city hospitals in Argentina from July

to October 2002 showed an HIV/syphilis co-infection rate of 59.7%. The prevalence of HIV/syphilis co-infection was significantly higher in men (13.6%) as compared to 1.7% in women (Grimberg *et al.*, 2006).

HIV prevalence trends and STDs among HIV-positive MSM in Western Europe were reviewed in an article which showed HIV prevalence among MSM diagnosed with syphilis in 11 countries to be in the range of 14–59% (Dougan *et al.*, 2007). In a study conducted among HIV infected patients in 13 dermatological and medical centres throughout Germany revealed 1.33% being co-infected by syphilis. Most of the syphilis patients were male (93.4%); females being only 6.6% (Schofer *et al.*, 1996). In a study conducted among HIV patients in Spain revealed 7.1% being affected by syphilis on the basis of MHA-TP test. The positivity of MHA-TP was more frequent among those presenting criteria of the AIDS at the diagnosis; 18% versus 5.6% (Pulido Ortega *et al.*, 1993). A similar research carried out in a hospital in Spain showed 5% of the HIV infected patients were infected with syphilis whereas only 0.9% of the HIV negative patients were infected with syphilis showing syphilis was more frequent in HIV infected than non-infected persons (Joyanes *et al.*, 1998). A retrospective investigation carried out in Italy (January 2000–February 2007) revealed a significant increase of syphilis in HIV-1 infected individuals and in the population 84 cases of syphilis were reported during the period; 25 out of 84 (29.7%) were HIV-1 positive (Celesia *et al.*, 2007).

A study conducted among the prisoners in an Ethiopian prison showed syphilis was strongly associated with HIV positivity as 62% of the HIV positive were syphilis positive as compared to only 30% in HIV negatives (Kebede *et al.*, 1991). A prospective cross sectional hospital based study conducted among pregnant women in Ethiopia showed an HIV/syphilis co-infection rate of 2.2% (Mulu *et al.*, 2007). A study was conducted among women attending antenatal clinics (ANC) in Tanzania during the 2003/2004 ANC surveillance. The prevalence of HIV/syphilis co-infection was found to be 0.7% (Swai *et al.*, 2006). Among blood donors, a study was conducted in a hospital in Tanzania which revealed syphilis was the only infection that occurred more

frequently among HIV infected (12.1%) than non-infected (4.6%) blood donors and whose prevalence increased with age (Matee *et al.*, 2006). A study over 3 months (February–April 1996) among blood donors in Mozambique revealed an HIV/syphilis co-infection rate of 1%. The proportion of patients with a positive serum VDRL was higher in HIV positive than in HIV negative patients; 10.5% vs. 5.9% (Tattevin *et al.*, 1997). In a study conducted among HIV positive individuals in North Central Nigeria showed the prevalence of HIV/syphilis co-infection to be 3.3%. The prevalence of HIV/syphilis co-infection among females was 1.9% and 5.8% among males. The ratio between males and females with HIV/syphilis co-infection was approximately 1.61 (Forbi *et al.*, 2009). A similar study was conducted among 200 HIV/AIDS patients in North-Eastern Nigeria, of which 18 were seropositive for syphilis giving an overall co-infection rate of 9% (Olokoba *et al.*, 2008). A case-control survey was conducted using RPR test and confirmatory Immunochromatographic test among HIV positive and HIV negative Nigerians. A total of 35 (14%) of 250 HIV-positive and 5 (2%) of 250 HIV-negative individuals studied were seropositive for syphilis revealing syphilis was more frequent in HIV infected than uninfected individuals (Uneke *et al.*, 2006). A study conducted in a district hospital in Zimbabwe revealed a higher prevalence of specific antibodies to *T. pallidum* in HIV positive than in HIV negative patients. Of the 110 HIV positive sera, 46 (42%) were RPR positive, 56 (51%) were TPHA positive and 54 (49%) were FTA positive. This compared with 21 (32%), 18 (27%) and 13 (20%) respectively for the HIV negative sera (Mason *et al.*, 1992). A study conducted among factory workers in Zimbabwe gave syphilis prevalence rate of 2.3%. HIV prevalence rate was 19.8%. There was a strong association between syphilis, whether active/incident/historic, and HIV. Men who were HIV positive had a 3-fold higher risk of having serological evidence of active syphilis (Gwanzura *et al.*, 1999). A cross-sectional study conducted among FSWs in Ivory Coast showed syphilis was more frequent in HIV infected than uninfected women; 27% vs. 17% (Ghys *et al.*, 1995). Studies conducted among rural populations in Uganda and Burundi revealed 64% and 21.4% of all HIV positive cases tested positive for syphilis respectively as compared to 25.8% and 1.6% of HIV negative cases respectively (De Lalla *et al.*, 1990).

A few researches on HIV/syphilis co-infection have been carried out in Asia. Cross sectional surveys among community recruited transgender and male sex workers and self recognized MSM undertaken in mid-2002 in Jakarta, Indonesia revealed a strong relation between early syphilis and HIV infection. Early syphilis was 3.8 times higher in HIV positive individuals than in the HIV negative. Conversely, those with early syphilis were 4.7 times as likely to be HIV infected as those without such infection (Pisani *et al.*, 2004). A study carried out in Japan among the patients of six national hospitals revealed 14.1% of the HIV infected population co-infected by syphilis. The rate of STDs, especially syphilis in HIV infected persons was higher than that in general population (Isao *et al.*, 2003). A retrospective study conducted among blood donors in Thailand revealed syphilis being prevalent in 2.2% of the HIV positive individuals as compared to only 0.34% in HIV negative individuals (Santibhavank *et al.*, 1998). Different studies are conducted in India with respect to HIV/syphilis co-infection. A research was conducted among patients with genital lesions attending the STD clinic in a hospital in India. Syphilis cases showed higher rate of HIV positivity (15.38%) than others; indicating that the disease increases the risk of acquiring HIV (Anand Kumar *et al.*, 2003). A study conducted among FSWs in 2005/06 in India showed 14.28% of the total HIV positive patients co-infected by syphilis (Shetwala *et al.*, 2009). Among blood donors, a study conducted in India showed HIV/syphilis co-infection rate of 6.67% (Mittal *et al.*, 1994). Other studies conducted in India among HIV infected patients had revealed the syphilis co-infection rate to be in the range of 4.34–21.6% (Mahajan *et al.*, 2008; Dhanvijay, 2000). Syphilis was the highest prevalent disease (13.55%) among the HIV positive individuals revealed by a study conducted in Bangladesh (Munushi *et al.*, 2008). In the context of Nepal, few studies had been carried out with respect to HIV/syphilis co-infection. A study conducted among HIV-infected sex-trafficked women and girls in Nepal revealed an HIV prevalence rate of 30.1% and syphilis prevalence rate of 20.4%. Those who were HIV positive were more likely than those who were HIV negative to be infected with syphilis; 31% vs. 15.9%, respectively (Silverman *et al.*, 2008). A study conducted by New ERA and SACTS in 1999 among sex workers and truckers in Nepal showed sex workers and truckers with syphilis had a

ten-fold higher risk of getting HIV than those without syphilis (New ERA/SACTS, 2000).

3.3.3 Diagnosis of syphilis in HIV infected patients

The diagnosis of syphilis may be more complicated in HIV infected patients, and this calls for: i) careful physical examination of HIV infected persons, ii) closely monitoring the treatment for syphilis in HIV infected patients, and iii) identification and treatment of sexual partners (Pialoux *et al.*, 2008). The documented serologic diagnostic confusions in HIV infected syphilis are: i) more BFPs as a result of polyclonal activation of B-lymphocytes and the state of hyper-gamma-globulinemia that the virus produces in infected subjects, ii) lack of serologic response in patients with a clinically confirmed case of active syphilis, iii) rapid progression to late stages of syphilis and neurologic involvement even after treatment of primary or secondary syphilis, iv) failure of non-treponemal titre to decline after treatment, v) disappearance of treponemal reactivity over time after treatment (Larsen *et al.*, 1995).

For the diagnosis of syphilis in HIV infected patients the clinician should seek confirmatory evidence from any available source, including the patient's history, clinical findings, direct examination of lesion material for spirochetes, and serologic tests for syphilis. If at all feasible, the clinician should perform dark-field examination or DFA staining of exudates from suspicious lesions of primary syphilis. For patients with suspicious lesions but negative serologic results, positive findings on dark-field examination or DFA stain can be diagnostic (Hall *et al.*, 2006). Despite several reports of unusual serologic responses in HIV infected patients, the interpretation of both treponemal and non-treponemal serologic tests for syphilis seems to be the same in HIV infected and uninfected patients (Lynn *et al.*, 2004). A positive RPR or VDRL test result may be biologically false positive if the confirmatory test is negative. A negative RPR or VDRL test result may not rule out syphilis in patients with HIV infection. In addition, a negative treponemal test may not rule out syphilis. If asymptomatic patients have a positive non-treponemal test and negative confirmatory treponemal test results, it

is very unlikely that they have active syphilis. Thus, it may be useful to consider both a non-treponemal and a treponemal test as a diagnostic strategy in newly infected persons with suspicious lesions (Hall *et al.*, 2006). False negative serologic tests in both primary and secondary syphilis have been reported in HIV/syphilis co-infected persons. The prozone phenomenon may cause a false negative non-treponemal test result, usually during secondary syphilis, when a high concentration of treponemal antigens hinders the formation of detectable antigen/antibody complexes. When clinical findings are suggestive of syphilis despite negative serologic test alternative test (dark-field microscopy) can be considered (Pialoux *et al.*, 2008).

In recent years the reliability of the serological tests for diagnosis of syphilis in HIV infected individuals has been questioned and some have discussed that false positive treponemal test results occur less frequently than false positive cardiolipin antigen tests (Bhat, 2005). Besides HIV, Lyme disease, certain types of pneumonia, malaria and systemic lupus erythematosus (SLE) may cause a false positive serologic test for syphilis (Levin, 2007). The different cause of false positive serologic tests for syphilis is presented in a tabulated form in Appendix IX. Many persons with HIV infection have anti-cardiolipin, anti-lecithin antibodies and polyclonal gammopathy. Thus, in such cases positive non-treponemal test result might be biologically false positive and does not represent active syphilis infection (Turbadkar *et al.*, 2007; Hall *et al.*, 2006). The BFP rate may be as high as 50% in selected populations, though it is expected to be of the order of 1–2% in a healthy population (Larsen *et al.*, 1995; Smikle *et al.*, 1990). A study was carried out to screen syphilis among pregnant women in Nigeria. The study showed 70% of the patients who were initially screened to be syphilis positive were found to be negative to syphilis antibodies (Taiwo *et al.*, 2007). Further, it is well documented that false positive rates for RPR are greater in HIV positive patients when compared to HIV negative patients (Augenbraun *et al.*, 1994; Rompalo *et al.*, 1992; Joyanes *et al.*, 1998). In a study conducted in Spain one quarter of the IDUs with infection by HIV presented false positive results to the reaginic test, thus leading to the recommendation that therapeutic measures should not be initiated without confirmation

with a treponemic test (Pulido Ortega *et al.*, 1993). Since syphilis and HIV infection are associated with each other at a higher rate than expected by chance, it is recommended that all patients who have HIV positive sera have to be investigated for syphilis and *vice versa* (Stevenson *et al.*, 2006; Pialoux *et al.*, 2008).

3.3.4 Treatment goals for syphilis in HIV infected patients

Treatment of syphilis in HIV co-infected patients is complicated, and success may depend on the integrity of host immune response as much as it does on antibiotic effect (Kassuto *et al.*, 2003). Treatment is largely supportive (Zetola *et al.*, 2007). The treatment of early syphilis in HIV-infected patients may need to be more intensive than has previously been recommended, because the immuno-suppression induced by HIV can accelerate the pace of the infection and increase the risk of progression to neurosyphilis. After treatment, careful and frequent follow-up is essential so that the often irreversible consequences of late syphilis can be avoided (Hicks, 1991). Benzathine penicillin G, administered parenterally, continues to be the drug of choice for all stages of syphilis in HIV-infected patients (Hall *et al.*, 2006; Karp *et al.*, 2009). It should be noted that the serologic response to therapy, though often less predictable in HIV positive individuals than in HIV negative individuals, doesnot necessariliy indicate a poorer clinical response (Birnbaum *et al.*, 1999). Besides careful long-term follow-up, repeated courses of therapy may be needed for patients infected with HIV who have syphilis (Malone *et al.*, 1995). Treatment failure which has been reported in HIV infected patients at all stages of syphilis and with all of the recommended regimens can be reduced by the use of ARV therapy which restores immune function (Ghanem *et al.*, 2008). The co-infected patients should be evaluated clinically and serologically for treatment failure at 3, 6, 9, 12 and 24 months after therapy for primary, secondary, and early latent syphilis and at 6, 12, 18 and 24 months after treatment for late latent syphilis or syphilis of unknown duration, which is probably the most important part of clinical management (Horn *et al.*, 2002; Hall *et al.*, 2006). Clinicians must evaluate patients using the same non-treponemal test on each occasion because titres from the VDRL and RPR tests are not interchangeable (Hall *et al.*, 2006).

CHAPTER – IV

4. MATERIALS AND METHODS

4.1 Materials

A complete list of materials including equipments, test kits and glasswares used during the study is listed in Appendix I.

4.2 Methods

4.2.1 Research/Study design

The study was a cross-sectional study.

4.2.2 Study site

The study was carried out based at National Public Health Laboratory (NPHL), Teku, Kathmandu, Nepal. NPHL is the referral centre for the diagnosis and treatment of HIV cases. The voluntary testing and counselling of HIV is done at National Centre for AIDS and STD Control (NCASC), Teku, Kathmandu. Anti-Retroviral Therapy (ART) is being provided from Teku Hospital from February 2004. As of July 2010, total number currently on ART coverage is 4,509. CD4 count service is available at 13 sites, while 4 sites have CD4 calibre (NCASC, 2010). CD4 count service is also available at NPHL. So, HIV suspected individuals visit NPHL frequently. Hence, NPHL was chosen as the site for this study.

4.2.3 Study population/Patient selection criteria

Individuals of all age groups and both sexes visiting NPHL for HIV diagnosis were included in the study.

4.2.4 Study period

The study was carried out from November 2008 to April 2009.

4.2.5 Sample size

Sample size estimated was based on the daily sample load at NPHL (1094 samples were selected who attended for the diagnosis of HIV). Duplicate samples were excluded (to exclude sampling bias). Sample size for syphilis (303) was calculated by Winpepi Version 3.8 (Appendix X).

4.2.6 Sample rejection criteria

The samples that were without or improper labeling, without form, with leakage and with any visual contamination were excluded in the study.

4.2.7 Data collection tools

A brief clinical history was collected from the study participants prior to sample collection by direct interview using a structured questionnaire (Appendix XI).

4.2.8 Data entry, analysis and interpretation

Data were entered into the excel sheet along with their results and statistically analyzed using WinPepi Version 3.8 (alpha = 0.05 was set for significance level). Chi-square test was applied to the data for association test whenever appropriate. Final results were interpreted after statistical analysis using different charts, figures and tables.

4.2.9 Laboratory procedure

The laboratory procedures were carried out as described below.

4.2.9.1 Sample collection

About 5ml venous blood was collected from each individual by a laboratory technician. After dispensing the blood sample in a clean labelled tube, it was allowed to clot without disturbing the tubes for 30 minutes. Serum (about 2ml) was separated as supernatant by centrifuging blood at 3000 rpm for 5 minutes. The separated serum was transferred carefully to a labelled clean and dry screw capped tube using a micropipette. Sera were immediately stored at -20°C, if not tested on the collection day.

4.2.9.2 Sample processing and storage

All the reagents were brought to room temperature and the sera were tested serologically by commercially available rapid test kits (Determine and Capillus tests) and ELISA (General Biological Corporation, Taiwan) kit for the detection of antibodies to HIV following WHO suggested algorithm for diagnosis of clinical cases (WHO/CDC, 2003). Serum samples which were found to be HIV positive were preserved at -20°C until tested for syphilis.

For syphilis, two tests were carried out first a non-treponemal test (Rapid Plasma Reagin card test) and second a treponemal test (*T. pallidum* Haemagglutination Assay). HIV positive samples that were reactive by RPR card test were further tested by TPHA. Manufacturer's instructions were strictly followed while processing the samples for both HIV and syphilis tests. The details of the kits content and procedure are given in Appendix II.

4.2.9.3 Quality control

Appropriate positive and negative controls were run with each batch of testing for quality control. Doubtful results were re-tested for reliability.

4.2.9.4 Result interpretation/Reporting

The individual result was interpreted using the test algorithm for HIV and syphilis adopted from the WHO (WHO/CDC, 2003). According to the WHO testing algorithm for HIV, serum that is reactive by test 1 and test 2 was considered positive. If test 1 was found to be positive and test 2 to be negative, further test 3 is to be done. If test 3 was found to be positive then, it is to be considered as HIV positive. Test 1 is ELISA technique, test 2 is Determine test and test 3 being Capillus test. According to the WHO testing algorithm for syphilis, the samples reactive on both the non-treponemal and treponemal tests are regarded as positive to syphilis (i.e. an active infection) whereas the samples reactive to RPR test but non reactive to TPHA test are reported as false positives (Larsen *et al.*, 1995).

4.2.9.5 Laboratory analysis (flow-chart)

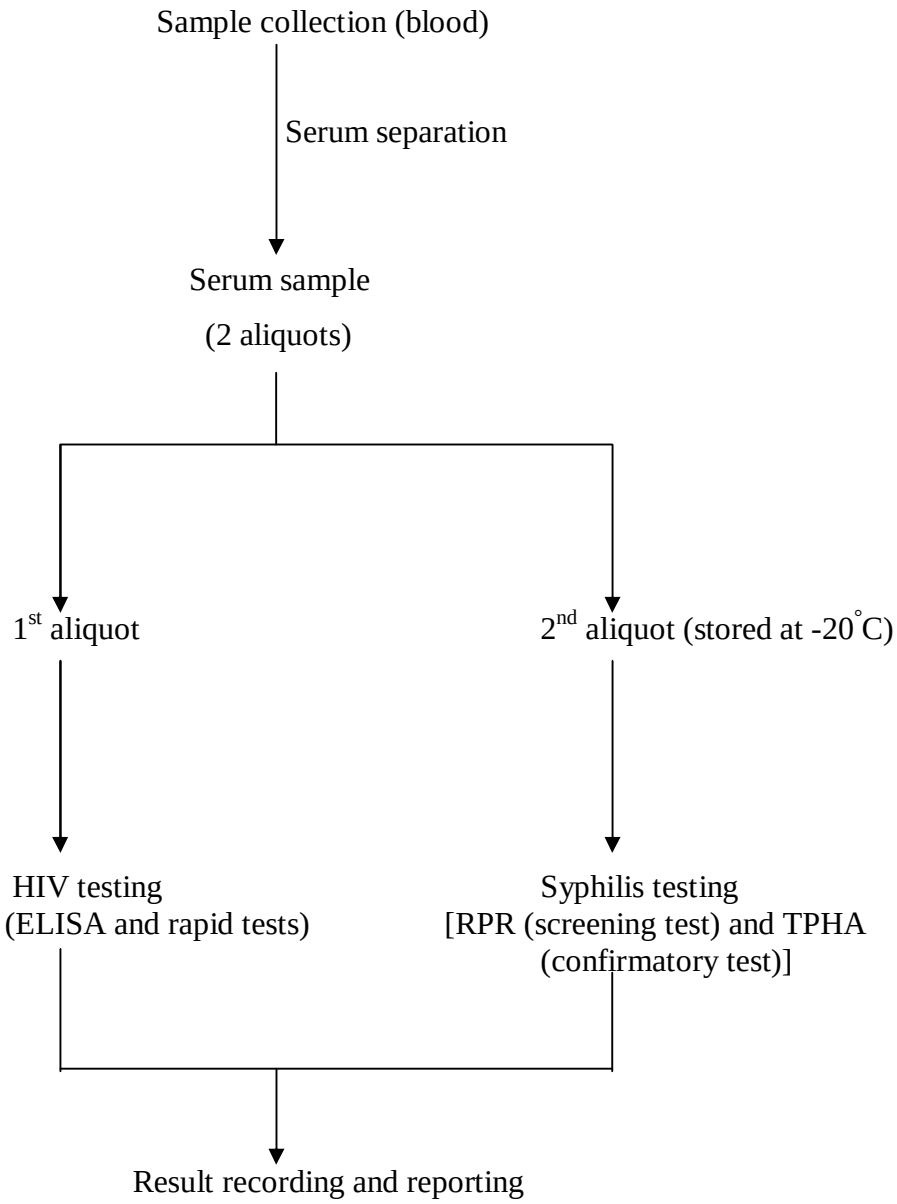

Figure 2: A simplified flow-chart for HIV and syphilis testing.

4.2.9.6 Testing algorithm for HIV

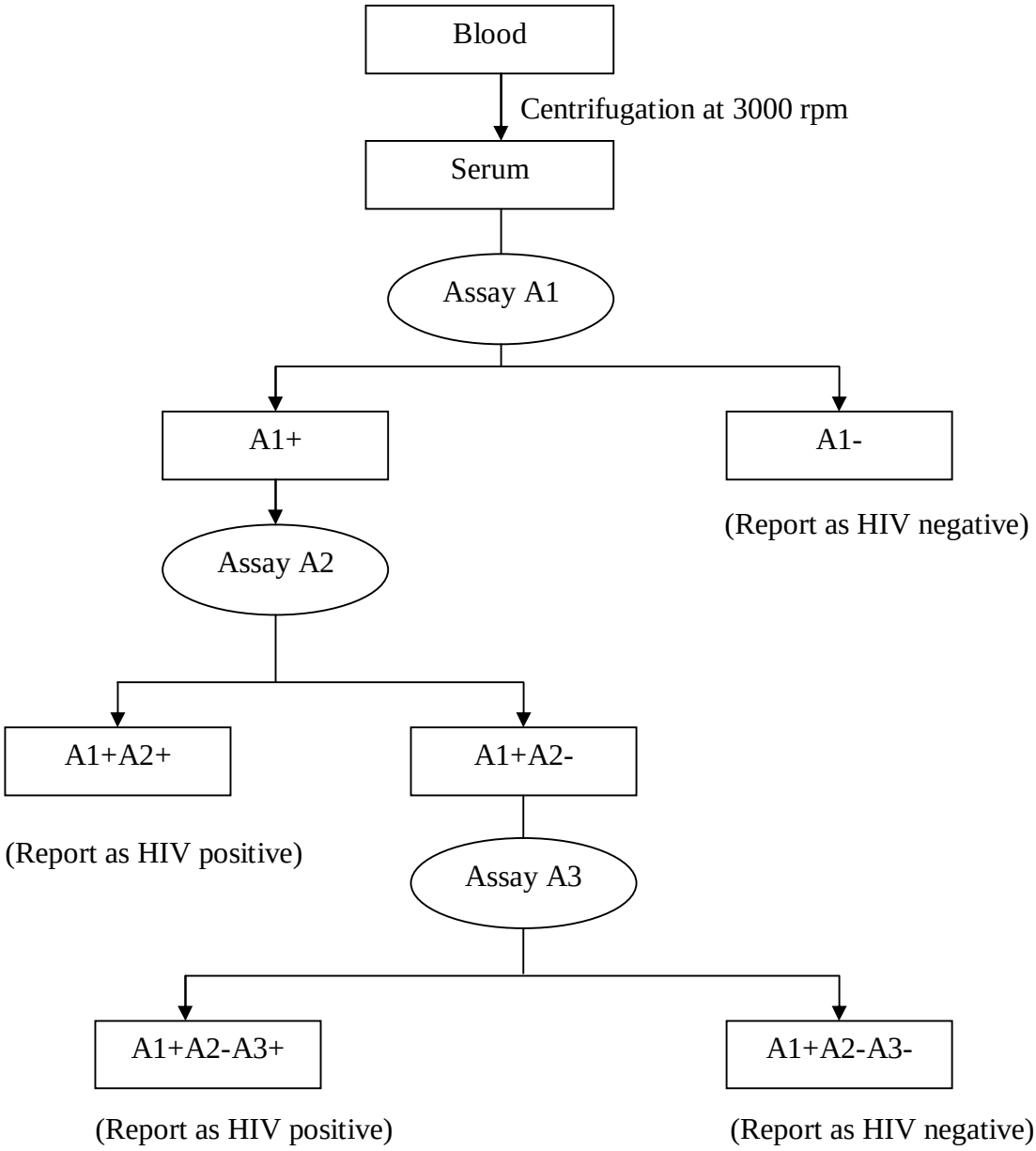

Figure 3: Testing algorithm for HIV (WHO/CDC, 2003)

Assay A1: ELISA

Assay A2: Rapid test (Determine)

Assay A3: Rapid test (Capillus)

4.2.9.7 Flow-chart for syphilis testing

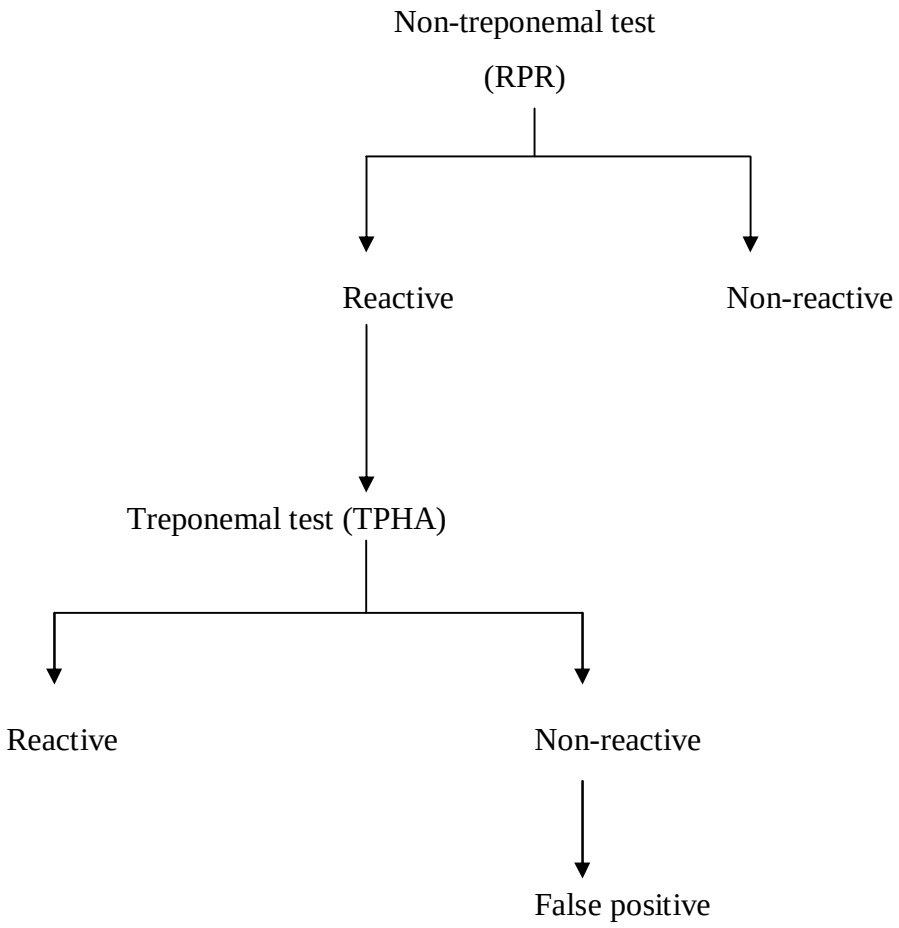

Figure 4: Routine screening scheme for syphilis

CHAPTER – V

5. RESULTS

5.1 Sero-prevalence of HIV among the individuals tested

Out of 1094 samples tested, 335 (30.6%) were HIV positives and remaining 759 (69.4%) were HIV negatives (Table 4).

Table 4: Sero-prevalence of HIV

Total samples tested Number (percent)	HIV status of the individuals tested	
	HIV positive Number (percent)	HIV negative Number (percent)
1094 (100.0)	335 (30.6)	759 (69.4)

5.1.1 Genderwise distribution of HIV

Out of 335 HIV positive individuals, higher numbers of HIV positive cases were found in males (61.5%) as compared to females (38.5%) (Table 5). The HIV positive cases were statistically significant among males as compared to females (p = 0.041).

Table 5: Genderwise distribution of HIV

Sex	HIV status of the individuals tested		Total	p-value
	HIV positive	HIV negative		
Male	206 (61.5)	515 (67.9)	721 (65.9)	0.041
Female	129 (38.5)	244 (32.1)	373 (34.1)	
Total	335 (100)	759 (100)	1094 (100)	

(Figures within parenthesis are percentages within cases of HIV in HIV individuals tested).

5.1.2 Agewise distribution of HIV

Among the studied population, the highest percentage of HIV positive individuals was observed in age group 25–34 years (46.9%) followed by 35–44 years (28.9%) and 15–24 years (9.0%). Children <15 years comprised of 8.4% of the total HIV positives (Table 6). The HIV positive cases were statistically highly significant among the age groups 25–34 years as compared to other age groups ($p<0.001$).

Table 6: Agewise distribution of HIV

Age groups (in years)	HIV status of the individuals tested		Total	p-value
	HIV positive	HIV negative		
<15	28 (8.4)	45 (5.9)	73 (6.7)	<0.001
15–24	30 (9.0)	247 (32.6)	277 (25.3)	
25–34	157 (46.9)	310 (40.8)	467 (42.7)	
35–44	97 (28.9)	90 (11.9)	187 (17.1)	
≥45	23 (6.8)	67 (8.8)	90 (8.2)	
Total	335 (100)	759 (100)	1094 (100)	

(Figures within parenthesis are percentages within cases of HIV in HIV individuals tested).

5.2 Syphilis sero-prevalence among HIV positives by RPR test

Out of 303 HIV positive samples tested for syphilis, 88 (29%) were reported as RPR reactive samples whereas remaining 215 (71%) were reported as RPR non-reactive samples (Table 7).

Table 7: Syphilis sero-prevalence among HIV positives by RPR test

Total HIV positive samples tested [(Number (percent)]	RPR reactive samples [(Number (percent)	RPR non-reactive samples [(Number (percent)]
303 (100.0)	88 (29.0)	215 (71.0)

5.3 Syphilis sero-prevalence among HIV positives by TPHA test (among RPR reactive samples)

Out of 88 RPR positive samples tested by TPHA test to confirm syphilis infection, 43 (48.9%) were reported as TPHA positive whereas remaining 45 (51.1%) were reported as TPHA negative samples (Table 8).

Table 8: Syphilis sero-prevalence among HIV positive individuals by TPHA test (among RPR reactive samples)

Total RPR reactive samples tested [(Number (percent)]	TPHA positive [Number (percent)]	TPHA negative [(Number (percent)]
88 (100.0)	43 (48.9)	45 (51.1)

True syphilis sero-prevalence was therefore 14.2% (43/303 HIV positive samples) while remaining 85.8% (260/303) were confirmed to be syphilis negative.

5.4 Genderwise distribution of syphilis (RPR and TPHA positives) among HIV positives

Out of 43 syphilis infected individuals, higher numbers of syphilis cases were found among males (62.8%) as compared to females (37.2%) (Table 9). However, the syphilis positive cases were found to be statistically insignificant between males and females (p = 0.729). Out of 88 RPR reactive samples 45 (51.1%) were confirmed as TPHA negatives which were slightly more in females (53.3%) as compared to males (46.7%) (Table 9).

Table 9: Genderwise distribution of syphilis among HIV positives

Sex	HIV positive individuals				Total	p-value
	Syphilis positive	Syphilis negative				
		RPR reactive + TPHA negative	RPR non-reactive	Total		
Male	27 (62.8)	21 (46.7)	135 (62.8)	156 (60.0)	183 (60.4)	0.729
Female	16 (37.2)	24 (53.3)	80 (37.2)	104 (40.0)	120 (39.6)	
Total	43 (100)	45 (100)	215 (100)	260 (100)	303 (100)	

(Figures within parenthesis are percentages within cases of syphilis in HIV positive individuals).

5.5 Agewise distribution of syphilis (RPR and TPHA positives) among HIV positives

The highest proportion of syphilis/HIV co-infection was observed in age group 25–34 years (60.4%) followed by 35–44 years (25.6%). Not a single case of co-infection was found in age group <15 whereas 7% each were observed in groups 15–24 and >44 (Table 10). However, the syphilis positive cases were found to be statistically insignificant between 25–34 years and other age groups (p = 0.195).

Table 10: Agewise distribution of syphilis among HIV positives

Age group (in years)	HIV positive individuals				Total	p-value
	Syphilis positive	Syphilis negative				
		RPR reactive + TPHA negative	RPR non-reactive	Total		
<15	0 (0)	0 (0)	24 (11.2)	24 (9.2)	24 (7.9)	0.195
15–24	3 (7.0)	6 (13.3)	16 (7.4)	22 (8.5)	25 (8.3)	
25–34	26 (60.4)	23 (51.1)	96 (44.7)	119 (45.8)	145 (47.8)	
35–44	11 (25.6)	14 (31.1)	65 (30.2)	79 (30.4)	90 (6.3)	
≥45	3 (7.0)	2 (4.5)	14 (6.5)	16 (6.1)	19 (6.3)	
Total	43 (100)	45 (100)	215 (100)	260 (100)	303 (100)	

(Figures within parenthesis are percentages within cases of syphilis in HIV positive individuals).

CHAPTER – VI

6. DISCUSSION AND CONCLUSION

6.1 Discussion

A total of 1094 individuals visiting NPHL during November 2008 to April 2009 were involved in the study. Among these populations, 335 (31%) were confirmed to be infected with HIV by serological tests i.e., an ELISA and two rapid methods (Determine and Capillus test kits). The WHO algorithm was followed for HIV determination (WHO/CDC, 2003). The prevalence of HIV was quite high as shown by the study, but this does not represent the general population because most of the patients actually test for HIV elsewhere and come to NPHL for confirmation (mostly the positive cases). Due to this fact HIV prevalence was as high as 31%, and therefore further studies need to be done among the general population so as to determine the true prevalence, as it has been already mentioned that the prevalence of HIV in Nepal is around 0.5%. Another reason for the high prevalence of HIV in this study might be due to the visit of risk groups such as sex workers and IDUs to NPHL. HIV epidemic in Nepal has changed from a "low level" epidemic to a "concentrated" epidemic within specific sub-groups of sex-workers and IDUs (Chander *et al.*, 2004). It has been reported that in most of the Asian countries, IDUs are the first community to be affected by HIV and Nepal was the first developing country to establish a harm reduction program with needle exchange for IDUs. The prevalence of HIV in these groups is obviously high as they are endulged in high risk behaviours. Different studies have revealed high prevalence of HIV among these groups. The most recent behavioural surveys (in 2005) showed HIV prevalence among injecting drug users to be 52% in Kathmandu, 32% in the Eastern Terai districts, 22% in Pokhara and 12% in the Western Terai districts (UNAIDS/WHO, 2008a). 17.1% of FSWs operating in Kathmandu valley were found to be HIV positive in 2001 (SACTS, 2001). HIV is spreading particularly fast among 15–39 year olds, with prevalence expected to reach 2% by 2015 in Nepal (USAID, 2005).

Out of 335 HIV positive cases, 206 (61.5%) were HIV sero-positive males, whereas only 129 (38.5%) were females. The HIV positive cases were found to be statistically significant among males as compared to females ($p = 0.041$). Males stay outsides and may practice unsafe sex, which may be the reason behind the high prevalence of HIV infection in males. When male members of family get ill, they have quick and easy access to the clinics/doctors and visit the health centres independently whereas women visit to modern health care facilities less than men because of social pressure or stigma, female members of family has to depend on other male or senior members of family to access to health centres. Moreover, females cannot freely express their health problems, which may be another reason behind the high prevalence HIV in males. Higher prevalence of HIV in male in this study is also supported by Chander *et al.*, (2004). The reason behind this gender differentiation might be due to exposure of male to the external environment than female which is attributed to more risky sexual behavior practiced by males due to prevailing socio-cultural-economic scenario, and female has still low value, which may be due to social prestige/restrictions to have sex with other people in their own location.

Out of 335 HIV sero-positive individuals, the highest percentage of HIV positives were detected in age group 25–34 years (157; 46.9%), followed by 35–44 years age groups with 97 (28.9%) and age groups 45 years and above with 23 (6.8%). The HIV positive cases were found to be statistically highly significant among age groups 25–44 years as compared to other age groups ($p<0.001$). So, the disease studied is fairly age specific and behaviour dependent. People of age 20–40 years are sexually and economically the most active group and the diseases are at high prevalence in those groups. In the study, highest percentage of HIV positive population were in age group 25–34 followed by 35–44 age groups and then 15–24 age groups with 9%. This variation in prevalence between groups may be attributed to the variation in duration and opportunity for risk exposure. But with the productive age, HIV infection increased gradually and started to decline after 45 years, which was seen in the study. Active age individuals are exposed to opportunity in search of work and other activities so these age groups are at higher

risk of HIV infection. Also, in the studies done before, highest HIV sero-positive patients were found in active age group, which has been supported by the study of Joshi *et al.*, (2003) and Moges *et al.*, (2006). From the report of cumulative HIV situation in Nepal as of August 2010, the highest HIV infection by age group was shown in 30–39 years (NCASC, 2010). As this study was conducted at NPHL, where mostly IDUs and sex workers who are adults come for HIV testing, the high prevalence of HIV might have been revealed in this age group. Foreign employees, who come under active age group and visit NPHL for HIV testing, are also the major risk groups for acquiring HIV infection. These groups might also contribute in raising the prevalence of HIV. Being separated from life partners for long period during their stay out of home for employment, they may in force them to be involved in sexual activities with the sex partners. For the economic survival of many households in both rural and urban areas migration to different countries from Nepal has been a necessity. Removal from traditional social structures, such as family, has been shown to promote unsafe sexual practices, such having multiple sexual partners and engaging in commercial sex. According to the informal source, more than 200,000 Nepalese workers are working in the Gulf region (Joshi *et al.*, 2003).

It has been reported that majority of HIV infection in Nepal is through sexual transmission, followed by injecting drug use and perinatal route of HIV transmission (NCASC, 2010). This fact has been supported by the result that quite low prevalence (8.4%) was observed among children (<15 years) as compared to sexually active population. Almost all HIV infected children acquire the virus from their mothers before or during birth or through breast-feeding. Perinatal transmission is the most common cause of HIV infection in paediatric population <15 years. MTCT (mother to child transmission) was the probable source of infection in 1.8% cases reported in India from 1986–2001. In the year 2003, out of 57,781 AIDS cases reported, 1551 (2.6%) cases were due to MTCT (Kapoor *et al.*, 2004). Up to 50% of infants born to HIV infected mothers are likely to be infected at birth or in the neonatal period (McCutchan, 2008). HIV infection in childhood following transfusion of blood from HIV infected

donor has also been reported. There had been speculations on the possible role of improperly sterilised syringes and needles in transmission of HIV infection in Africa (Ramachandran, 1988). The main limitation of this study was the lack of information about risk behaviour such as unprotected sex or drug use. Therefore, the study was unable to determine the relative impact of behavioural risk factors among the patients. However, these results will have important public health implications.

Among 335 HIV positive cases, only 303 could be further tested for syphilis; out of which, only 43 (14.2%) were confirmed to be co-infected with syphilis. Non-treponemal test used in the study was RPR card test and treponemal test used was TPHA test. The RPR test was performed qualitatively i.e. tested undiluted. Quantitative tests for RPR were not performed. Typically, a quantitative TPHA was performed at a unique serum dilution of 1:80 in the diluent supplied by the manufacturer. The samples that are reactive in both RPR and TPHA tests are regarded as having active/recent syphilis. Initial screening of syphilis when done by RPR card test revealed 88 of the 303 HIV positive individuals (29%) to be infected with syphilis. When RPR positive samples were further tested by TPHA test, only 43 were found to be infected with syphilis; revealing a high percentage of false positive reactions (51.1%; 45 out of 88). A literature review showed that anti-cardiolipin antibodies (aCL) occur frequently in viral infections, particularly in HIV (49.75%), HBV (24%) and HCV (20%) which reveals false positive reactions of non-treponemal test in the patients co-infected with these viral infections (Sene *et al.*, 2008). Different studies had shown a high percentage of false positive reactions among HIV positive individuals than their HIV negative counterparts. Researches done by Augenbraun *et al.*, 1994 (6.9% vs. 0.9%); Rompalo *et al.*, 1992 (4% vs. 0.8%) and Joyanes *et al.*, 1998 (15% vs. 1.2%) are few of them. Among 45 false positive samples, 21 (46.7%) were of males and remaining 24 (53.3%) were of females showing false positive reactions were slightly higher among females than in males. Chronic false-positive reactions have been associated with connective tissue diseases such as SLE or diseases associated with immunoglobulin abnormalities, which are more common in women; thus, chronic false-positive reactions are more

common in women than in men (Larsen *et al.*, 1995). This might be the reason that false positive reactions were observed at a slightly higher percent in females than in males.

The prevalence of HIV/syphilis varies widely in different studies, mainly due to the variation in the distribution of risk factors, geographic location, etc. of the study population. In this study, syphilis co-infection which was found to be 14.2% is similar to the study conducted by Isao *et al.*, 2003; Uneke *et al.*, 2006; Munushi *et al.*, 2008 and Shetwala *et al*., 2009.

Lower percentage of HIV/syphilis co-infection than the present study have been reported by Venegas *et al.*, 1991 (2.2%); Santibhavank *et al.*, 1998 (2.2%); Malone *et al.*, 1995 (8.3%); Schofer *et al*., 1996 (1.3%); Signorini *et al*., 2007 (2.7%); Mahajan *et al.*, 2008 (4.3%); Olokoba *et al.*, 2008 (9%); Forbi *et al.*, 2009 (3.3%); Huhn *et al.*, 2008 (10.1%); Pulido-Ortega *et al.*, 1993 (7.1%); Joyanes *et al.*, 1998 (5%); Matee *et al.*, 2006 (12.1%) and Tattevin *et al.*, 1997 (10.5%). These variations might be due to geographical variations, risk behaviours of the population under study, kits and strategy used for tests, period of study and donor selection criteria or even awareness status of the people in particular area.

Higher percentage of HIV/syphilis co-infection than the present study have also been reported by Kebede *et al.*, 1991 (62%); Grimberg *et al.*, 2006 (59.7%); Ansell *et al.*, 1994 (31.7%), Ghys *et al.*, 1995 (27%); Dhanvijay, 2000 (21.6%); Rodriguez *et al.*, 2004 (20.3%) and Anand Kumar *et al.*, 2003 (15.3%). The higher percentage of co-infection might be due to the use of non-treponemal test only as its only use cannot exclude syphilis in HIV positive individuals. Differences in seroprevalence can be attributed to population's lifestyle, sensitivity and specificity of test kits used along with preference of diagnostic algorithms.

Different studies have shown that a much higher prevalence of syphilis infection among HIV infected patients exist than in HIV negative individuals. For example, studies

conducted by Silverman *et al.*, 2008 (31% vs. 15.9%); Joyanes *et al.*, 1998 (5% vs. 0.9%); Ansell *et al.*, 1994 (31.7% vs. 5.9%); Matee *et al.*, 2006 (12.1% vs. 4.6%); Tattevin *et al.*, 1997 (10.5% vs. 5.9%); Rodriguez *et al.*, 2004 (20.3% vs. 11.9%); Kebede *et al.*, 1991 (62% vs. 30%) Uneke *et al.*, 2006 (14% vs. 2%); Ghys *et al.*, 1995 (27% vs. 17%) and Santibhavank *et al.*, 2009 (2.2% vs. 0.3%) are few of them. Because syphilis infection is associated with significant increases in the HIV viral load and significant decreases in the CD4 cell count, it is very important to prevent and promptly treat syphilis in HIV-infected individuals (Buchacz *et al.*, 2004). Several reports indicate that HIV co-infected patients may be at higher risk for syphilis relapse than HIV negative patients (Rolfs *et al.*, 1997). Because the possibility of clinical relapse after syphilis therapy may be slightly higher in HIV infected patients, the importance of closely following HIV-infected patients with syphilis cannot be overstated (Hall *et al.*, 2006; Karumudi *et al.*, 2005; Lynn *et al.*, 2004).

Of the 43 HIV/syphilis co-infected patients, 27 were males (62.8%) whereas 16 were females (37.2%). However, the syphilis positive cases were found to be statistically insignificant between males and females ($p>0.05$). The results obtained were similar to the study conducted by Forbi *et al.*, 2009 and Grimberg *et al.*, 2006 which revealed a higher prevalence of HIV/syphilis co-infection among males than in females. The ratio between males and females with HIV/syphilis co-infection was approximately 1.6:1. Therefore, HIV-infected men are 1.6 times more likely to be syphilis-positive than HIV-infected women. This is in line with the North Central Nigeria and Rio de Janeiro studies where a larger number of men than women were co-infected with HIV and syphilis (Forbi *et al.*, 2009; Signorini *et al.*, 2007).

Among 43 HIV/syphilis co-infected cases, the highest co-infection rate was reported in age group 25–34 years with 26 (60.4%), followed by 35–44 years with 11 (25.6%). However, the syphilis positive cases were found to be statistically insignificant between the age group 25–34 years and the other age groups ($p>0.05$). These age groups constitute the sexually active groups in the population. Seropositivity for syphilis in

them may therefore indicate that HIV in them has been contracted from high risk sexual activity. None of the patients under 15 had co-infection of HIV and syphilis. 7% of the co-infected patients were in the age group 15–24 years and ≥45 years. In the absence of effective interventions in time, the HIV and syphilis can be the leading cause of death of the population of productive age group over the coming years and can lead to a vicious cycle of poverty and vulnerability.

As per the WHO and international recommendations, the RPR and TPHA tests should be used in parallel for syphilis screening (Young *et al.*, 2000; Larsen *et al.*, 1995), which will give the highest degree of sensitivity, indispensable to select possible blood donors, while maintaining a good degree of specificity (D'Errico *et al.*, 1996). This is very useful in limiting the false positive results as non-treponemal tests are very likely to give the false positive reactions. The diagnosis of syphilis may be more complicated in HIV infected patients as up to 50% of the individuals co-infected with HIV/syphilis might show the false positive reactions in non-treponemal tests (Larsen *et al.*, 1995; Sene *et al*., 2008). Thus, non-treponemal tests alone cannot be used to diagnose syphilis in HIV positive patients. The test, however, is very useful in screening syphilis. Because RPR test can not confirm syphilis, it is a very good practice to use the TPHA test for those samples (especially HIV positive samples) that are reactive in RPR test. Many laboratories only use RPR test which might be mostly false positive (as shown by this study) and has very worse impact on patients such as unnecessary medication, mental torture, etc. In general, positive non-treponemal test results, particularly with titers greater than 1:8, should be interpreted as indicating active infection, with interval testing to assess delayed seroreactivity of the confirmatory test (Hall *et al.*, 2004; Larsen *et al.*, 1995). Because titer was not determined in the study, this might be the reason for the higher percent of false positive reactions of syphilis in the HIV positive samples. Both the tests, therefore, should be conducted in parallel to conclude or exclude syphilis (RPR alone can not be used), both in normal individual and co-infected patients. The choice of the RPR card test in this study was because it is widely used as a screening test in the developing world, easy to perform, does not need advanced

equipment, and is inexpensive. Since antibody response to non-treponemal antigens rise in response to infection and decline with successful treatment, these tests remain the only tools for the diagnosis of active or untreated infection to date (West *et al.*, 2002). The confirmatory test (TPHA) has equivalent sensitivity and greater specificity than the screening test (RPR) and independent methodologically, which reduce the chance of coincident false positive reactions (Egglestone *et al.*, 2000). Treponemal assays are not generally used for screening because they are not as sensitive as non-treponemal tests in the first 2–3 weeks of the primary stage of syphilis. These are also not useful for monitoring treatment effectiveness (Peeling *et al.*, 2004; Keren, 2006) as titres of the treponemal tests do not correlate with disease activity (Birnbaum *et al.*, 1999).

This study confirms the existence of co-infection of syphilis in patients with HIV/AIDS. Therefore, there is a need to screen patients with HIV/AIDS for syphilis infection. As already mentioned, the main limitation of this study was the lack of information about risk behaviours of the study subjects. Also, the prevalence data are not necessarily representative of the total population but these surveys provide useful estimates, and must be interpreted with caution. However, these results will help to implement screening for syphilis in all HIV patients. Understanding the prevalence of these diseases in the high risk population is important in planning and framing public health policy. The findings of the present study could be very valuable for meta-analysis in future. The findings highlight the necessity of improved state and national surveillance systems for syphilis among HIV positive individuals and HIV infections among persons with syphilis. Expanded screening, preventive strategies, and surveillance for co-infections among at-risk and known HIV infected individuals are vital to controlling the intersecting HIV and syphilis epidemics. People's negligency on syphilis testing (only do HIV tests but not syphilis) will have adverse effects on the health status of the country, as an emerging epidemic of HIV in a developing country like Nepal has made STI control as one of the strategies imperative and probably the most important one to decrease HIV transmission. Proper treatment of syphilis will go long way in preventing HIV infection. Persons with HIV infection acquired through sexual route, thus, should

be encouraged to test for the presence of not only syphilis but all other STDs that could co-infect the individual which, if not diagnosed in time, could lead to different complications, and all patients with STIs should be counselled for HIV testing. This will help in proper management of patients having STIs and HIV co-infection and reduces the risk of developing AIDS. This approach is a must so that the individual live longer and be healthier. Syphilis prevention, thus, could reduce HIV incidence rates resulting in substantial reductions in future HIV/AIDS medical costs. Thus, it should be made mandatory in Nepal to screen every HIV/AIDS patient for syphilis co-infection and *vice versa* for early detection and a simultaneous treatment besides HIV infection management to combat the menace of these dreadful diseases. The use of non-treponemal test along with treponemal test should be encouraged in diagnosing syphilis in HIV co-infected patients as a high percentage of false positive reactions do occur in the co-infected patients.

In addition to contributing to the spread of HIV, untreated syphilis could also contribute to poor health outcomes resulting from the consequences of latent stages of the disease and maternal infant transmission with resultant congenital syphilis. Thus, prevention of syphilis infections would help to stem the rising numbers of new HIV infections in the world and would certainly prevent congenital syphilis in developing nations like Nepal. Nepal, being a developing nation, could not under-estimate the ill effects of syphilis which go far beyond the disease's effect on the individual infected. The prevalence of both syphilis and HIV is increasing. Medical professionals are likely to encounter an increasing number of HIV co-infected patients in the coming years. Awareness of the subtle differences in presentation, diagnosis and management in syphilis and HIV co-infected patients is imperative to help curb the current pandemic of each disease (Narula *et al.*, 2010). Nepal faces numerous challenges in effectively addressing the HIV prevention and treatment to the epidemic. Despite, numerous efforts by Nepal Government, HIV prevention and treatment services are not able to reach the at-risk populations because there is a gap between top levels to grass root level. Thus, the policy makers seriously need planning to anticipate and translate the plan into action to

prevent and treat the increasing numbers of people living with HIV/AIDS. There is urgent need to address those issues and challenges and strengthen the whole spectrums of health systems through collaborative approach to achieve the millennium development goals. Research on sexual networking is urgently required to guide HIV control in Nepal. There is also a need for further good quality epidemiological studies of HIV seroprevalence.

STI control programs have been and will continue to be in the forefront of public health management. Early STI detection and treatment in the high risk group could be particularly effective and cost-beneficial in reducing HIV transmission for three reasons most STIs promote increased shedding of HIV; the number of HIV-infected person is smaller than the number of person at risk for becoming infected and HIV-infected persons often are receiving regular medical care. Early detection and treatment of other STIs should be a critical component of national, state, and local strategies to prevent HIV infection and AIDS, in concert with the behavioural and other interventions that constitute a comprehensive HIV prevention approach (CDC, 1998). This preventive approach is a must for a developing nation like Nepal if the rapid spread of HIV and STIs is to be controlled. HIV/AIDS is increasingly straining Nepal's public health system. It has been estimated that, if prevalence continues to increase at the current rate, AIDS could be the major cause of death in Nepal by 2010. UNAIDS predicts that, without effective treatment and care programs, AIDS will soon claim the lives of between 10,000 and 15,000 Nepalese per year (USAID, 2005).

Besides the negative impact on socio-economic development and loss of productive life, the burden of these diseases would change dramatically in geometric ratio over next 10 years that could add tremendous stress to our society and country. So, the timely interventions with multi-sectoral engagement and broad political commitment are strongly desirable. At last but not the least, continuation of prevention to care programs taking the human right based approach into consideration is also the demand of the time.

6.2 Conclusion

The study revealed the rate of HIV/syphilis co-infection to be 14.2%. Males were found to be more infected than females in both HIV and HIV/syphilis co-infected cases. Highest HIV and HIV/syphilis co-infection, both, were found in age groups 25–34 years followed by 35–44 years. A high percentage of false positive reactions (51.1%) were found while screening syphilis by RPR in the co-infected patients. The continous serological screening of pregnant woman, blood donors and “high risk groups” is helpful in the detection and control of HIV/syphilis co-infection. Aggressive control and proper treatment of syphilis may offer one very feasible approach to reducing transmission of HIV. The findings strongly indicate the need for syphilis screening for HIV infected persons and HIV screening for syphilis infected persons. The study results clearly indicated that TPHA should be considered as the confirmatory test for syphilis as RPR reactive samples may have TPHA positives as well as false positive cases.

CHAPTER – VII

7. SUMMARY AND RECOMMENDATIONS

7.1 Summary

This dissertation work was conducted in NPHL, Teku, Nepal (from November 2008 to April 2009) with general objectives to determine HIV sero-prevalence among the suspected individuals and estimating the syphilis co-infection among HIV positive individuals visiting NPHL. The prevalence was studied on the basis of sex and age groups. Major findings of the study can be summarized as follows:

1. A total of 1094 individuals were involved in the study. Serum samples were processed for the detection of HIV and HIV/syphilis co-infection. Serum samples were processed for HIV diagnosis by using ELISA test and Rapid methods (Determine test and Capillus test); and syphilis diagnosis in HIV positive individuals was performed by the help of two serological tests; RPR (non-treponemal test) and TPHA (treponemal test).

2. Out of 1094 serum samples, 721 (65.9%) were of males and remaining 373 (34.1%) were of females. 335 samples (30.6%), out of 1094, were found to be HIV positive. 206 out of 335 (61.5%) were HIV positive males, whereas remaining 129 (38.5%) were HIV positive females. The HIV positive cases were statistically significant among males as compared to females ($p = 0.041$).

3. Highest HIV prevalence (46.9%) was found in age groups 25–34 years followed by 35–44 years (28.9%) and 15–24 years (9%). 8.4% of the children (<15 years) were infected with HIV. Only 6.8% of the individuals with age 45 and above were infected with the virus. The HIV positive cases were statistically highly significant among the age groups 25–34 years as compared to the other age groups ($p<0.01$).

With the productive age, HIV infection increases gradually and started to decline as the age increases above 45.

4. Out of 335 HIV positive cases, only 303 samples (183 males and 120 females) were further tested for syphilis to determine the HIV/syphilis co-infection rate. Initially, 88 samples were found to be infected with syphilis when tested with RPR card test. The samples reactive on RPR test, when further tested by TPHA test, gave only 43 samples to be infected with syphilis, giving a true prevalence of 14.2%. Out of 45 samples that were false positives, 21 (46.7%) were of male individuals whereas remaining 24 (53.3%) were of female individuals.

5. Out of 43 samples, 27 (62.8%) were HIV/syphilis co-infected males whereas remaining 16 (37.2%) were co-infected females. However, the syphilis positive cases were statistically insignificant between males and females ($p>0.05$).

6. Regarding the age, the highest percentage of co-infection was observed in the age group 25–34 years (60.4%; 26 out of 43), followed by 35–44 years (25.6%; 11 out of 43). Not a single case of co-infection was observed among children (<15 years). However, the syphilis positive cases were statistically insignificant between the age groups 25–34 years and the other age groups ($p>0.05$).

7. From the study, it was observed that age and sex were important factors in the distribution of syphilis co-infection among HIV positive individuals. Sero-prevalence data indicate the high potential for a generalized epidemic in Nepal. As a public health measure, the need to intensify efforts on the promotion of safer sexual behavior particularly among adolescents and provision of effective, accessible treatment for STDs in Nepal can not be overstated. Transforming such measures into public health policy is indispensable to the success of HIV/STD interventional programmes as even a “low to moderate growth scenario” would make HIV/AIDS the leading cause of death in the 15–49 year old population over the coming years.

7.2 Recommendations

Based on the findings of this study, following recommendations have been made:

1. All diagnosed HIV cases should be tested for syphilis infection. Prevention, care and public awareness programs are needed to work in close collaboration for both infections. Those who are infected by syphilis should also be tested for HIV as both the diseases act synergistically to co-infect the host.

2. The high percentage of false positive results to the reaginic test in HIV infected patient leads to the recommendation that therapeutic measures should not be initiated without confirmation with a treponemic test (TPHA). Government should develop a guideline on syphilis screening to increase the reliability of the diagnostic tests.

3. RPR test should be considered only for screening. RPR reactive cases should be confirmed with TPHA.

4. Further study with bigger number of samples representing whole country may be useful in making national policy and planning.

CHAPTER – VIII

8. REFERENCES

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APPENDIX I

MATERIALS

1. EQUIPMENTS

a) Centrifuge (Gemmy Industrial Corporation, Taiwan)
b) Pharmaceutical refrigerator (Sanyo Pharmaceuticals, Japan)
c) Incubator (Narang Scientific Works Pvt. Ltd., New Delhi)
d) Micropipettes of size 50µl, 100µl (Human, Germany)
e) ELISA reader (Humareader, Japan)
f) ELISA washer (Humareader, Japan)
g) VDRL rotator

2. DIAGNOSTIC TEST KITS

a) HIVASE 1+2 direct sandwich ELISA kit (General Biological Corporation, Taiwan)
b) Rapid test kit (Determine; Abott, Japan)
c) Rapid test kit (Capillus; Trinity Biotech, Ireland)
d) Rapid plasma reagin (RPR) test kit (New Market Laboratories Ltd., United Kingdom)
e) *Treponema pallidum* haemagglutination assay (TPHA) test kit (Human, Germany)

3. GLASSWARES AND OTHERS

a) Test tubes and test tube stands
b) Disposable gloves
c) Sodium hypo-chlorite solution and distilled water
d) Beaker, conical flask and measuring cylinder
e) Disposable micropipette tips
f) Marker and blotting paper
g) Adhesive seals and clock with timer

APPENDIX II

TESTS PROCEDURES

1. DIAGNOSIS OF HIV BY ELISA (HIVASE 1+2)

1.1 Principle of the test

The reagent kit, HIVASE 1+2, developed by General Biologicals Corporation (GBC) adopts the "direct sandwich principle" as the basis for the assay to detect antibodies to HIV-1 and/or HIV-2.

The GBC's HIVASE 1+2 is a sandwich enzyme immunoassay which employs recombinant HIV-1 and HIV-2 antigens for the detection of antibodies to HIV-1 and HIV-2 in human serum or plasma. These rDNA antigens which are reactive with the predominant antibodies to HIV-1 and HIV-2, constitute the solid phase antigenic absorbent. When human serum/plasma is added to the well, the HIV-1/2 antigens and Anti-HIV 1/2 is present in the specimen. After washing, the well is filled with a solution containing conjugate of rDNA HIV-1/2 antigens and horseradish peroxidase (HRPO), allowing the formation of (HIV-1/2) • (Anti-HIV1/2) • (HIV1/2 • HRPO) complex. After washing out the unbound conjugate, TMB solution (3, 3', 5, 5'-tetramethylbenzidine) is added for color development. The absorbance of the color development is a measurement of the anti-HIV1 and/or anti-HIV2 content in the sample.

1.2 Test procedure

1. All the reagents and specimens were brought to room temperature (20–30°C) before beginning the assay.
2. The two wells for blanks, two wells for negative control, two wells for each anti-HIV1 and anti-HIV2 positive control, and one well for each specimen was prepared.
3. 100µl of each control and specimen was added into each appropriate well.

Note:

Use a new pipette tip for each sample to avoid cross contamination.

Avoid touching the edge of the well during each pipette step.

4. Plate was sealed with an adhesive slip and the plate was incubated at 37±1°C in a water bath or humidified incubator for 30 minutes.
5. Diluted conjugate was prepared by following the **conjugate preparation chart**.
6. At the end of the incubation period, adhesive slip was removed and discarded and the plate was washed by the **plate washing procedures**.
7. 100µl of the diluted conc. HIV antigen conjugate was added in each well (except blank wells).
8. Again, the plate was sealed with an adhesive slip and the plate was incubated at 37±1°C in a water bath or humidified incubator for 20 minutes.
9. Step 6 was repeated.
10. 50µl of TMB substrate solution A and 50µl of TMB substrate solution B was added into each well including 2 blanks and gently mixed well.

    Note: The color of the blank should be colorless to light yellow; otherwise the test result is invalid. Test has to be repeated.
11. Plate was then covered with black cover and was incubated at room temperature for 15 minutes.
12. The reaction was stopped by adding 100µl of 2N H_2SO_4 to each well, including the blank wells.
13. Optical density was measured within 15 minutes by a precision ELISA reader. The instrument was blanked by using the lighter one of the two blanks. Absorbance was read at wave length of 450nm or 450/650nm.

Plate washing procedures

Note: Dilute Washing Solution D (20X) Concentrate with distilled or de-ionized water to 1:20 dilution. Do not use tap water.

Automatic or Semi-automatic microplate washing procedure

a. Any commercial automatic microplate washer or other liquid aspirating/dispensing devices can be used for washing purpose. The user should test the devices to determine the proper volume of water and wash cycles to insure proper washing. It suggests 6 cycles with at least 0.5ml washing buffer per well per wash and soaking at least for 20 seconds.

b. Blot dried by inverting the plate and tapping firmly onto absorbent paper. All residual wash buffers should be blotted dry.

Warning: Improper washing will cause false results.

Conjugate preparation chart:

Number of wells used	Volume of conjugate diluent needed (ml)	Volume of conc. HIV antigen conjugate needed (µl)
8	1	10
16	2	20
24	3	30
32	4	40
40	5	50
48	6	60
56	7	70
64	8	80
72–80	9	90
81–96	10	100

1.3 Calculation and determination

1.3.1 Calculation of NCx

Example:	NC	Absorbance
	1	0.022

2	0.024

NCx = (0.022+0.024)/2=0.023

1.3.2 Calculation of anti-HIV 1 PCx and anti-HIV 2 PCx

Example:	Anti-HIV 1 PC	Absorbance
	1	1.045
	2	1.170
	Anti-HIV 1 PCx	= (1.045+1.170)/2
		= 1.108

	Anti-HIV 2 PC	Absorbance
	1	1.116
	2	1.097
	Anti-HIV 2 PCx	= (1.116+1.097)/2
		= 1.107

Both Anti-HIV1 PCx and Anti-HIV2 PCx should be ≥ 0.5; otherwise, the test is invalid.

1.3.3 Determination of cut-off value

Cut-off value = NCx + 0.10

Example:

Cut-off value = 0.023 + 0.10

= 0.123

1.4 Interpretation of result

Non-reactive result

Sample absorbance < Cut-off value

Reactive result

Sample absorbance > Cut-off value

2. DIAGNOSIS OF HIV BY RAPID METHOD (DETERMINE™ HIV-1/2)

2.1 Principle of the test

Determine HIV-1/2 is an immunochromatographic test for the quantitative detection of antibodies to HIV-1 and HIV-2. Sample is added to the sample pad. As the sample migrates through the conjugate pad, it reconstitutes and mixes with the selenium colloid-antigen conjugate. This mixture continues to migrate through the solid phase to the immobilized recombinant antigens and synthetic peptides at the patient window site.

If antibodies to HIV-1 and/or HIV-2 are present in the sample, antibodies bind to the antigen-selenium colloid and to the antigen window, forming a red line at the patient window site.

If antibodies to HIV-1 and/or HIV-2 are absent, the antigens/selenium colloid flows past the patient window and no red line are formed at the patient window site.

2.2 Test procedure

1) The protective foil cover was removed from each test.
2) For serum/plasma samples:
    a) 50µl of sample (precision pipette) was applied to the sample pad (marked by the arrow symbol).
    b) After a minimum of 15 minutes (up to 60 minutes) result was read.
3) For whole blood samples:
    a) 50µl of sample (precision pipette) was applied to the sample pad (marked by the arrow symbol).
    b) After a minute one drop of chase buffer was applied to the sample pad
    c) After a minimum of 15 minutes (up to 60 minutes) result was read.

2.3 Interpretation of result

Non-reactive result (one bar)

One red bar appearing in the control window of the strip and no red bar appearing in the patient window of the strip

Reactive result (two bars)

Red bars appearing in both the control window and the patient window of the strip

Invalid (no bar)

No red bar in the control window of the strip. Even if a red bar appears in the patient window of the strip, the result is invalid and should be repeated.

3. DIAGNOSIS OF HIV BY RAPID METHOD (CAPILLUS^{TM} HIV-1/2)

3.1 Principle of the test

The majority antigens from the envelope proteins of HIV-1 and HIV-2 have been identified and cloned using recombinant DNA technology. These HIV-1 and HIV-2 proteins have been expressed and purified. The Trinity Biotech capillus HIV-1/2 employs these two proteins bound to polystyrene latex beads to form the basis of a direct latex aggregation assay for the detection of antibodies to HIV-1/2 in human serum, plasma or whole blood. The slide consists of a well area for mixing of latex reagent and sample. At one end of the mixing well, there is a capillary flow channel which leads to a viewing window. The latex reagents and test sample are mixed in the mixing well on the slide. The mixed reagents are drawn to the flow channel and the reagents begin to flow by capillary action towards the viewing window. Samples with HIV-1/2 specific antibodies will cause the antigen coated latex to aggregate. The capillary flow enhances the binding of specific antibodies to the latex and hence promotes aggregation. The reaction is read visually when the latex solution reaches the viewing window. Aggregation in the viewing window should be considered as initially reactive. A smooth milky white appearance is considered non-reactive.

3.2 Test procedure

1. Reagents and patients samples were brought to room temperature (18–25°C) before use.
2. Patient's sample identification numbers were recorded.
3. The latex reagent was mixed well by gently agitating the bottle to insure that the latex suspension is homogeneous. Foaming of the latex reagent was avoided and also the latex was drawn up and down a few times with the graduated dropper to insure good mixing before latex is dispensed on to the slide.
4. The latex reagent was drawn to the latex calibration mark (120µl volume approximately). Drawing of air bubbles should be avoided. The reagent was dispensed on to the slide at the edge of the mixing well furthest away from the capillary chamber. Contact of the graduated dropper with the slide was avoided when dispensing the reagent.
5. The pre-calibrated pipette was used with a fresh disposable pipette provided in the kit and retrieved the test sample or control (10µl volume).
6. The sample was then dispensed directly into the latex solution. Using the pipette the sample and the latex was mixed by pumping the mixture in and out of the tip three times and was stirred in a circular motion at least five times.

   Note: Effective mixing of sample in the latex is critical to insure reproducible and accurate test results.
7. The pipette tip was continued to use to move the well mixed samples and latex solutions to the opening of the channels until the capillary flow begins.
8. The latex mixture was allowed to flow through the entire capillary channel and into the viewing window before interpreting the result (3–7 minutes approximately).

3.3 Interpretation of result

Reactive result

Samples demonstrating any latex aggregation

Non-reactive result

Samples showing no aggregation

4. DIAGNOSIS OF SYPHILIS BY RAPID PLASMA REAGIN TEST KITS

4.1 Principle of the test

The New Market Laboratories RPR kits use carbon particles with a mixture of lipid antigens, which will combine with antibody present in patient's serum or plasma. The particles are suspended in a medium containing components to eliminate non-specific reactions. Positive reactions are shown by macroscopic aggregation of the particles. Although the kit is intended for use primarily as a qualitative test, antibody levels may be titrated by doubling dilution. Agglutination patterns are interpreted by eye.

4.2 Test procedure

1. All reagents, controls and specimens were brought to room temperature prior to use.
2. 50µl of sample or control was placed into a circle on the test card.
3. The sample was spread evenly over the test circle area.
4. The vial of RPR antigen was shaken to ensure thorough mixing.
5. The dropping needle was attached to the plastic dropping bottle and the RPR antigen was taken up by suction.
6. The dropping bottle was inverted and squeezed gently to expel air from the needle.
7. Holding the dropping bottle vertically over the test specimen, a single drop of antigen was dispensed.
8. Test card was placed on a card rotator and was rotated at 100 rpm for 8 minutes.
9. Results were read and interpreted visually in good light.
10. Unused antigen was returned from dropper bottle to glass vial.
11. Dropper bottle and needle was cleaned with distilled water, and was allowed to dry before re-using.

    Note:

    Specimens that are grossly contaminated, excessively haemolysed, extremely turbid or lipaemic must not be used.

The kit positive and negative controls must be run with each batch of tests.

4.3 Interpretation of result

Strong reactive (SR)

Large clumps of carbon particles with a clear background

Reactive (R)

Large clumps of carbon particles somewhat more dispersed than in strong reactive

Weak reactive (WR)

Small clumps of carbon particles with light grey background

Trace reactive (TR)

Slight clumping of carbon particles typically seen as a button of aggregates in the centre of the test circle or dispersed around the edge of the test circle

Non-reactive (NR)

Typically a smooth grey pattern or a button of non-aggregated carbon particles in the centre of the test circle

Reactive samples were recorded as antibody positive and were subjected to further tests to determine the presence or absence of specific anti-treponemal antibody.

For the assay to be valid the positive control provided should give a strong positive pattern and the negative control provided should give a clearly negative result.

5. DIAGNOSIS OF SYPHILIS BY TPHA LIQUID

5.1 Principle of the test

Syphilis TPHA liquid is an indirect haemagglutination test for the detection of specific antibodies against *T. pallidum.* Avian erythrocytes are coated with *T. pallidum* antigen. In the presence of syphilitic antibodies the sensitized cells will agglutinate to form characteristic pattern in microtitration plates. Antibodies to non-pathogenic Treponemas are absorbed by an extract of Reiter's Treponemas included in the cell suspensions.

5.2 Test procedure

1. Using a micropipette 100µl of diluent (DIL) was placed in well 1 and 25µl in well 2 and 3 each.
2. 25µl of serum sample or positive control (PC), negative control (NC) was added to well 1. The contents of well 1 was mixed with a 25µl micropipette and 25µl was transferred to well 2 (control well). The contents of well 2 was again mixed with a 25µl micropipette and 25µl was transferred to well 3 (test well). The content was again mixed and 25µl was discarded from well 3.
3. 75µl of the carefully re-suspended control cells (SCC) was added to well 2 and 75µl of the carefully re-suspended test cells (STC) was added to well 3.
4. The plate was gently shaken to ensure the contents were thoroughly mixed.
5. The plate was placed on a white level surface, away from vibration and direct sunlight. It was left for 45–60 minutes before reading the result. The plate may be left overnight.

5.3 Interpretation of result

Negative result

Compact button of non-agglutinated cells, with or without a very small hole in the centre

Indeterminate result

Button of cells with a small hole in the centre (appearance of a well defined, thick dense ring with a clear background)

Positive result

Partial or total agglutination of cells (appearance of an even layer of agglutinated cells, possibly surrounded by a circle of cells); weak positive samples show a ring with a frayed border surrounded by agglutinated cells.

APPENDIX III

COURSE OF UNTREATED HIV INFECTION

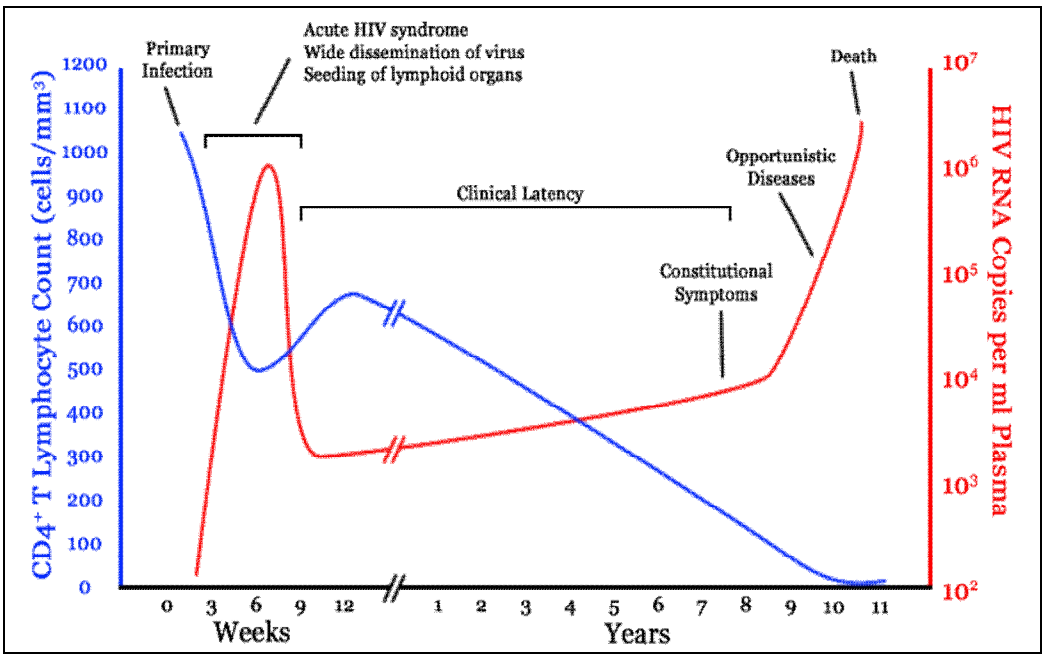


Figure I: A typical course of untreated HIV infection (Butel, 2007)

APPENDIX IV

GLOBAL MODES OF HIV TRANSMISSION

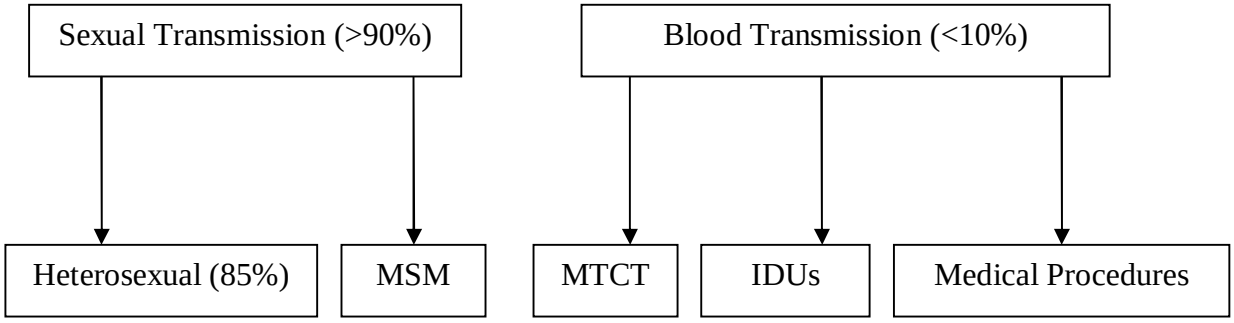


Figure II: Global modes of HIV transmission (Global Health Council, 2010)

APPENDIX V

OUTCOMES OF INFANTS BORN TO HIV INFECTED MOTHERS

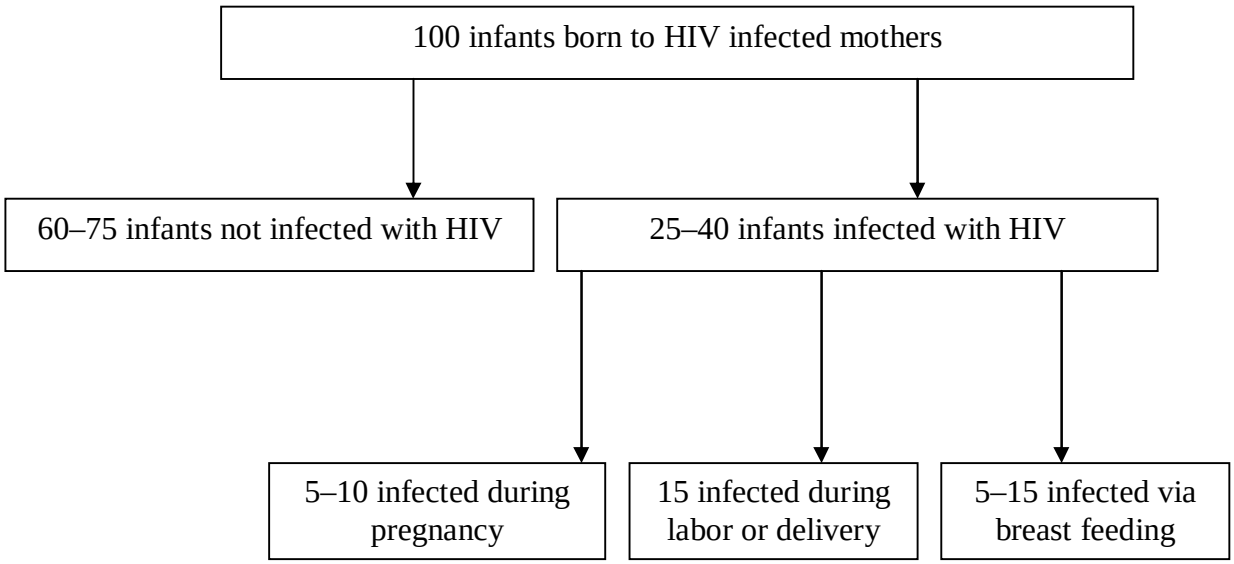


Figure III: Outcome of infants born to HIV infected mothers (Global Health Council, 2010)

APPENDIX VI

MAJOR ROUTES OF HIV TRANSMISSION IN NEPAL

Table I: Route of HIV transmission in Nepal

Route of transmission	Total number of infections	Percent
Sexual intercourse	12475	76.7
Blood or Organ recipients	49	0.3
Injecting drug use	2617	16.1
Perinatal	1037	6.4
Unidentified	84	0.5
Total	16262	100.0

Source: NCASC, 2010

APPENDIX VII

NATURAL HISTORY OF UNTREATED SYPHILIS

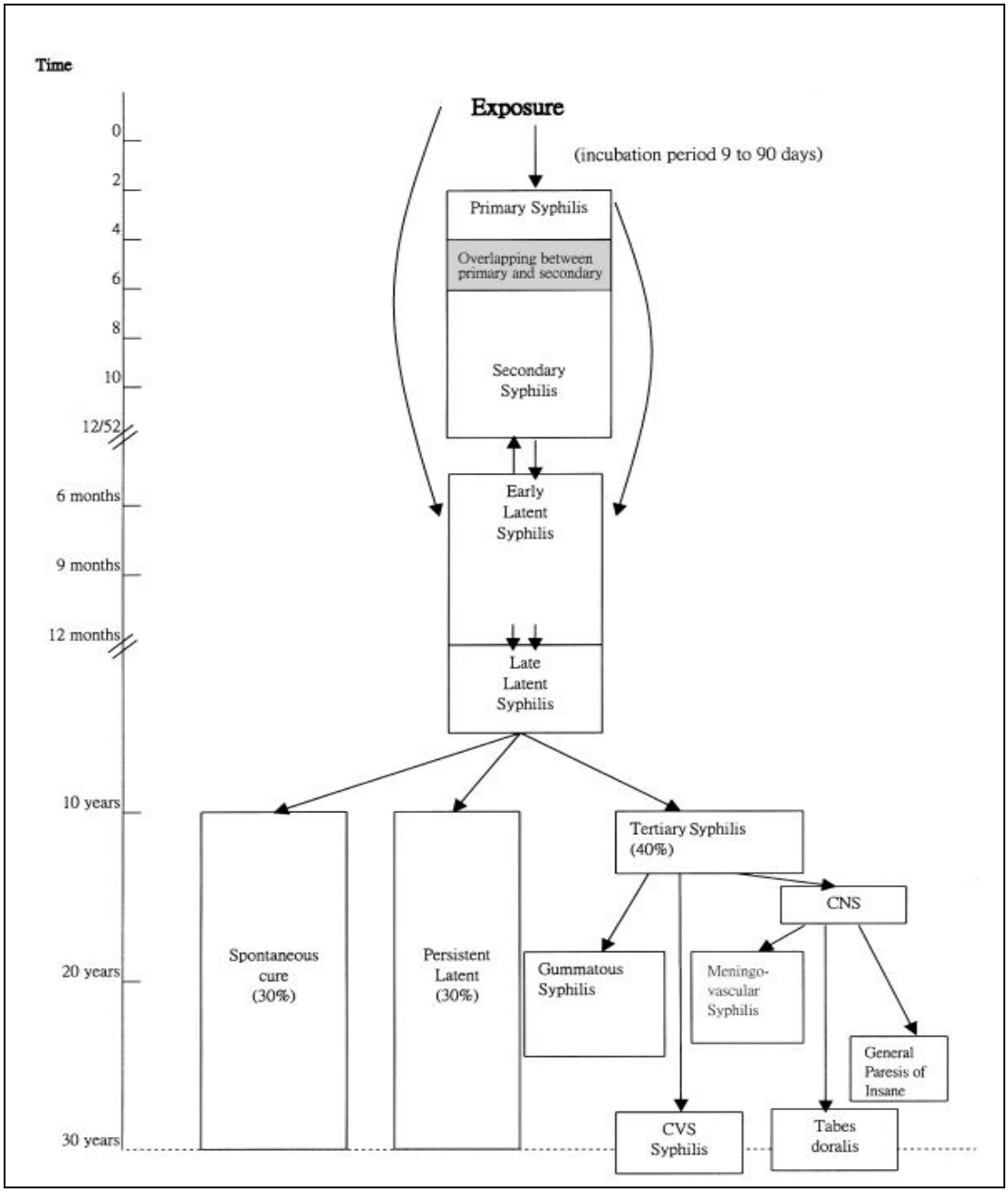


Figure IV: Natural course of syphilis (Ho, 2002)

APPENDIX VIII

FALSE POSITIVE SEROLOGIC TESTS FOR SYPHILIS

Table II: Causes of false positive serologic tests for syphilis

Tests	Acute conditions (<6 months)	Chronic conditions (>6 months)
Non-treponemal	Pneumonia (Viral, Pneumococcal, Mycoplasma), Hepatitis, Tuberculosis, Mononucleosis, Chancroid, Chicken pox, HIV infection, Measles, Malaria, Immunizations, Pregnancy, Laboratory error	Liver disease, Malignancy, Intravenous drug use, Aging, Connective tissue disorders, Multiple blood transfusions
Treponemal	Mononucleosis, Lyme disease, Leprosy, Malaria	Systemic lupus erythematosus (SLE)

Source: Birnbaum *et al.*, 1999

APPENDIX IX

SAMPLE SIZE CALCULATION

Using **WINPEPI Version 3.8**, sample size for the dissertation entitled **"Sero-prevalence of syphilis among HIV positive serum samples obtained from National Public Health Laboratory"** was calculated with,

Confidence Interval:	95%
Error:	5%
Prevalence (probability):	15% (Waseem *et al.*, 2009)

Thus, sample size of 196 calculated using WINPEPI Version 3.8.

Limitation

Data drop-out was due to:

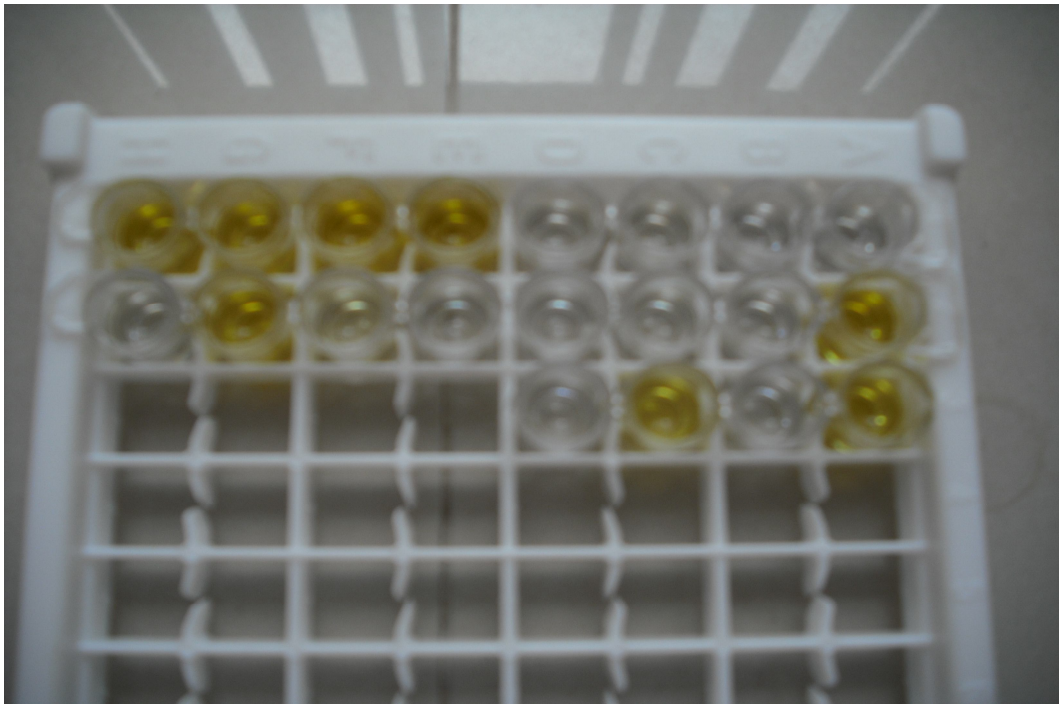
a) Loss of serum sample due to haemolysis
b) Insufficient amount of serum sample

Thus, total sample size taken for the syphilis study was 303 out of 335.

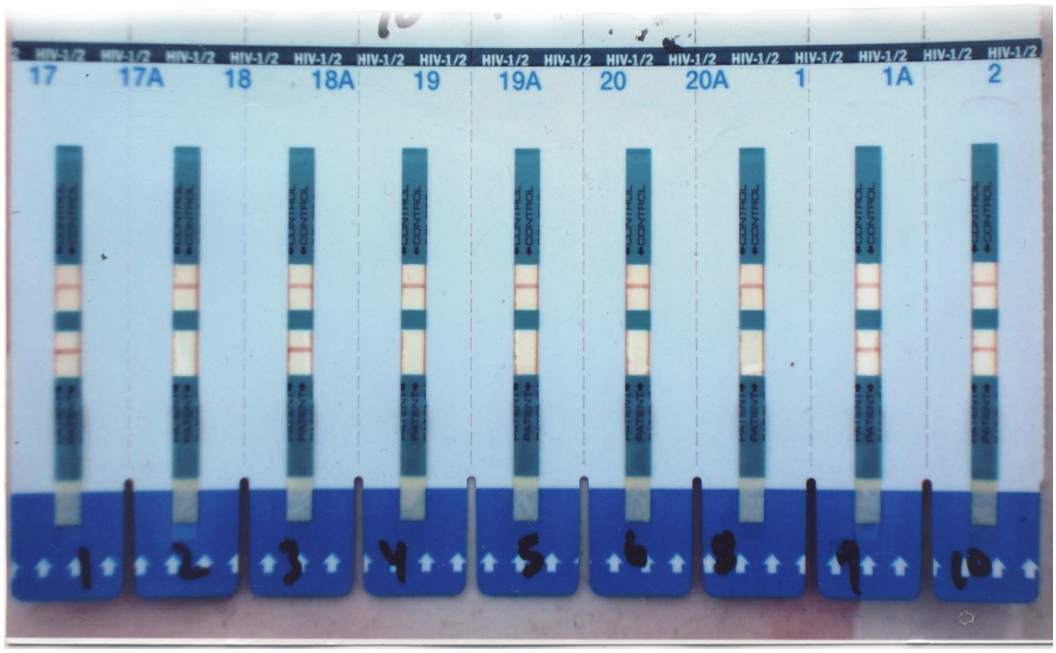
APPENDIX X

QUESTIONNAIRE

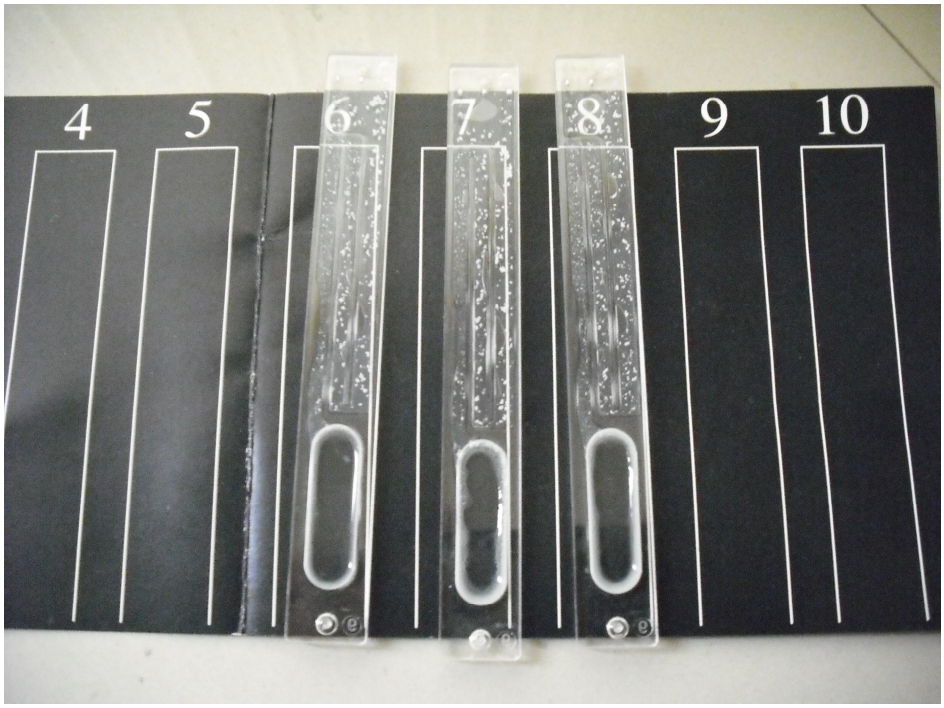
1. Date of sample testing: _____
2. Laboratory number/Unique ID: _____
3. Name of the patient: _____
4. Address: _____
5. Age: _____ Sex: _____
6. Recommended test: _____
7. Short clinical history: _____



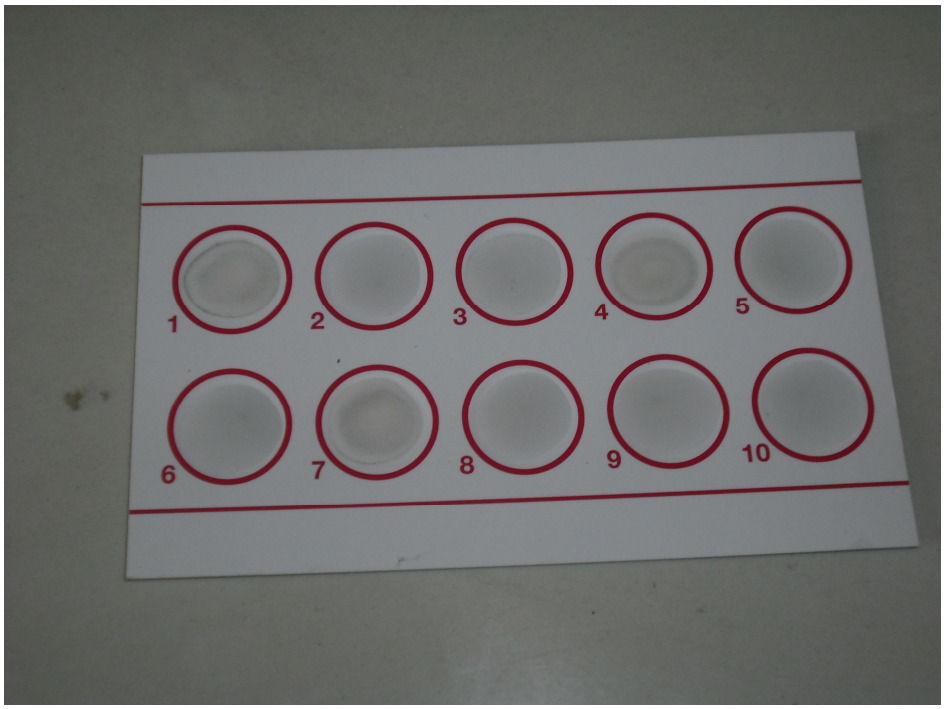
Photograph 1: HIV sero-positive (yellow-colored samples) and sero-negative (colorless samples) reactions shown by ELISA technique



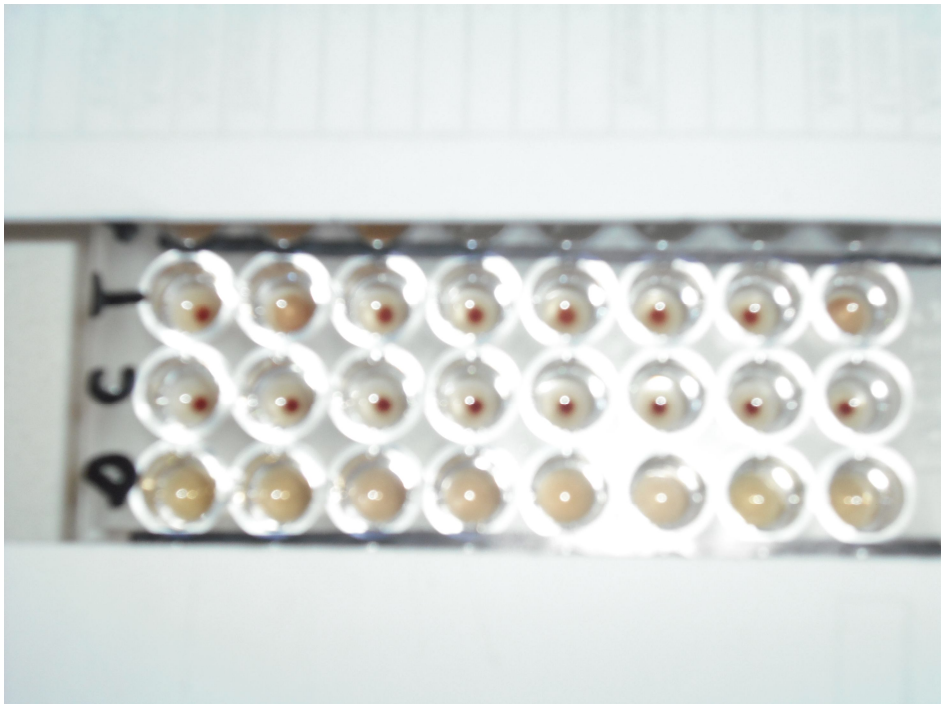
Photograph 2: HIV sero-positive (1, 3, 9 and 10) and sero-negative (2, 4, 5, 6 and 8) reactions shown by Rapid test (Determine)



Photograph 3: HIV sero-positive reactions shown by Rapid test (Capillus)



Photograph 4: Syphilis sero-positive (4 and 7) and sero-negative (3, 5, 6, 8, 9 and 10) reactions shown by RPR card test; 1-positive control, 2-negative control



Photograph 5: Syphilis sero-positive (agglutination of cells in ‘T’ column; row 2 being positive control) and sero-negative (compact button of non-agglutinated cells in ‘T’ column; row 1 being negative control) reactions shown by TPHA test