PHYLOGENY AND SEROLOGY OF HUMAN IMMUNODEFICIENCY VIRUS AMONG MOTHERS AND CHILDREN IN KIMPESE, RURAL CONGO (FORMERLY ZAIRE)

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DECLARATION

The work presented in this thesis was conducted solely by myself and I alone have composed this thesis, except where stated otherwise.

"Attitudes are more important than abilities

Motives are more important than methods

Character is more important than cleverness

Perseverance is more important than power

And the heart takes precedence over the head"

(Denis Burkitt, 1911-1994)

DEDICATION

Isosono lokasi lo,

eoka yá losámo lwa sango la nyango'ami,

la bofoka wau ndo ekesa mbi njaso ya wewe

konda mbile engaliki mbi wainènge*.

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* I would like to dedicate this thesis to mum and (late) dad for their love and courage to teach me great values of life since my very early age.

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ABSTRACT

It is over 15 years since HIV was identified as the causal agent of AIDS, and the global epidemic continues to expand at an alarming pace. A cumulative number of 27.9 million people have been infected with HIV since the beginning of the pandemic. Of these, 68% have lived in sub-Saharan Africa. Of the 3 million infants born in the world with HIV, over 90% have been born in Africa. It is in Africa that there are still many problems about the diagnosis of HIV infection and management of infants born to seropositive women. In addition, the molecular features of HIV-1 and their relevance to the transmission from mother-to-child remain poorly understood. The research described in this thesis deals with these two important issues:

1. **Early diagnosis of HIV-1 infection in children born to HIV-1 infected women:** The diagnosis of HIV infection in children born to HIV-1 infected mothers is hampered by the passive transfer of maternal IgG across the placenta which is detectable up to 12 or even 15 months of age. In the first months of life, it is not possible to discriminate the mother's IgG from the child's by using commercially available ELISA techniques. This delays the diagnosis of HIV-1 in children until 18 months of age. Alternatively, several techniques can achieve the diagnosis of HIV-1 infection during the first few months of life. These include the Polymerase Chain Reaction (PCR), measurement of *in vitro* antibody production and co-cultivation of the lymphocytes with stimulated donor cells. All these techniques are expensive and technically unsuitable for small laboratories in Developing Countries. Therefore simple, cheap, sensitive and specific tests, the Antibody class A specific capture enzyme immuno assay (AAC-EIA) and class M specific capture enzyme immunoassay (MAC-EIA) were employed for the detection of

IgA and IgM anti HIV-1 in children born to seropositive mothers. Contrary to the currently held dogma, it was demonstrated in the present study that IgA and IgM DO CROSS the placental barrier. The maternally derived IgA and IgM class anti-HIV-1 decline and are undetectable by 3 months of age, but after this time, detection of these antibodies (IgA and IgM) in infant samples is a strong indicator of HIV infection.

2. **The genotype, the serotype and the level of antibodies against the vertically-transmitted virus:** The relative role of different subtypes of HIV-1, the nature of the antibodies and indeed their amounts in mothers who do and do not transmit infection to their children is largely unknown. All previously described methods on serotyping of HIV-1 are subject to a high level of cross-reactivity. In this study, a *de novo* method for serotyping HIV-1 was developed and applied. It was demonstrated that immunodominant antibodies to the principal neutralising domain of HIV-1, the V3 loop, are not subtype specific and correlate poorly with HIV-1 genotype. Significant cross-reactivity of V3 loop based peptides to react with heterologous peptides may increase the sensitivity of serological assays. In addition, it was shown that there was a lack of correlation between the level of antibodies to V3 loop in maternal samples and the outcome of HIV-1 infection in children. At the molecular level, there was no evidence of preferential mother-to-child transmission of one subtype of HIV-1 over the others. However, a remarkable molecular heterogeneity was observed in a small number of patients examined in this study, with at least 7 subtypes (A, C, D, F, G, H and J) and another possible new subtype of HIV-1 present in the population examined.

ABBREVIATION

AACELISA	Antibody class-A capture Enzyme linked immunosorbent assay
AIDS	Acquired Immunodeficiency Syndrome
ARV	AIDS-related virus
AZT	3'-azido-3' deoxythymidine
BIV	Bovine immunodeficiency virus
bp	base pair(s)
BPC-ELISA	Branched peptidecompetitive enzyme linked immunosorbent assay
BSA	Bovine serum albumine
BW	Buffered water
^{0}C	Degree centigrade
CD4	Cluster determinant 4
CDC	Centers for Disease Control and prevention, Atlanta, USA.
cDNA	Complementary deoxyribonucleic acid
CIZA (CILU)	Cimenterie du Zaire (change recently to CILU, Lukala)
CO (CUT)	Cut off
CTL	Cytotoxic T lymphocyte
CMV	Cytomegalovirus
DRC	Democratic Republic of Congo
dATP	Deoxyadenosine-5'-triphosphate
dCTP	Deoxycytidine-5'-triphosphate
ddATP	2', 3'-dideoxyadenosine-5'-triphosphate
ddCTP	2', 3'-dideoxycytidine-5'-triphosphate
ddGTP	2', 3'-dideoxyguanosine-5'-triphosphate
ddNTP	2', 3'-dideoxynucleoside-5'-triphosphate
ddTTP	2', 3'-dideoxythymidine-5'-triphosphate
dGTP	Deoxyguanosine-5'-triphosphate
DEPC	Diethylpyrocarbonate
DMF	Dimethylformamide
DMSO	Diethylsulphoxide

DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DNAML	Maximum likelihood
dNTP	Deoxynucleoside-5'-triphosphate
DTT	Dithiothreitol
EBV	Epstein-Barr Virus
ECSP	Early Childhood Screening profile
EDTA	Ethylenediaminetetraacetic acid
EIA	Enzyme immuno assay
ELISA	Enzyme linked immunosorbent assay
FIV	Feline immunodeficiency virus
gag	Group antigen
GACELISA	G antibody capture enzyme immuno sorbent assay
GDE	Genetic Data Environment
gp	Glycoprotein
HIV	Human immunodeficiency virus
HBV	Hepatitis B virus
HTLV-I	Human T-cell leukaemia virus type I
HTLV-II	Human T-cell leukaemia virus type II
HTLV-III	Human T-cell leukaemia virus type III
HSV	Herpes simplex virus
HIVIG	Human immunodeficiency virus immunoglobulin
HPLC	High-performance liquid chromatography
HMA	Heteroduplex mobility assay
IDAV	Immunodeficiency associated virus
ICTV	The International Committee on the Taxonomy of Viruses
IgA	Immunoglobulin A
IgM	Immunoglobulin M
IgG	Immunoglobulin G
IME	Institut Médical Evangélique
IVAP	*In vitro* antibody production

KS	Kaposi's sarcoma
kb	Kilobase
kd	Kilodalton
K-ABC	Kaufman Assessment Battery for Children
LTR	Long Terminal Repeat
LAV	Lymphadenopathy associated virus
LIP	Lymphoid interstitial pneumonitis
LP	Liquid phase
µCi	microCurie
µl	microlitre
µg	microgramme
MCT	Mother-to-child Transmission
MA	Matrix
MIP	Macrophage inflammatory protein
MEGA	Molecular Evolutionary Genetics
MACELISA	Immunoglobulin M class-specific capture ELISA
MPEIA	Monomeric peptide enzyme immuno assay
MPP	Mother positive with positive child
MPN	Mother Positive with negative child
MPI	Mother Positive with indeterminate child
MNN	Mother negative with negative child
Min	Minimum
Max	Maximum
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
nm	Nanometre
NN	Negative children of negative mothers (control)
NC	Negative control
NSI	Non-syncytium inducing

OH-	Hydroxyl group
OPD	(Ortho)1-2-phenylenediamine dichloride
OD	Optical density
OD/CO	Optical density divided by the cut off
%	Percentage
^{32}P	Phosphorous emitting β isotope
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PGL	Persistent and generalised lymphadenopathy
PND	Principal neutralising determinant
pol	Polymerase
PB	Primer binding site
PHYLIP	Phylogenetic Inference Package
PBS	Phosphate-buffered saline
PP	Positive children of positive mothers
PN	Negative children of Positive mothers
PI	Indeterminate children born to HIV positive mothers
PC	Positive control
RNA	Ribonucleic acid
RT	Reverse transcriptase
RRE	*Rev* responsive element
RPM	revolution per minute
^{35}S	Sulphur β emitting isotope
STD	Sexually transmitted diseases
SIV	Simian immunodeficiency virus
SP	Solid phase
SI	Syncytium inducing
TAR	Trans-activation response element
TMB	3,3',5,5'-tetra methylbenzidine
TEMED	N,N,N'N'-Tetramethylethylenediamine
TE	Tris-EDTA buffer

TFA	Trifluoroacetic acid
U3	Unique region at 3' end of the genome
U5	Unique region at 5' end of the genome
UNAIDS	United Nations Programme on HIV / AIDS
USA	United States of America
UK	United Kingdom
UV	Ultraviolet
V1-5	variable region 1 through 5
W/V	weight per volume
WHO	World Health Organisation

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PUBLICATIONS AND PRESENTATIONS

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1. **Royal Society of Tropical Medicine and Hygiene** *Genetic and Antigenic variation of HIV-1.* University of Glasgow, March 1997.
2. **Abstracts at IInd International conference on HIV in Mothers and Children, Edinburgh, September 1993.** *Convenient diagnosis of vertically acquired HIV infection by IgA anti-HIV EIA.* **Mokili JLK**, Connell JA, Parry JV, Green SDR, Davies AG, Cutting WAM. **Poster: P2.11.**
3. **Oral Presentation at IInd International conference on HIV in Mothers and Children, Edinburgh, September 1993**: BCG and HIV infection in infants in rural Zaire. Green SDR, Cutting WAM, Davies AG, Nganga A, **Mokili JLK. Abstract O40.**

CHAPTER 1

INTRODUCTION: THE HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 (HIV-1)

1.1. CLINICAL ASPECTS OF HIV-1 INFECTION

1.1.1. HISTORIC OVERVIEW: THE DISCOVERY OF HIV-1

The first few cases of a novel disease, which soon was called the 'acquired immunodeficiency syndrome' (AIDS), were reported in June, 1981 by the Centers for Disease Control (CDC) (Gottlieb *et al.,* 1981.a; Gottlieb *et al.,* 1981.b). The patients concerned in the early reports were mainly homosexual men presenting with *Pneumocystis carinii* pneumonia and mucosal candidiasis (Gottlieb *et al.,* 1981a; Gottlieb *et al.,* 1981b; Colon *et al.,* 1996), hence AIDS was known for some time as a homosexual or 'gay' disease. Coincident with the epidemic in the homosexual community were reports of cases of AIDS among Haitians immigrants to the United States of America (USA), with no history of homosexuality (Vieira *et al.,* 1983). In 1982, the first cases of AIDS in very young children (Centers for Disease Control, 1982; Rubinstein *et al.,* 1983) provided the first clues as to the possibility of an infectious agent being the cause of AIDS. Other cases were also reported among haemophiliacs, transfusion recipients and the sexual partners of high-risk individuals (intravenous drug users and homosexuals). At that time, viruses such as cytomegalovirus (CMV), hepatitis B virus (HBV) and human T-cell lymphotrophic virus type 1 (HTLV-1) were thought to be possible causative agents of AIDS since enlarged lymph nodes were common features among AIDS patients (Gottlieb *et al.,* 1981.b). However, it seemed unlikely that HTLV-1 (or HTLV-2) could be the cause of AIDS as it is unable to kill lymphocytes, but tends to immortalise them (Miyoshi *et al.,* 1981). Conversely, the depletion of CD4 lymphocytes is a characteristic of

advanced HIV-1 infection (Barré Sinoussi *et al.,* 1983; Dalgleish *et al.,* 1984; Rubinstein *et al.,* 1983).

In early 1983, a team led by Montagnier of the Institut Pasteur in Paris isolated a virus from a patient with persistent generalised lymphadenopathy (PGL) and this new virus was given the name of Lymphadenopathy Associated Virus (LAV) (Barré Sinoussi *et al.,* 1983). Subsequently, the same virus was isolated by a different group led by Gallo in the United States of America (USA) (Gallo *et al.,* 1984). They called this 'novel' agent the Human T-cell Lymphotropic Virus type III (HTLV-III) (Gallo *et al.,* 1984). In addition, other names, including AIDS related virus (ARV), immunodeficiency associated virus (IDAV) were also used by other research groups.

The International Committee on the Taxonomy of Viruses (ICTV) agreed on one name, the Human Immunodeficiency Virus (HIV). In 1986, another human retrovirus distinct from HIV, yet with similar biological and morphological properties, was isolated from West African patients and subsequently in Europe and North America (Clavel *et al.,* 1986a; Clavel *et al.,* 1986b; Guyader *et al.,* 1987). The original virus was specified as HIV-1 and the newly identified one as HIV-2.

HIV-2 is transmitted through the same routes and causes a similar spectrum of disease as HIV-1. Whereas HIV-1 is more prevalent and widely distributed throughout the globe, HIV-2 strains have been more limited to patients in several parts of West Africa. There are small numbers of HIV-2 cases in Europe, United States and South America often in individuals with a West African connection (Levy, 1994). Furthermore, HIV-2 appears to be less pathogenic and less transmissible than HIV-1 (Travers *et al.,* 1995; Marlink *et al.,* 1994). The average incubation period

from infection with HIV-2 to the development of AIDS appears to be longer than is is the case for HIV-1. However, data about HIV-1 and HIV-2 infected individuals followed up from infection to the development of AIDS are scarce.

1.1.2. EPIDEMIOLOGY OF HIV-1 INFECTION

1.1.2.1. PREVALENCE AND TRENDS OF HIV/AIDS

Enormous progress has been made in the elucidation of the epidemiology of HIV-1. It is now over 15 years since HIV was identified as the causal agent of AIDS. Although the risk factors for the transmission of HIV-1 are known, the global epidemic continues to expand at an alarming pace. As of July 1996, nearly 1.4 million AIDS cases - in adults and children - had been reported to the World Health Organization (WHO) (UNAIDS, 1997). This represents approximately a 19% increase from the 1,169,811 cases reported by June 1995. Allowing for under-diagnosis, incomplete reporting, and reporting delay, the United Nations Programme on HIV / AIDS (UNAIDS) estimated that over 7.7 million AIDS cases have occurred worldwide since the pandemic began. Due to the average 10 year incubation period, the overall number of AIDS case may merely reflect the trend of HIV infection of a decade ago (UNAIDS, 1997). The UNAIDS estimates the global trends for HIV infection to be 3.1 million new cases during 1996. This figure suggests that there were over 8,500 new infections occurring daily - 7,500 adults and 1,000 children (UNAIDS, 1997). As of November 1997, UNAIDS estimated that about 21 million adults, and more than 800 thousand children were living with HIV / AIDS . Of adult cases, 90% were living in developing countries. Among children, HIV / AIDS prevalence was estimated to be 35 times greater in the developing world than

industrialized nations (UNAIDS, 1997). In addition, over 9 million children under age 15 have lost their mothers to AIDS, and one in three children orphaned by HIV / AIDS is younger than 5 years old. However, in some areas in the developing countries, the epidemic has shown signs of stability (Green *et al.,* 1994) and even a decrease (Nelson *et al.,* 1996).

1.1.2.2. TRANSMISSION ROUTES OF HIV-1

The routes of transmission of AIDS were known well before the identification of the aetiology. It is generally admitted that HIV is transmitted (i) through sexual contact, (ii) by parenteral routes and (iii) from an infected mother to her offspring.

1.1.2.2.1. SEXUAL AND PARENTERAL ROUTES OF HIV-1 TRANSMISSION

The transmission through anal intercourse was suggested in early 1981 following an unusual outbreak of *Pneumocystis carinii* among homosexual men as a result of an unknown infectious agent responsible for inducing immunodeficiency (Gottlieb *et al.,* 1981.b; Masur *et al.,* 1981). Anal intercourse between men has been the common mode of transmission of HIV in the United States, whereas heterosexual transmission is the predominant mode of transmission in Africa, in Asia and in Central and South America. Heterosexual transmission of HIV has direct consequences in that women at child-bearing age also become infected with the virus may also transmit the virus to their offspring (see section 1.1.2.2.2.). Social and economic changes currently observed in many African countries may have adverse effects on the HIV epidemic. Mulder *et al.* (1994) measured the excess death attributable to the HIV epidemic in rural Uganda. In this study the mortality rate among

seronegative adults was 7.7 per 1000 compared with mortality rate as high as 115.9 per 1000 seropositive adults of the same population. Also, 50-80% of all deaths in this population were associated with HIV-1 infection. Similar findings by Ryder *et al.* (1994a) showed higher rates (5-10 fold) among seropositive women, their partners and children than among HIV-1 seronegative control women. These high mortality rates have direct effects upon economic growth, demographic and social stability in many countries. For example, Ryder *et al.* (1994b) in another study assessing the socioeconomic consequences of HIV, note that children born to seropositive mothers are at greater risk of becoming orphans than children born to seronegative mothers. Once again the burden lies within developing nations, with 90% of maternal orphans living in sub-Saharan Africa (UNAIDS, 1997).

Parenteral transmission through the transfusion of contaminated blood or blood products is another important route of transmission. The importance of this route in the United States and Western industrialised countries was substantially reduced when HIV-1 antibody testing was introduced (Selik *et al.,* 1993). However, in developing countries, many transfusions are still being carried out without prior screening for HIV-1 antibodies in donated blood units. In addition, transmission by the parenteral route can occur through the use of contaminated and shared needles among drug users (review in Kaplan, 1989; Hoelscher *et al.,* 1994). The risk of needle-stick injuries with infected blood among health care workers appears to be low. In one study, only 0.36% of cases (n=1103) seroconverted following accidental injury with HIV contaminated needles (Tokars *et al.,* 1993). However, people who accidently prick themselves with contaminated needles need better standards of care.

1.1.2.2.2. MOTHER-TO-CHILD TRANSMISSION OF HIV-1

Another important route of transmission of HIV-1 is from an infected mother to her child: mother-to-child transmission (MCT). Of the estimated cumulative number of 3 million infants born in the world with HIV since the beginning of the epidemic, over 90% have been born in Africa (World Health Organisation, 1996; UNAIDS, 1997). Among children, MCT represents the main source of infection though transmission attributable to parenteral routes is also possible.

It is difficult to determine the time when MCT of HIV occurs. Transmission can occur *in utero*, and/or at the time of delivery, and/or *intrapartum*, and/or postnatally through breast-feeding. However, the relative contribution of these periods is not precisely known. Generally, the timing of HIV transmission from mothers to their children is very variable and at present no method can determine with accuracy the time and mechanism of transmission. In most cases occurring in the developing countries a large proportion of all vertical transmission of HIV occurs before delivery. Krivine *et al.* (1992) using highly sensitive tests were unable to detect HIV-1 infection at birth in almost 70% of babies who subsequently were found to be infected. These findings suggested an active replication of HIV during the first weeks of life and support the hypothesis that transmission of HIV-1 takes place either at the end of pregnancy or at delivery. A number of *in utero* infections occur at an early gestational period. This was confirmed by the detection of virus antigens by immunohistochemistry and *in situ* hybridization in abortus material as early as 8 weeks (Lewis *et al.,* 1990) and from a 15 week old fetus (Schafer *et al.,* 1986). However, these findings still need to be confirmed by independent investigators. Nevertheless, studies in Africa have shown

that early transmission of HIV may adversely affect pregnancy outcome: fetal wastage, prematurity, stillbirth and neonatal death (Braddick *et al.,* 1990; Temmerman *et al.,* 1990; Ryder and Temmerman, 1991). Other studies (Gloeb *et al.,* 1988; Selwyn *et al.,* 1989), however, failed to show any of these outcomes to be attributable to HIV in pregnant women.

In studying a cohort of children born to HIV-1-infected mothers in Kinshasa, former Zaire, Bertolli *et al.,* (1996) estimated 23% of infected children might have had intrauterine and 65% intrapartum/early postpartum transmission. In this study, labour and delivery played a significant role in MCT of HIV-1. Some gynecological conditions like *placenta previa*[1], when a large amount of blood may enter the fetal circulation, may be a major source of infection with HIV at the end of pregnancy. During labour, uterine contraction may drive maternal blood into the fetal circulation, particularly if the labour is intense, difficult or prolonged. Tissue damage may occur as the result of labour or obstetric intervention, e.g. forceps delivery which increases the risk of blood-to-blood contact between the mother and the fetus. It is likely that children are susceptible to HIV infection when they pass through the birth canal, where they may ingest viral particles present in maternal blood and other fluids.

In addition to MCT of HIV-1 *in utero* or during delivery, there is an additional risk of postnatal infection through breast-feeding. HIV-1 was detected by culture in breast milk of 3 asymptomatic women (Thiry *et al.,* 1985) and raised the concern about postnatal transmission through breast feeding. Confirming this theory, Ziegler *et al.,* (1985) reported a case of HIV infection in a breast-fed child whose mother had

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[1] a placenta which develops in the lower uterine segment, in the zone of dilatation accompanied with painless haemorrhage in the last trimester, particularly during the eighth month.

acquired the virus by blood transfusion after caesarian section. Others (Behets *et al.*, 1991) found no significant role of breast-feeding but have shown that breast milk confers protection against gastrointestinal and upper respiratory illness in children born to infected mothers. Another study by Van de Perre *et al.* (1993) showed that detection of integrated viral DNA in the mother's cells (in breast milk) is a risk factor for infectivity but the presence of anti-HIV-1 specific IgA and IgM conferred protection against postnatal transmission of HIV-1. Because 1 in 7 breast-fed children of HIV positive mothers acquire post-natal HIV, this protective effect is often insufficient. Computer models have been developed to assess the impact of breast-feeding, HIV infection and socioeconomic factors on under-five mortality and to assist in evaluating implications of breast-feeding policy. In the model proposed by Del Fante *et al.* (1993), analysing data from a hypothetical West African country, it was shown that in urban areas, under-5 mortality would increase by 27% and 108% among children born to seropositive and children born to seronegative mothers, respectively, if breast-feeding ceased. In general, not breast-feeding would increase the mortality rate in that hypothetical setting by 82%. Dunn *et al.* (1992) estimated the total risk of infection through breast-feeding by seropositive mothers: when mothers have been infected prenatally, the additional risk of postnatal transmission of HIV-1 through breast-feeding, above transmission *in utero* and *intrapartum* is estimated at 14%. When mothers become infected postnatally, the risk of transmission is 29% (Dunn *et al.*, 1992). A possible explanation of the increased risk of transmission through breast milk when maternal infection has occurred post-natally, may be the viraemia associated with the primary infection period.

The protective properties of breast milk in the presence of HIV-1 infection have been previously documented (Victoria *et al.*, 1987; Howie *et al.*, 1990). In general, the protection provided by breast-feeding against a variety of illnesses is independent of associated personal and social factors (Cunningham (1979). In addition to psychosocial and child-spacing benefits, breast milk contains protective elements including immunological components (IgA, IgM, IgG, cells) and biochemical factors (lysozyme,

lactoferrin, lactoperoxidase, bile salt stimulated lipase). It also contains factors like complement, *Lactobacillus bifidus* growth factor and nutrients that make breast milk the best food for the new born especially in developing countries (Cunningham, 1979).

The rate of vertical transmission of HIV varies widely from study to study (Dabis *et al.,* 1993). It is higher in developing countries than in the industrialised World. In Africa for example, the rates of vertical transmission of HIV may range between 25% and 48% whereas in Europe and North America figures between 13% and 32% have been reported (Dabis *et al.,* 1993). No direct comparison could be made between these studies because the methods of calculation of mother-to-child transmission rates were different. Therefore a standardized method has been proposed by the Ghent Working Group of Mother-to-Child Transmission of HIV to allow all studies to report rates of transmission in a comparable fashion (Dabis *et al.,* 1993).

1.1.2.2.3. MATERNAL FACTORS INFLUENCING MCT OF HIV-1

Not all children born to HIV-seropositive women are infected. About 60-80% of such children remain uninfected and it is difficult to predict accurately the outcome of children born to HIV-infected mothers. Maternal factors have been suggested to play an important role in MCT of HIV. These include p24 antigenemia (StLouis *et al.,* 1993), high viral RNA load (Dickover *et al.,* 1996), impaired immune system determined by low CD4 count (<700/mm^3), or CD4-to-CD8 ratio (<0.6) or the stage of clinical AIDS (Newell *et al.,* 1992; Ryder *et al.,* 1989; StLouis *et al.,* 1993). However, asymptomatic seropositive pregnant women, with no indicators that maternal infection is advanced, may also transmit HIV to their offspring. This has been shown in a study carried out in Congo (then Zaire) that HIV-1 infected women with normal CD4 counts

but higher CD8 counts (>1800) were at increased risk of transmitting HIV, although some of them were at the early stage of infection (StLouis *et al.,* 1993). Becoming pregnant during the year of seroconversion is another risk factor for MCT of HIV (Hague *et al.,* 1993). This may be explained by the fact that this particular period is characterised by intense replication of HIV as indicated by p24 antigenemia (Newell *et al.,* 1992; Krivine *et al.,* 1992).

The structure of the placental barrier if damaged by chorioamnionitis may facilitate infection of the fetus. This inflammation of the placental membrane may be due to intercurrent infections including sexually transmitted diseases (STD) during pregnancy. In our own study (Green *et al.,* 1991) as in others (St Louis *et al.,* 1993), placental membrane inflammation was associated with perinatal transmission. In addition, the synergistic effect of pregnancy and HIV may cause reactivation of several infectious agents including herpes simplex virus (HSV) and cytomegalovirus (CMV). HSV and other STDs such as syphilis (*Treponema pallidum*) and chancroid (*Hemophilus ducreyi*) cause cervicitis and genital ulcers and these agents may enhance the risk of both intrauterine and *intrapartum* transmission of HIV. In a population of high prevalence of HIV-1, unprotected sexual intercourse with multiple partners before and during pregnancy was shown to increase the likelihood of MCT of HIV-1 (Bulterys *et al.,* 1993).

Several studies have been conducted to determine the role of maternal antibodies in the reduction of MCT of HIV. Results on this topic remain conflicting. Whereas some studies (Scarlatti *et al.,* 1993a; Scarlatti *et al.,* 1993d; Rossi *et al.,* 1989; Devash *et al.,* 1990) reported that maternal antibodies to the principal neutralising domain (PND) of the envelope glycoprotein gp120 and gp41, protect the fetus from

infection, others (Parekh *et al.,* 1991; Halsey *et al.,* 1992; Robertson *et al.,* 1992) failed to confirm the findings. This discrepancy may be due to lack of sufficient information about the neutralising activity of the antibodies or the choice of strains or peptides to make up assays. In the present thesis, the role of maternal antibodies to PND using a cocktail of peptides from different subtypes of HIV-1 was also investigated (for more information see chapter 6).

1.1.2.2.4. PREVENTION AND INTERVENTION TO REDUCE MCT OF HIV-1

The development of strategies to prevent vertical transmission of HIV is an important objective to reduce and even eliminate the possibility of MCT of HIV. The first priority in preventing MCT of HIV is to prevent women in childbearing age becoming infected with HIV. This can be achieved by education programmes for AIDS awareness in juvenile populations before they become sexually active. Routine antenatal screening of pregnant women and early diagnosis of HIV infection in the newborn are important for appropriate counselling. Named (rather than unnamed and unlinked) testing of pregnant women as shown in a study from Sweden (Lindgren *et al.,* 1993) can provide valuable data on the importance of spread of the epidemic and opportunities for counselling to reduce vertical transmission of HIV in later pregnancies.

A number of studies of intervention strategies to reduce vertical transmission of HIV are currently underway. An option for preventing perinatal transmission of HIV, consists of the treatment of pregnant women at the end of pregnancy or *intrapartum* with antiretroviral drugs. The AIDS Clinical Trial Group using the protocol 076 (ACTG076) have shown some benefit in the use of zidovudine (AZT) in order to

reduce the rate of mother-to-child transmission of HIV-1. A reduction (by 60%) of MCT was observed as a result of of intervention using AZT (Wiznia *et al.,* 1996; Connor *et al.,* 1994). Long term harmful effects of AZT on the fetus or on the pregnancy remain unclear. A particular concern is AZT's teratogenicity[1]. In a study by Toltzis *et al.* (1994) a direct effect on developing mouse embryos was reported, when AZT was administered in early pregnancy.

With the existing evidence that HIV immune plasma can neutralise circulating infectious HIV-1 (Karpas *et al.,* 1990 and 1994), passive immunisation of pregnant women was suggested as another option for prevention of MCT of HIV-1. This involves administration of anti-HIV immunoglobulins (HIVIG) to HIV-1 positive pregnant women. However, there are conflicting reports about the value of maternal immunoglobulins in the prevention of MCT of HIV.

Active immunisation of pregnant women during pregnancy is another type of intervention. In a previous study by Vanrompay *et al.* (1996), active immunisation of pregnant rhesus macaques successfully prevented infection with simian immunodeficiency virus (SIV). It was suggested that strategies such as active immunization of HIV-infected pregnant women may decrease the rate of perinatal HIV infection. However, the lack of understanding of the immune responses necessary to prevent and contain HIV-1 constitutes the most basic problem in the development of an HIV vaccine (Ada, 1992). It is not clear whether or not there is a protective immune response to HIV due to intracellular transmission. A number of candidate vaccines have been developed and their clinical trials have been initiated

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[1] Fetal hematologic toxicity

(table 1.1). It will take several years before the value of these vaccines is established and they become readily available for use worldwide.

There is increasing evidence that most MCT of HIV occurs close to delivery or when the baby passes through the birth canal (Newell *et al.,* 1992; Goedert *et al.,* 1991; Krivine *et al.,* 1992). Elective caesarean section has been suggested as a possible intervention procedure to reduce MCT rate of HIV-1 (Newell *et al.,* 1992; Newell *et al.,* 1994). The European Collaborative Study found that the likelihood of MCT of HIV-1 was higher in vaginal delivery than when babies were delivered by elective caesarean section. In this study, there was no statistical difference between the two modes of delivery, possibly due to confounding factors (Newell *et al.,* 1992). In another study, when confounding factors such as maternal clinical stage were taken into account, it was clear that caesarean section could halve the rate of MCT of HIV-1 (Newell *et al.,* 1994).

Table 1.1. HIV vaccines relevant to MCT [§]

Trial	Vaccine	HIV-1 strain	Target population
AVEG 102	rgp160	LAI	HIV infected pregnant women
AVEG 104	rgp120	MN	HIV infected pregnant women
ACTG 185	Hyper HIV IgG	Pooled HIV+ plasma	HIV infected pregnant women
AVEG 007B	rgp120	SF-2	HIV uninfected individuals
AVEG 003	rgp160	LAI	HIV uninfected individuals
AVEG 009	rgp120	MN	HIV uninfected individuals
Army	rgp160	LAI	HIV uninfected individuals
AVEG 103	rgp120	SF-2	HIV infected individuals

[§] modified from McElrath and Corey, 1994.

Other preventive measures such as cleansing of the birth canal before membrane rupture have been suggested as a way to reduce the risk of transmission for infants born to HIV-1 infected women (Goedert *et al.,* 1991). The efficacy of such a procedure in reducing perinatal transmission was examined by Biggar *et al.* (1996) in Malawi. In that study, the intervention had no significant impact on HIV transmission rates, except when membranes were ruptured more than 4 hours before delivery (transmission 25% in the intervention group vs. 39% in the control group).

The only effective way to prevent postnatal transmission of HIV-1 occurring through breast-feeding is by providing alternative methods of feeding to new born babies. However, this recommendation is not universal. It depends on circumstances, and varies with the environmental and domestic circumstances of the mother (World Health Organisation, 1992).

1.1.3. NATURAL HISTORY OF HIV-1 INFECTION

Without intervention, infection with HIV-1 is a slow, progressive and irreversible process, from the initial infection to what has been designated as 'full-blown AIDS'. The clinical manifestations of HIV-1 infection have been grouped by the Centers for Disease Control and Prevention (CDC) into four clinical stages: group I, II, III and IV (Table 1.2; Centers for Disease Control, 1987). This classification was modified in 1992, to include CD4+ cell count and other immunological and clinical conditions (Centers for Disease Control, 1992). However, at present few laboratories in the developing countries can afford the cost for CD4 estimation which makes it difficult to compare data from different studies.

The early phase of HIV-1 infection (group I) starts from the time the individual comes in contact with the virus until the production of antibodies or illness. This period may vary between 1 to 4 weeks for the 'flu-like' illness and up to 6 weeks after which some neurological conditions can be observed (Ho *et al.,* 1985). A wide variety of symptoms and signs can be observed during the early stage of HIV-1 infection, the commonest of which are fever, malaise, diarrhoea, myalgia, sore throat, headaches and maculo-papular rash (Ho *et al.,* 1985; Kinlochdeloes *et al.,* 1995). During this phase, the plasma HIV-1 titre (as measured by p24 Ag, plasma RNA) can show a substantial increase (Clark *et al.,* 1991; Daar *et al.,* 1991). The fact that the observed plasma viremia is only transient suggests that the immune system, and more precisely, the cellular cytotoxic T lymphocytes (CTL) may play an important role in containing the replication of HIV-1 (Autran *et al.,* 1996). Occasionally, the CD4 counts in some patients can show a decrease (Fahey *et al.,* 1990; Bofill *et al.,* 1996). The appearance of CTL activity coincides with a decrease in viral load as monitored by plasma viremia (D'Souza and Mathieson, 1996).

TABLE 1.2: Centers for Disease Control case classification of HIV infection

CDC Stage	Clinical manifestation
Group I	Acute infection: flu-like illness
Group II	Asymptomatic infection
Group III	Persistent generalised lymphadenopathy (PGL)
Group IV	
Subgroup A	Constitutional disease: weight loss (>10% of the normal body weight), fever (>38^{0}C), diarrhoea (>2weeks).
Subgroup B	Neurological disease: HIV encephalopathy, myelopathy, peripheral neuropathy.
Subgroup C	Secondary infectious diseases:
	C1: AIDS defining secondary infectious disease (*Pneumocystis carinii* pneumonia, cerebral toxoplasmosis, cytomegalovirus retinitis)
	C2: Other specified secondary infectious diseases (oral candidiasis, pulmonary tuberculosis, multidermatomal varicella zoster).
Subgroup D	Secondary cancers: Kaposi's sarcoma, non-Hodgkin's lymphoma.
Subgroup E	Other conditions (lymphoid interstial pneumonitis).

(Centers for Disease Control, 1987)

Early high virus load with sustained viremia is often accompanied, in both adults and infants, by the inability to mount an effective immune response, resulting in rapid disease progression (D'Souza and Mathieson, 1996). Seroconversion with the production of detectable antibodies, takes place approximately 4 weeks after infection. Antibodies to the envelope glycoprotein gp120 are the first to be detected followed by antibodies to p24 (*gag*), p53 and p64 (*pol*) proteins. The role of these antibodies in the neutralisation of the virus has been reported by several investigators (Poignard *et al.*, 1996; McElrath *et al.*, 1996; Sattentau, 1996; Mascola *et al.*, 1996; Halsey *et al.*, 1992). However, the time at which neutralising antibody appears is variable and unpredictable (D'Souza and Mathieson, 1996). It appears that the main target for the neutralising antibodies is the gp120 glycoprotein, particularly the principal neutralising determinant (PND), the V3 loop, situated at the tip of the envelope hypervariable region (Kuiken *et al.*, 1993; Zwart *et al.*, 1991; Wolfs *et al.*, 1991; Javaherian *et al.*, 1990). Another hypervariable region, V2 (Warrier *et al.*, 1994; Gorny *et al.*, 1994; Moore *et al.*, 1993), and the glycoprotein gp41 (Purtscher *et al.*, 1994; Muster *et al.*, 1994; Back *et al.*, 1993) have also been reported to elicit the production of neutralising antibodies. However, the mechanism of neutralisation still remains unclear.

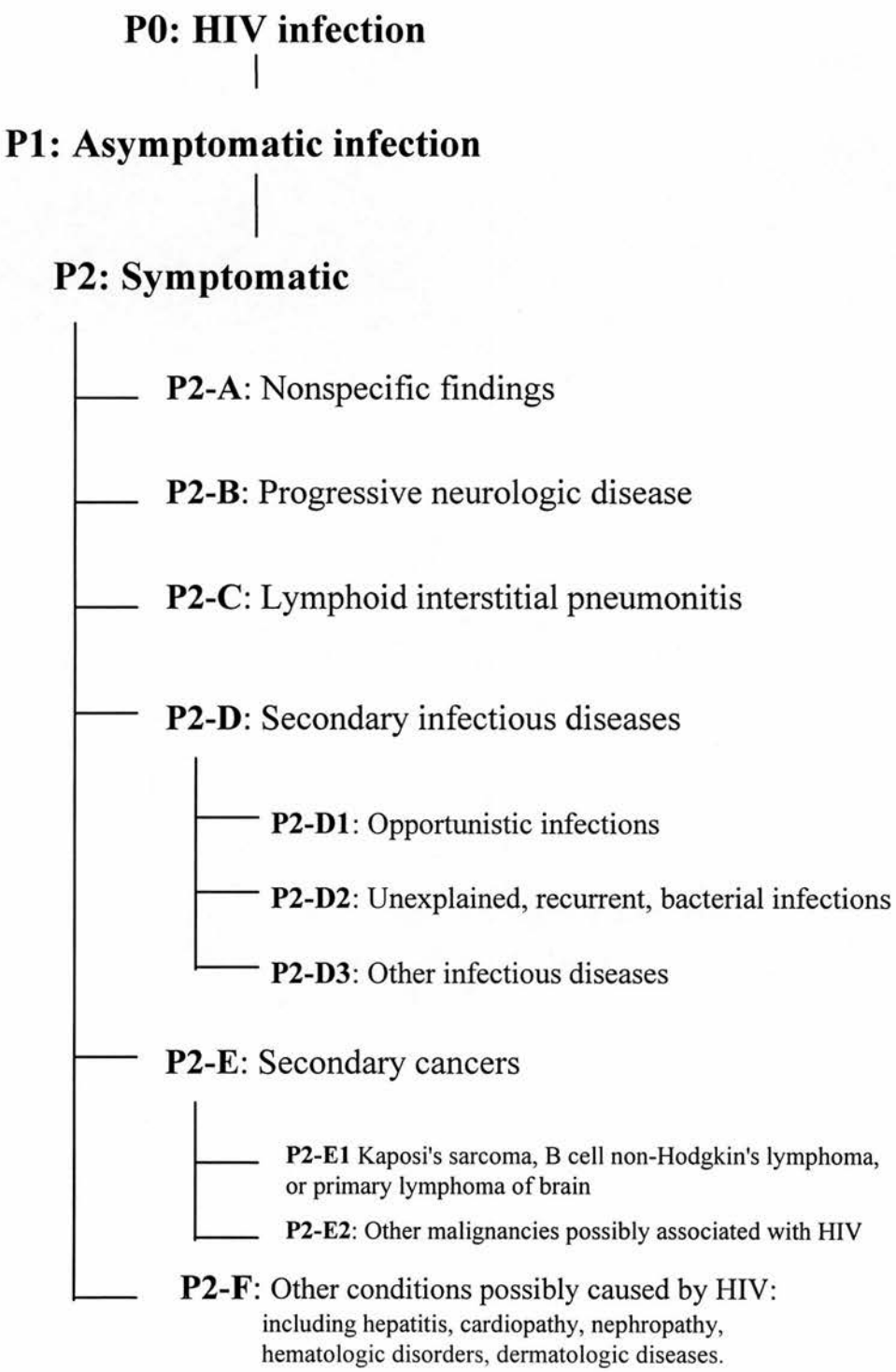
Patients in group II present no clinical symptoms or signs (hence asymptomatic HIV infection). From a virological point of view, this period is characterised by a high viral turnover of infected cells (Wei *et al.*, 1995; Ho *et al.*, 1989). The asymptomatic period can last from a few months to several years until the development of painless systemic, enlarged lymph nodes- a state called persistent generalised lymphadenopathy (PGL). PGL is the main characteristic of the CDC stage III. Occasionally, night sweats

and weight loss can be observed in addition to the markedly asymmetrical and enlarged lymph nodes.

During the CDC IV stage, a wide range of clinical illnesses can be noted, and have been subdivided into subgroups A to E (Table 1.2). Individuals classified within the CDC group IV-A present with one or more of the following symptoms: fever (>1 month), diarrhoea (>1 month) and/or involuntary weight loss (>10% of the individual normal weight) as a direct consequence of HIV infection. The subgroup B includes individuals presenting with neurological conditions including dementia, myelopathy and peripheral neuropathy, which are a direct consequence of HIV-1 invading the cerebro-spinal system. The group C includes patients presenting with secondary infectious diseases and this subgroup is divided into two categories: C1 and C2. Patients classified in the C1 category present one or more AIDS defining secondary infectious diseases (*Pneumocystis carinii* pneumonia, cerebral toxoplasmosis, cytomegalovirus retinitis). C2 includes individuals with other specified secondary infectious diseases such as oral candidiasis, pulmonary tuberculosis and/or varicella zoster. Subgroups D and E are associated with the secondary cancers (Kaposi's sarcoma, non-Hodgkin's lymphoma) and other clinical conditions (such as lymphoid interstitial pneumonitis) (Table 1.2).

The natural history of HIV infection in children born to seropositive mothers requires additional consideration and needs to be interpreted from a paediatric point of view (table 1.3). In general, HIV-infected children appear well for the first few months although some immune abnormality can be noticed. Some infected children can remain well for more than 5 years. The CDC classification for children born to seropositive mothers is summarised in Figure 1.1 (details see appendix 2). The clinical features in children are classified by categories or classes: class P-0, class P-1, and class P-2. The class P-0 includes children (<15 months old), in whom the HIV status cannot be established, by lack of definitive evidence of HIV infection or AIDS. The P-0 is followed by the asymptomatic infection stage (class P-1), including children with normal immune function (P-1, subclass A) or those with apparent immune disfunction (P-1, subclass B) including hypergammaglobulinemia, CD4 lymphopenia, decreased CD4-to-CD8 ratio or absolute lymphopenia. The asymptomatic P-1 class is followed by the class P-2, symptomatic infection phase and is subdivided into 6 (A-F) subclasses.

FIGURE 1.1. Classification for HIV infection in children (CDC, 1992; Appendix 3).



1.1.4. LABORATORY DIAGNOSIS OF HIV INFECTION

The wide variability of the time when MCT occurs makes the diagnosis of HIV infection, the management of children and counselling of mothers very complicated. Most proposed clinical criteria for paediatric classification of HIV infection, including those proposed by the CDC classification have low sensitivity, specificity and positive predictive value especially in very young children, in developing country settings. The diagnosis of HIV infection in children born to seropositive mothers is more accurate if based on laboratory findings. In adults, serological diagnosis of HIV is based on the detection of IgG antibodies in plasma or serum or other body fluids of infected individuals. Unfortunately, the early diagnosis of HIV infection in children is hampered by the passive transfer of maternal antibodies into childrens' circulation. These maternal antibodies persist in the child's circulation for a mean time of 10 months (Mok *et al.,* 1987) and in some cases they can be detected at up to 2 years (Newell and Peckham, 1993). Generally, 15 months is the cut-off time after which, if a baby is found to have HIV specific IgG, he or she may be considered as infected.

To overcome the difficulty associated with this delay, alternative serological methods for early diagnosis have been sought. These are based on 2 principles: a) detection of antibodies that have a short half-life and b) detection of antibodies that 'do not cross placenta'. Based on the hypothesis that maternal IgA and IgM do not cross the placenta, their detection has been used to establish a diagnosis of HIV-1 infection of children (reviewed in Sison and Campos, 1992). Evaluation of the sensitivity and specificity of this method is hampered by various factors including the variation of time of infection of the baby and the level of B cell impairment that might result in

hypogammaglobulinemia. Early diagnosis of HIV by detection of IgA and IgM in the baby was studied in this thesis and the results are presented in chapter 4.

Detection of p24 antigen with or without preliminary acid dissociation treatment is another possible means of diagnosis of HIV in children (Quinn *et al.,* 1993). However, it is essential that p24 is dissociated from the antibody so that it can be identified and is not masked in the complex. By prior treatment of the sample with acid (acid dissociation), the sensitivity of the test can be increased from 18 % to 90% (Quinn *et al.,* 1993).

The *in vitro* antibody production (IVAP) technique is another research tool for HIV antibody detection in the new-born (De Rossi *et al.,* 1988; Sison and Campos, 1992). It relies on the principle that during HIV infection, B cell lymphocytes are activated to produce antibodies. *In vitro*, peripheral blood lymphocytes from a patient suspected of HIV infection are isolated and cultured. Antibody production from sensitised cells is activated by mitogen such as pokeweed or a virus such as Epstein-Barr virus (EBV) which induces polyclonal B cell activation. The presence of HIV-1 is confirmed by testing the culture supernate for the presence of HIV-1 antibodies. IVAP has been used by DeRossi et al (1988) for the diagnosis of HIV infection in children. They identified 92.5% of 27 infected children but there is not much information on the evaluation of this technique on a larger sample and its use may be limited to well-equipped laboratories.

Viral culture can also be used for the diagnosis of vertically acquired HIV infection in infants. The sensitivity and specificity of the HIV culture by 6 months has approached 100%, but varied with reports of 38-50% at birth and increasing thereafter (Hutto *et al.,* 1991; Rogers *et al.,* 1991; Sison and Compos, 1992). This variation may

be related to the time of infection and the amount of virus in the baby at the time of culture. Culture can be of limited value in small laboratories in developing countries as it needs to be performed in well equipped laboratories with adequate safety measures. However, HIV culture is not a rapid diagnostic test, as it can take up to 28 days to complete (Sison and Compos, 1992).

The most successful method for early diagnosis of vertically acquired HIV in children is probably the polymerase chain reaction (PCR) (Paul *et al.,* 1996; Newell *et al.,* 1995; Roques *et al.,* 1995; Newell *et al.,* 1996; Bertolli *et al.,* 1996; Sison and Campos, 1992; Krivine *et al.,* 1992). The PCR technique allows the amplification of the viral nucleic acid by a million - to a billion fold. The method is dealt with at length in the present study (see Materials and Methods) and the results are presented in chapter 5.

1.2. VIROLOGICAL ASPECTS OF HIV-1

1.2.1 OVERVIEW OF THE *RETROVIRIDAE*

A unique feature of the retroviruses is that they contain an enzyme called reverse transcriptase (RT), which enables them to replicate "backwards" (hence *retro*), from RNA to DNA. The cryptogram code of the retroviruses is:

[R/1: 7-10/2: Se/* : V/C, I, O, R].

This code summarises the general features of the members the retrovirus family: virus contains a single strand of RNA of 7-10 million molecular weight (about 2% of the infective particle). The virus particle is essentially of spherical symmetry and is enveloped. The systematic classification of *Retroviridae* is shown in Table 1.3. Based on the viral structure, the utilisation of particular cell receptors, the presence or absence of oncogenes and other pathogenetic properties, the ICTV subdivides retroviruses into 3 subfamilies. These are the oncoviruses, the spumaviruses and the lentiviruses.

The oncoviruses include a variety of viruses (types A-D) associated with the activation of certain cell genes leading to tumour development. They cause disease in both animals and humans. The spumaviruses are readily isolated from humans and animals (particularly primates) but have not been associated with any specific disease. The lentivirus genus includes viruses responsible for a range of slow progressive (hence *lenti-* which means slow) neurological and immunological diseases; the viruses are not directly implicated in malignancy. The prototype member of the *Lentivirinae* is the visna virus of sheep which can cause slow but progressive pathological conditions in the brains of infected animals. Other members

of the subfamily of lentiviruses include the equine infectious anaemia virus, the caprine arthritis-encephalitis virus, the feline and bovine immunodeficiency viruses (FIV and BIV) and the human and simian immunodeficiency viruses (HIV and SIV) (Clements *et al.,* 1988; Levy, 1994).

TABLE 1.3. The family of Retroviridae

Subfamily	Example
Oncoviruses: *associated with immortalisation of host cells*	
Type A	Mouse intracisternal type A
Type B	Mouse mammary tumour virus
Type C	Murine leukaemia virus
	Human T cell lymphotropic virus type I and II
	Feline leukaemia virus
	Bovine leukaemia virus
Type D	Mason-Pfizer virus
Spumavirus: *Isolated in primates and human. Not associated with any specific disease*	
	Simian foamy virus
	Human foamy virus
Lentivirus: *Produce slow and progressive destruction of the immune system*	
	Feline immunodeficiency virus
	Equine infectious anaemia virus
	Bovine immunodeficiency virus
	Simian immunodeficiency virus
	Human immunodeficiency virus
	• HIV-1
	Group M: subtype A, B, C, D, E*, F, G, H, I**, J**.
	Group O
	HIV-2: subtype A, B, C, D and E.

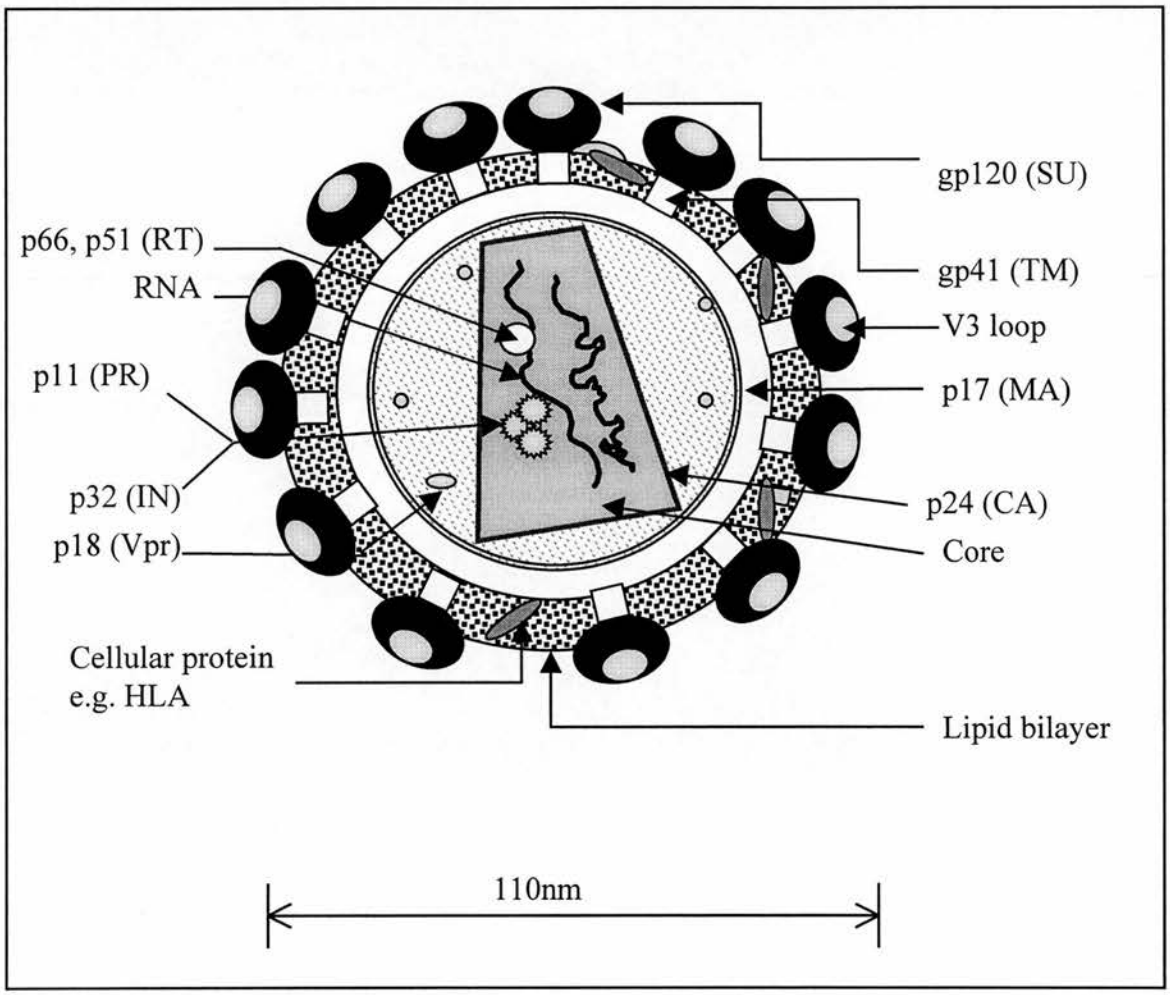
(Table modified from Fields Virology, 1996. * isolates classified as subtype E have been classified as such based on the analysis of the *env* gene of HIV-1 and were shown to be subtype A when the *gag* genes were analysised. **Subtypes I and J are still provisional as more sequences are needed to confirm their classification as distinct cluster).

1.2.2. VIRION STRUCTURE

A schematic diagram of the structure of the HIV-1 virion is represented in figure 1.2. HIV-1 has several characteristics in common with HTLVs and other retroviruses. These include morphological and structural features, biochemical and biological properties (Gallo *et al.,* 1984). The viral particle is about 110nm in diameter. When viewed by electron microscopy, it shows a cone-shaped core which is the viral capsid (or nucleoid) composed of the viral p24 *gag* protein (CA) (Gelderblom *et al.,* 1989; Gelderblom *et al.,* 1987). On its surface, the virus is studded with 70-80 protrusions that project radially. These knobs derive from a structural protein (gp160) consisting of two protein sub-units (gp41 and gp120) encoded by the envelope (*env*) gene (McCune *et al.,* 1988). Each knob has a diameter of about 15nm, a height of 9nm and plays an important role in viral infectivity (Gelderblom *et al.,* 1989; Gelderblom *et al.,* 1987). In addition, the envelope contains other proteins which derive from the host (such as human leukocyte antigens, HLA) which the virus may have acquired when budding through the cell membrane (Cantin *et al.,* 1996; Desantis *et al.,* 1996). These host components may affect virus-host immune system interaction.

The nucleocapsid, or core, is roughly spherical to conical shape measuring about 100nm in length. The viral matrix protein, gag p17 (MA), is attached to the inner surface of the lipid bilayer. The conical capsid protein surrounds two other nucleocapsid proteins (p7 and p9), enzymes (integrase, p11; reverse transcriptase, p66 and p51; and protease, p15) and the two identical copies of the HIV RNA genome (Gelderblom et al., 1987).

FIGURE 1.2: **Structure of the HIV-1 virion shown in cross section** (modified from Levy, 1994)



In brackets are abbreviations of viral proteins: NC, nucleocapsid; PR, protease; RT, reverse transcriptase; IN, integrase; Vpr, regulatory of mature virus particle; SU, surface glycoprotein; TM, transmembrane glycoprotein; MA, matrix; CA, capsid.

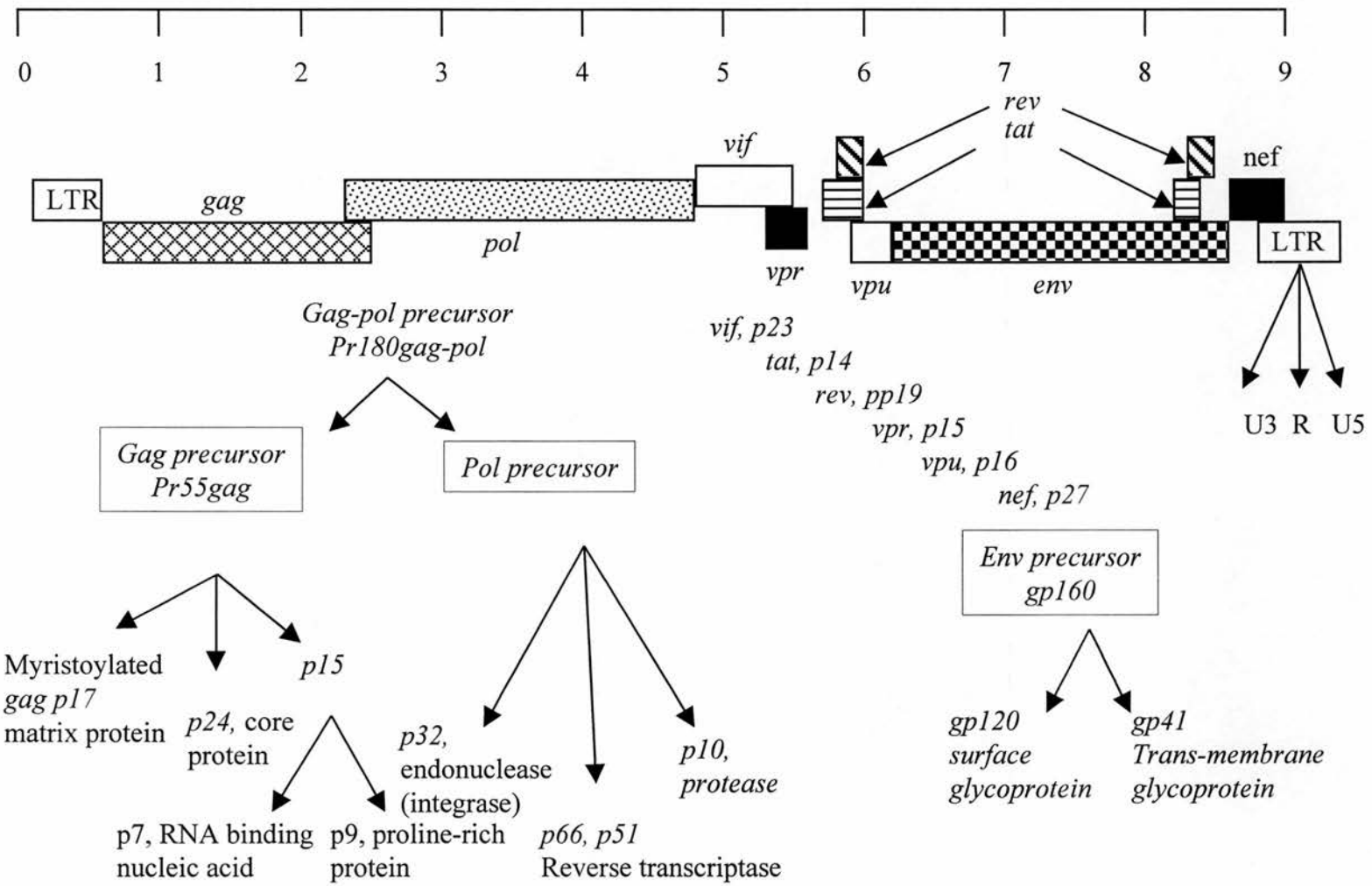
1.2.3. GENOME STRUCTURE AND ORGANISATION

Figure 1.3 shows the schematic representation of the structure of the genome and proteins encoded by various HIV-1 genes. The genome is relatively small, about 9.2 kilobases (kb) long, yet its function is very complex. The genes encode for three major structural proteins (*gag, pol* and *env*) and at least 6 additional proteins involved in the regulation of the viral gene expression. There are also two non-coding regions, known as long terminal repeats (LTRs), at each end of the genome which have identical nucleotide sequences (Wain Hobson *et al.,* 1985; Ratner *et al.,* 1985).

1.2.3.1. THE NON-CODING REGIONS: LONG TERMINAL REPEAT REGIONS (LTR)

The non-coding LTR regions are located in terminal regions (figure 1.3), which contain important recognition signals for the synthesis of DNA or RNA. The LTR has a redundant (R) section, which is the same in both the 3' and 5' ends of the genome. The R sequence plays an important role during reverse transcription by transferring the nascent DNA from one end of RNA to the other. The unique information region (U5) is the first to be translated into DNA and becomes U3 at the 3' end of LTR. There is a simple variable untranslated region separating the *env* and the beginning of the U3, which may play the role of the *rev* gene (Bray *et al.,* 1994). It is thought that some U5 sequences are essential for initiation of RT. Other regions of the LTR include the primer binding site (PB) and the leader site (L) (Rosen *et al.,* 1985; Sodroski *et al.,* 1985a; Sodroski *et al.,* 1985b).

FIGURE 1.3. **Genomic organisation of HIV-1** (modified from Fields Virology, 1996)



1.2.3.2. HIV STRUCTURAL, ENZYMATIC AND REGULATORY GENES

The structural and regulatory genes of HIV-1, their approximate size and the function they play in the viral life cycle are summarised in table 1.4.

A. STRUCTURAL AND ENZYMATIC GENES

The *env*elope (*env*) gene encodes a protein (p85), which is glycosylated to form the envelope precursor protein gp160. It is a monospliced message from the viral mRNA. The cleavage of this gp160 yields the surface envelope gp120 and the transmembrane gp41 glycoproteins (Stein and Engleman, 1990). The attachment of the virus to the cellular receptor (CD4) is mediated by the gp120. In addition, gp41contains amino acids which play an important role in syncytium formation (Wang *et al.,* 1996; Hart *et al.,* 1996). Other spliced mRNA genes of the *env* region encode regulatory and accessory proteins, which play, at various levels, a role during the viral replication (Figure 1.3; Table 1.4.).

The *pol* gene encodes a precursor protein, which is cleaved into the reverse transcriptase (RT), integrase and protease. The RT also has an RNase H activity which degrades the RNA template during the synthesis of DNA (Varmus, 1988a; Varmus, 1988b).

The group specific antigen, *gag,* encodes a 55kd protein precursor that is cleaved by the viral protease into three proteins, the myristoylated matrix protein (p17), the phosphorylated capsid protein (p24) and the nucleocapsid binding protein (p6/7 and p9) (Veronese *et al.,* 1988). The p7 interacts with the viral RNA through its 'zinc finger' (Veronese *et al.,* 1988), and supports the structure of the membrane

(Veronese *et al.,* 1988; Green and Chen, 1990). The p17 region has been the focus of the molecular analysis of HIV-1 isolates described in this thesis.

TABLE 1.4. Structural and regulatory genes of HIV-1

(Modified from Fields Virology, 1996)

Name	***Size***	***Function***
Genes encoding structural proteins		
Env (envelope)	gp160	Precursor of gp120/41
	gp120	Outer *env* glycoprotein
	gp41	Transmembrane glycoprotein
Gag (core)	p55	Precursor of gag proteins
	p24	Capsid structural protein
	p17	Matrix structural protein
	p15, p9, p7	Other gag proteins
Pol (polymerase)	p66	Reverse transcriptase
	p51	Reverse transcriptase
	p31	Integrase (endonuclease).
	p10	Protease
Genes encoding regulatory proteins		
Tat	p14	Transactivator of RNA synthesis
Rev	p19/20	Regulation of viral mRNA expression
Nef	p27	Negative factor (pleiotropic)
Vif	p23	Infectivity factor
Vpu	p16	Virus release
Vpr	p18	Virus replication

B. REGULATORY GENES

The genome of HIV contains at least 6 additional genes that encode genetic information for the synthesis of regulatory proteins. These proteins are synthesised via translation from multiply spliced genes, which play an important role in virus gene expression (Table 1.2). For example, the transactivator protein, *tat,* is a major protein involved in up-regulation of HIV replication. It interacts with the loop formed at the 3' LTR of RNA called the *tat* responsive element (TAR) (Herrmann and Rice, 1993). Another gene involved in the regulation of viral protein expression, *rev*, interacts with the *cis*-acting RNA loop structure called *rev* responsive element (RRE) (Malim *et al.,* 1989). This interaction helps the viral mRNA to migrate from the cell nucleus to enter the cytoplasm where synthesis of viral protein takes place (Sodroski *et al.,* 1986a). The *nef* gene or 'negative factor' gene is so-called for its negative influence on viral expression (Ahmad and Venkatesan, 1988; Niederman *et al.,* 1989). This was shown experimentally by Luciw *et al.* (1987) by deleting the *nef* gene from a wild virus SF2 which resulted in a variant that replicated to higher titre and produced a more pronounced cytopathic effect than the original SF2 isolate. However, these findings need to be confirmed.

In addition HIV-1 has a 23kd *vif* protein which has been found to be essential for the spread of HIV-1 in peripheral blood lymphocytes and in primary cultures of macrophages, as well as in some established T-cell lines. It is required at the stage of viral particle formation, for cell-to-cell as well as for cell-free transmission of HIV-1 (Vonschwedler *et al.,* 1993). The role of the accessory gene product of *vpr* during

HIV-1 infection remains unclear. As for *vif*, the product encoded by the *vpr* gene possibly plays a role during the late phase of the production of infectious viruses. The *vpr* protein is incorporated into the virion at the C- terminal end of the gag protein precursor Pr55 (*gag*) (Li *et al.,* 1996). Sato *et al.* (1996) demonstrated a direct association between *vpr* and the C-terminal region of matrix protein p17 within the mature virion. The interaction plays a direct role in the nuclear transport of the viral pre-integration complex in non-dividing cells such as macrophages. Far from being merely called 'non-essential' or 'accessory genes', Balliet *et al.* (1994) showed that *vpr, vpu* (and *nef*) may be related to each other in facilitating productive infection in macrophages. In fact, the loss of vpr or vpu genes reduced viral antigen production in macrophages by up to 1000- fold, while replication in lymphocytes was only marginally affected. However, the loss of *nef* had little or no effect on infection of lymphocytes, but decreased the viral replication in macrophages to a small extent.

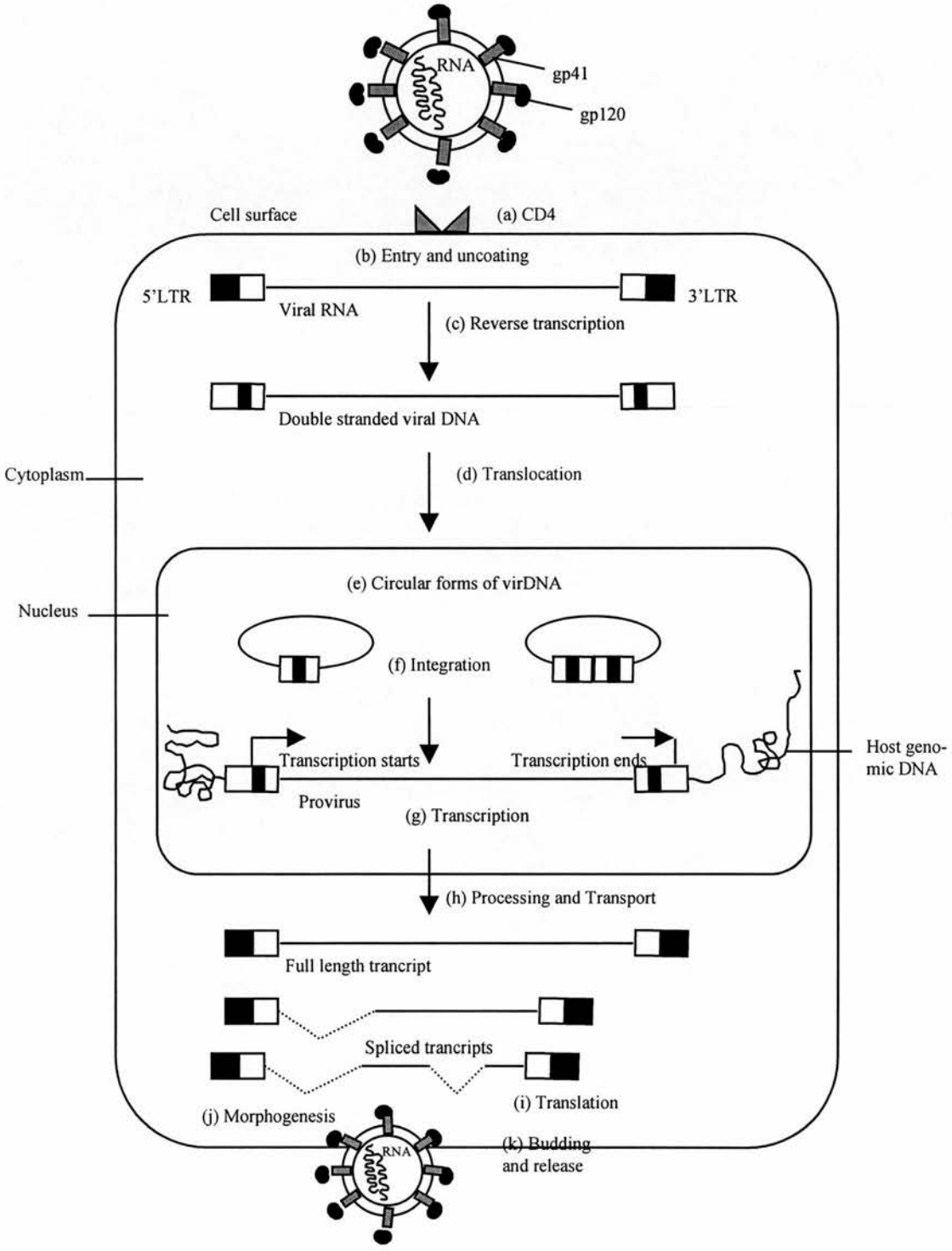
1.2.4. VIRAL REPLICATION CYCLE

The life cycle of HIV-1 is illustrated in figure 1.4. Like all viruses, the replication of HIV-1 is critically dependent on a host cell, which provides essential conditions for the virus to reproduce itself.

FIGURE 1.4. Life cycle of HIV-1 (modified from Peterlin, 1988)

During the life cycle, HIV attaches to cells by specific interactions between gp120 and CD4 (a). After penetration and uncoating (b), virion associated reverse transcriptase (c) converts genomic viral RNA into double-stranded linear DNA, which is translocated (d) from the cytoplasm to the nucleus and (e) converted to supercoiled molecules containing one or two copies of the long terminal repeat (LTR). Efficient retroviral growth requires integration (f) of a DNA copy of the viral RNA genome into a chromosome of the host. It is not known whether in some circumstances HIV replicates without integration of the viral genome into a host chromosome. In the nucleus, HIV gene expression (g) involves synthesis of viral transcripts by host-RNA polymerase II and other cellular and viral factors. Primary transcripts are differentially spliced (h) and transported to the cytoplasm. Full-length transcripts are messenger RNAs for *gag* and gag-pol precursors and genomes for assembly of virion. Spliced transcripts are translated into viral proteins (i). Morphogenesis involves formation of two molecules of HIV genomic RNA complexed with *gag* and *pol* gene products. This core buds through the cellular membrane and acquires a coat containing *env* glycoproteins.

FIGURE 1.4. **Life cycle of HIV-1** (modified from Peterlin, 1988)



1.2.4.1. ATTACHMENT AND ENTRY

The main cellular receptor for HIV-1 is a determinant cluster 4 (CD4) glycoprotein (Isobe *et al.,* 1986; Maddon *et al.,* 1986; Dalgleish *et al.,* 1984). The CD4 is a 54kd transmembrane glycoprotein expressed on various cells including helper T lymphocytes, on B cells and cells of monocyte-macrophage lineage and some cells of the central nervous system (Maddon *et al.,* 1986). The first step of HIV-1 infection is the binding of the virion gp120 to the CD4 receptor, although the mechanisms of fusion and entry remains poorly understood (Dalgleish *et al.,* 1984; Klatzmann *et al.,* 1984). However, specific sites within the V3 loop domain of gp120 and the transmembrane gp41 protein have been shown to be critical for virus fusion and infectivity (Freed *et al.,* 1991; Page *et al.,* 1992). The introduction of the nucleocapsid into the host cell cytoplasm was initially thought to occur by endocytosis of the receptor-virus complex (Pauza and Price, 1988). Another line of evidence suggests that HIV-1 entry to the host cytoplasm occurs by direct fusion and this pathway appears to be acidic pH-independent (Stein *et al.,* 1987).

The hypothesis, that 'CD4 positive cells are the host of HIV' was supported by the finding that a substantial decline of CD4 positive cells was observed in individuals following infection with HIV. In addition, pre-incubation of CD4+ lymphocytes with CD4-specific monoclonal antibodies prevents the binding and entry of the virus into these cells (Klatzmann *et al.,* 1984). Several co-receptor molecules, however, have been suspected. For example, HIV-1 can only bind to, but not enter some murine cells which naturally express the CD4 molecule (Maddon *et al.,* 1986). Nevertheless, if fused with human cells, murine cells become infectable

with HIV-1, which provides more evidence of the existence of co-receptors of HIV-1 entry (Alkhatib *et al.,* 1996). More compelling pieces of evidence were reported by Feng *et al.* (1996) who identified the existence of fusin, a protein which is a putative G-protein-coupled chemokine receptor. In fact, the infection of lymphocytes by T-cell-line-tropic isolates is successfully blocked by antibodies against fusin (Feng *et al.,* 1996). In addition, Cocchi *et al.* (1995) identified molecules called (i) RANTES (regulated upon activation, normal T expressed and secreted), and (ii) macrophage inflammatory proteins (MIP)-1α and MIP-1β, which are chemokines released by CD8 positive T-cells. They found that these chemokines suppressed infection of CD4+ cells by HIV-1. Another important discovery is the identification of CCKR5, a protein that belongs to the same family as fusin, and serves as receptor for the β-chemokines RANTES, MIP-1α and MIP-1β. The last three molecules block HIV-1 infection by interfering with the binding of CC-CKR5 to gp120 or by sending signals which down regulate their own production (Deng *et al.,* 1996; Dragic *et al.,* 1996; Alkhatib *et al.,* 1996; Choe *et al.,* 1996; Doranz *et al.,* 1996). Several cells, other than CD4+ human T lymphocytes, including CD8+ T-cells, macrophages, and dendritic cells have also been shown to be susceptible to HIV but the mechanism of infection remains unclear (Schnittman *et al.,* 1989; Dalgleish *et al.,* 1984; Klatzmann *et al.,* 1984; Livingstone *et al.,* 1996). It also is possible that an HIV-antibody complex binds to macrophages through the constant fragment (Fc) during the natural killing process (Homsy *et al.,* 1989; Jouault *et al.,* 1989).

1.2.4.2. REVERSE TRANSCRIPTION AND INTEGRATION OF PROVIRAL DNA

After fusion of the viral and cellular membranes, the viral core is delivered into the cell cytoplasmic matrix (Bukrinsky *et al.,* 1993). Initiated by various tRNA molecules as primers, the reverse transcription is mediated by the *pol* gene product, the reverse transcriptase (RT) (Lund *et al.,* 1993). The RT has two major functions: the DNA polymerase and the ribonuclease H (RNase H). DNA polymerase activity enables the RT to use the RNA and DNA as templates to synthesise the proviral DNA. Then through RNase H activity, the viral RNA from an RNA-DNA hybrid molecule is selectively degraded allowing the synthesis of the second strand of DNA (Varmus, 1988a; Varmus, 1988b). As a result of RT activity, there are three forms of DNA in the cell cytoplasm. The linear molecule appears to have all HIV-1 genes in the order they are found in the genome and flanked by two LTRs. There are in addition two circular forms and it remains unclear whether these forms play a role in the life cycle (Bukrinsky *et al.,* 1993).

Efficient retroviral growth requires integration of a DNA copy of the viral RNA genome into a chromosome of the host. It is not known whether in some circumstances HIV replicates without integration of the viral genome into a host chromosome. In one study, although integration was found to be necessary for viral replication, a limited amount of virus replication seemed to occur without integration (Sakai *et al.,* 1993). Following its translocation to the nucleus, the linear double-stranded viral DNA is inserted into the cellular DNA using the virus *pol* gene encoded integrase (Bushman *et al.,* 1990). Several other viral components, including

the protease, and more importantly the integrase also enter the nucleus. Prior to integration, the integrase cleaves the colinear double-stranded proviral DNA at the 3' end of both strands which results in the loss of 2 bases (Varmus, 1988a; Varmus, 1988b). At the repeat bases located at the end of the LTR region is an important recognition site for the integration. Mutations within this region have a negative effect on proviral DNA integration.

1.2.4.3. TRANSCRIPTION AND TRANSLATION

After integration, transcription of the full-length viral RNA, initiated by cellular proteins (transcription factors), occurs as the proviral DNA takes over the cellular transcription process. The viral RNAs evolve into two pathways. One group of RNA molecules becomes the mRNA for production of various viral proteins; the other group of full-length RNA molecules plays the role of viral genome. At an early phase of the viral expression, the multiply spliced mRNA, which encodes regulatory proteins (*tat, rev and nef*) is transported into the cytoplasm (Kim *et al.,* 1989). The *nef* protein is not required for the replication of the virus *in vitro.* However, *tat,* the transactivating protein, enhances the transcription activity of the HIV-1 and *rev* plays a significant role in the cytoplasmic transport of transcript of the viral structural proteins (Malim *et al.,* 1989; Malim *et al.,* 1988; Sodroski *et al.,* 1985a; Sodroski *et al.,* 1986b). In addition to *tat*, various cell factors bind to *tat* or co-operate with it in order to process the RNA polymerase II, initiate the viral transcription, increase the level of initiation and enhance RNA elongation (Cullen, 1986).

The late expression of viral genes is also mediated by the *rev* protein. At this stage, the gag-pol mRNA and the singly spliced *env* mRNA are translated into

precursors of viral structural and enzymatic proteins. These precursors are subject to proteolytic cleavage, glycosylation, phosphorylation and myristoylation. Other regulatory proteins, *vif, vpu and vpr* intervene during the late phase of the viral gene expression. Their late expression suggests that they may be involved in the processing or the assembly of the various viral and structural proteins. Particularly, the *vpu* protein, only found in HIV-1 and not in HIV-2 (where it is replaced by *vpx*), is required for maturation and increases the release of virions from infected cells (Klimkait *et al.,* 1990). Once the late expression of viral genes is completed, mature virus particles are packaged and released by budding through the cell membrane (Popovic *et al.,* 1984). During the budding process, the virus particles acquire the outer lipid bilayer, which contains the external and transmembrane envelope glycoproteins (figure 1.4).

1.2.4.4. ASSEMBLY AND RELEASE

The packaging of the newly replicated HIV virions is the most poorly understood aspect of the replication cycle. During assembly, RNA genomes are assembled by highly specific interaction with p55 and p160 *gag-pol* polyprotein precursor molecules. Prior to packaging, the precursors of viral proteins undergo various modifications. The p55 and p160 *gag-pol* are modified after their translation by the attachment of a myristoyl group onto their N-termini, which may anchor the constituent of the nascent virus to the cytoplasmic side of the cell membrane. In addition, the cleavage and glycosylation of the glycoprotein gp160 takes place in the endoplasmic reticulum and the golgi complex to produce the gp120 and the gp41 glycoproteins (Stein and Engleman, 1990); their incorporation is not well understood.

Naturally, the budding of the viral component from the cellular membrane will result in the death of the host cell.

1.2.5. GENETIC HETEROGENEITY OF HIV-1

Retroviral replication is a highly error-prone process with varying estimates of about $7x10^{-6}$ to $1.4x10^{-4}$ base-pair substitutions per nucleotide per replication cycle (Dougherty and Temin, 1988; Leider *et al.,* 1988; Pathak and Temin, 1990a). The observed mutation rates are the result of an error-prone reverse transcriptase, which in the case of HIV-1 has a misincorporation error rate of approximately 10^{-4} per base per replication cycle (Roberts *et al.,* 1988; Preston *et al.,* 1988). Additional factors contributing to viral heterogeneity are the frequent occurrence of insertion and deletion (Dougherty and Temin, 1988; Pathak and Temin, 1990a; Pathak and Temin, 1990b) and the commonly observed hypermutation expressed by frequent G-to-A transitions (Fitzgibbon *et al.,* 1993; Pathak and Temin, 1990a; Vartanian *et al.,* 1991). Furthermore, recombination constitutes another factor contributing to the high genetic heterogeneity observed in HIV-1 strains (Srinivasan *et al.,* 1989). Recombination has been observed to occur *in vivo* in a baby dually infected with two different strains (Diaz *et al.,* 1995). With a viral half life of about 2 days in infected individuals (Ho *et al.,* 1995), the combined effect of hypermutation, base substitution, recombination and intergenomic recombination can produce a large number of variants within the same individual. These are called viral 'quasispecies' (Goodenow *et al.,* 1989; Simmonds *et al.,* 1991; Holmes *et al.,* 1992; Balfe *et al.,* 1990).

Phylogenetic analysis of HIV strains from different parts of the world resulted in the division of HIV in two super-groups, namely HIV-1 and HIV-2. Genetically, HIV-1 is subdivided into 2 distinct groups, the main (M) group and the outlier (O) group (Myers *et al.,* 1995). Group M viruses appear to be the most prevalent and have a worldwide distribution. At least 8 group M subtypes (A- H) have been identified based on the analysis of the *gag* region. Usually, HIV-1 strains grouped together in clades (subtypes) when analysed on the basis of the *gag* and *env* region sequences (Myers *et al.,* 1995). However, all currently published *env* subtypes E do not group as a distinct clade, but cluster within subtype A on the basis of the *gag* gene (Sharp *et al.,* 1994; Myers *et al.,* 1995). It is not yet known whether the two new subtypes provisionally assigned the letters I and J based on the *env* phylogeny (Kostrikis *et al.,* 1995; Leitner *et al.,* 1995) will constitute distinct clusters if they are analysed in the gag region. In addition to group M clades, it is believed that HIV-2 may also be divided into five subtypes (A-E) (Chen *et al.,* 1996; Albert *et al.,* 1996).

AIMS OF THE STUDY

The present study was originally designed to provide basic information on the natural history of HIV-1 infection among mothers and children in a rural community in Kimpese, Congo. In the course of the recruitment process, it was shown that the seroprevalence of HIV-1 among child-bearing mothers was both low and remained stable over a 5 year period (Green *et al.*, 1994). In addition, the vertical transmission rate was observed to be one of the lowest ever reported in Africa (Dr AG Davies, personal communication). Reasons for both situations remain poorly understood. Part of the explanation could be the social behaviour within the population and it required a social scientist to carry out such an investigation[1]. The nature of the virus was also suggested as a possible avenue for study. It was therefore important to define as early as possible the study group (HIV-1 infected patients) and matched controls (HIV-1 uninfected patients). For adults, there were commercial tests for detection of IgG anti-HIV-1. However, in children, the diagnosis of HIV-1 is hampered by the presence of maternally derived IgG anti-HIV-1, which can persist beyond 12 months of age (Newell *et al.*, 1995). In developing countries, highly sensitive techniques for diagnosis of HIV-1 in children such as the polymerase chain reaction (PCR) and virus culture are not always available. The few laboratories where these methods are used cannot cope with the rising numbers of HIV-1 infections in children.

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[1] Dr Louise de la Gorgendiere, of the Department of Sociology, University of Edinburgh, visited Kimpese to carry out a pilot study on social behaviour which could explain the low and stable seroprevalence of HIV-1 in the region. No specific marker was found to be associated with the stability in the region.

The aims of this project were:

- Diagnosis: to develop a simple and inexpensive method for early diagnosis of HIV-1 infection in children (Mokili *et al.,* 1996).
- Subtype: to investigate the distribution of HIV-1 subtypes in Kimpese, and to establish their biological significance in mother-to-child transmission. Detailed analyses of the nucleic and amino acid sequences of the p17 *gag* region were planned to determine the specific characteristics of HIV-1 variants of transmitting and non-transmitting mothers.
- Serotype: Because of the large number of cases studied in this project, the determination of HIV-1 subtypes by sequencing was too expensive and time consuming. It was planned to develop a serological method to subtype a large number of samples containing antibodies to the principal neutralising domain of HIV-1. The same system would also measure the level of antibodies to V3 loop in transmitting and non-transmitting mothers.

This study would therefore lay foundations for future research, including the development and evaluation of a future HIV-1 vaccine.

CHAPTER 2

MATERIALS AND METHODS

2.1. SITES OF INVESTIGATIONS

2.1.1. KIMPESE

The samples analysed in the present study were collected from mothers and children in Kimpese, a rural town in the Democratic Republic of Congo (DRC), formerly Zaire (Figure: 2.1). Kimpese is a town of 30,000 people, situated on the main road between the capital Kinshasa (220 kilometre, km), and the port city of Matadi (140 km). The economy of Kimpese is based upon subsistence agriculture with local trading and a few industries. Kimpese is for many local people a commercial centre. Immediately surrounding the hospital where the study was based is a cattle farm, 'Les Grands Elevages de Kimpese'. Farmers bring their crops to sell them at Kimpese's weekly Sunday market and to buy necessary provisions to take back to their villages. The hospital, Institut Medical Evangélique (IME), is a joint enterprise between American, British, Canadian, Swedish and Congolese missionaries. It was founded in 1950 as a small rural health centre but now the hospital has expanded to be the reference hospital of a health region, which covers a population of over 120,000 people. With 400 beds, the hospital recorded over 9,000 admissions in 1985. The number of admissions dropped to 4,800 in 1993, probably due to growing socio-economic difficulties, which make it difficult for poor villagers to get to hospitals or afford the cost of treatment. However, those who cannot afford to pay for hospital treatment are subsidised by charitable funds available as part of the hospital mission. Specialities include internal medicine, surgery and orthopaedics (with three operating theatres), obstetrics, gynaecology and paediatrics.

There are four antenatal clinics where all the patients involved in this study have been recruited (IME, Lamba, Cité and CIZA-Lukala[1]). In addition, specialities like ophthalmology, a dental unit, a tuberculosis sanatorium and a leprosy unit make IME probably the biggest rural hospital complex in the Democratic Republic of Congo.

2.1.2. UNITED KINGDOM

The construction of branched peptides (discussed below) was carried out at the Medical Research Council (MRC), Institute of Virology, University of Glasgow, under the supervision of Dr Howard Marsden. The serological testing was performed at various sites in UK, including the Regional Virus Laboratory, City Hospital, Edinburgh, the Public Health Laboratory Service, Colindale (by coutesy of Dr John V Parry) and at the AIDS Clinical Trials laboratory, St. Mary's Hospital, London (by courtesy of Dr Rachanee Cheingsong-Popov). The molecular work was carried out at the Molecular Virology laboratory, Department of Medical Microbiology, University of Edinburgh by courtesy of Dr Peter Simmonds.

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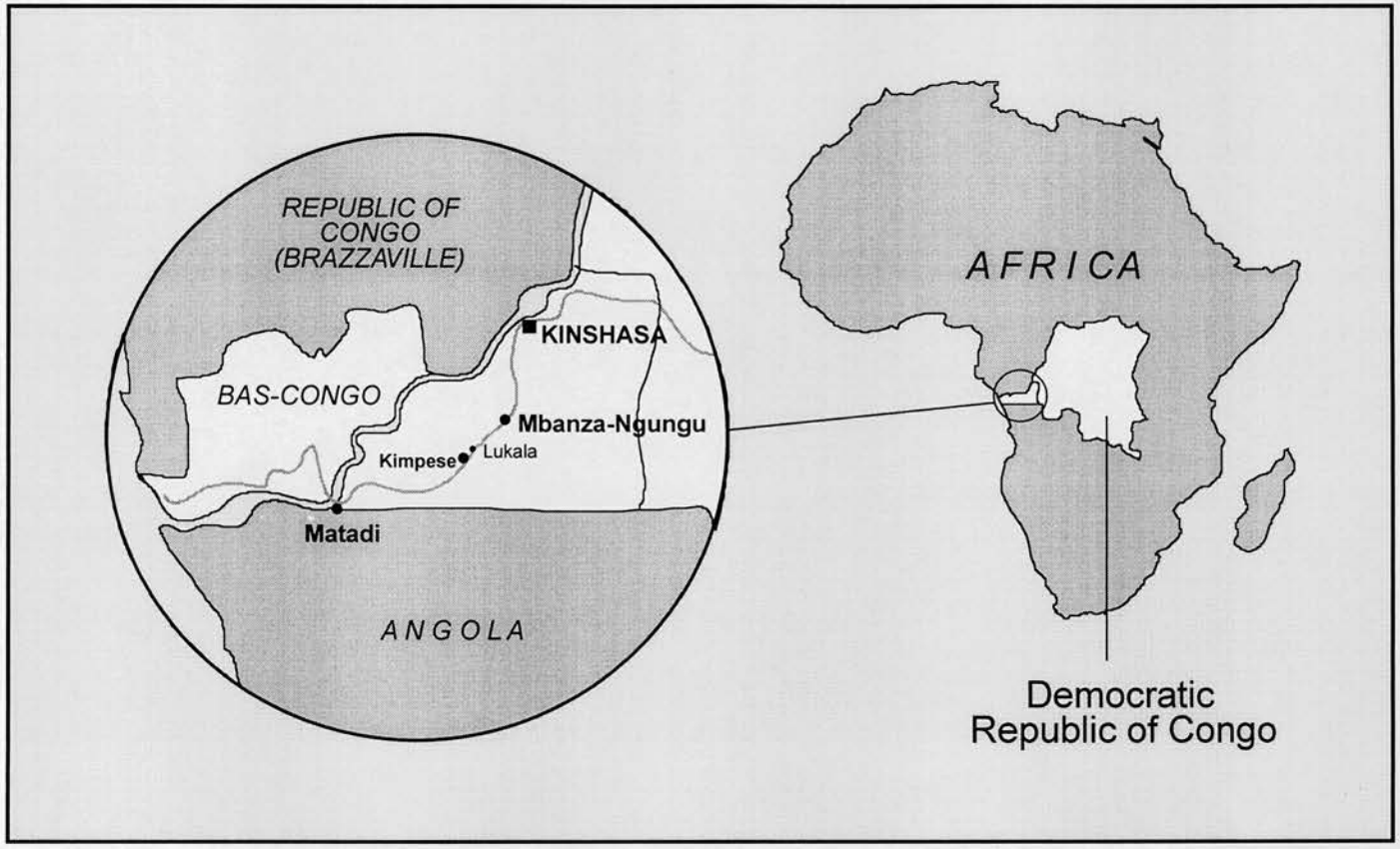
[1] CIZA-Lukala: cimenterie zaïroise de Lukala, recently changed to CILU, cimenterie de Lukala.

FIGURE ***2.1*** *Map of Africa showing the location of Kimpese in the Democratic Republic of Congo*



2.2. RECRUITMENT AND ASSESSMENT OF PATIENTS

The protocol for this study was approved by the hospital, the (former) Zairian Ministry of Health and, in the UK, by the regional paediatric ethical committee in Edinburgh (Appendix 1). The recruitment of patients was carried out in two phases.

During phase I of the study, which spanned August 1988 and December 1991, pregnant women attending antenatal care were screened for HIV-1 antibodies at four maternity clinics (IME, Lamba, Cité and CIZA-Lukala) in Kimpese. The screening continued at these four antenatal clinics until December 1990. In June 1991, the screening was discontinued at Cité and CIZA-Lukala clinics due to workload created by the follow up of mothers and children recruited for the HIV-1 mother-child transmission study. At the end of phase I, 9129 mothers had been screened in the Kimpese laboratory for antibody to HIV-1 using the Abbott recombinant HIV-1/HIV-2 enzyme linked immunosorbent assay (ELISA) (Abbott Diagnostic Division, Minehead, Berckshire, UK). Samples reactive at this stage were re-tested using the same ELISA test and a competitive ELISA (Wellcozyme, manufactured by Murex, formerly Wellcome Diagnostics, Dartford, Kent, UK) for HIV-1 only.

All HIV-positive mothers were invited to enrol in the project and gave signed consent before entry (Appendix 2). A control group of HIV negative pregnant women was recruited at the same time. Infected and control mothers were group matched for age, parity, social background and residence (village, town, or city). It was explained that the project was a study of childhood illness including HIV infection. All necessary medical care of the children was provided free of charge.

Phase II: February 1993-February 1994. In order to increase the number of

children in this study, the screening of antenatal mothers was resumed at IME and Lamba. During this period 2,183 antenatal mothers were tested for antibody to HIV-1. It is possible that during phase I, some women undergoing seroconversion were missed by the Abbott HIV-1/HIV-2 ELISA as this test only detects IgG antibodies. A test that could detect both IgG and IgM anti-HIV was therefore needed to address this problem. The first line screening tests, Murex Base Pack GE01/02/03 and Detection Pack GE11/12/13 for detection of IgG and IgM anti HIV-1 and HIV-2, were kindly provided by Dr Urszula Beckford of Murex Biotech (Murex Biotech limited, Central Road, Temple Hill, Dartford, Kent England).

Mothers were asked to bring their children to the study clinic at ages 3, 6, 9, 12, 15, 18 and 24 months for full assessment by a paediatrician and for blood tests. The children were also seen by a study nurse at each of the intervening months and by a nurse or doctor when brought to the clinic because of illness. Clinicians were blind to the HIV status of mother and child unless this became evident from clinical manifestations. The HIV staging of infected mothers and children was based on the medical history and physical examination during the puerperium (CDC, 1987). In total there were 292 live births in the study including six pairs of twins, and six stillbirths, five of them to infected women. Fourteen babies were lost to follow up from birth. The remaining 278 (91%) were followed to death or at least 12 months of age, 81% to at least 15 months, and 62% to 24 months.

PLACENTAL HISTOLOGY

In order to study placental histology, portions of placenta, from 43 HIV infected and from 54 uninfected women, were fixed in formalin, embedded in

paraffin wax, sectioned, and stained with haematoxylin and eosin. The same pathologist (Dr Ann Nelson, University of Kinshasa), blind to the HIV status of the mothers and children, examined sections from all placentae for malaria parasites and pigment, chorioamnionitis and funisitis. Each feature was graded in two categories: absent or slightly abnormal, and moderately or markedly abnormal.

ESTIMATION OF VERTICAL TRANSMISSION RATE

The rate of vertical transmission was estimated by the direct and indirect methods recommended by the Working Group on Mother-to-Child Transmission of HIV (Dabis *et al.,* 1993). These methods were described in order to facilitate comparisons between different studies. The direct method uses the HIV antibody status of the child at or after 15 months, by which age maternal antibody has disappeared from the blood of virtually all children of HIV-infected mothers. Clinical assessment of HIV status is used in children for whom the presence of antibody at age 15 months cannot be ascertained. When clinical assessment is also not possible, the child's status is classified as indeterminate. For the **direct method**, three rates of the transmission rate are made: a minimum rate which assumes that the indeterminates are not infected, a maximum rate which considers all indeterminate as infected, and an intermediates rate which disregards the indeterminates. The **indirect method** relies on antibody status at 15 months together with an estimate of the excess mortality amongst the children of HIV positive women in comparison with a control group of children born to uninfected women. The excess is computed by life table analysis. The advantage of this method is that it obviates clinical assessment of

whether children under 15 months are infected and whether early deaths are HIV related.

2.3. SEPARATION AND STORAGE OF BLOOD SAMPLES

At the antenatal and pediatric clinics, 5ml of blood drawn by venepuncture was collected in a tube containing EDTA-K and four drops were dried on card and sealed (Guthrie cards, kindly provided by Dr Anthony Girdwood, Scottish Neonatal Screening Laboratory, Stobhill Hospital, Glasgow). Whole blood samples were transported to the Kimpese laboratory and prepared for storage within 4 hours of venepuncture. After centrifugation at 1,200 revolution per minute (RPM) for 15 minutes, plasma samples were separated from cells and were first stored at 4-8^{0}C until they were tested by ELISA (2-3 days), then at -20^{0}C until they were transported to the Regional Virus Laboratory in Edinburgh, United Kingdom (UK).

Official permission for 'export of biological research materials' was obtained from the national committee for AIDS research in Congo (then Zaire). Samples were shipped to UK frozen and thawing time was kept minimal. The confirmatory testing was performed by Mr Tom Shaw of the Regional Virus Laboratory, City Hospital, UK. This included measurement of p24 core antigen (Abbott, without acid dissociation technique) and anti-p24 antibodies (Abbott) and western blot (Organon Teknika).

Buffy-coat containing peripheral blood mononuclear cells (PBMC) was either stored immediately at -20^{0}C or the lymphocytes were separated using ficoll gradients. The Lymphoprep^{TM} (Nycomed Pharma As, Diagnostic Division, Norway) was used for cell separation. The technique is based on the aggregation of

erythrocytes and granulocytes in sodium metrizoate and polysaccharide, thereby increasing their sedimentation rate (Ting and Morris, 1997). The isolation of the peripheral blood mononuclear cells was carried out as follows: 1.5ml of buffy coat was mixed with 1.5 ml distilled water containing 0.9% of sodium chloride (NaCl). Lymphoprep (1.5ml) was measured in a 5ml sterile tube. Three ml of blood was carefully layered on top of the lymphoprep avoiding mixing the blood with the separation fluid. The tube was capped and centrifuged at 800 x g for 20 minutes at room temperature, in a swing-out rotor. PBMCs were harvested from the interface between plasma above and aggregated cells below, using a pasteur pipette. The harvested cells were washed in 0.9% NaCl solution and pelletted at 250 x g for 10 minutes. Samples were then stored at -20^{0}C in Kimpese. Samples stored this way were adequate for PCR and sequencing performed later in the UK.

MOLECULAR METHODS

2.4. EXTRACTION OF DNA FROM PBMC

REAGENTS

- DNA lysis buffer (EDTA, 0.05M; Tris-Cl, 0.1M, pH 7.5; NaCl, 0.05M) (, Sigma: Poole, UK).
- Proteinase K (10mg/ml) (Sigma: Poole, UK).
- N-lauroylsarkosyl (10%, w/v in sterile distilled water) (Sigma: Poole, UK).
- Chloroform-isoamylalcohol (50:1) (Merck Ltd, Merk House, Poole, Dorset, UK)
- Phenol (Merck Ltd, Merck House, Poole, Dorset, UK)
- Ethanol, 100% (Rathburn Chemicals Ltd, Walkburn, Scotland)
- Sodium acetate (Sigma-Aldrich Company Ltd, Fancy Road, Poole, Dorset, UK).

PROCEDURE

Stored PBMCs (400µl) were resuspended in 400µl of DNA lysis buffer containing 4µl of proteinase K, 44µl N-lauroylsarkosyl (10%, w/v in sterile distilled water) and incubated at 65^0C for 2 hours. The suspended cells were mixed with an equal volume of phenol (Rathburn Chemicals Ltd, Walkburn, Scotland) and vigorously shaken for 10 minutes then spun at 15,000 RPM for 10 minutes. After centrifugation, the supernate was vigorously mixed with chloroform-isoamylalcohol (50:1) (both supplied by Merck Ltd, Merk House, Poole, Dorset, UK). The mixture was centrifuged for 10 minutes, at 15,000 RPM. The nucleic acid present in the supernate was precipitated in 840µl solution containing 800µl of 100% ethanol

(Rathburn Chemicals Ltd, Walkburn, Scotland) and 40µl sodium acetate (Sigma-Aldrich Company Ltd, Fancy Road, Poole, Dorset, UK). After incubation at -40^{0}C for 2 hours (or overnight at -20^{0}C), the precipitated DNA was collected by centrifugation at 15,000 RPM (0^{0}C) for 10 minutes. The nucleic acid was then resuspended in 100-200 µl of distilled water. An aliquot (5µl) of the DNA was analysed to determine the yield and the purity of DNA using a spectrophotometer set at 260 and 280nm (ultra violet spectrophotometer Cecil 2000).

2.5. EXTRACTION OF RNA FROM PLASMA

REAGENTS

- TNE buffer: 0.11M NaCl, 55mM Tris, pH 8.0; 1.1 mM EDTA, pH 8.0; 0.55%SDS
- Proteinase K (10%, w/v in sterile water)
- Poly A RNA carrier (Sigma-Aldrich Company Ltd, Fancy Road, Poole, Dorset, UK).
- Chloroform-isoamylalcohol (50:1) (Merck Ltd, Merk House, Poole, UK)
- Phenol (Merck Ltd, Merk House, Poole, Dorset, UK)
- Ethanol, 100% (Rathburn Chemicals Ltd, Walkburn, Scotland)
- Sodium acetate (Sigma-Aldrich Company Ltd, Fancy Road, Poole, Dorset, UK).

PROCEDURE

A protocol modified by Mr Neil Adams (Molecular Virology Unit, Med. Microbiology, University of Edinburgh) for the extraction of RNA was used successfully in the extraction of RNA from plasma samples. Briefly, 100µl of plasma was mixed with pre-heated (37^{0}C) 400µl of extraction buffer containing 350µl TNE,

42 µl proteinase K (10%), and 8µl poly A RNA carrier. The mixture was incubated for 10 minutes at 37^0C, then phenol-chloroform extraction and precipitation of the nucleic acid was carried out as described under 2.2.4 (Extraction of DNA from PBMC).

2.6. REVERSE TRANSCRIPTION (RT) OF VIRAL RNA TO cDNA

MATERIALS

Reverse transcription buffer:

50mM Tris-Cl, pH 8.0 (Sigma-Aldrich Company Ltd, Poole, Dorset, UK).

5mM $MgCl_2$ (Sigma-Aldrich Company Ltd, Poole, Dorset, UK).

5mM DTT

50mM KCl (Rathburn Chemicals Ltd, Walkburn, Scotland)

0.05µg/µl BSA

600µl of each dNTP; 20% DMSO; 150 ng of outer antisense primer;

10 units RNAsin (Promega, Southampton, UK)

10 units AMV reverse transcriptase

Water-bath or thermocycler (set at 42^0C).

PROCEDURE

Prior to the polymerase chain reaction, it is essential to reverse transcribe the RNA extracted from patient's plasma or serum to a complementary DNA (cDNA). To promote the reaction, a specific viral primer, an antisense primer used during the primary PCR, is needed. The extracted RNA (5µl) was added to 15 µl of

RT buffer and incubated in a water bath or a thermocycler set at 42^0C for 30 minutes. The resulting cDNA product was allowed to reach room temperature for 20 minutes before use or stored at -20^0C.

2.7. PRIMER DESIGN

The design of the primers was based on the consensus sequence of the most conserved regions of the *gag* gene of 72 aligned sequences from various parts of the world compiled in the Los Alamos database (Myers *et al.,* 1995). The primers were synthesised by OSWEL DNA service of the University of Southampton (Boldrewood, Southampton, SO16 7PX, UK). To ensure that primers can target only HIV proviral DNA, a search of identical or similar sequences (to the primers) contained in the Genbank was performed using the programme Blast (Miyoshi *et al.,* 1981). Care was taken to (i) avoid primers being complementary with each other and (ii) minimise the mismatch of the primer and template by not having a T at the terminal base at the 3' end. The annealing temperature was determined using the equation,

$$T=4(G+C)+2(A+T)$$

where A,T, G and C are the number of those bases in the oligonucleotide. The primer sequences and their positions relative to HIV_{HXB2} isolate are depicted in table 2.1.

TABLE 2.1: *Primers for PCR amplification and sequencing*

(Positions relative to the HIV_{HXB2} genome)

ID	Sequence	Position
Set 0:		*gag*
a: 531	GCGAGAGCGTCAGTATTAAGCGG	795-817
b: 532	GGGAAAAAATTCGGTTAAGGCC	835-857
c: 533	CTTCTACTACTTTTACCCATGC	1248-1269
d: 534	TCTGATAATGCTGAAAACATGGG	1296-1318
Set 1:		*gag*
a: S1P1	ATGGGTGCGAGAGCGTCAGTA	789-808
b: S1P2	CATNTAGTRTGGGCAAGCAG	885-904
c: S1P3	CYTCTAYYACYTTYACCCATGC	1248-1269
d: S1P4	TCTGRTAATTGCTGWRAACATG	1298-1318
Set 2:		*gag*
e: S2P1	GACACCAARGAAGCYYTAGA	1074-1093
f: S2P2	AGYAAATTAYCCYATAGTRCA	1173-1195
g: S2P3	ATACATYCTTACTATTTTATTTAA	1596-1619
h: S2P4	GGYCCTTGTYTTATRTCCARAATGC	1630-1654

ID	Sequence	Position
Set 3:		*gag*
i: S3P1	CARATRAGRGARARCCAAGGGGAAG	1467-1489
j: S3P2	GAYATAGCAGGAACTACTAGTA	1491-1512
k: S3P3	CCTTCYYTGCCACARTTGAARCA	1962-1984
l: S3P4	GAYCAGGRGCCYTGCAA	2000-2016
Set 4:		*gag*
m. S4P1	TCCAAAAYGCRAAYCCAGAYTGTAA	1757-1781
n. S4P2	TAGAAGAAATGATGACAGGATGYCA	1817-1841
o. S4P3	GCCARTKYTCCTAAAAAATTAGC	2080-2104
p. S4P4	TGGCCTYCCCTTGYKGGAAGG	2104-2123
Set 5:		*env*
E1	TACAATGTACATGGAATT	6957-6976
E2	TGGCAGTCTAGCAGAAGAAG	7009-7028
E3	ATTCGCATGGGAGTGTG	7465-7482
E4	GGAGGGGCATACATTGC	7520-7537

Outer primers: set 0: a, d; set 1: a, d; set 2: e, h; set 3: I, l; set 4: m, p; set 5: E1,E4
Inner primers: set 0: b, c; set 1: b, c; set 2: f, g; set 3: j, k; set 4: n, o; set 5: E2, E3.
Code for ambiguities: R: A or G; Y: C or T; K: T or G; W: A ot T.

2.8. NESTED POLYMERASE CHAIN REACTION (PCR)

MATERIALS:

- Reaction mixture (primary PCR: 45µl, secondary PCR: 20µl)

50mM KCl, (Promega, Southampton, UK)

10mMTris-HCl (pH 8.0) (Promega, Southampton, UK)

0.1% Triton X-100 (Promega, Southampton, UK)

3.3µM of each dNTP (Boehringer Mannheim UK Ltd, BN7 1LG, UK)

0.5µM of each of the primers (outer primers, Primary PCR, inner primers: secondary PCR) synthesised by OSWEL DNA service of the University of Southampton (Boldrewood, Southampton, SO16 7PX, UK)

20 units/ml of *Taq* polymerase (Promega, Southampton, UK)

- DNA or cDNA (1µg)

PROCEDURE

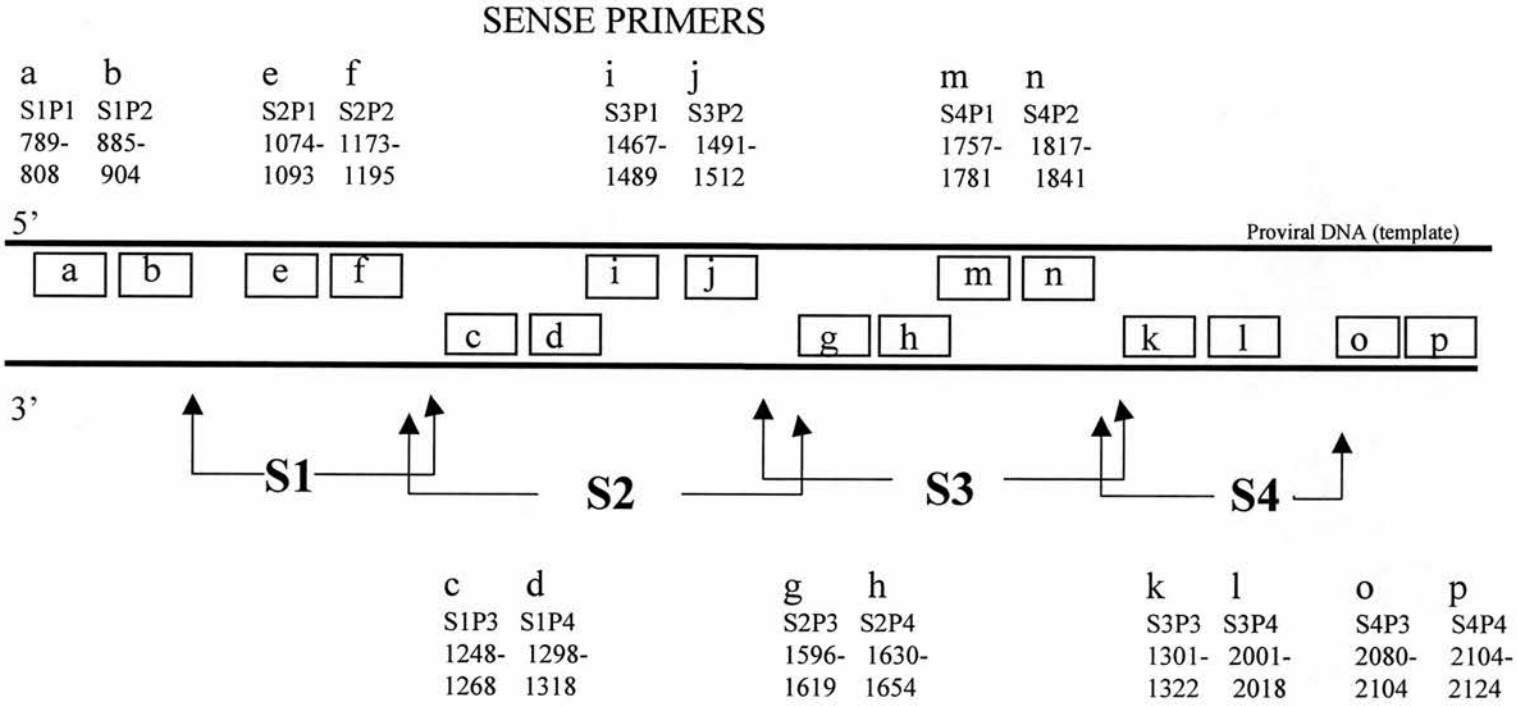
Approximately 1µg DNA per reaction was subjected to nested PCR amplification as previously described (Simmonds *et al.,* 1990). The amplification of the selected section of the *gag* or the *env* (V3-V4) gene was performed in two steps: the primary PCR and the secondary PCR. During the primary PCR, two outer primers, (e.g. set 0: a, sense and d, antisense; table 2.1) are needed to screen the desired region of the genomic DNA in which the proviral DNA has been inserted. The secondary PCR requires primers of the same set (table 2.1) (e.g. set 0: b, sense and c, antisense) specific to the inner section of the region amplified during the primary PCR. The PCR amplification cycles consist of denaturation of double stranded DNA, annealing of

primers, synthesis of cDNA; the reactions were carried out at 95^0C for 0.6 min., 45^0C for 1.5 min and 68^0C for 3 min respectively. The reaction mixture for the first round PCR (50µl) contained 50mM KCl, 10mMTris-HCl (pH 8.0), 1.5mM $MgCl_2$, 0.1% Triton X-100; and 3.3µM of each dNTP; 0.5µM of each of the outer primers; 20 units/ml of *Taq* polymerase and approximately 1 µg of DNA or cDNA. One drop of liquid paraffin was added before subjecting the tubes to 30 cycles of amplification reactions. At the end of 30 cycles, as high as 10,000 fold amplification of the template can be achieved (Simmonds *et al.,* 1990). One µl of primary PCR was subjected to another amplification, the secondary PCR. This second amplification step was performed with 2 inner primers (e.g. primers b and c in table 2.1) and with the same ingredients and cycling conditions as for the primary PCR but in 20µl volume, for 40 cycles. The set 0 and set 1 of primers (Table 2.1) were used to screen the presence of HIV proviral DNA in the sample. If the amplification failed using these primers, alternative primers (Table 2.1: sets 2, 3 and 4; figure 2.2) specific to the *gag* gene were used. PCR was also performed to amplify the region encompassing the hypervariable domain of the envelope gene (*env)* using the set 5 primers (Table 2.1). Prior to PCR amplification of the V3-V4 region, the template was serially diluted in order to perform the sequencing on approximately one single copy of the virus.

FIGURE ***2.2.*** *Primers for PCR amplification and sequencing*

The primers were designed to enable the sequencing of the full length of the *gag* region. The lines in bold indicate the DNA template. Positions of primers are relative to HIV_{HXB2} genome. Primers binding on the top and bottom templates are sense and anti-sense primers, respectively. Arrows indicate the region spanning between sense and antisense primers which can be sequenced using 4 sets of primers:S1, S2, S3 and S4. Primer Set 1: a, b, c, d; set 2: e, f, g, h, set 3: i, j, k, l; and set 4: m, n, o, and p, respectively (Table 2.1).

FIGURE 2.2. **PRIMERS FOR PCR AMPLIFICATION AND SEQUENCING**
(POSITIONS OF PRIMERS RELATIVE TO HIV$_{HXB2}$)



2.9. BIOTINYLATED PCR

MATERIALS

- 13µl PCR buffer: 50mM KCl, 10mM Tris-HCl (pH8.0), 1.5mM $MgCl_2$ 0.1% Triton X-100 and 3.3µM of each dNTP (Boehringer Mannheim Ltd, BN7 1LG, UK)
- 0.05µM of each of the outer primers, one of which is biotinylated (Figure 2.2)
- 2 units/ml of *Taq* polymerase (Promega, Southampton, UK)
- 1 µg of primary PCR product
- Diethylpyrocarbonate (DEPC) treated-water

PROCEDURE

The biotinylated PCR was a modification of the PCR described under 2.2.8. The secondary PCR was carried out in 100µl (instead of 20µl) of PCR mix containing 13µl of PCR buffer, 0.05µM of each of the outer primers, 2 units/ml of *Taq* polymerase (Promega, Southampton, UK) and approximately 1 µg of the primary PCR product. Only one biotinylated primer at a time was needed in each biotinylated PCR. If the desired sequencing product is a sense fragment, the antisense primer used during the secondary PCR was biotinylated. The total reaction mix (100µl) was subjected to an amplification reaction for 40 cycles.

2.10. VISUALISATION OF AMPLIFIED PCR PRODUCT

REAGENTS

- 2% (w/v) Agarose (SIGMA, Poole, Dorset, UK).
- TBE: 0.089M Tris-borate and 0.089M boric acid, 0.01M EDTA;

pH 8.2-8.9) (SIGMA, Poole, Dorset, UK).

- Ethidium bromide (10mg/ml) (SIGMA, Poole, Dorset, UK).

PROCEDURE

A 2% agarose solution was prepared by dissolving 6g of agarose in 300ml of 1x TBE (0.089M Tris-borate and 0.089M boric acid, 0.01M EDTA; pH 8.2-8.9). The gel solution was heated at 100^0C for 5 minutes, then cooled down to about 42-46^0C. 25µl of ethidium bromide (10mg/ml) was added and mixed (final concentration of 0.8µg/ml). The mixture was poured onto a pre-levelled 20 x 20 cm electrophoresis gel plate with mounted combs and left to polymerise at room temperature. Once the gel was set, the PCR product was put in holes occupied by the comb during the polymerisation. The electrophoresis was carried out at 150 volts (90.5mA) for 5 minutes without immersing the gel in TBE. The gel was then flooded with TBE and electrophoresis carried out for a further 10 minutes. The electrophoresis system included a gel tank and a power supply (TriPak 400/200, Bioscience services, UK or BioRad model 200/2.0). A transluminator was used to view the amplified PCR product and a Polaroid camera (MP-4 land camera) was used to photograph the gel.

2.11. SOLID PHASE EXTRACTION OF SINGLE STRANDED DNA FROM PCR PRODUCT

MATERIALS

- Dynabeads (6.7 x 10^8 beads/ml; Dynal) coated with streptavidin

- Bovine serum albumin [BSA, 0.1% (w/v) in phosphate buffered saline, pH 7.2]

- Buffered water (BW) (10 mM Tris-HCl, pH 7.5; 1mM EDTA and 2M, NaCl)

- Sodium hydroxide (NaOH, 0.15M)

- Tris-EDTA solution (TE: 10 mM Tris-HCl, pH 7.5 and 1mM EDTA)

- Biotinylated PCR product (double stranded DNA)

PROCEDURE

The PCR product of the biotinylated PCR containing double-stranded DNA was subjected to treatment to remove the non-biotinylated single stranded DNA using a modified Dynal DNA separation protocol (Dynabead, M280, Dynal). A suspension of beads (20µl at 6.8 x 108 beads/ml) was washed with 40µl bovine serum albumin (BSA, 0.1% in phosphate buffered saline, pH 7.2) and 40µl buffered water (BW) (10 mM Tris-HCl, pH 7.5; 1mM EDTA and 2 M NaCl) using a magnetic stand. Beads were then resuspended in 40µl BW at a concentration of approximately 5µg beads per µl BW. Forty µl of the biotinylated PCR product containing double-stranded DNA was mixed with washed beads and incubated at room temperature for 15-20 minutes with occasional resuspension. The supernate was removed, and the double stranded DNA (now immobilised on beads by biotin-streptavidin affinity) was washed with 40µl BW. The DNA was then denatured with 40µl sodium hydroxide (NaOH, 0.15M) and the non-biotinylated single-stranded DNA discarded. The biotinylated single stranded DNA was washed with 50µl BW, then with 50 µl TE (10 mM Tris-HCl, pH 7.5 and 1mM EDTA). The washed biotinylated single stranded DNA was resuspended in 30 µl of TE and stored at 4^0C until sequenced.

2.12. DIRECT SEQUENCING OF PCR PRODUCT

MATERIALS

- Biotinylated single stranded DNA immobilised on beads

- Annealing mix (10% DMSO, 200mM Tris-HCl pH 7.5; 100mM $MgCl_2$; 250mM NaCl; 10ng primer) (United States Biomedical, USA)

- **Extension mix:** (United States Biomedical, USA)

    - 0.025M DTT

    - Labelling mix:

        dGTP

        dCTP

        dTTP

        α-^{35}S-dATP

        2 units sequenase

- **Termination mix:** (United States Biomedical, USA)

    - 80µM dNTP (dGTP, dATP, dCTP, dTTP)

    - 50mM NaCl

    - 8mM ddNTP (ddGTP, ddATP, ddCTP, ddTTP)

- **Stop solution**

    95% Formamide,

    0.02 M EDTA

    0.1% Bromophenol Blue

    0.1% Xylene Cyanol FF

PROCEDURE

Biotinylated PCR product was sequenced directly from single strand DNA immobilised on streptavidin-coated beads using the dideoxy-chain termination method according to the Sequenase protocol (Sequenase, United States Biochemical). After the annealing of a primer to the template, the DNA polymerase synthesises the complementary strand of DNA using deoxyribonucleotide triphosphates (dGTP, dATP, dCTP and dTTP) the same way as during an ordinary PCR reaction. The elongation reactions take place in 4 wells of a microtitre plate. In addition, modified dNTPs, called dideoxynucleotide (ddNTP) are also incorporated in each reaction well, one at time. Dideoxynucleotides have a hydrogen (H+) instead of the hydroxyl group (OH-) at the 3' position on their sugar component. This causes the elongation of the DNA molecule to stop (termination) after ddNTPs have been incorporated by the DNA polymerase. In practice, 5µl of DNA was mixed with the annealing mix and incubated for 2 minutes at 67^0C. The mixture was left to cool down slowly to reach the room temperature. A labelling and extension mix was added to the tube containing the annealed template. For each sample, the labelling extension mix contained 1 µl dithiothreitol (DTT, 0.1 M), 2 µl of labelling nucleotide mix (1:20), 0.5 µl of [α-^{35}S] or [α-^{32}P]dATP (5µCi) and 2 µl diluted sequenase polymerase (1:8, approximately 3.25 units). For each sample, 2.5 µl of dideoxy termination mixture was put in each of four V bottomed wells labelled 'G', 'A', 'T' and 'C' of a microtitre plate. Each termination mix contained 80µM dNTP (dGTP, dATP, dCTP, dTTP) 50mM NaCl and 8µM of one of the 4 ddNTP (ddGTP or ddATP or ddTTP or ddCTP). After a 5 minute incubation at 37^0C, the extension and termination

reactions were stopped by adding the stop solution (95% Formamide, 0.02 M EDTA, 0.1% Bromophenol Blue and 0.1% Xylene Cyanol FF).

2.13. POLYACRYLAMIDE GEL ELECTROPHORESIS

MATERIALS

Gel

- 20ml acrylamide/bisacrylamide solution (30%; 1.034%, respectively), Easygel, Scotlab, UK.
- 50g urea (BDH laboratory supplies, Poole, UK)
- 32ml distilled water
- 0.1 g ammonium persulphate (Sigma, Poole, Dorset, UK)
- 10ml 10x TBE (Sigma, Poole, Dorset, UK)
- TEMED (N, N, N'N'-Tetramethylethylenediamine, Sigma, Poole UK)

Fixative

-7% acetic acid (Rathburn Chemical Ltd, Scotland)

-7% methanol (Rathburn Chemical Ltd, Scotland)

- Tap water

PROCEDURE

The product of the sequencing reaction is a mixture of different sizes of single stranded DNA. During the sequencing reaction, the synthesis of the complementary DNA is made possible by incorporation of dNTPs. This reaction was terminated every time ddNTPs are incorporated during sequenase activity. Because DNA is negatively charged, it was possible to separate different fragments of DNA by

running the sequencing reaction product electrophoretically through a 5.3% polyacrylamide gel (containing 0.18% bisacrylamide). Throughout this study, wedged gels (thickness: 0.4mm at the top and 1.2mm at the base of the gel) were used. For 112ml of 5.3% gel, 50g urea, 20 ml of acrylamide/bisacrylamide solution [(30% and 1.034% w/v, respectively), Easygel, Scotlab, UK], 0.1 g ammonium persulphate and 10ml 10x TBE (pH 8.8) and 32ml distilled water were mixed under hot tap water. The mix was allowed to cool to room temperature, then 25µl of TEMED was added to the gel mix before pouring the gel between two cleaned glass plates separated by two wedged spacers and levelled horizontally. The even distribution of the gel between the glass plates was achieved by capillary motion. The gel was allowed to set for between 20 minutes and up to 24 hours. Prior to loading, the sequencing products were heated at 95^0C for 2 minutes to denature any double stranded DNA in the sequencing product, then loaded between spaces left by inserting a comb on top of the gel. The electrophoresis was set to run at 75 watts in a tank containing TBE, until bromophenol dye reached the bottom of the gel. The gel was fixed in tap water containing 7% acetic acid and 7% methanol for 10-15 minutes. The gel was then dried in a gel dryer (Bio-Rad, model 583) for 3 hours at 80^0C, and then exposed to X ray film (Kodak) in a dark chamber for about 24 hours.

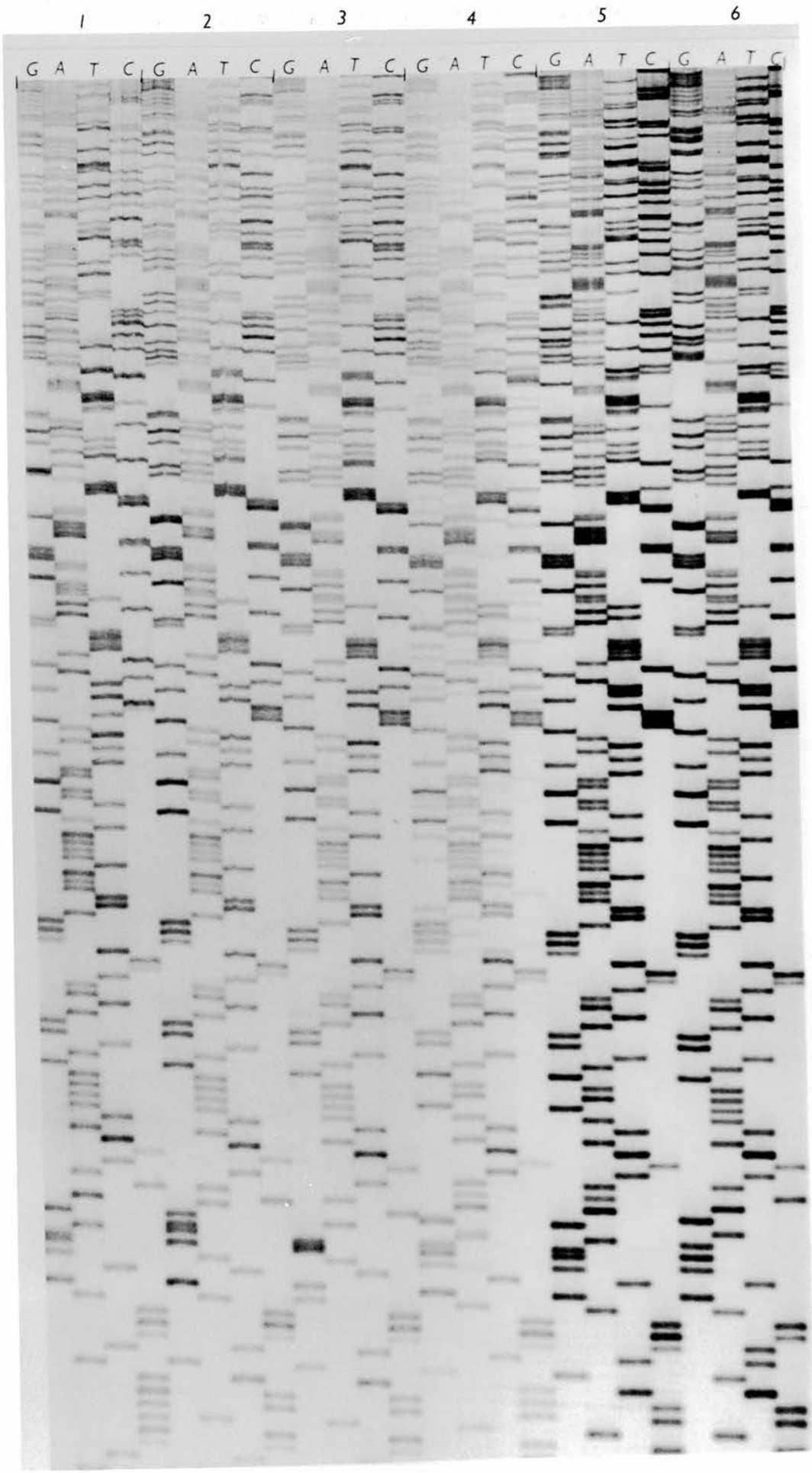
2.14. AUTORADIOGRAPHY

The films were developed using an automatic X-ray processor. Figure 2.3. shows an autoradiograph exposed overnight. From each patient, there were 4 sequencing reactions. The bands at the bottom of the figure represent shorter DNA fragments, with less nucleotide incorporated than those at the top of the gel. The

ddNTPs were incorporated after only a few dNTPs were joined together to form the complementary DNA. To read an autoradiograph, it is necessary to record the position of each band from the fastest moving to the heavy and slow migrating DNA molecules in four lanes. size in the four columns labelled. Each band in the column represents fragments of a DNA (same size) terminated by the incorporation of a dideoxynucleotide (ddNTP). There is one band for each level of the four columns. The sequences are read from the bottom (shorter DNA framents) to the top (longer DNA fragments) and noting for each level one band at a time.

FIGURE 2.3: *Autoradiograph showing nucleotide sequences*

The sequencing was carried out on single stranded biotinylated PCR products. The proviral DNAs were primarily amplified using the S3P1 and S3P4 during the primary reaction and S3P2 and S3P3 in secondary reaction (Figure 2.2 and Table 2.1). The numbers on top represents the patients from whom the samples were obtained (1: Sq61M; 2: Sq62M-1; 3: Sq62M-2; 4: Ped12; 5: Sq13M; 6: Sq63M). For each sample, there are four columns labelled G, A, T and C (2'-deoxyguanosine, 2'-deoxyadenosine, 2'deoxythymidine and 2'-deoxycytidine). Each band in the column represents a fragment of a DNA terminated by the incorporation of a dideoxynucleotide (ddNTP). There is one band for each level of the four columns. The sequences are read from the bottom (shorter DNA framents) to the top (longer DNA fragments) and noting for each level one band at a time.


1
2
3
4
5
6
G A T C G A T C G A T C G A T C G A T C G A T C

2.15. PHYLOGENETIC ANALYSIS

Sequences from this study were entered in a computer using Word Perfect computer software (Wordperfect Corporation, Orem, Utah, USA). The on-screen view applications were modified such that each letter representing a different nucleotide was shown in different colours (G: yellow, A: green, T: white and C: red). This allowed easy manual alignment during the reading of the nucleotide sequences. The alignment was carried out relative to sequences available in the Los Alamos database (Myers *et al.,* 1995). The Genetic Data Environment (GDE) package was also used to check the alignment (Smith *et al.,* 1994). Preliminary analyses were performed using Molecular Evolutionary Genetics (MEGA) software (Kumar *et al.,* 1993) using the Jukes-Cantor distance estimation method. Final phylogenetic analysis was carried out using the programmes available in the Phylogenetic Inference Package (PHYLIP) provided by Dr J. Felsenstein (Felsenstein, 1993). Phylogenetic trees were constructed using the neighbour-joining (Saitou and Nei, 1987) (program NEIGHBOR) and Fitch-Margoliash (Program Fitch) (Fitch and Margoliash, 1967) distance method, with nucleotide sequence distances calculated for all pairwise sequence comparisons using the generalised two-parameter (maximum likelihood) model (program DNADIST) or the Kimura two-parameter method using the MEGA software (Kumar *et al.,* 1993). The Kimura method estimates the distance between two sequences as if one has evolved from another. To calculate the divergence of two sequences (pairwise distance), the Kimura model takes into account two forms of substitution of nucleotides which can happen by

transition (purine replaced by purine or pyrimidine replaced by pyrimidine) or by transversion (purine replaced by pyrimidine, and *vis versa*). Assuming that one sequence has evolved from another, the estimated number of nucleotide substitution (K) was calculated by the expression:

$$K = -1/2 \ln[(1-2P-Q)\sqrt{(1-2Q)}]$$

where P is the number of sites at which the sequences differ by transition and Q the fraction of which they differ by transversion. The comparison was performed for each pair of sequences (interhost nucleotide distance) contained in the dataset and was generated as a matrix table (see appendix 5). The analysis of the distance matrix between and within subtypes was performed using the Systat computer programme (Systat for Windows, version 5.03, Evanston Illinois, USA). Bootstrap resampling (Felsenstein, 1985) (programs SEQBOOT and CONSENSE) was employed to assign support to the neighbour-joining tree. One hundred to 2,000 bootstrap replications were performed. The presentation of the phylogenetic trees was carried out using the Treeview computer programme kindly provided by Dr Roderic D.M. Page of the the Institute of Biomedical and Life Sciences (Glasgow, UK).

SEROLOGICAL METHODS

2.16. MUREX IgM-IgG ANTI-HIV-1+2 ELISA

(Base Pack GE01/02/03 and Detection Pack GE11/12/13)

The Murex assay used microwells coated with a mixture of rabbit anti-human-IgG and -IgM. Serum or plasma samples were incubated in the wells and a

proportion of human IgG and IgM is captured. Unbound antibody was removed by thorough washing. A conjugate made of HIV-1 recombinant antigens was added which bound to anti-HIV-1/HIV-2 IgM and IgG captured on the well. Unbound conjugate was washed away and a substrate containing 3, 3', 5, 5'-tetra methylbenzidine (TMB) and hydrogen peroxide was added. Wells with bound conjugate developed a purple colour, which turned into an orange colour when the reaction was stopped with sulphuric acid. The amount of the colour was determined spectophotometrically and was proportional to the amount of IgG and IgM present in the sample. Reactive samples were tested again with the same Murex test and confirmed with a second generation Abbott ELISA for antibody (IgG) to HIV-1 and HIV-2. All assays were performed according to manufacturers' instructions.

2.17. DETECTION OF ANTI-V3 LOOP ANTIBODIES

2.17.1. MONOMERIC PEPTIDES

The monomeric peptides, representing the principal neutralising domain of 5 HIV-1 subtypes (A, B, C, D, E), were kindly provided by Dr H.C. Holmes of the National Institute for Biological Standards and Control and by Dr R. Cheingsong-Popov of St Mary's Hospital, London. These peptides have previously been used for serotyping HIV-1 in relation to HIV-1 genotype (Cheingsong-Popov *et al.,* 1994). The amino acid sequences are shown in table 2.2.

2.17.2. BRANCHED PEPTIDES

The synthesis of the branched peptides was carried out under the supervision of Ms Karen MacEachran and Dr Howard Marsden at the Institute of Virology, University of Glasgow, UK. The branched peptides correspond to consensus sequences of deduced amino acid sequences encompassing the V3 loop region of subtype A-F sequences available in the Los Alamos database (Myers *et al.,* 1995). In addition, another peptide called Zr was derived from the amino acid sequence of the V3 loop of a highly divergent strain identified in the present study. The branched peptides (A-F, and Zr) were synthesised by continuous-flow Fmoc (9-fluorenylmethoxycarbonyl) chemistry. Each peptide was synthesised in a four-branched molecular structure with four glycine spacers between each V3 branch (monomeric peptide) and the lysine linker complex attached to the solid phase resin core (4BTGA) (Table 2.2). Peptides constructed in this way (branched structure with 4 glycine spacers) have an advantage over monomeric peptides, in that smaller amounts of peptides are necessary to detect traces of antibodies in serum or plasma (Marsden *et al.,* 1992).

The synthesiser used was a PSSM-8 model, which was designed to be able to perform simultaneously multiple solid-phase peptide synthesis. The amino acids were prepared and put at the right sequence order for the PSSM-8 to use in peptide synthesis. The synthesising scale was graduated in micromole (5-50 μmol for each of the eight reaction vessels) and synthesis performed using information entered in a computer software. The necessary amino acids needed were weighed and dissolved in dimethylformamide (DMF, cat. number: PST6020; Rathburn Chemical Ltd, Scotland).

TABLE 2.2. Monomeric and branched peptides

I. Monomeric peptide	***Sequence***
A:	KSVHIGPGQAFYAT
B:	KSIHIGPGRAFYTT
C:	KSIRIGPGQTFYAT
D:	RQRTHIGPGQALYTT
E:	DTSITIGPGQVFYRT
II. Branched peptide	
V3-BP-A	$(KSVHIGPGQAFYATG_4)_4K_3A$
V3-BP-B	$(KSIHIGPGRAFYTTG_4)_4K_3A$
V3-BP-C	$(KSIRIGPGQTFYATG_4)_4K_3A$
V3-BP-D	$(RQRTHIGPGQALYTTG_4)_4K_3A$
V3-BP-E	$(DTSITIGPGQVFYRTG_4)_4K_3A$
V3-BP-F	$(RKSIHLGPGQAFYTTG_4)_4K_3A$

Amino acid sequences of V3 loop monomeric and branched peptides. Peptides A-F peptides correspond to consensus sequences of deduced amino acid sequences of subtype A-F sequences available in the Los Alamos database (Myers *et al.*, 1995). Each branched peptide was synthesised by continuous flow Fmoc chemistry based on the corresponding monomeric peptide representing the same subtype.

To synthesise simultaneously 5 branched peptides (A-F), 54.8 ml of piperidine were dissolved in 127.8 ml DMF then the solution was added to appropriate tube in which the synthesis reaction took place. Amino acids were put in tubes and aligned in the order in which they are incorporated into the peptide. This process was carried out for 16 hours.

The cleavage of the resin residue from the peptide was carried out as follows. Each peptide-resin complex was washed with 2-3 ml of DMF. The peptide-resin complex was dried up by removing the DMF using nitrogen gas to flush it through the syringe containing the complex. The same procedure was repeated with tert-amyl alcohol (2-methyl-2 butanol, cat number: PST6005; Rathburn Chemical Ltd). A series of washes was carried out, using acetic acid, tert-amyl alcohol and di-ethyl ether. A mixture of reagents (81.5% Trifluoroacetic acid; 5% Thioanisol; 5% Phenol; 2.5% EDTA and 1% Triisopropylsilane and water) designated reagent K was prepared for the cleavage reaction of the complex. The reagent K (2 ml) was added to the washed resin-branched peptide complex and the mixture was left to cleave for 6 hours. The peptide solution was collected in a 15 ml falcon tube ready for precipitation of the peptide. The precipitation of the branched peptide was carried out by adding diethyl ether to the solution, mixing and centrifuging for 5 minutes at 2,500 revolutions per minute (rpm). At the end of the centrifugation, the supernate was poured off (without disturbing the pellet, peptide). The process was repeated twice.

Each peptide was dissolved in 40 ml of distilled water and transferred to a 200 ml round bottom flask. Prior to dry-freezing, the flask containing the peptide solution was shell frozen by rotating it on a bowl of dry ice and ethanol. The flask was then attached to a freeze-dryer and left overnight.

Dried peptide was collected from the flask and weighed. A small amount of peptide (0.5 mg diluted in water, 1mg/ml) was analysed for purity by high-performance liquid chromatography (HPLC). The evaluation of the purity of peptide by HPLC is based on the separation principle in that peptide and impurities in the synthesis product will separate as a result of their interaction with the insoluble support, or stationary phase. In addition, there was a cylindrical column that contained the derivatised support upon which separation occurred and a mobile phase (liquid) by which the separation is developed. The binding of the component in solution (peptides and debris) to the solid phase occurred through hydrophobic interactions with the column support of a two-system reverse-phase HPLC. The column was equilibrated in 0.05% trifluoroacetic acid (TFA) for sample loading and elution was produced by an increasing gradient of acetonitrile as a water miscible organic modifier.

2.17.3. OPTIMISATION OF ELISA CONDITIONS

To optimise the conditions of the ELISA, plasma samples from 23 HIV-1 infected individuals and 16 HIV-1 negative controls were tested under different conditions:

Concentrations of the branched peptide on the solid phase:

- 10 μg/ml,
- 1μg/ml,
- 0.1 μg/ml,
- 0.01μg/ml and
- 0.001μg/ml);

Incubation time;

- 1 hour,
- 30 minutes

Buffer: with or without Tween20.

2.17.4. INDIRECT BRANCHED PEPTIDE-BASED ELISA

MATERIALS

- Branched peptides (A-F and Zr)

- Flat bottomed plates: Nunc-F, cat. No. 67466.

- Carbonate coating buffer: 20mM sodium carbonate, pH 9.8

- Phosphate buffer saline (PBS), pH 7.4: NaCl 8g/l, KCl 0.2g/l, Na_2HPO_4 anhydrous 1.15g/l KH_2PO_4 0.2g/l. Alternatively, commercially available tablets were used (Dulbecco PBS, cat. 2810305, Sigma, UK)

- Blocking buffer or sample diluent (PBS, pH 7.4 containing 0.01% chloracetamide, 5% dried skimmed milk and 10% heat inactivated new born calf serum)

- Dried skimmed milk (Tesco, UK)

- Heat inactivated newborn calf serum (Life Technologies, cat 023-0610)

- Tween 20 (Polyethylene sorbitan monolaurate)

- Conjugate: Anti-human IgG $F(ab)_2$ (Sigma A2290) conjugated with peroxidase.

- 0.1M citrate phosphate buffer, pH 5.

    Citric acid 6.7g/l

    Disodium monohydrogen phosphate, 9.46g/l

- 30% Hydrogen peroxide

- 1-2-phenylenediamine dichloride (OPD, Dakopatts, Denmark, cat. no. S2000).

- goat antihuman IgG peroxidase conjugate (cat. A2290, Sigma, UK)

- Stop solution: 1M H_2SO_4

PROCEDURE

Each peptide was diluted in carbonate coating buffer (1μg/ml) and 100μl of the mix was added to each well (1ng/well) of an ELISA plate (Nunc-F immunomodule, cat. No. 67466). The plates were allowed to coat for 24 hours at room temperature, and were then washed with PBS and blocked with the blocking solution for 2 hours at room temperature. The plates were washed again and were ready for use. If plates had to be stored, 200μl of PBS containing 0.01% sodium azide was added to each well and plates were kept at 4^0C until use. Plates prepared this was could be stored for up to 3 months.

Each sample was tested against a panel of V3 loop branched peptides (A-F) coated in each of 6 wells of the same plate (Nunc-F, cat. No. 67466) and a cocktail-well containing all 6 peptides. Plasma (0.5μl), diluted 1:200 in sample diluent (phosphate-buffered saline, PBS) pH 7.4 containing 5% dried skimmed milk, and

10% heat-inactivated new born calf serum), was added to each well and incubated at 37^0C for 60 min. The plates were then washed with phosphate buffer saline (PBS). Anti-human IgG $F(ab)_2$ (Sigma A2290), conjugated with peroxidase was diluted 1:10,000 in sample diluent. This conjugate (100µl) was added to each well and incubated at 37^0C for 1 hour. After washing, the colour was developed for 20 minutes using ortho-phenylenediamine dihydrochloride substrate (OPD, Dakopatts, Denmark, cat. no. S2000). The reaction was stopped by the addition of 100µl sulphuric acid (1M), and the optical density (OD) measured spectrophotometrically at 492nm. Twelve HIV-1 negative samples were included in each plate. The cut-off (CO) value was determined as (mean of negative samples + 3 SD) x 2. The average cut-off lay between 0.15 to 0.3 OD units. The ratio of specific antibody bound to the peptide to the cut-off value was determined (OD/CO). The highest ratio of antibody binding to a particular peptide among the panel of A-F was determined, and the serotype was ascribed to that peptide.

2.17.5. V3 LOOP COMPETITIVE ELISA

The ELISA consisted of two phases (Table 2.3). The solid phase consisted of a panel of 6 peptides (A-F) and two peptide cocktails of all A-F peptides immobilised in wells of an ELISA plate (Nunc-F, cat. No. 67466) at a concentration of 1µg/ml in carbonate buffer. The liquid or competitive phase, contained a cocktail of branched peptides (10µg/ml) excluding the peptide found on the solid phase. The sample (0.5µl) was mixed with peptides in the competitive phase, then incubated with the

peptide on the solid phase for 1 hour (at 37^0C). Any cross-reactive antibodies in the samples are blocked by the high concentration of peptides in the liquid phase.

Each sample was controlled by two cocktail-control wells, the negative and the positive control wells. The negative control well was coated with a cocktail of all peptides (A-F) (1μg/ml carbonate buffer) and the sample tested in this well was pre-incubated with a cocktail of peptides at 10μg/ml (10x the concentration of peptides on the solid phase). In the positive control well, the sample was tested without the inhibition of the cross-reactive antibodies to V3 peptides. In addition, there were 4 negative controls which were tested in the same way as the patient samples and were used to determine the cut-off (mean OD of 32 negative wells plus 2 standard deviations).

The sample with highest OD/CO after inhibition of cross-reactive antibodies was recorded and the serotype assigned to the subtype corresponding to the peptide on the solid phase. The highest OD/CO had to be greater than the reading of the well with complete inhibition of antibodies (Table2.3, reading in well 8). If the reading in well 8 was over 1 (positive), the values of the individual A-F wells had to be subtracted prior to assignment of the subtype.

TABLE 2.3: Template showing the panel of peptides found on the solid phase (SP) and the liquid phase (LP) of the competitive ELISA.

	MICROTITRE WELLS							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Peptide on SP	A	B	C	D	E	F	A-F	A-F
Peptide on LP	B-F	A, C-F	A-B, D-F	A-C, E-F	A-D,F	A-E	-	A-F

2.17.6. MONOMERIC PEPTIDE-BASED ELISA

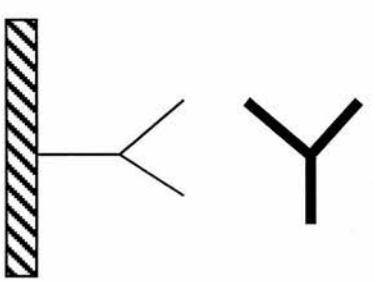
Antibody binding to a panel of V3 loop monomeric peptides representing the consensus sequences of subtypes A-E was tested as previously described (Cheingsong-Popov *et al.,* 1994). Briefly, flat bottomed microtitre plates (Nunc-F, cat. No. 67466) were coated with monomeric peptides at a concentration of 10μg/ml in 20mM carbonate buffer, pH 9.6, and stored at room temperature (RT) for 48 hours. The plates were washed, blocked with blocking buffer [phosphate-buffered saline (PBS) pH 7.4 containing 5% dried skimmed milk, and 10% heat-inactivated new born calf serum] for 2 hours at RT then washed twice. As for branched peptides, if plates were to be stored, 200μl of PBS containing 0.01% sodium aside was put in each well and kept at 4-8^{0}C until use. The ELISA procedure was the same as previously described (Cheingsong-Popov *et al.,* 1994) except that the conjugate was diluted 1:10,000 instead of 1:100.

2.18. ANTI-HIV-1/HIV-2 IgG, IgM AND IgA CAPTURE ELISAs

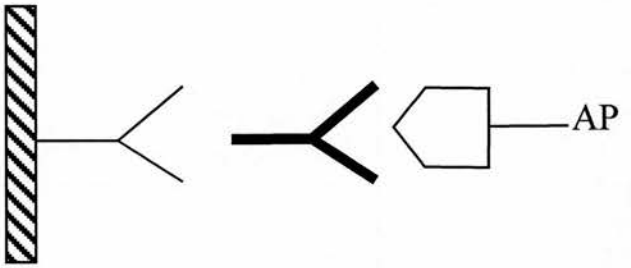
The immunoglobulin G class-specific capture ELISA (GACELISA) previously described by Connell et al (1993) was designed for detection of low levels of antibody (less than 0.1μg/ml) in samples such as saliva and urine. The test was adapted for the detection of anti-HIV-1 and HIV-2 IgA and IgM for early diagnosis of HIV-1 infection in children (Mokili *et al.,* 1996). The principle of GACELISA is illustrated in figure 2.4. Briefly, each well in U-bottomed plates (Nunc Immunomodule Maxisorb, cat.no. 4-77631) was coated with 100 μl of high affinity

anti-human IgG (DAKO, cat. no. A424) at a concentration of 5.3 mg/L of freshly prepared carbonate/bicarbonate buffer (1.6g Na_2CO_3, 2.94g $NaHCO_3$ in 1 litre of distilled water, pH 9.6). The plates were then left for antibody to adsorb at room temperature overnight in a moist chamber. Two hundred µl of 5% Sol-u-pro (Dynagel Inc, Illinois) was added and left at room temperature for 2 hours. The plates were washed and dried at 37^0C overnight. Plates prepared in this way could be stored, sealed in plastic bags for up to 3 months. Quality control of every new batch of plates was performed against previously prepared plates.

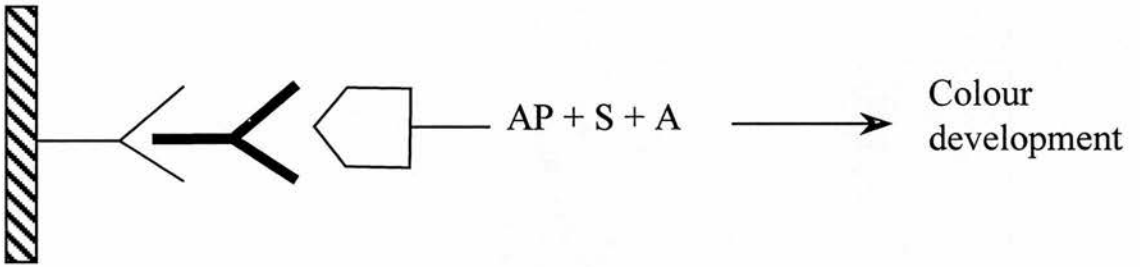
FIGURE 2.4. Schematic representation of the principle of the class-specific antibody ELISA



a) Solid phase coated with anti-human IgG capturing human IgG in plasma or serum



b) Detection of anti-HIV antibodies by proteins conjugated to alkaline phosphatase(AP)



c) Detection of captured conjugate by amplifying substrate (NADP) dephosphorylating to NAD. The reaction results in colour development.

A: amplifying reagent; AP: Alkaline phosphatase; NAD: nicotinamide adenine dinucleotide; NADP: nicotinamide adenine dinucleotide phosphate; S: Substrate.

The test consists of capturing IgG in the patient's plasma or serum with anti-human antibodies coated on the solid phase. Captured HIV antibodies are detected by adding recombinant core and envelope proteins conjugated to alkaline phosphatase. After removal of unbound materials by washing, the conjugate bound to the solid phase is detected by an amplifying substrate, nicotinamide adenine dinucleotide phosphate (NADP), which is dephosphorylated to nicotinamide adenine dinucleotide (NAD).

The immunoglobulin M class-specific capture ELISA (MACELISA) and the immunoglobulin A class-specific capture ELISA (AACELISA) were designed in the same format except that, for the detection of IgM and IgA, the solid phase was coated with anti-human IgM and anti-human IgA, respectively.

Five µl of sample was diluted in 95µl of sample diluent and put in a microtitre plate well containing anti-human antibodies bound on the solid phase. Three negative and two positive controls (serum containing HIV-1 and HIV-2 antibodies) were also added to the wells at a volume of 50 µl. Samples and controls were incubated for 1 hour at 37^0C (Fig. 2.3.a). After plates were washed three times and dry blotted, a 50µl conjugate was added into each well and incubated at 37^0C. The conjugate contained immunodominant gp41 and p24 antigen molecules linked to alkaline phosphatase (Figure 2.4.b.). A substrate was added (50µl) to each well and incubated for 20 minutes at 37^0C. The reaction enzyme-substrate was enhanced by adding 100µl of amplifier reagent in each well and left in a dark chamber at room temperature (Figure 2.4.c). The colour develops in wells corresponding to samples of patients infected with HIV. The intensity of the colour in each well was measured by spectrophotometry at a wavelength of 492nm.

CHAPTER 3

DETERMINANTS OF VERTICAL TRANSMISSION OF HIV-1 IN KIMPESE, RURAL DEMOCRATIC REPUBLIC OF CONGO, FORMERLY ZAIRE

3.1. INTRODUCTION

By the end of 1995, more than 6 million women had been infected with HIV in sub-Saharan Africa, out of a world total of nearly 9 million women (World Health Organisation, 1996). This total is expected to increase to 14 million by the year 2000. Aproximately one third of the babies born to HIV-infected women are also infected. The likelihood of transmission is higher in developing countries where reported rates vary between 20 and 48%, compared with 13 to 25% for industrialised countries (Dabis *et al.,* 1993).

Vertical transmission is increased when there is a heavy maternal viral load, as is found in advanced HIV disease and when the CD4 count (<700/mm^3), or CD4-to-CD8 ratio (<0.6) are low (Newell *et al.,* 1992; European Collaborative Study, 1992; Ryder *et al.,* 1989; Stlouis *et al.,* 1993; Peckham & Gibb, 1995). Breast-feeding is an important potential mode of transmission (European Collaborative Study, 1992). Placental inflammation and placental malaria may also increase the risk of transmission (Zachar *et al.,* 1994; Bloland *et al.,* 1995). Some studies suggested that high levels of maternal antibody to peptides in the V3 loop of HIV RNA protect against vertical infection (Rossi *et al.,* 1989; Scarlatti *et al.,* 1993a; Scarlatti *et al.,* 1993d), while others have not (Halsey *et al.,* 1992; Parekh *et al.,* 1991; Robertson *et al.,* 1992).

In the present study the rate of mother-to-child transmission was estimated in a population served by medical facilities in a small town in Bas-Congo (former Zaïre), where the maternal seroprevalence has remained stable at 4% between 1988 and

1993 (Green *et al.*, 1994). Evidence has been sought of a relationship between vertical transmission and breast-feeding, stage of the mother's infection, placental pathology, and maternal antibody response to V3 loop peptides.

3.2. RESULTS

3.2.1. MATERNAL FACTORS FOR MCT OF HIV-1

3.2.1.1. AGE AND PARITY

Two hundred and eighty seven mothers enrolled, of whom 208 were infected with HIV. Seven infected women and one uninfected woman enrolled in two pregnancies. The mean age of transmitting mothers did not differ significantly from that of infected women who did not transmit (24.6 vs. 26.2 years, Kruskal-Wallace, p = 0.06), neither did the mean number of previous pregnancies differ significantly (2.5 vs. 3.1, Kruskal-Wallace, p = 0.1).

3.2.1.2. MORBIDITY AND MORTALITY

Because of small numbers at different clinical stages, mothers were grouped according to whether they were symptomatic or not. Similar proportions of transmitting and of non-transmitting mothers had symptoms of HIV infection (27% vs. 29%, $\chi^2 = 0.02$, P = 0.9). During the period of study, eleven of the 196 infected mothers and one of the 80 uninfected mothers died (Fisher exact test, p = 0.09).

3.2.1.3. PLACENTAL CHANGES

Moderate or large numbers of malaria parasites and moderate or large amounts of malarial pigment were seen in higher proportions of placentae from HIV positive women than from negative women, but chorioamnionitis and funisitis were not significantly associated with maternal HIV infection (Table 3.1). There was no significant association between vertical transmission and any of these histological conditions.

TABLE 3.1. *Proportions of placentae showing malaria parasites and pigment, chorioamnionitis and funisitis*

Mother	n	**Parasites**	**Pigment**	**Chorioamionitis**	**Funisitis**
HIV positive	43	30%[a]	26%[b]	26%	17%
• Transmitting	14	36%	21%	29%	8%
• Non-transmitting	29	28%	28%	24%	21%
HIV negative	54	7%	6%	29%	15%

Comparison was made between 1) HIV positive and negative mothers and 2) mothers who transmitted and those who did not. a: $\chi^2 = 8.6$, P = 0.003 for HIV positive vs. HIV negative; b: $\chi^2 = 7.8$, p = 0.005 for HIV positive vs. HIV negative.

3.2.2. DETERMINANTS OF VERTICAL TRANSMISSION OF HIV-1 IN CHILDREN

3.2.2.1. LENGTH OF GESTATION AND BIRTH WEIGHT

The mean length of gestation did not differ significantly in uninfected babies according to the HIV status of their mothers, but was slightly less in infected babies compared with all uninfected (39.3 vs. 39.7 weeks, P = 0.01). Babies born before 40 weeks were 2.3 times as likely to be infected (95% CL 1.2, 4.6). Birth weight did not differ significantly with the status of either the mother or the baby (mean for infected babies: 2,740g, uninfected: 2,860g, P = 0.5).

3.2.2.2. MORTALITY

In the 24 months from birth, there were 35 (16.5%) known deaths in the 211 children born to HIV positive mothers and 6 (7.4%) deaths in the 81 children of negative mothers. Comparison of the two groups by Kaplan-Meier life table analysis indicated a significantly increased mortality in the HIV-infected group in the first two years of life (Lee-Desu statistic = 4.4, p=0.04). The causes of death are given in Table 3.2.

TABLE 3. 2. Causes of child deaths

HIV STATUS		No. of	Principal condition
MOTHER	**CHILD**	**Deaths**	**(age of death in months)**
Infected	Infected n=34	12	AIDS (4, 11, 12, 22, 22) chronic enteritis (2, 11, 17, 23) bronchopneumonia (19) pulmonary tuberculosis (3) unknown (9)
Infected	Uninfected n=108	4	septicaemia (14) Chronic lower resp. Infection (23) Malaria (10) unknown (15)
Infected	Indeterminate n=67	19	Septicaemia (0, 0, 0, 2, 7, 12) Pneumonia (3, 4) respiratory insufficiency (2) pulmonary tuberculosis (10) chronic lower respiratory infection (2) malaria (8, 9, 12) gastro-intestinal malformation (2) anaemia (1) perinatal death (0, 0) unknown (4)
Uninfected	Uninfected n=81	6	prematurity (0, 0) perinatal death (0, 0) malaria (9) retinoblastoma (16)

3.2.2.3. COGNITIVE AND MOTOR DEVELOPMENT

Eleven children born to HIV-1 positive mothers and who were vertically infected (PP) were compared with 15 seroreverters (children who lost maternal antibodies) (PN) matched for age, gender, educational level of the mother and general economic status of the home environment as assessed from a home evaluation. In addition, 15 children born to HIV-1 negative mothers (NN) who were also HIV-1 negative as determined by Abbott recombinant HIV-1/2 ELISA, were also included as a control group. The NN children were also matched with the PP children as described for PN children. The cognitive and motor assessment was performed by a trained nurse blind for children's HIV status. The methods for the assessment of the children included the Quaker arm circumference (determined by the upper arm circumference divided by the height of the child x100), the Early Childhood Screening profile (ECSP), and the Kaufman Assessment Battery for Children (K-ABC) (Boivin *et al.,* 1995).

The results of the assessment of these children have been reported elsewhere (Boivin *et al.,* 1995). In assessing the global ECSP indicator of cognitive or language development, there was no significant differences among the PP, PN and NN children except for the motor development. Both PN and NN children scored higher than PP children (Table 3.3). The poor motor development by the PP children could be explained by their impaired general physical development as indicated by the QUAC (Boivin *et al.,* 1995)

TABLE 3.3: Cognitive and motor development of children born to HIV infected mothers and control

	PP (n=11)		PN (n=15)		NN (n=15)		
	Mean	*SD*	*Mean*	*SD*	*Mean*	*SD*	p
Language	96.0	10.3	102.3	13.9	97.5	18.6	>0.05
Cognitive	79.1	9.3	82.8	7.4	84.6	14.0	>0.05
Motor	83	14.2	104.6	24.7	115.29	29.4	<0.05

Between-group comparisons of the vertically infected children (PP, n=11), seroreverters born to HIV-1 infected mothers (PN, n=15) and control children born to seronegative mothers (NN, n=15) on the standardised measures from the early children screening profiles (Boivin *et al.,* 1995).

3.2.2.4. ESTIMATION OF VERTICAL TRANSMISSION RATES

By the criteria of the Working Group on Mother-to-Child Transmission (Dabis *et al.*, 1993), 34 of the 209 offspring of infected women were infected, 108 were uninfected, and the remaining 67 were of indeterminate status. The lower estimate of vertical transmission rate given by the direct method, making the assumption that the indeterminates were not infected, was 16.2% (95% confidence limits: 11.7, 22.1). The intermediate estimate, ignoring indeterminates, was 23.9% (17.3, 32), and the upper estimate, assuming that all indeterminates were infected, was 48.3% (41.4, 55.3). The indirect method, using the children's antibody results and the excess mortality of children born to infected women, was 25.1% (16.8, 33.4).

Nine infants lost IgG antibody as measured by GACELISA and/or Abbott ELISA and then became positive again. As none had received a blood transfusion, they were assumed to have been infected postnatally as a result of breast feeding. Of these children who lost maternal antibodies, two became positive again by the age of 6 months, two more by 9 months, a further two by 12 months and the remaining one by 18 months. The rate of pre- and intranatal transmission, ignoring the presumed postnatal infections, is 13.6% (9.1, 19.6), 18.8% (12.7, 26.7) and 50% (42.5, 57.4) for the lower, intermediate and upper direct estimates, and 20.4% (12.3, 28.6) for the indirect estimate.

3.3. DISCUSSION

The rate of vertical transmission was 23.9% using the intermediate direct method recommended by the Working Group on Mother-to-Child Transmission (Dabis *et al.,* 1993) and 25% by the indirect method. These figures are in the lower part of the range found in developing countries, but are higher than in most reports from industrialised countries (McIntosh *et al.,* 1994; Nesheim *et al.,* 1994; Rodriguez *et al.,* 1996; Dabis *et al.,* 1993). Ignoring babies who appeared to be postnatally infected, the calculated rate for pre- and intranatal transmission, which accounted for roughly three quarters of infections, was similar to vertical transmission rates in industrialised countries.

Postnatal transmission due to breast-feeding was not thought to be important in 11 of the 13 studies reviewed by the Working Group and in a subsequent Indian study (Kumar *et al.,* 1995; Dabis *et al.,* 1993). However, late seroconversion or the recurrence of positive status after a period of seronegativity indicated that vertical infection due to breast-feeding occurred in studies in France and Uganda, as well as in the present study (Blanche *et al.,* 1989; Datta *et al.,* 1994).

A review of transmission of HIV through breast-feeding concluded that the risk was greatest when the mother became infected post-natally (Dunn *et al.,* 1992), presumably because she is likely to have a high level of virus in body fluids, including breast milk, shortly after being infected. The World Health Organisation

and the United Nations Childrens' Fund have recommended that HIV infected women should not breast-feed if artificial feeding is practicable and safe (World Health Organisation, 1992). Breast-feeding by infected women is still advocated in developing countries where death from other infectious diseases and malnutrition is a greater threat than death from HIV. For our mothers and babies breast-feeding was the only option.

Lower rates of breast-feeding in industrialised countries are likely to be one reason for lower reported rates of vertical transmission in the latter. Another contributory factor is the greater prevalence of vitamin A deficiency in developing countries which increases the likelihood of HIV-1 DNA being present in breast milk and doubles the rates of vertical transmission overall (Semba *et al.,* 1994; Nduati *et al.,* 1995). In addition, selection bias may exaggerate rates reported from developing countries (Ryder and Behets, 1994). Most studies in the latter have been done in major hospitals where women with symptomatic HIV infection are more likely to be confined than asymptomatic women; symptomatic women are liable to have high viral loads (Wang *et al.,* 1995) and therefore to be transmitters (Dickover *et al.,* 1996).

The indirect method of estimating the rate of vertical transmission is of particular value if lack of diagnostic facilities make it difficult to classify children of HIV infected mothers as either infected or not. This was evident in a study in Haiti

(Halsey *et al.,* 1990), where the intermediate direct estimate of the vertical transmission rate was 13%, compared with a more probable indirect figure of 21% (Dabis *et al.,* 1993). However, indirect estimates, because they rely on the difference in mortality between the babies of HIV-infected and uninfected mothers, will be biased if mortality of babies is increased by infected mothers being too ill to feed or care for their babies adequately. The Study Group recommended that in the indirect method the control group of babies born to uninfected mothers should be at least as large as the group from infected mothers. This was not so in our study, so the confidence limits are probably wider than they would have been otherwise.

A minor source of bias in the direct method is that infected children are classified by IgG antibody level at age fifteen months, whereas the uninfected are classified at nine months. Thus, if there are many children between these ages at the end of a study, the uninfected will be classified but the infected will be indeterminate, so giving a falsely low transmission rate.

A number of studies have shown that vertical transmission is related to viral load in the mother's blood (Peckham and Gibb, 1995; Dickover *et al.,* 1996). Attempts to measure this association was carried out in this study. Unfortunately, pressed by shortage of funds and time, it was not possible to standardise our in-house limiting dilution method. In addition, the fact that samples were collected from a region with a high genetic heterogeneity (see chapter 5), some samples from known HIV-1 infected individuals failed to be amplified although a wide range of different primers was used (see materials and methods, page 62; 65-66). This difficulty was

also encountered by Alaeus *et al.*, (1997) in testing plasma from individuals infected with different subtypes of HIV-1.

Though chorioamnionitis appears to be associated with maternal HIV infection and preterm birth by infected women and may predispose to vertical transmission (Nelson *et al.,* 1992; Gichangi *et al.,* 1993; Nair *et al.,* 1993), there was no indication of this in the present study.

When maternal plasma was examined for neutralising antibodies directed against V3 loop consensus peptides representing five HIV-1 subtypes, the samples reacted with at least one peptide in most infected women (details in chapter 6). It is possible that cross-reactivity of the antibodies inflated the number of women appearing to have antibodies to different V3 loop peptides, but it has been shown in this thesis that a number of subtypes are present in this African population (Chapter 5). There was no evidence that any of the five antibodies protected against transmission. Previous studies of the effect of anti-V3 loop antibodies on vertical transmission have given conflicting results, but another report indicates that antibodies of maternal origin may protect against transmission when present on the infant's peripheral blood mononuclear cells (Wang *et al.,* 1995).

This study has not identified factors whose modification might reduce mother-to child transmission of HIV. However, 9 of the 34 infected infants appeared to acquire infection postnatally as a result of breast-feeding. As breast-feeding is essential for most babies in developing countries, there is an urgent need for the timing and other possible determinants of infection by this route to be fully investigated.

CHAPTER 4

DIAGNOSIS OF VERTICALLY ACQUIRED HIV-1 INFECTION BY CLASS-SPECIFIC IgG, IgM and IgA CAPTURE ASSAYS

4.1. INTRODUCTION

In any individual case, the timing and mechanism of mother-to-child transmission of HIV are not clear. There is evidence for infection *in utero* (Specher *et al.,* 1986; Mano and Chermann, 1991), just prior to or during delivery (Krivine *et al.,* 1992), and by breast-feeding (Datta *et al.,* 1994; Van de Perre *et al.,* 1993; Ziegler, 1993). In the developing world breast-feeding is recommended by the World Health Organisation (World Health Organisation, 1992), but it nevertheless increases the risk of HIV transmission from mother-to-child (Dunn *et al.,* 1992). A number of laboratory tests, reviewed by Sison and Campos (1992), may detect transmission which has occurred before or during the neonatal period, including polymerase chain reaction (PCR), HIV culture, and *in vitro* antibody and *in vitro* antigen production. However, most are expensive, technically demanding and unsuitable for use in laboratories in developing countries. A possible exception is the detection of HIV p24 antigen. While Andiman *et al.* (1992) found only 20% of samples collected from infected babies in the first month of life to be HIV antigen positive, Miles *et al.* (1993), employing a preliminary acid dissociation step, reported p24 antigen in 80% of HIV infected neonates. Ascher *et al.* (1992) enhanced the detection of p24 antigen by acid pre-treatment of the sera to dissociate immune complexed antigen. However, when Fauvel *et al.* (1993) further assessed the value of acid pre-treatment of the sample they concluded that although a small improvement in sensitivity was possible

in the first two months of life acid treatment of specimens made confirmation of reactive samples essential. This increases the volume of specimen needed and the cost.

The presence of IgA anti-HIV in at-risk infants has been shown to be predictive of HIV infection in the child. Immunoblots have been employed to identify IgA and IgM anti-HIV in samples collected at and following birth, but removal of competing IgG from the samples by absorption with protein G prior to testing was necessary (Weiblen *et al.,* 1990). Many of the published data have been obtained using Western blot or immunoblots which incorporate recombinant HIV antigens (Weiblen *et al.,* 1990; Martin *et al.,* 1991; Quinn *et al.,* 1991; Portincasa *et al.,* 1992; Re *et al.,* 1992). In these studies, when samples collected between birth and 3 months have been tested, the sensitivity for IgA detection has been low (range 16 to 18%). In one study, however, IgA anti-HIV was detected in all 10 HIV-infected children tested at birth and in 2 further infected children tested at 2 months of age, but not in any of 5 uninfected children at risk (Portincasa *et al.,* 1992). This study assumes that maternal IgA anti-HIV does not cross the placenta in which case transmission must have occurred in all cases long enough before birth for the babies to mount an IgA response. This assumption conflicts with the current consensus that a substantial proportion of transmissions occur perinatally (Ehrnst *et al.,* 1991; Krivine *et al.,* 1992). In another study, however, IgA anti-HIV was detected in

similar proportions of uninfected (12/37, 32%) and infected (4/12, 33%) neonates born to HIV infected mothers (Connell *et al.,* 1992). Its presence was associated with strong IgA anti-HIV reactivity in the mothers' serum, suggesting that acquisition of maternal IgA had occurred. With the exception of one study (Portincasa *et al.,* 1992), these studies indicate that IgA anti-HIV production commences in the infected infants between 3 and 6 months (22 to 66% IgA anti-HIV positive), and that most are producing IgA anti-HIV by 9 months (range 54 to 99%). IgA anti-HIV has rarely been found in uninfected infants of 3 months and older.

In the present study, immunoglobulin class specific antibody capture EIAs for IgA, and also for IgM and IgG anti-HIV were devised and evaluated for the diagnosis of HIV infection in children at risk of infection from their mothers. These assays are simple, cheap and require only small volumes of untreated serum or plasma specimens. They can be readily used by laboratory services in developing countries where, because of breast-feeding, children continue to be at risk of HIV infection into the second year of life and beyond (Datta *et al.,* 1992).

4.2. PATIENTS

Thirty-four children born to seropositive mothers were diagnosed as infected (PP group) by the presence of IgG anti-HIV at eighteen months of age, the detection of p24 antigen, AIDS defining illness before eighteen months of age, detection of

proviral DNA or HIV-related death (Centers for Disease Control, 1987). There were two control groups of children: 92 who did not develop the infection during the period of follow up but whose mothers were HIV positive (PN group) and 21 children born to anti-HIV negative mothers (NN group). Plasma samples were collected from the three groups of children in the neonatal period (cord blood and/or peripheral blood) and at 3, 6, 9, 12, 18 and 24 months of age. Because no alternative was available the mothers were not advised to stop breast-feeding.

4.3. RESULTS

4.3.1. OPTIMISATION OF THE ELISA CONDITIONS

For the IgG test (GACELISA), three negative controls (NC1, NC2 and NC3) and 2 positive controls (PC1 and PC2) were included in each test. The negative controls were samples pre-calibrated by MUREX diagnostics, containing HIV-1 positive samples diluted in plasma from seronegative individuals (Dr Urszula Beckford, personal communication). The positive controls were collected from HIV infected individuals, CP1 from HIV-1 and CP2 from HIV-2 infected patients. On one occasion, the negative control had an OD above 0.3. This value was rejected and the calculation of the cut-off was determined with two negative controls. The summary of the performance of negative and positive controls in 51 ELISAs is shown in table 4.1.

TABLE *4.1. Repeatability of negative and positive control results in 51 tests (G-EIA)*

Controls	Mean OD	Minimum	Maximum	STD	St. Error
NC1	0.192	0.109	0.687	0.08	0.01
NC2	0.186	0.131	0.336	0.045	0.006
NC3	0.183	0.109	0.291	0.043	0.006
HIV1	1.532	0.707	2.438	0.5	0.072
HIV2	1.616	0.312	2.656	0.5	0.081

*Student's 't', testing whether mean differs from zero.

For the IgA and IgM tests, positive controls were kindly provided by Dr Jeff Connel of the Public Health Laboratory Service, Colindale, London. The controls were previously found to be reactive and were serially diluted to provide a measurable scale (0.1, 1, 3, 10 and 30 units). The threshold for positivity was determined by adding the arbitrary value of 0.2 to the mean of three negative controls for GACELISA, MACELISA and AACELISA. The positive controls had to be reactive for the test to be considered as valid. For the AACELISA and MACELISA, the positive results could be negative with the 0.1 units and weakly reactive on the 1 unit positive controls but the 3, 10 and 30 unit positive controls had to be positive for the test to be valid.

4.3.2. IgA, IgM and IgG ANTI-HIV-1 IN CONTROL CHILDREN

A total of 142 sequential samples were collected from 21 anti-HIV negative children born to HIV uninfected mothers. These samples were all negative when tested by IgA, IgM and IgG ELISAs giving a 100% specificity of each of the tests.

4.3.3 IgA, IgM and IgG ANTI-HIV IN CHILDREN BORN TO HIV INFECTED MOTHERS

The samples tested from children were collected at birth and at 6 weeks, 3, 6, 9, 12, 15, 18 and 24 months. During the phase I study, samples were not collected at 6 week and 15 months of age

4.3.3.1. NEONATAL SAMPLES

A total of 261 samples (cord blood n=113 and venous blood n=148) were collected from neonates born to HIV-1 positive mothers during the perinatal period. Ignoring the cord blood, the average age of the babies when the 148 venous blood samples were taken was 2.9 days (median 3, minimum 0 and maximum 17 days).

Of 109 neonatal samples (56 cord and 53 venous blood) 3 samples (all cord) collected from HIV-1 positive mothers were reactive when tested for anti-HIV-1 IgG by Abbott recombinant HIV-1+2 and GACELISA (137/137). One of the three children with a negative result in the cord blood sample was born to a mother who was seroconverting around the delivery period. There was no obvious explanation for the other two children in whom IgG was not detected in cord blood. Nevertheless, one of these two children died of cryptococcal meningitis at the age of three months, a strong indication of immunodeficiency at that age.

Surprisingly, IgM anti-HIV-1 antibodies were found in 61/103 and in 61/128 cord blood and samples collected perinatally from babies born to HIV-1 infected mothers, respectively. Similarly, anti-HIV-1 IgA was detected in 58/129 (45%) neonatal venous blood samples and in 63/103 (61.1%) cord blood samples from babies of HIV-1 infected mothers. The proportions of samples that were IgA positive versus those that were IgM positive in cord or venous neonatal samples did not differ statistically (Mantel-Haenszel test: $p>0.5$) (Table 4.2). While the proportion of

samples that were IgM anti-HIV-1 positive did not differ significantly whether cord or venous bloods were tested, there was a statistically significant higher proportion of IgA anti-HIV-1 in cord blood than in neonatal venous blood (Mantel-Haenszel test, p=0.014). However, the level of the reactivity for reactive samples was not significantly different. Indeed, the mean of optical density over the cut off was 1.9 (range: 1-6.9) for IgM and 2.2 (range: 1-8.7) for IgA. Sixteen (19.2%) samples which were IgA anti-HIV positive were not reactive when tested for IgM anti HIV-1. On the other hand, 13 samples were reactive with the IgM but not with IgA anti-HIV capture assay.

The presence of class anti-HIV IgA and IgM in neonatal samples and the outcome of HIV infection in children

The proportions of IgA and IgM detected in cord and venous blood of children born to HIV positive mothers who subsequently were shown to be infected (PP children) and PN children (born to HIV-1 positive mothers but not infected) are shown in figure 4.1. In PP children, of 20 cord blood samples available, 17 (85%) and 15 (75%) were reactive with anti-IgA and IgM assays, respectively. Samples collected by venepuncture were also shown to be reactive by anti-IgA and anti IgM assays [12/17 (70.6%) and 10/17 (58.8%), respectively). However, in PN children, out of 47 cord blood samples available for testing, 30/47 (63%) were IgA positive and 31/47 (66%) had detectable IgM. Half of the venous samples were positive for both IgA and IgM (24/48; 50%). The proportion of infected children who had

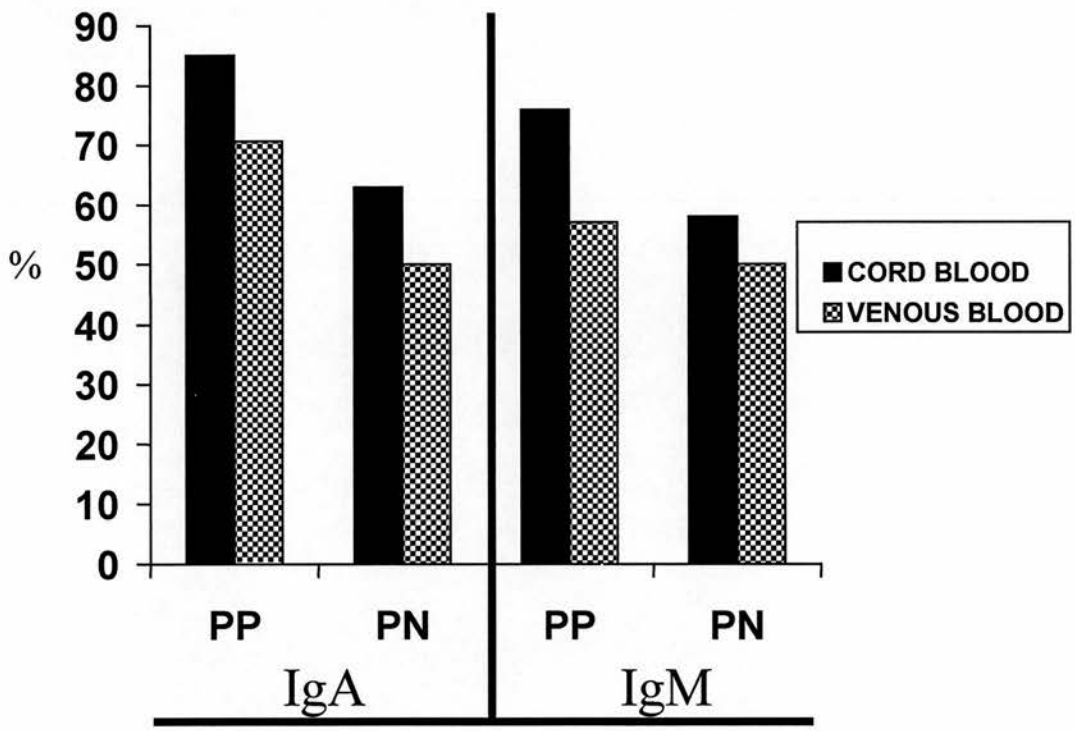
detectable IgA and IgM anti-HIV-1 in cord blood or in venous samples did not differ statistically to that of PN children ($p=0.07$ for IgA; $p=0.4$ for IgM).

TABLE 4.2: Comparison of reactivity of cord vs venous blood in IgA and IgM test

	IgM	IgA	p value*
Cord blood	61/103	63/103	0.7
Venous blood	61/128	58/129	0.6
p value	0.06	**0.014095**	

*p value calculated by Mantel-Haenszel test.

FIGURE 4.1: *Proportion of IgA and IgM class anti-HIV in cord and venous blood samples*



Samples (cord blood and venous blood) were collected from children born to HIV-1 infected mothers. The comparison was made between 20 children who were infected with HIV-1 (PP) and 47 who were not (PN).

TABLE 4.3. A. IgA and IgM class anti-HIV detected in cord and venous blood of infected (PP) and uninfected (PN) children born to HIV-1 positive mothers.

	PP CHILDREN		PN CHILDREN	
	CORD	VENOUS	CORD	VENOUS
IgA	17/20 (85%) [A]	12/17 (70.5%) [B]	30/47 (63.8%) [C]	24/48 (50%) [D]
IgM	15/20 (75%) [E]	10/17 (58.8%) [F]	31/47 (65.9%) [G]	24/48 (50%) [H]

Note high proprotion of IgA and IgM being in neonatal samples.

Comparison between proportions:

CELLS	Chi^2	p value
• A vs. B	0.44	0.508
• C vs. D	1.85	0.173
• E vs. F	1.1	0.294
• G vs. H	2.48	0.115
• A vs E	0.16	0.69
• B vs. F	0.52	0.47
• C vs. G	0.05	0.82
• D vs. H	0	1

TABLE *4.3.B. IgA, IgM and IgG class anti-HIV in uninfected children born to HIV-1 infected mothers (PN) at different ages.*

	AGE (at time of collection of samples)						
	6w	3m	6m	9m	12m	18m	24m
IgA	3/24 12.5%	0/80 0%	0/71 0%	0/65 0%	0/77 0%	0/48 0%	0/33 0%
IgM	6/24 25%	0/80 0%	0/70 0%	0/65 0%	0/77 0%	0/48 0%	0/34 0%
IgG	23/27 85%	69/80 86.2%	9/74 12.2	9/65 13.8%	1/90 1.1%	0/49 0%	0/35 0%

(PN, negative children born to HIV-1 infected mothers; w: weeks; m: months)
Note IgA and IgM detected at 6 weeks and not after, consistent with the suggestion that there is passive transfer of IgA and IgM across the placenta. Also note gradual decline of the proportion of IgG anti-HIV-1; no IgG anti-HIV-1 detected at 18 and 24 months of age.

TABLE 4.3.C. IgA, IgM and IgG in positive children infected with HIV-1 born to mothers (PP) who are also infected with HIV-1.

	AGE						
	6w	3m	6m	9m	12m	18m	24m
IgA	1/7 14.2%	4/31 12.9%	17/29 58.6%	20/27 74%	22/30 73.3%	28/28 100%	9/12 75%
IgM	1/7 14.2%	3/31 8.3%	10/29 48%	15/26 57.7%	17/30 56.7%	28/28 100%	8/12 66.6%
IgG	7/7 100%	24/27 88.9%	19/26 73%	21/22 95.4%	24/30 80%	28/28 100%	12/12 100%

(PN, negative children born to HIV-1 infected mothers; w: weeks; m: months)

Because IgA and IgM anti-HIV were still detected in uninfected children at 6 weeks of age (Table 4.3.b), detection of IgA and IgM from 3 months onwards was a strong indication of HIV infection in children.

Class-specific IgA and IgM anti-HIV-1 in neonatal samples by maternal stage of HIV-1 infection in mothers

Stratified data analysis was performed to determine maternal factors in HIV-1 infected mothers associated the presence of IgA and IgM in pediatric samples (Table 4.4). The factors included particularly maternal age, the stage of disease of the mother and histological features of the placenta (funisitis, malarial parisite, malarial pigment, and chorioamnionitis). Because samples from children born to seronegative mothers were all non-reactive with the IgA and IgM capture assays, only maternal factors of HIV-1 positive mothers were considered in the present analysis.

Maternal Age (data not shown) and stage of HIV infection (Table 4.4) were not associated with presence of IgA and IgM in neonatal samples. There was no statistically significant relationship between chorioamnionitis or funisitis and the presence of IgA-class and IgM-class antibodies in pediatric samples (Table 4.4.a.). In addition, no association was observed between the presence of IgA and IgM class-specific antibodies in neonatal samples with malarial pigment found in placenta of the mothers. However, there was an association between malarial parasite in placenta and IgA in neonatal samples ($p=0.0285$) (Table 4.4.b). It is intriguing that the presence of malaria parasite should prevent passage of IgA across the placenta. It is likely that there are some other confounding factors which explain the association between IgA and IgM in neonates.

Tables 4.4: Placental histopathology, HIV stage and IgA/M class-specific antibodies in neonatal samples

A. Association between placentatis and presence of IgM and IgA in neonatal samples

		Funisitis*				Chorioamnionitis*			
		0	**1**	**2**	**3**	**0**	**1**	**2**	**3**
IgM	**Neg**	13	1	1	2	8	2	4	3
	Pos	21	3	3	1	19	1	4	2
		P=0.6				P=0.3			
IgA	**Neg**	18	3	2	2	13	5	4	3
	Pos	26	5	2	1	24	2	4	2
		P=0.8				P=0.23			

A. Association between malaria pigments and parasites in placenta and presence of IgM and IgA in neonatal samples

		M. pigment*				M. parasite*			
		0	**1**	**2**	**3**	**0**	**1**	**2**	**3**
IgM	**Neg**	6	3	6	2	8	1	2	6
	Pos	13	9	6	0	14	6	6	2
		P=0.157				P=0.074			
IgA	**Neg**	12	5	5	2	11	4	2	7
	Pos	18	8	8	0	21	5	7	1
		P=0.36				P=0.0285 ←			

B. Association between the stage of HIV infection and presence of IgA and IgM in neonatal samples

		HIV stage**			
		2	**3**	**4**	
IgM	**Neg**	11	4	2	
	Pos	23	1	7	
		P=0.07			
IgA	**Neg**	21	2	2	
	Pos	27	3	7	
		P=0.48			

* 0= no evidence, 1= minor changes, 2= moderate, 3= marked

** The stage of HIV infection in mothers was classified by 0= uninfected, 1= early seroconversion illness, 2=asymptomatic, 3=symptomatic pre-AIDS, 4=AIDS.

\# M= malarial. Arrow indicates association.

4.3.3.2. IgA, IgM and IgG CLASS ANTI-HIV-1 IN POST-NATAL SAMPLES OF CHILDREN BORN TO HIV-1 POSITIVE MOTHERS

The samples were taken at 6 weeks of age from a small group of children during the second phase of the study (between 1993 and 1995). In samples available from 27 PN children at that age, IgG could still be detected in 23 (85.2%). In comparison to what was found in neonatal samples, the proportions of specific IgA and IgM in PN children showed a significant decline from 30/47 (63%) and 31/47 (66%), respectively, to 3/24 (12.5%) and 6/24 (25%) at 6 weeks. In seven HIV-1 infected children tested at 6 weeks, the IgG tests were all positive. Only one out seven PP children was still reactive for IgM and IgA at 6 weeks of age. By the age of 3 months, while IgG anti-HIV-1 was still detectable in 69/80 (86.3%), none of the samples from 80 PN children was IgA or IgM positive at 3 months ($p<0.0001$). Infected children also had detectable IgG at 3 months at a similar proportion with the PN children (24/27; 88.9%) but IgM and IgA anti-HIV were found in 3 (8.3%) and in 4 (12.9%), respectively, of 31 children tested.

At 6 months of age, 7 of PP 26 children (26.9%) were non-reactive for IgG as tested by GACELISA and the commercially available Abbott recombinant HIV-1+2 while the remaining 19 samples were still reactive. At the same age, the proportions of samples with IgA and IgM class anti-HIV were significantly higher than those observed at 3 months in PN children and were 17/29 (58.6%) and 10/29 (48%),

respectively. At 9 months of age, 21/22 (95.4%) PP children were IgG anti-HIV reactive. The reactivity of the IgM test was relatively lower as 15 (57.7%) of 26 infected children were shown to have detectable antibodies. However, the IgA test had a higher sensitivity to detect HIV-1 infection in children tested at 9 months of age. With this assay, 20 of 27 (74%) samples from infected children were reactive for IgA anti-HIV-1 antibodies. In addition, seroreversion was more apparent in the PN children at 9 months. While 9 (13%) out of 65 PN children were still reactive with IgG assay at 9 months, none of the PN group appeared to be reactive with IgA and IgM assays.

Four PP children did not have detectable IgA anti-HIV in any sample collected between 3 and 12 months of life. These children also lost IgG anti-HIV reactivity during their first 12 months, suggesting that infection occurred well after birth. Two of them had symptoms possibly due to HIV at 6 months, the third had non-specific findings and the fourth child was asymptomatic throughout the first year. The presence or absence of HIV-associated symptoms in the first year of life did not correlate with the presence or absence of IgA anti-HIV. Sequential PCR testing on children who lost maternal antibodies and were anti-HIV-1 IgA and IgM negative between 6 and 12 months also showed transient negative PCR results before the first PCR was positive.

IgG, IgA and IgM in PP and PN children at 12 and 18 months of age:

Whereas the reactivity of PP children at 12 months for IgG anti-HIV was 80% (24/30), IgM was detectable in 17/30 (56.7%) and IgA in 22/30 (73.3%). At this age, 1 (1.1%) of 90 PN children still had detectable maternal IgG anti-HIV while neither IgA nor IgM anti-HIV-1 was detected in 77 samples tested. Four samples were available from PP children at 15 months and were IgG and IgA anti-HIV-1 reactive. The control PN children were unreactive for IgG, IgM and IgA anti-HIV at 15 months of age.

IgG, IgA and IgM in PP and PN children at age 18 months of age and over:

None of the 90 seroreverters, was reactive with either IgG, IgA or IgM anti-HIV-1 assays. This provides a high specificity (100%) of these assays after seroreversion. All 28 samples from the PP children were IgG, IgA and IgM positive at 18 and 24 months.

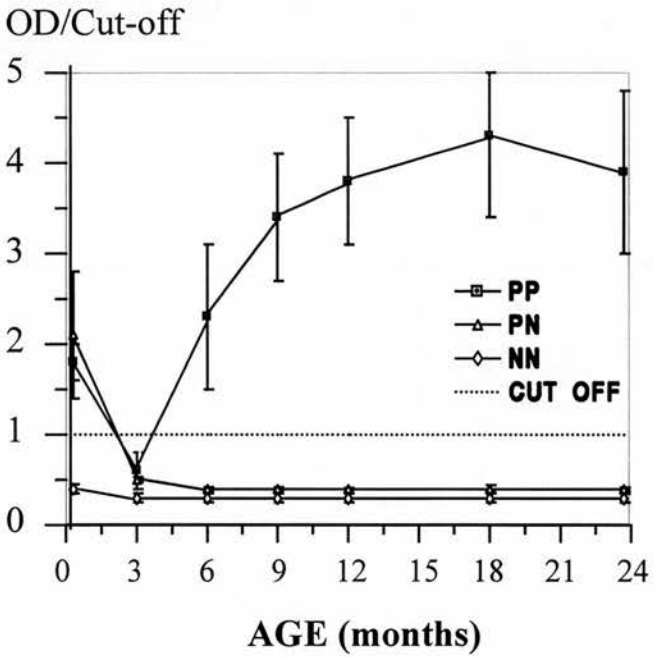
4.3.3.3 PROFILES OF IgG, IgM and IgA CLASS ANTI-HIV-1 IN CHILDREN

The profiles of IgG, IgM and IgA class anti-HIV in the three groups of children (PP, PN and NN) are presented in figure 4.2. Anti-HIV-1 IgA (Figure 4.2.a), IgM (Figure 4.2.b) and IgG (Figure 4.2.c) antibodies were detected in children born to HIV-1 infected mothers at birth. IgA and IgM showed a rapid decline to complete clearance at 3 months of age in uninfected children. This suggested that the IgA and IgM anti-HIV-1 detected in the infants' neonatal samples were of maternal

origin. The presence of specific IgA and IgM from 3 months onwards was a strong indication of HIV infection in children. Clearance of maternal IgG was more rapid in uninfected than in infected children.

FIGURE 4.2. **IgA (4.2.a), IgM (4.2.b) and IgG (4.2.c) class immunoglobulins to HIV-1 in PP, PN and NN children.**

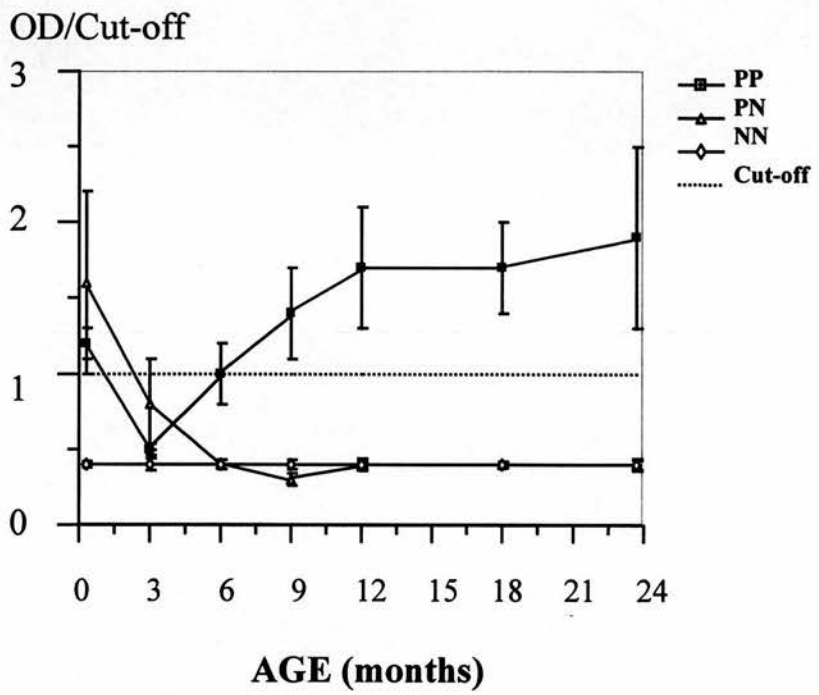
4.2.a: IgA class anti-HIV-1 in PP, PN and NN children*



*The analyses were performed on sequential samples collected at birth, 3, 6, 9, 12, 18 and 24 months of age from 21 HIV-1 infected children born to seropositive mothers (PP), 21 seroreverters born to HIV-1 infected mothers and 21 seronegative children born to HIV-1 negative mothers. The mothers of the control children (PN and NN) were matched with the mothers of infected children for age, parity and stage of disease (CDC stage). Antibodies were detected by in-house capture ELISAs. Standard error bar: 95% confidence limit of the mean.

Figure 4.2. (continued)

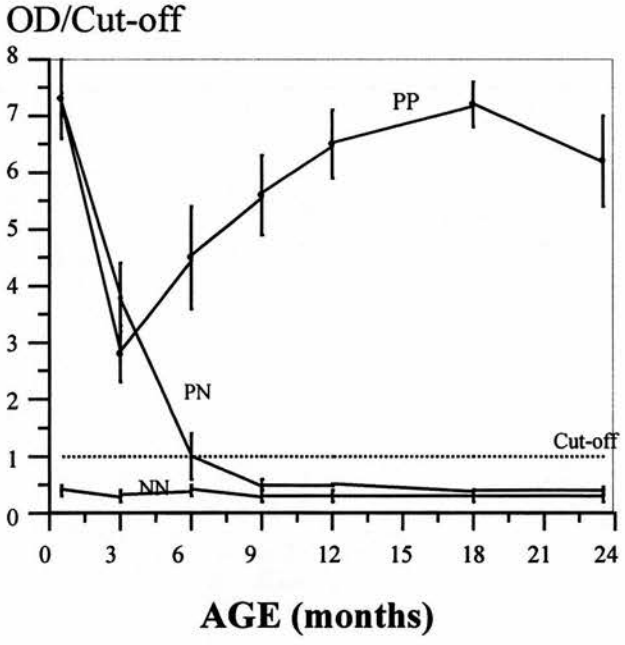
4.2.b. IgM class anti-HIV-1 in PP, PN and NN children*



* see note in figure 4.2.a

Figure 4.2. (continued)

4.2.c. IgG class anti-HIV-1 in PP, PN and NN children*



* see note in figure 4.2.a.

4.4. DISCUSSION

It is commonly accepted that 'only' IgG readily crosses the placental barrier to the fetal circulation. This passively transferred antibody is important as it provides immune protection to the newborn. Unfortunately, the presence of maternal IgG in children confounds the use of assays for IgG class anti-HIV in the early diagnosis of HIV-1 infection in newborns. An alternative is the detection in neonates and young children of specific immunoglobulins such as IgA and IgM which 'do not' cross the placental barrier (Portincasa *et al.,* 1992; Sison and Campos, 1992; Miles *et al.,* 1993). The lack of passive placental transfer of IgA and IgM has been explained by the fact that these antibodies are larger in molecular weight as a result of their dimeric (IgA) and pentameric (IgM) structure (Avrech *et al.,* 1994).

In this study, the investigation of IgA and IgM class anti-HIV-1 was carried out in sequential samples collected from children born to HIV-1 infected and uninfected mothers. These antibodies were found at high proportion in children born to seropositive mothers, irrespective of whether they were subsequently shown to have been vertically infected or seroreverted after losing maternal IgG. In an earlier study of European babies, it was shown that the simple and inexpensive class-specific antibody capture assays employed in this study were sensitive and specific (Connell *et al.,* 1992). In that study 20 (83%) of 24 HIV-infected babies of between 3 and 12 months of age were IgA class anti-HIV positive by AACELISA.

Substantial proportions of uninfected infants between birth and 3 months of age, but not older, also had IgA class anti-HIV in their blood and it was suggested that this antibody had been passively transferred from the mother. The presence of IgA and IgM class anti-HIV in the neonatal blood of some uninfected infants born to HIV positive mothers was confirmed in this study of African children. In view of the earlier findings, however, it was initially surprising to find an apparently lower sensitivity for IgA and IgM anti-HIV detection in these Central African children who were subsequently confirmed to be HIV infected. Overall, only 62 of 116 (53.4%) bloods collected from them between 3 and 12 months (inclusive) of age were IgA anti-HIV positive, many of them weakly so. Tests for the presence of IgM anti-HIV also proved less sensitive and they seemed not to augment the IgA findings. The IgA findings were also at variance with those of Quinn et al (1991) and Weiblen et al (1990) who used immunoblot procedures to detect IgA anti-HIV in HIV infected infants.

Why should this be? Two factors may have accounted for this discrepancy. Firstly, all of the babies in this study, unlike others, were breast-fed by their mothers. Whereas breast-feeding had been thought until recently to carry only a small risk of mother-infant transmission of HIV (Behets *et al.,* 1991) it has now become apparent that up to half of mother-child transmissions in breast-fed children occur by this route (Dunn *et al.,* 1992). Such transmissions are delayed compared with those *in utero*

and at birth so that IgA anti-HIV may appear later. Secondly, it was apparent in this study and others (Re *et al.,* 1992; Weiblen *et al.,* 1990) that an IgA anti-HIV response is absent in many, but not all, children who experience rapid HIV-disease progression. This could account for poor IgA response in 3 of the HIV-infected children studied here. In testing European children, for whom breast-feeding is discouraged, it has not been shown thus far that a child who was subsequently diagnosed as HIV-infected can lose reactivity for IgG anti-HIV and then regains it (Dr JV Parry, personal communication). In the series of HIV-infected children reported here, however, 8/34 (24%) children lost anti-HIV as measured by the GACELISA and/or Abbott EIA at some stage in the first year of life; several others became only weakly reactive. With the exception of 2 children who died, all later became unequivocally seropositive. This is evidence that perhaps a quarter of the HIV-positive infants studied here were infected well after birth, presumably through breast-feeding, and is in accord with other findings (Dunn *et al.,* 1992). The 4 infants in whom an IgA anti-HIV response was not detected between 3 and 12 months all showed transient loss of IgG anti-HIV reactivity over the same period.

For the PN group IgG anti-HIV reactivity was lost earlier in the GACELISA assay than in the indirect (Abbott) assay. This confirms similar findings in another study (Parekh *et al.,* 1993) and may prove to be a useful feature. Particularly in babies who have not been breast-fed the GACELISA may permit earlier

identification of babies who have escaped HIV infection compared with a conventional anti-HIV screening test.

Unfortunately, there is no information on the frequency or duration of breast-feeding in the earlier published work on IgA anti-HIV detection in at-risk children. If, as seems likely, many children in these other studies were wholly or largely bottle-fed, infection might only rarely have occurred postnatally. By contrast, in this study, transmission could have occurred at any time from birth until the children who escaped intrauterine or perinatal infection were completely weaned (Van de Perre *et al.,* 1993; Datta *et al.,* 1992; Ziegler, 1993). Risk of transmission by breast-feeding may accrue with time due to the duration of exposure of the child. It is possible that the decline of the mother's immune competency can lead to higher levels of viral replication and lower levels of partially protective maternal antibody and the emergence of more transmissible strains of HIV in the mother. Moreover, it might be that the dynamics of the immune response in a child infected orally under 'cover' of maternal antibodies is suppressed compared to that of adults or of children infected at or before birth.

If it is the case that a specific IgA response is delayed or absent in breast-fed babies infected postnatally, is there any value in attempting early diagnosis by IgA antibody capture anti-HIV testing? I believe there is, for the following reasons. First, in this study and others, it has been demonstrated that, when present, IgA anti-

HIV (and IgM anti-HIV) in the blood of children of 3 months and older is highly predictive of HIV infection in that child. By contrast, other anti-HIV reactivity in screening tests, predominantly IgG, is often present in uninfected children even beyond 12 months of age, making it difficult to distinguish infected from uninfected children. Second, the method employed in this study is robust and simpler and cheaper than tests for IgA anti-HIV which require preliminary removal of IgG and the immunoblot assay. The IgA antibody capture ELISA employs conventional ELISA methods and its reagent costs should be no more than 2 to 3 dollars (US): immunoblot reagents cost approximately 10 fold more. Third, although the precise time of transmission was not known for 34 HIV-infected children in this study, it is clear that postnatal transmission had occurred frequently. In such circumstances it would not be possible to determine at what point other tests, e.g. for HIV p24 Ag or proviral DNA might become positive relative to IgA anti-HIV seroconversion. It can be assumed that p24 antigen detection and PCR would also have shown an apparently low sensitivity in the first few weeks or months of life as, in some cases, transmission would not yet have occurred. Fourth, even in situations where PCR and HIV p24 antigen testing can be afforded and done to a high standard of accuracy, some laboratories in developed countries have found it valuable to have a serological method, such as detection of IgA anti-HIV, to confirm HIV infection in children who are positive for these other markers of HIV infection (JV Parry, personal

communication). Fifth, in a previous study, Tosswill *et al.* (1994) have experienced failures both of HIV p24 Ag testing and PCR to diagnose HIV infection in HIV-infected babies of over 3 months of age whose serum was shown to contain IgA class anti-HIV. Sixth, it is often difficult to obtain sufficient blood from an infant or young child to perform HIV p24 Ag testing and/or PCR. As the IgA capture method requires only tiny volumes (5µl or less), it can be performed on specimens which are insufficient for a p24 Ag test (often use 200µl of plasma). Although not formally demonstrated here, the IgA capture assay, like the IgG capture assay, would probably function equally well on eluates prepared from dried blood spots.

In summary, the main findings of this study were the observation that IgA and IgM class anti-HIV can be passively transferred through the placenta like IgG. This hampers early diagnosis of HIV-1 in children. However, unlike IgG which can remain detectable up to 12 months and beyond, passively acquired IgA and IgM clear quickly. From three months of age their detection is a strong indication of HIV infection. A simple, inexpensive assay for IgA anti-HIV, such as described here, would be ideally suited to laboratories that are unable to perform complex and expensive tests, such as PCR and viral culture, to diagnose mother-to-child transmission of HIV. Although the sensitivity of the method in breast-fed infants is imperfect, it is highly specific. In circumstances where other approaches are not

available the method will, by 6 months of age, permit the identification of three-quarter of babies who become infected *in utero*, perinatally or postnatally. Others will need to be followed up for longer, especially if breast-fed. Early diagnosis can reduce the stress to parents of not knowing their child's HIV status and and allow early introduction of appropriate prophylactic therapy and monitoring of the infected child initiated.

CHAPTER 5

MOLECULAR CHARACTERISTICS OF THE p17 gag REGION: IDENTIFICATION OF DIFFERENT HIV-1 SUBTYPES AND THE ROLE OF FUNCTIONAL NUCLEIC AND AMINO ACID SIGNATURES IN THE MOTHER-TO-CHILD TRANSMISSION OF HIV-1

5.1. INTRODUCTION

Why some children born to HIV positive mothers become infected and others do not is an area of intense investigation and much controversy. The risk factors that affect the transmission of HIV-1 from infected mothers to their offspring can be assembled in two groups: the endogenous and exogenous factors. It is possible that these factors influence transmission in a synergistic manner. This issue was discussed in Chapter One. Briefly, the endogenous factors include all the variables related to the host - the mother and to some extent, the child. For example, the clinical stage of HIV infection of the mother is an important factor for vertical transmission of HIV-1. It has been shown that women with symptomatic HIV-1 infection or with low CD4 counts and those who had a previous child with AIDS may be at more likely to transmit HIV to their offspring (Ryder *et al.,* 1989; St Louis *et al.,* 1993). St Louis *et al.* (1993) examined various maternal factors and showed that the risk of MCT ranged from 7 to 71%. In that study, it was shown that when mothers had p24 antigenemia and CD8+ lymphocyte counts of at least $1.8x10^9$/L the risk of mother-to-child transmission was high. In addition, in women with neither high CD8+ nor low CD4+ lymphocyte counts, placental membrane inflammation was strongly associated with MCT. A role for maternal neutralising ability has been identified by some groups (Goedert *et al.,* 1989; Rossi *et al.,* 1989), and not by others (Halsey *et al.,* 1992; Parekh *et al.,* 1991; Robertson *et al.,* 1992).

On the other hand, there are exogenous factors which are the biological properties of the agent: HIV-1. HIV-1 variants vary in the way they affect cells. Some variants are fast replicating [with syncytium inducing (SI) phenotype] and others cause little (or no) cytopathic effect [non-syncytium inducing (NSI) phenotype]. NSI isolates appear to be transmitted preferentially from mother to infant even when the mother's strain is predominantly SI (Scarlatti *et al.,* 1993b; Spencer *et al.,* 1994). These biological phenotypes are mirrored in the genetic sequences of the viruses.

The phylogenetic analysis of HIV has identified two genetically distinct types, HIV-1 and HIV-2. There is clear evidence that there are biological differences between these two types. Although both cause AIDS, HIV-1 transmission has resulted in a global pandemic, whereas HIV-2 has remained relatively isolated geographically. In some areas of West Africa where the two viruses co-circulate, it has been observed that the seroprevalence of HIV-1 was increasing while that of HIV-2 was decreasing (DeCock *et al.,* 1993).

Strains of HIV-1 have been grouped into at least eight genotypic subtypes (A-D, F-I) within group M (main) and the more divergent outlier (O) group (Myers *et al.,* 1995). Another group M clade has been provisionally classified as 'J' and is awaiting additional independent reports of strains with similar genetic features (Leitner *et al.,* 1995). Very little is known about the effect of genetic diversity on

viral infectivity. Subtype B is the predominant subtype in Europe and North America. It is intriguing to speculate why this subtype is rare in Africa where higher numbers of infected people are found. More recent and more compelling evidence about the functional differences of HIV-1 subtypes, has shown that subtype B is less able than subtype E to infect Langerhans' cells (SotoRamirez *et al.,* 1996) which are found in the mucosa of the cervix, the vagina and the penile foreskin. These findings were suggested as an explanation for the preferential distribution of subtype B among drug users and subtype E among sex workers in Thailand (Ou *et al.,* 1993; Kalish *et al.,* 1995). More interestingly, a case control study of Thai men and their female sex partners supported the suggestion that subtype E is more transmissible sexually than subtype B. Using the differences between HIV-1 and HIV-2 and also the transmissibility difference between subtype B and subtype E as a background, it was decided to determine the distribution of genetic strains of HIV among pregnant women and their children in the present study of a Congolese population in Africa.

To date, most studies aiming to determine the functional significance of different HIV-1 subtypes have been based on genetic information extracted from the region encoding the envelope glycoproteins gp120, particularly the hypervariable region, the V3 loop (Briant *et al.,* 1995; Wolinsky *et al.,* 1992; Scarlatti *et al.,* 1993b; Mulderkampinga *et al.,* 1993). The focus on this gene can be justified by its high immunogenicity and its role in neutralisation by antibodies. For this reason, the

envelope region constitutes an important target for the development of an HIV-1 vaccine (Keefer *et al.,* 1996; Jelonek *et al.,* 1996; Girard *et al.,* 1995; Salmonceron *et al.,* 1995; Gorse *et al.,* 1996; OToole *et al.,* 1996; Ahlers *et al.,* 1996; Belshe *et al.,* 1993; Shirai and Klinman, 1993; McElrath *et al.,* 1996; Wagner *et al.,* 1996).

Analysis of HIV-1 quasispecies in mother-child pairs, has shown that either minor or major viral variant can be transmitted from mother-to-child (Ahmad *et al.,* 1995; Wolinsky *et al.,* 1992; Scarlatti *et al.,* 1993b). Wolinsky *et al.* (1992) have shown that sequences obtained from vertically infected babies lacked an N glycosylation site that was present in maternal sequences, suggesting that the loss of this site may in some way enhance the infectivity of the virus. However, only 3 of 7 children studied by Ahmed *et al.* (1995) and one of three children in another study (Mulderkampinga *et al.,* 1993) showed the absence of this N glycosylation site. Such a feature was not observed in any of the V3 sequences of the 10 children studied by Scarlatti *et al.* (1993b). These discrepancies between studies suggest that no specific association can be made between genetic features studied within the *env* region and the transmission of HIV-1 from mother-to-child.

It is important to analyse other regions of the genome to determine their relevance in MCT of HIV-1. In the present study, the emphasis was mainly on the p17 region for such an investigation and the rationale for this choice is as follows. First, it was shown by Demareuil *et al.* (1995) that the p17 region of an isolate from

the former Zaire HIV-1_{NDK} interacted with the *env* gene to define the viral phenotype. In that study, the variability in three regions, including p17 gag matrix, correlated with formation of large syncytia. It was suggested that the p17 matrix protein is capable to influence virus-cell fusion during formation of syncytia (Demareuil *et al.,* 1995). Secondly, the p17 region plays an important role during viral replication. It has been suggested that the myristoylated N terminal region of p17 *gag* plays an important role during the assembly of the viral particle (Spearman *et al.,* 1994; Reicin *et al.,* 1995). In addition, the p17 region is involved in the formation of the preintegration complex and in the genomic transport of the genome to the cell nucleus, which allows the virus to infect non-dividing cells (Karageorgos *et al.,* 1993). Changes in the p17 region may affect the transport of the cytoplasmic complex and hence influence viral infectivity. Gallay *et al.* (1995) demonstrated that phosphorylation of the C-terminal tyrosine of p17 is a critical step during infection of new cells. Thirdly, compared with the V3 region, the p17 region is less variable and more suitable for molecular epidemiological mapping of HIV-1 (Holmes *et al.,* 1993; Myers *et al.,* 1995). Finally, a study by Narwa *et al.* (1996) based on a small group of mothers and children, suggested that there are motifs within the p17 region which may be associated with mother-to-child transmission of HIV-1 (Narwa *et al.,* 1996). This study has thus far not been confirmed.

In the present study, sequences were determined of HIV-1 variants from infected mother-child pairs and were compared with sequences obtained from non-transmitting mothers. The phylogenetic analysis was performed in order to determine the subtype of each HIV-1 variant and additional analyses were carried out to determine the association of p17 amino acid motifs with vertical transmission of HIV-1. It was also of interest to see whether one particular subtype was more prevalent than others, and therefore responsible for the low and stable seroprevalence and low MCT rates observed within the study population.

5.2. RESULTS

5.2.1. IDENTIFICATION OF HIV-1 SUBTYPES

A total of 72 sequences were determined, of which 63 were from epidemiologically unlinked individuals[1] (57 mothers and 6 children) and 9 from children born to seropositive mothers from whom sequences were also available. The sequences (225bp) in the *gag* region were aligned with reference sequences previously published, which are available in the Los Alamos database (Myers *et al.*, 1995). The nucleotide sequences were analysed to determine their placement in the previously defined phylogenetic tree (Myers *et al.*, 1995). As a prerequisite, it was important to determine whether the length of these sequences (225bp) and the region (*gag*) in which they were analysed were suitable for the classification of HIV-1 strains.

To investigate the suitability of the length (225bp) and the region used in phylogenetic analysis of Kimpese strains, a phylogenetic tree was constructed using a dataset of 83 reference sequences aligned relative to that of the HIV_{HXB2} isolate. These reference sequences were previously classified into well-defined subtypes by analysis of the full *gag* and/or the full *env* gene (Myers *et al.*, 1995). It was essential

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[1] Two individuals are designated as epidemiologically linked if there is a history of recent transmission of HIV between them. For example, an HIV-1 infected blood donor and a recipient or a mother and a vertically infected child can be so designated.

that all the reference sequences cluster within their originally defined clades with equidistance lineage and a star-like phylogeny (Myers *et al.,* 1995; Kostrikis *et al.,* 1995; Leitner *et al.,* 1995). Indeed, these requirements were met in that all the reference sequences strongly clustered within their original subtypes as previously documented (see appendix 4) (Myers *et al.,* 1995). This suggested that the regions and the length analysed were adequate for subtyping the HIV-1 strains in the study population.

Phylogenetic analysis was then carried out on sequences from the present study (length: 225bp) together with the Los Alamos sequences used here as references (Myers et al., 1995). A Fitch-Margoliash tree was constructed using the maximum likelihood distance method and is presented in figure 5.1. Interestingly, no subtype B sequence was found in the Kimpese cohort. The sequences obtained from 63 epidemiologically unlinked individuals examined in this study clustered with sequences grouped within 6 previously established subtypes (A, C, D, F, G and H). Twenty-eight epidemiologically unlinked individuals were infected with subtype A. These included 25 mothers (Sq3M, Sq6M, Sq9M, Sq11M, Sq12M, Sq13M, Sq27M, Sq29M, Sq31M, Sq32M, Sq34M, Sq36M, Sq37M, Sq38M, Sq39M, Sq40M, Sq43M, Sq46M, Sq48M, Sq49M, Sq51M, Sq52M, Sq54M, Sq55M) and 3 children (Ped2, Ped10 and Ped12). The average interhost-distance for sequences classified as subtype A sequences was 12.9% (range: 4.8%-23.3%). A sequence obtained from

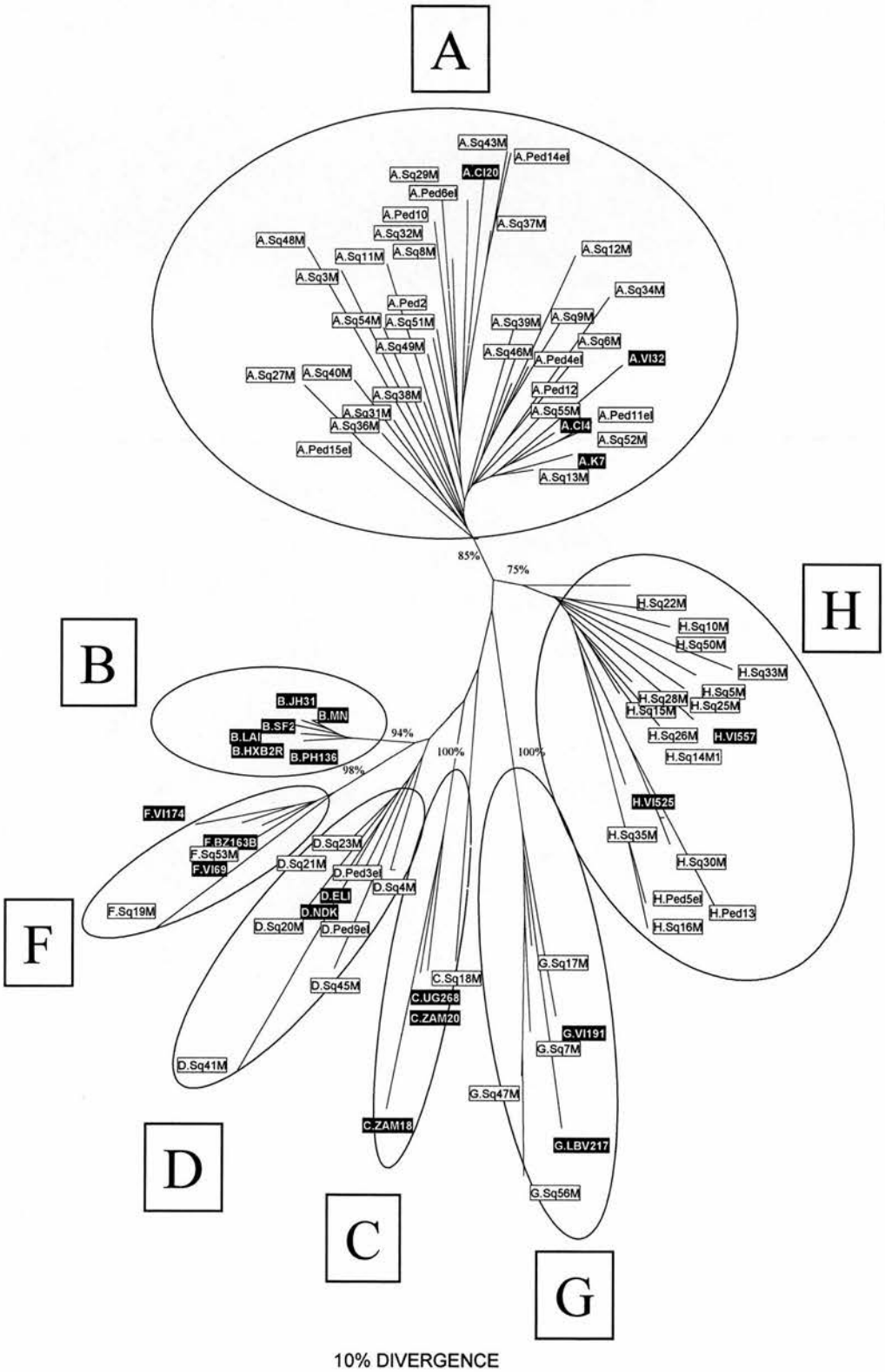
mother Sq18M clustered with subtype C reference sequences and 5 mothers (Sq4M, Sq20M, Sq21M, Sq23 and Sq41M) were infected with subtype D. Two other sequences (Sq19M and Sq53M) were grouped with subtype F sequences (VI174, BZ163 and VI69) (Myers *et al.,* 1995). In addition, Sq7M, Sq17M, Sq47M and Sq56M clustered together with two reference sequences, LBV217 and VI191, previously classified as subtype G (Myers *et al.,* 1995). Subtype H included a large number of sequences from Kimpese, with 13 representatives (Sq5M, Sq10M, Sq13M, Sq15M, Sq16M, Sq22M, Sq25M, Sq26M, Sq28M, Sq30M, Sq33M, Sq35M, Ped13). Thirty samples, which classified by the analysis of 215bp (figure 5.1), were also analysed with longer sequences (315bp). The subtype inference with the later dataset was the same as in the 215bp long dataset.

The Fitch-Margoliash tree shown in figure 5.1 was reconstructed as a Neighbor-joining tree using the maximum likelihood distance method. The resulting tree showed a similar topological distribution of sequences from Kimpese in clades (data not shown). The Fitch tree and the Neighbor-joining trees agreed to 98.4% (of total sequences, n= 63), as one sample (C.Sq18M) which was classified as subtype C in maximum likelihood tree formed a separate branch.

FIGURE 5.1. *Phylogenetic tree of HIV-1 variants from Kimpese.*

This is a Fitch-Margoliash tree constructed using sequences in the p17 region of HIV-1 variants from the study population and Los Alamos reference sequences (Myers *et al.,* 1996). The phylogenetic analysis was based on an unambiguously aligned 225bp region of the *gag* gene (position: 907-1131 in the HIV_{HXB2} genome). Maternal sequences from this study are labelled by a letter showing the subtype followed by SqN (Sq: Sequence, N: number) and the letter M (maternal sample). Pediatric samples are labelled with a letter indicating the subtype, followed by 'Ped' and a number. If a pediatric sample is followed by 'el', it indicates that patient was epidemiologically linked to a mother. The bootstrap values expressed in % are shown close to the corresponding branch and indicate the robustness of the grouping of a clade. Only bootstrap values ≥75% are shown. Letters inside boxes indicate the subtype. The scale bar indicates 10% of nucleotide substitutions. **Note** that at least one sequence from the study population clustered among the reference sequences of clades A, C-H and none among subtype B reference sequences.

FIGURE 5.1. **Phylogenetic tree of HIV-1 variants from Kimpese**



The degree of divergence of HIV-1 within the studied population was estimated by the Kimura two-parameter distance estimation method (Kumar *et al.*, 1993). The nucleotide distances between sequences in this study are shown in appendix 5. In summary, the interhost nucleotide distances between HIV-1 variants obtained from epidemiologically unlinked individuals was on average 18.3% and ranged between 1% (D.Sq21M-D.Sq23M, and H.Sq30M-H.Sq35M) to 37% (D.Sq56M and H.Ped13). The mean pairwise distance in 62 epidemiologically unlinked individual sequences was 18.5% and ranged between 0.9% to 37.7%. The degree of heterogeneity within the *gag* region of sequences from Kimpese is relatively higher than the average pairwise distances previously documented by Louwagie *et al.* (1993). In this study, the interhost distances within the same clade A, D, F, G and H were on average 13%, 11%, 14.6%, 11.2 and 13% respectively (Table 5.1; Appendix 5), higher than expected. The distance between individual sequence and reference sequences of the same subtype was in all cases lower than the distance of that sequence to reference sequences of a different subtype (Appendix 6).

The bootstrap resampling of the phylogenetic tree shown in Figure 5.1 was carried out to test the robustness of each clade on the phylogenetic tree. Although the statistical relevance of the bootstrap test has been debated (Felsenstein & Kishino, 1993; Hillis & Bull, 1993), the cut-off value of around 75% is generally considered to establish confidence in the phylogenetic classification groupings (Simmonds et al.,

1996). The clades A, B, C, F, G and H were supported with high bootstrap above the cut-off value (Figure 5.1).

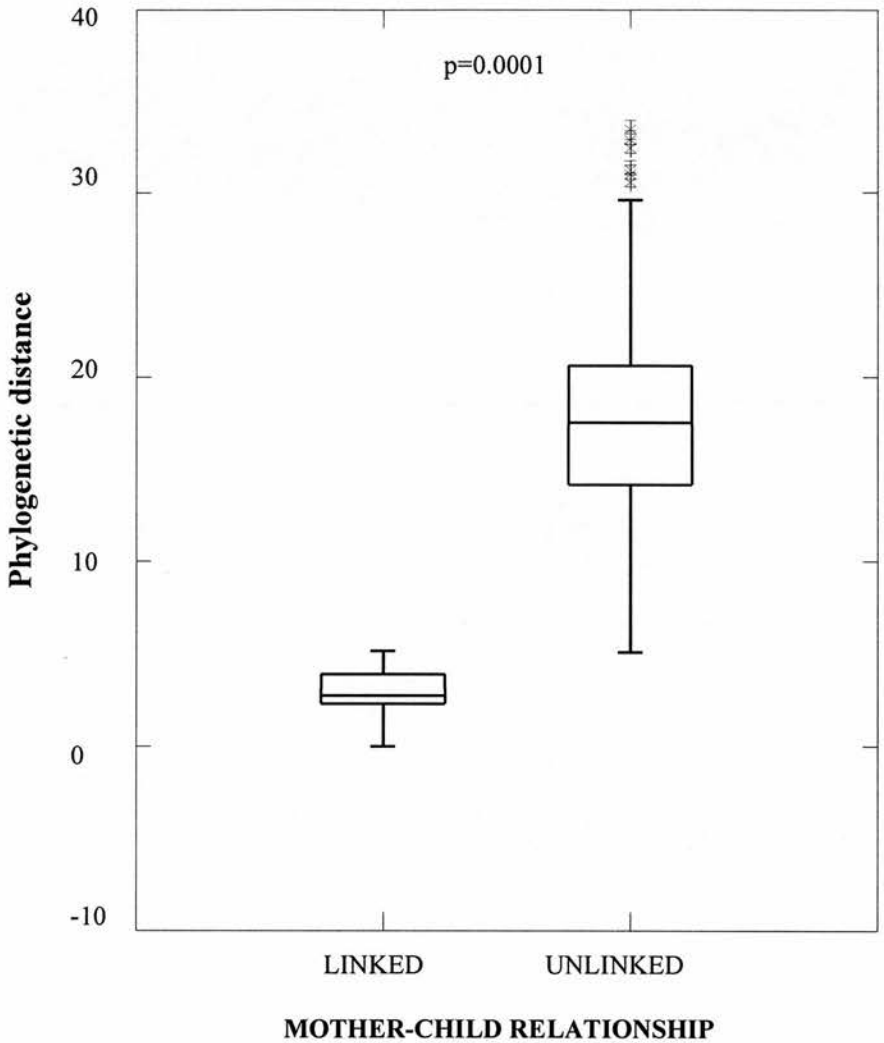
TABLE 5.1. *Average intersubtype and intrasubtype distances between classified HIV-1 sequences from epidemiologically unlinked individuals in Kimpese, Bas-Congo.*

Subtype						
	A	13.0				
	C	20.2	-			
	D	21.0	18.0	**11.1**		
	F	25.6	23.6	18.9	**14.6**	
	G	21.7	22.8	24.6	30.5	**11.2**
	H	18.6	18.0	22.6	25.1	25.9
		A	**C**	**D**	**F**	**G**
		Subtype				

5.2.2. PHYLOGENETIC ANALYSIS OF HIV-1 NUCLEOTIDE SEQUENCES FROM MOTHER-CHILD PAIRS

In most cases, nine sequences from vertically infected children (D.Ped3el, A.ped4el, H.Ped5el, A.Ped6el, D.Ped9el, A.Ped11el, APed14el, A.Ped15el and Q.Ped1) were topologically closer to their corresponding maternal samples (D.Sq4M, A.Sq9M, H.Sq16M, A.Sq32M, D.Sq45M, A.Sq52M A.Sq37M, A.Sq27M and Q.Sq1M, respectively) than to other samples to which they were unlinked. All mothers were classified in the same subtype as their vertically infected children. As depicted in figure 5.2, the distance between mother-child pairs was significantly lower than epidemiologically unlinked sequences ($p=0.0001$). The average of pairwise nucleotide distance between sequences of mothers and their vertically infected children was 2.5% (range 0-5.1%), compared with 17.5% (range 5.1-33.5%) which was the average pairwise distance between the sequences from the same children and epidemiologically unlinked individuals. Therefore, HIV-1 variants from vertically infected children were far more similar to their respective mothers than those they were not epidemiologically linked.

FIGURE 5.2. **Distribution of the distances between sequences obtained from mother-child pairs and epidemiologically unlinked individuals**



The comparison was made between nucleotide distances between 9 children and their mothers (epidemiolocally linked pairs) and distances between the same children and unrelated mothers (unlinked). The nucleotide distances are expressed in % of pairwise nucleotide differences. The distribution of nucleotide distances between sequences shown: horizontal line inside the box represents the median and the horizontal ends of the box represent the lower and upper hinges (the 25th and 75th percentiles). Asterisks represent outside values, which are data values outside the inner fences.

5.2.3. A NEW CANDIDATE SUBTYPE OF HIV-1

It was intriguing to find that HIV-1 variants from 4 patients (Sq1M, Sq2M, Sq24M, and Ped1) did not cluster with any on the *gag* gene subtypes documented in the Los Alamos database (Myers *et al.,* 1995) (Figure 5.3.). The sequences from Kimpese formed a distinct clade, designated 'Q' (query).

To meet the criteria for the documentation of a new subtype (Kostrikis *et al.,* 1995; Leitner *et al.,* 1995), it was important to determine if these patients were epidemiologically linked or not. Except patient Ped1, who was vertically infected from patient Sq1M, interview with the three other patients (Sq1M, Sq2M, Sq24M,) showed no evidence of possible transmission to each other. However, patient Sq1M was engaged in prostitution two years before the collection of the sample. Therefore, it is possible that she could be linked, although indirectly, to other women, including Sq2M and Sq24M, through her clients. The archival samples collected in 1988 and all available pediatric samples were sequenced and subtyped.

The child born to Sq24M was shown to be infected by detection of p24 Ag and IgA class anti HIV-1 IgA in the plasma (Mokili *et al.,* 1996). Samples from this child were not available for PCR and sequencing. The child born to Sq2M was shown to be uninfected after 30 months. The demographic and clinical data including the CD4 and CD8 data from the patients are shown in Table 5.2.

TABLE 5.2: Patients infected with an as yet undescribed HIV-1 subtype

Patients	**Age**	**Res**	**STM**	**Par**	**WBC**	LY	CD4	CD8	CDC stage
Sq1M	36	Kimp	SW	6	6.4	50	27.27	44.44	IVC1
Sq2M	31	Kimp	SM	5	ND	ND	ND	ND	II
Sq24M	26	Kimp	SM	3	7.7	17	13.21	ND	IVC1
Sq42M	32	Kimp	SM	5	ND	ND	ND	ND	ND

Age expressed in year. Res: place of residence. Kimp: Kimpese. STM: Marital status. SW: sex worker. SM: stable monogamic. Par: parity (number of previous pregnancies). WBC: white blood count, value shown x 10^9/l; LY: lymphocytes: percent of WBC; CD4 value is percent of LY; CD8, value shown is percent of LY and CDC: Centers for disease control surveillance case definition of AIDS (Centers for Disease Control, 1987).

Phylogenetic analysis was carried out using longer sub-genomic regions of the *gag* (337bp and 754bp, positions 904-1240 and 904-1898[1] in the HIV_{HXB2} genome) and the *env* genes (409bp). In addition to the Los Alamos sequences (Myers *et al.,* 1995), the recently documented sequences, provisionally designated as subtype I (*env*) and J (*env* and *gag*) (Kostrikis *et al.,* 1995; Leitner *et al.,* 1995), were also included in the analysis. Sequences known to have mozaic genomes (e.g. MAL, K124, LBV2310) were also used in the present analysis to assess their position vis-a-vis the Q clade. Additional phylogenetic analysis of the sub-genomic fragments of both the *gag* and the *env* genes consistently grouped the Sq1M, Sq2M, Sq24M and Ped1 into a separate clade [Figure 5.4. (337bp), Figure 5.5., 5.6.]. The bootstrap value supporting this new clade was always above 97%.

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[1] The Sq1M sample was amplified and sequenced using primers (see table 2.2). Three subgenomic regions (904-1144, 1233-1445 and 1589-1898) obtained were joined together. After gaps between these regions were removed, the sequence was aligned, and phylogenetic analysis carried out as described in the text.

The tree shown on FIGURE 5.3. was constructed using the maximum likelihood distance estimation method. The Alignment (gene: *gag;* length: 225bp, position as in Figure 5.1) included reference sequences from the Los Alamos database (Myers et al, 1996) and 4 sequences (Q.Sq1M, Q.Ped1, Q.Sq2M and Q.Sq24M) from Kimpese. Bold lines at both sides of the tree indicate different grouping of sequences of the same subtype. **Note** the placement of sequences from 4 patients from Kimpese. These sequences cluster separately from previously documented sequences (Myers et al, 1996) and the cluster is supported on this tree by a bootstrap value of 99% of 500 resamplings. The scale bar represents 10% divergence.

FIGURE 5.3. **Maximum likelihood tree showing the placement of unclassified sequences from 4 patients (Q.Sq1M, Q.Ped1, Q.Sq2M and Q.Sq24M) in Kimpese.**

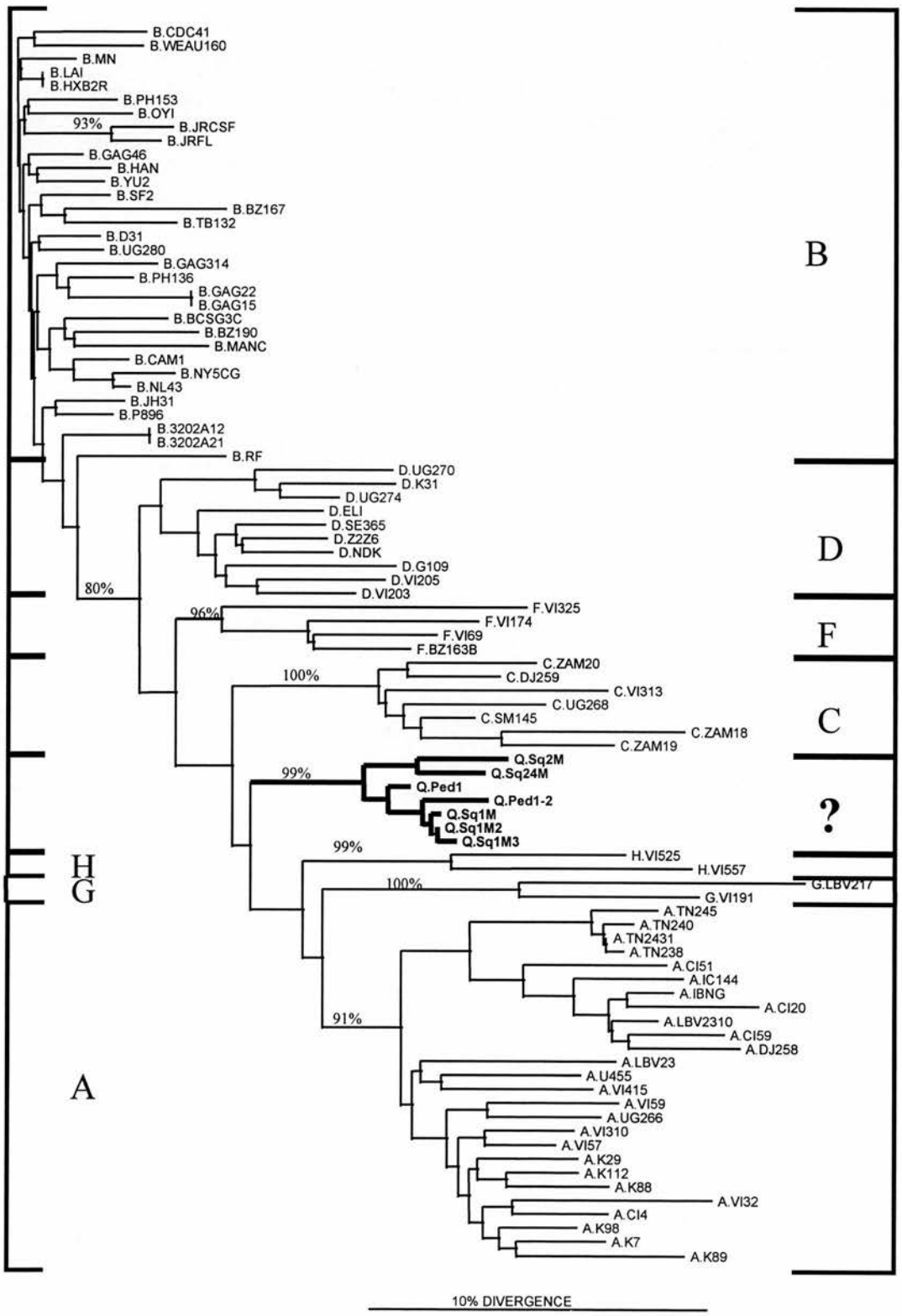
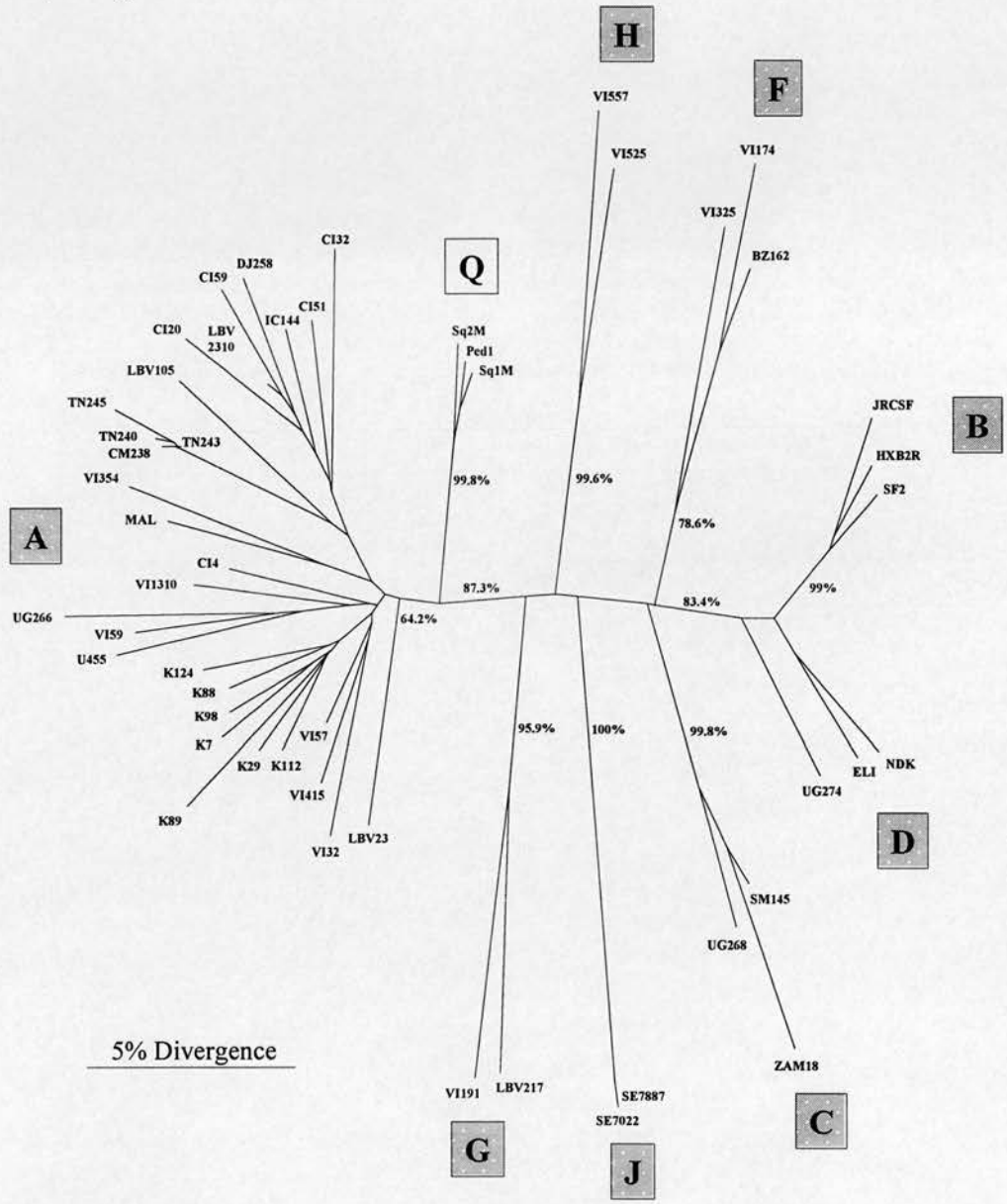


FIGURE 5.4. *Placement of Q sequences in neighbour-joining tree of the p17 gag gene phylogenies (337bp).*



The query (Q) sequences, Sq1M, Sq2M and Ped1, were aligned relative to all published full length sequences previously classified as subtype A and 2-3 representatives of the other subtypes. These included the recently described subtype 'J'. Gag sequences of the *env* subtype 'I' were not available . The *gag* gene phylogenetic tree is based on a 337bp unambiguously aligned region (positions 904-1240 in the HIV_{HXB2} genome). The scale bars correspond to 5 changes per 100 nucleotide positions. Bootstrap values, based on 2000 bootstrap replications, are expressed as a percentage.

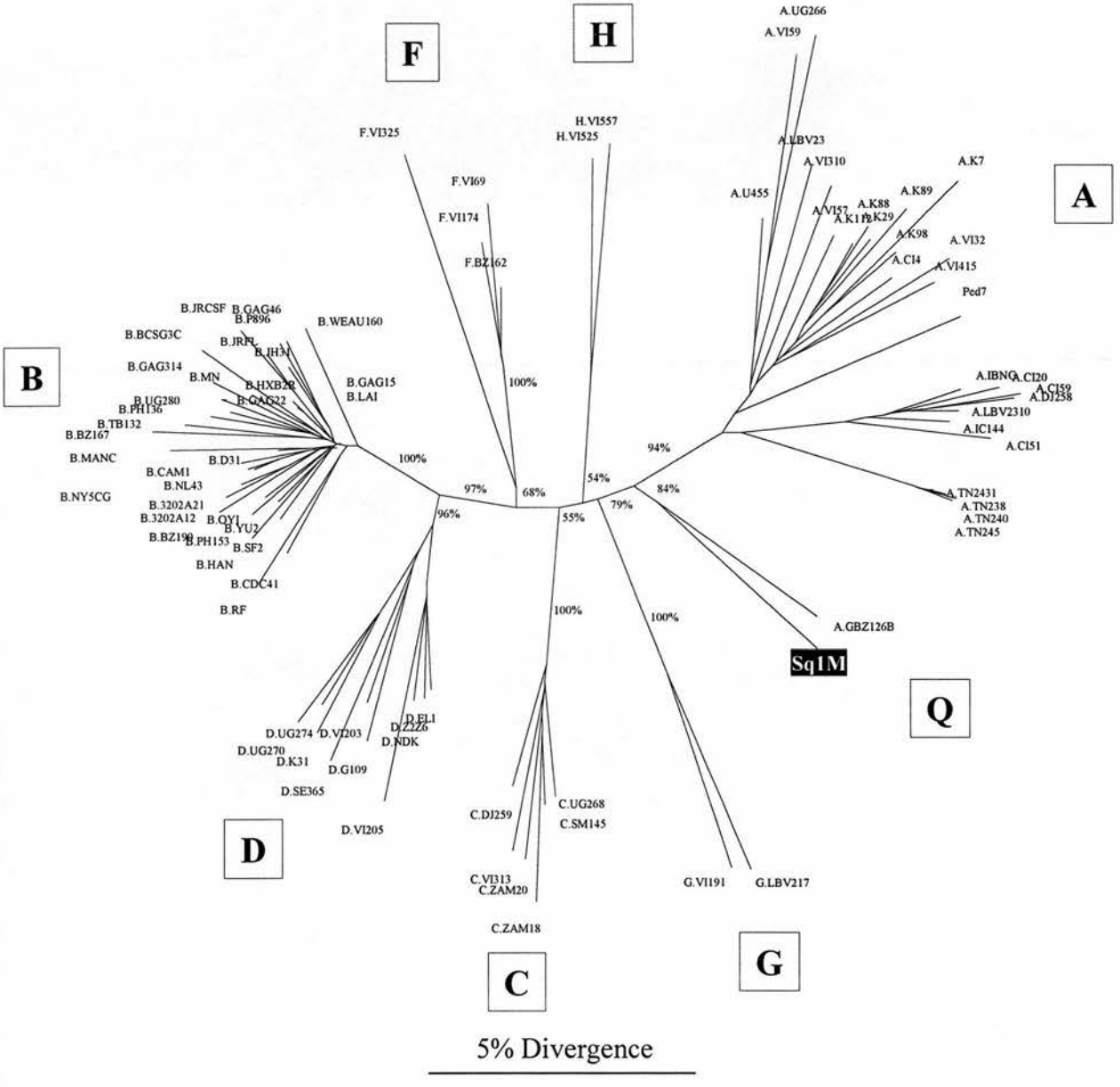
As shown in Figure 5.4, the branch grouping Sq1M, Sq2m and Ped1 clusters distinctly from all other subtypes. Although closely related, the Q cluster was distinct from the A clade as shown by the bootstrap support of 99.8% of 2000 resamplings. The bootstrap support for the A clade was 64.2%, probably due to the presence of recombinant sequences such as MAL, in the dataset. The low bootstrap support for the A clade observed in this study was comparable with a previous report by Louwagie *et al.* (1993) using the whole *gag* gene (66%). The placement of the Q clade with 337bp was consistent in two other phylogenetic analyses, the Fitch-Margoliash (Fitch and Margoliash, 1967) and maximum likelihood methods (Felsenstein, 1981). With both methods, the Q branch clustered distinctly from the A clade and all other subtypes (data not shown).

A likelihood test was used to test the significance of the main branch grouping together the A clade. This test was based on the neighbour-joining phylogeny which included all subtype A sequences and representatives of other subtypes. The new tree derived from the collapse of the A clade branch to 0 branch length was not significantly worse than the original tree at a 5% level of significance. In addition, bootstrap analyses using only sequences of the A, B and the Q clades showed similar bootstrap values to those obtained in the tree shown in figures 5.3 and 5.4. The bootstrap values for the A, B and Q clade were 67.3%, 100% and 98.3% respectively. The inclusion of reference sequences belonging to subtype D, a close clade to the B subtype but distant

from subtype A did not change the placement of the Q clade. Indeed, the bootstrap support of the Q clade remained high (98.1%) with a relative increase of support for the main A clade branch (69.2%).

Additional analysis was performed using a longer sequence (754 bp) of the *gag* region. Four sets of primers (see Materials and Methods: Figure 2.2) were used for PCR and sequencing of this fragment of proviral DNA from patient Sq1M. The sequence from this isolate was aligned with the Los Alamos reference sequences (Myers et al., 1996), relative to positions 904-1898 in the HIV_{HXB2} genome. Where nucleotide sequences were not available, the gaps in those regions (position 1145-1132 and 1446-1589) were removed from the analysis. As shown in figure 5.5, all the reference sequences clustered in their original clades. Again, Sq1M does not cluster in any other subtypes but forms a separate group with GBZ126B (Figure 5.5). GBZ126B is a sequence from Brazil that was originally classified as subtype F based on the analysis within the env and gag regions (Myers *et al.,* 1995). A more recent analysis from Los Alamos suggests that GBZ126B is a recombinant between A and C subtypes (Dr Brian Follet, Los Alamos, personal communication, 1997). A similar phylogenetic placement of Sq1M and GBZ126B was also resolved using both the Fitch-Margoliash (Fitch and Margoliash, 1967) and maximum likelihood methods (Felsenstein, 1981) (data not shown).

FIGURE 5.5. **Placement of Sq1M sequence in neighbour-joining tree of the *gag* gene phylogenies (754bp)**



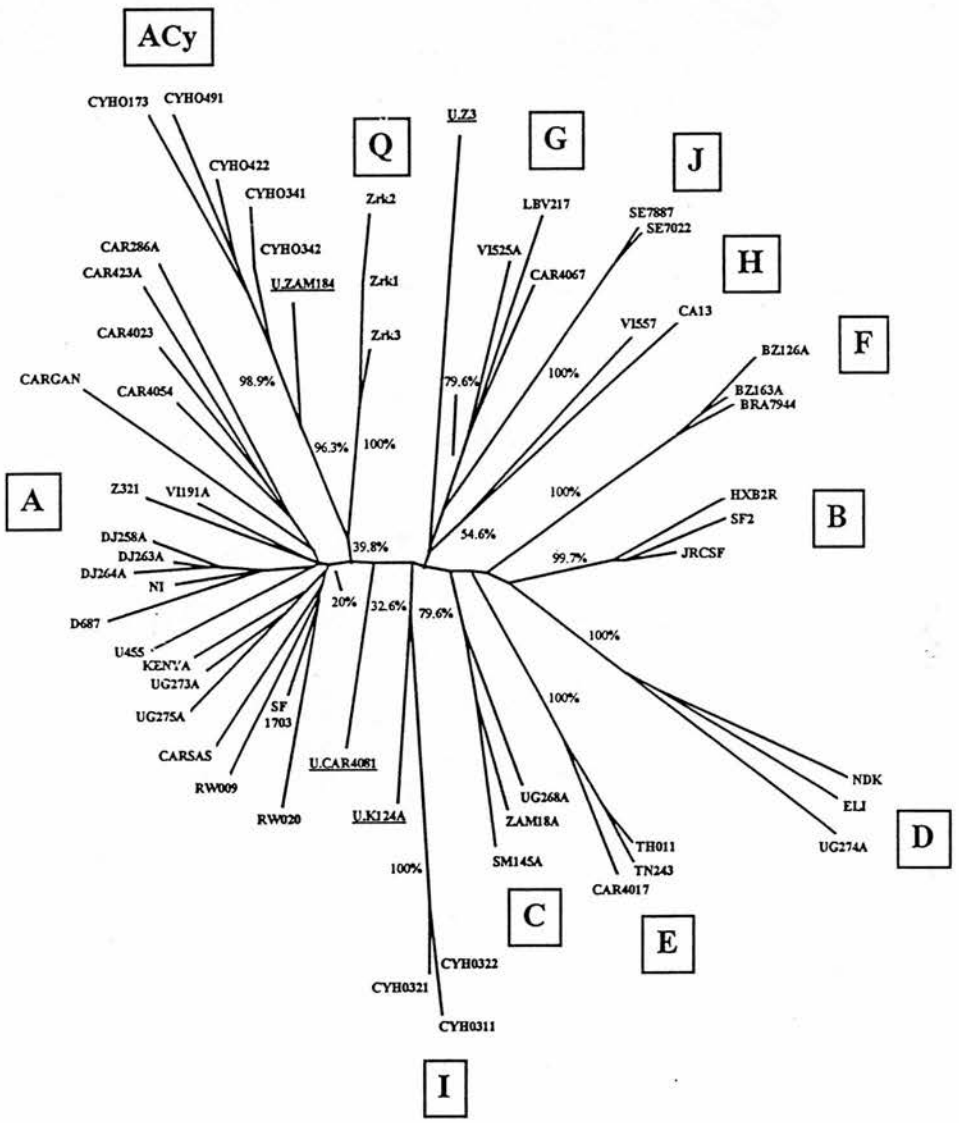
Neighbour-joining tree of partial p17 *gag* gene showing the placement of the Sq1M relative to previously classified sequences (reference) and Ped7 (from this study). The *gag* gene phylogenetic tree was based on a 754bp aligned relative to HIV_{HXB2} (positions 904-1898, gaps removed). The scale bar represents 5% diversity. Bootstrap value (% of 2000 resamplings) shown near the related branch.

The placement of the Q clade (patients Sq1M, Sq2M and Ped1) in HIV-1 group M phylogeny was further investigated by examining a region of the envelope gene. It is worth noting that the *env* region is located 5kb away from the *gag* region which resulted in phylogenetic trees presented in figures 5.1, 5.3-4). The *env* sequences of the patients in this study were obtained from single DNA molecules by a limiting dilution approach (Simmonds *et al.,* 1990). Analysis of the hypervariable region (V3/V4) of the envelope glycoprotein (gp120) was performed as for the *gag* region (see Chapter 2: Materials and methods). A phylogenetic tree was generated based on 409bp unambiguously aligned sites (relative to positions 7030-7439 in the HIV-1_{HXB2} genome). Fifty four sequences, representing 10 previously classified subtypes and several unclassified and recombinant sequences were aligned and phylogenetic analysis carried out with the Q sequences. The result of this phylogenetic analysis is shown in Figure 5.6 and is consistent with the previous observation. All the previously documented sequences (Myers *et al.,* 1996; Louwagie *et al.,* 1993a; Louwagie *et al.,* 1993b, Kostrikis *et al.,* 1995, Leitner *et al.,* 1995) clustered in their original clades with high bootstrap values (B: 99%, C: 80%, D: 100%, E: 100%, F: 100%, G; 80%, I: 100% and J: 100%). As expected, the two clades, A and H, which show very high values of intersubtype distances and have very close neighbouring branches of either unclassified sequences or clades, had relatively low bootstrap values (44% and 63%, respectively). The low bootstrap support for the clade A could be due to the presence

in the dataset of recombinant variants (e.g. ZAM184, CAR4081, K124A), consistent with previous studies (Louwagie *et al.,* 1993a, Louwagie *et al.,* 1993b). More interestingly, the sequences from this study (Sq1M, Sq2M and Ped1) clustered separately from all other previously classified subtypes (Figure 5.6), with this clade supported in 100% bootstrap replicates. Fitch-Margoliash (Fitch and Margoliash, 1967) and maximum likelihood (Felsenstein, 1981) analyses confirmed a consistent placement of the Q clade (data not shown).

The pairwise mean distances between Q sequences and the representative sequences of the previously described subtypes within *env* were calculated. The distances of the nucleotide variation within the V3/V4 region between the Q clade and the previously recognized subtypes were on average 31%, ranging between 26%% (Q to A clade) and 39.8% (Q to D clade). The distance between Q and A (26%) is comparable to the inter-subtype distance between B and D (mean: 28%).

FIGURE 5.6. ***Env* gene phylogeny (409bp)**



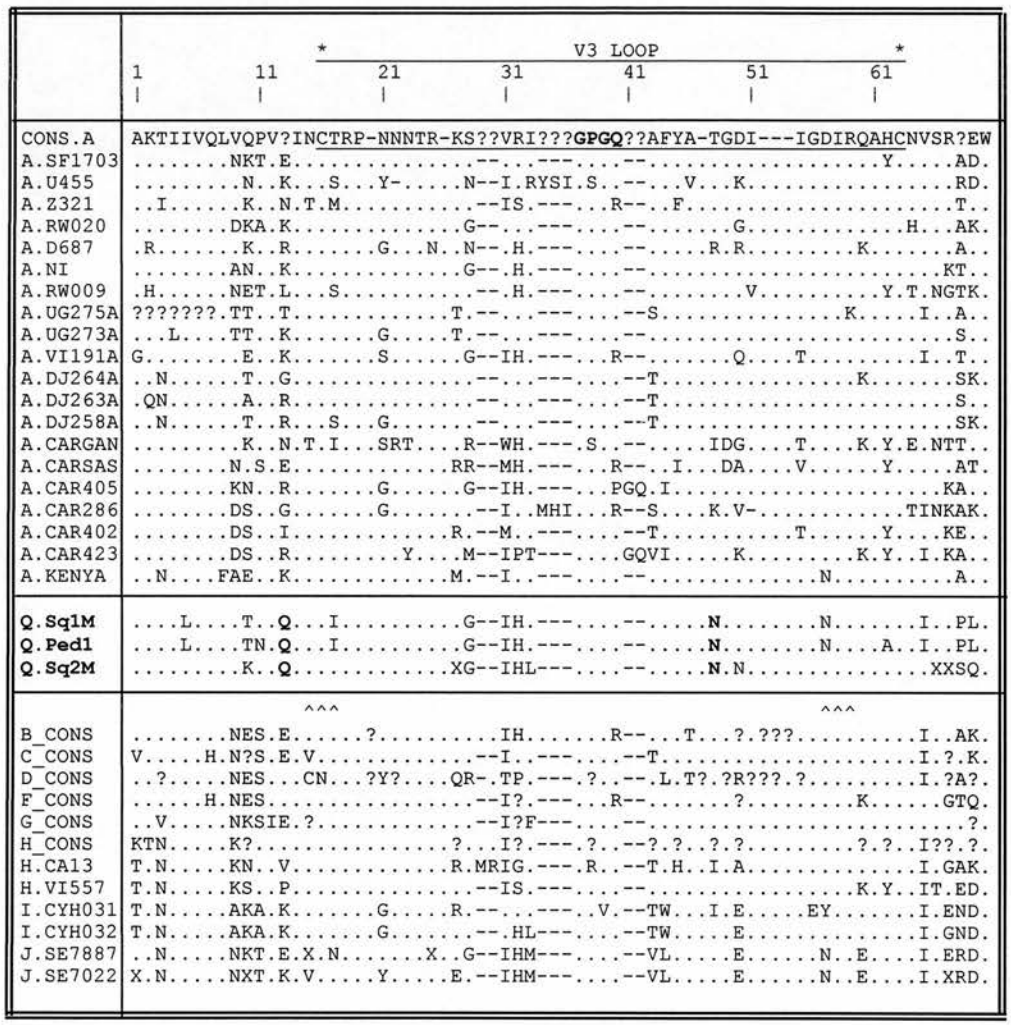
A reconstructed Neighbour-joining of HIV-1 *env* gene phylogeny showing the distribution of 54 previously publihed *env* subtypes. The phylogenetic tree is based on an unambiguously aligned 409bp region of the *env* gene (position: 7030-7439 in the HIV_{HXB2} genome). The scale bar corresponds to 5 changes per 100 nucleotide positions. Bootstrap values, based on 100 bootstrap replications, are expressed as a percentage.

AMINO ACID SEQUENCE CHARACTERISTICS OF THE Q SEQUENCES

(Sq1M, Sq2M and Ped1).

The predicted amino acid sequences of the Sq1M, Sq2M and Ped1 *gag* and *env* sequences relative to those of the previously documented subtypes are presented in Figures 5.7.a. and 5.7.b. Because of the observed proximity of subtype A sequences to Q sequences (Sq1M, Sq2M and Ped1) (Figures 5.3, 5.4, 5.5, 5.6), in addition to the A consensus sequence, individual deduced amino acid sequences were also included. Within the sequenced *env* region, the amino acids of the sequences from this study (Sq1M, Sq2M and Ped1) showed 4 residues which were not found in any subtype A reference sequence (figure 5.7a.). These consisted of glutamine (Q), asparagine (N), methionine (M) and threonine (T) at positions 12, 48, 111 and 113 respectively. In addition, a glycine (G) at position 131 was found in all the 3 sequences (Sq1M, Sq2M and Ped1) but was absent from the A reference sequences, with the exception of Z321. The principal neutralising domain situated at the crown of the V3 loop region was represented by the motif GPGQ in all three sequences. However, the 2 N-linked glycosylation sites normally found at both ends of the V3 loop were absent in 2 of the 3 Q sequences examined. In addition, three residues found in all Kimpese sequences (Sq1M, Sq2M and Ped1), but absent from all subtype A reference sequences used in the analysis, were identified in the sequenced *gag* region (Figure 5.7b.). These consisted of an arginine (R) at position 19, lysine (K) at position 52 and cysteine (C) at position 72.

FIGURE 5.7.a. *Env* (V3/V4) deduced amino acid of Q sequences (Sq1, Sq2 and Ped1) relative to sequences of previously described subtypes[1].



[1] The region of the *env* gene corresponds to positions 7064-7405 in the HIV_{HXB2} genome. The sequences from this study (labelled 'Q') are aligned relative to the consensus sequences of all previously characterised subtypes. In addition to the A consensus sequence, all individual subtype A reference sequences are included within the alignment. Where a subtype is represented by only two reference sequences, a consensus is not presented. Potential N-linked glycosylation sites are shown with '^^^' under the 'Q' sequences. Identical amino acid sequences are represented by a dot (.); deletion to allow alignment of sequences by a dash (-). A question mark (?) indicates there was not a predominant amino acid to be considered as consensus at this site.

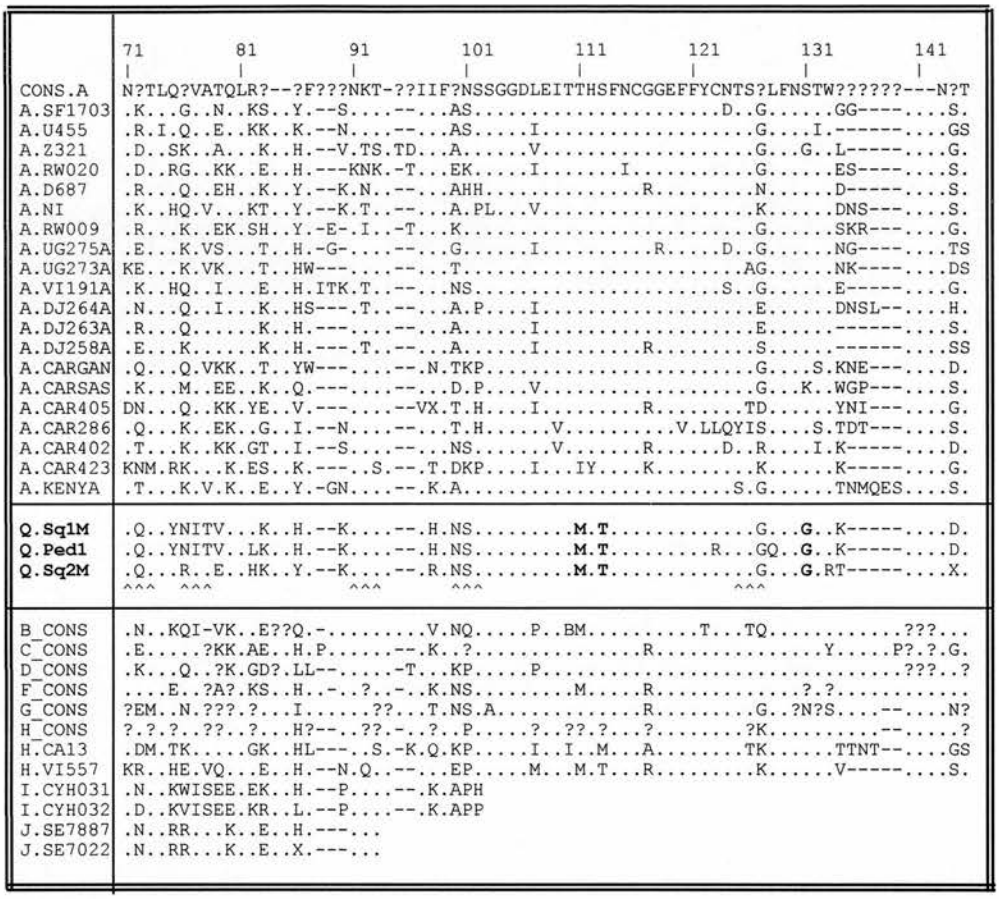
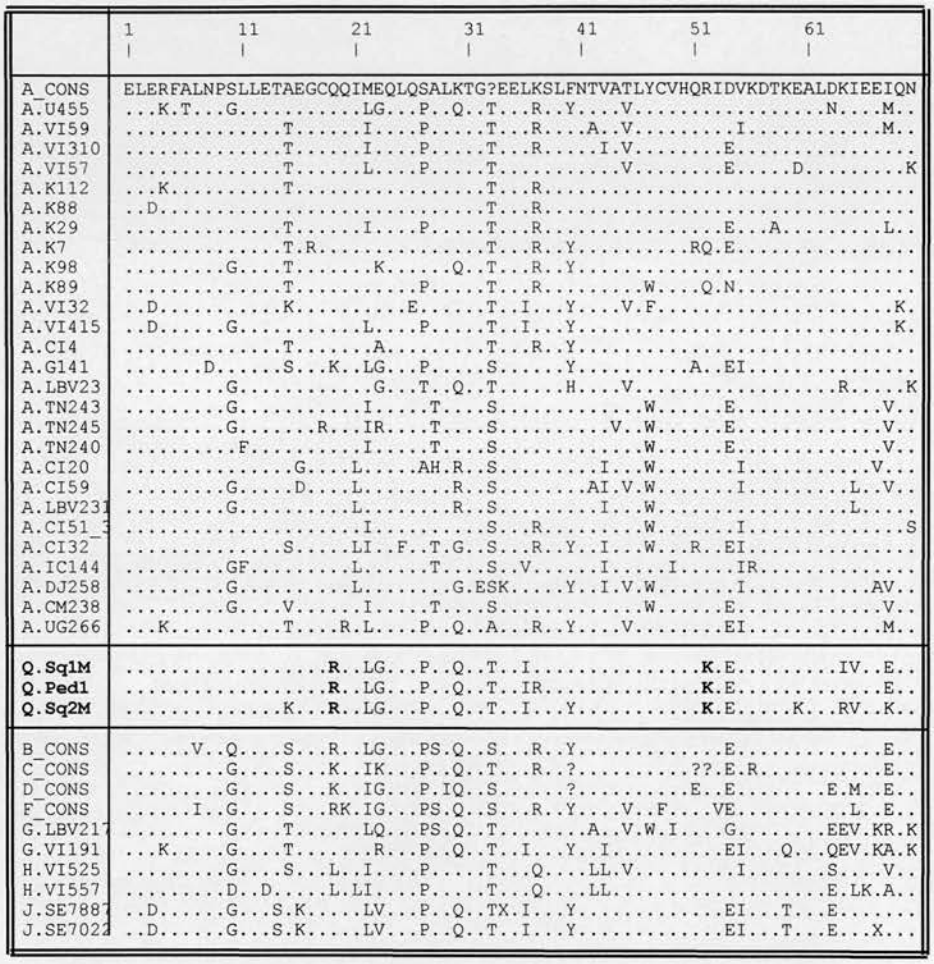


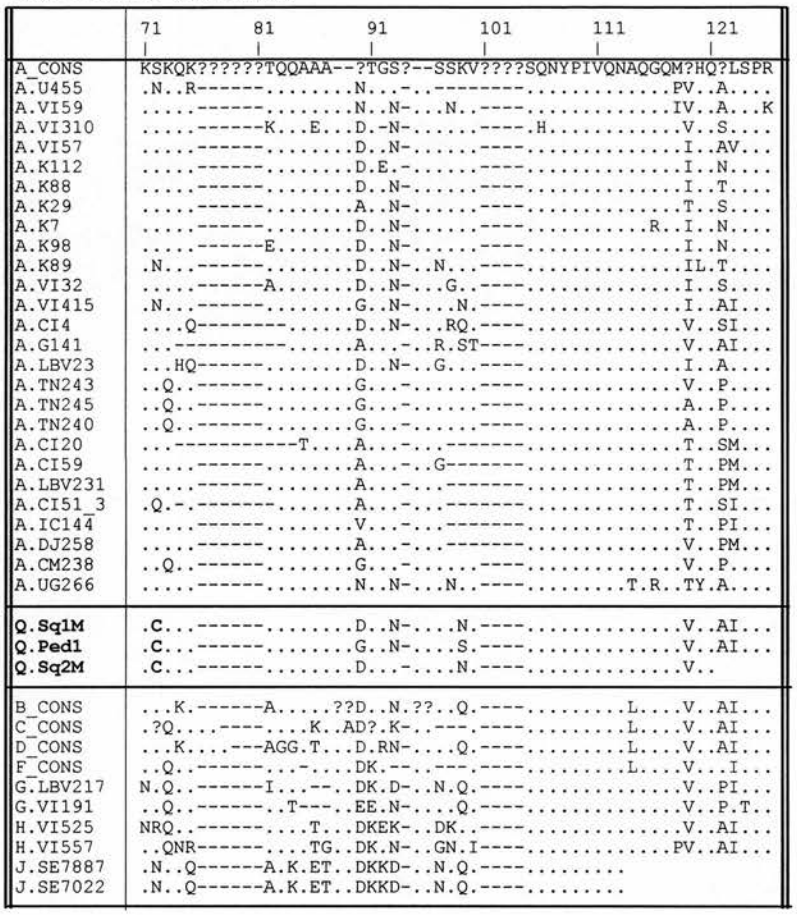
Figure 5.7.a. (continued)

FIGURE 5.7b. *Gag p17 deduced amino acid of Q sequences (Sq1, Sq2 and Ped1) relative to sequences of previously described subtypes*[1].



[1] The region of the *gag* gene corresponds to positions 904-1121 in the HIV_{HXB2} genome. The sequences from this study (labelled 'Q') are aligned relative to the consensus sequences of all previously characterised subtypes. In addition to the A consensus sequense, all individual subtype A reference sequences are included within the alignment. Where a subtype is represented by only two reference sequences, a consensus is not presented. Identical amino acid sequences are represented by a dot (.); deletion to allow alignment of sequences by a dash (-). A question mark (?) indicates there was not a predominant amino acid to be considered as consesus at this site.

Figure 5.7.b. (continued)



```
                71          81          91          101         111         121
                |           |           |           |           |           |
A_CONS          KSKQK??????TQQAAA--?TGS?--SSKV????SQNYPIVQNAQGQM?HQ?LSPR
A.U455          .N..R------........N...-..--------.............PV..A....
A.VI59          .....------........N..N-...N..----.............IV..A...K
A.VI310         .....------K...E...D.-N-.....----.H............V..S....
A.VI57          .....------........D..N-.....----..............I..AV...
A.K112          .....------........D.E.-.....----..............I..N....
A.K88           .....------........D..N-.....----..............I..T....
A.K29           .....------........A..N-.....----..............T..S....
A.K7            .....------........D..N-.....----..........R..I..N....
A.K98           .....------E.......D..N-.....----..............I..N....
A.K89           .N...------........D..N-..N...----..............IL.T....
A.VI32          .....------A.......D..N-...G..----..............I..S....
A.VI415         .N...------........G..N-....N.----..............I..AI...
A.CI4           ....Q--------......D..N-...RQ.----..............V..SI...
A.G141          ...----------......A...-..R.ST----..............V..AI...
A.LBV23         ...HQ------........D..N-..G...----..............I..A....
A.TN243         ..Q..------........G...-.....----..............V..P....
A.TN245         ..Q..------........G...-.....----..............A..P....
A.TN240         ..Q..------........G...-.....----..............A..P....
A.CI20          ...----------T....A...-...-------..............T..SM...
A.CI59          .....------........A...-..G-------..............T..PM...
A.LBV231        .....------........A...-...-------..............T..PM...
A.CI51_3        .Q.-.-------.......A...-...-------..............T..SI...
A.IC144         .....------........V...-...-------..............T..PI...
A.DJ258         .....------........A...-...-------..............V..PM...
A.CM238         ..Q..------........G...-.....----..............V..P....
A.UG266         .....------........N..N-...N..----.........T.R..TY.A....

Q.Sq1M          .C...------........D..N-....N.----..............V..AI...
Q.Ped1          .C...------........G..N-....S.----..............V..AI...
Q.Sq2M          .C...------........D...-....N.----..............V..

B_CONS          ...K.------A.....??D..N.??..Q.----.........L....V..AI...
C_CONS          .?Q....----....K..AD?.K-..---.----.........L....V..AI...
D_CONS          ...K....---AGG.T...D.RN-....Q.----.........L....V..AI...
F_CONS          ..Q..------...-....DK.--..---.----.........L....V...I...
G.LBV217        N.Q..------I...--..DK.D-..N.Q.----..............V..PI...
G.VI191         ..Q..-------..T---..EE.N-....Q.----..............V..P.T..
H.VI525         NRQ..------....T...DKEK-..DK..----..............V..AI...
H.VI557         ..QNR------....TG..DK.N-..GN.I----.............PV..AI...
J.SE7887        .N..Q------A.K.ET..DKKD-..N.Q.----.........
J.SE7022        .N..Q------A.K.ET..DKKD-..N.Q.----.........
```

5.2.4. IDENTIFICATION OF SUBTYPE 'J' IN KIMPESE

HIV strains from 2 patients (Sq42 and Sq57) could not be shown to cluster with the established (A-H) reference sequences of the Los Alamos database (Myers *et al.*, 1996). Additional phylogenetic analysis was performed using the Los Alamos database sequences (Myers et al., 1996) and the provisionally classified subtype J[1] published by Leitner *et al.* (1995). Interestingly, these sequences, Sq42, Sq57_1 and Sq57_2 (the latter two are from the same patient), clustered strongly with the sequences published by Leitner *et al.* (1995) and are supported by a bootstrap value of 89% of 2000 replicates (Figure 5.8.). The average distance between Sq42 and Sq57 was lower with the subtype J reference sequences (12.8%) than with any other subtypes (distance to subtypes A: 17.3%, B: 19.1%, C: 19.3%, D: 18.9%, F: 23.9%, G: 25.3%, H: 19.5%) and the Q clade (16.4%). Data of the envelope from these Kimpese subtype J sequences were not available to confirm their classification in the present observation.

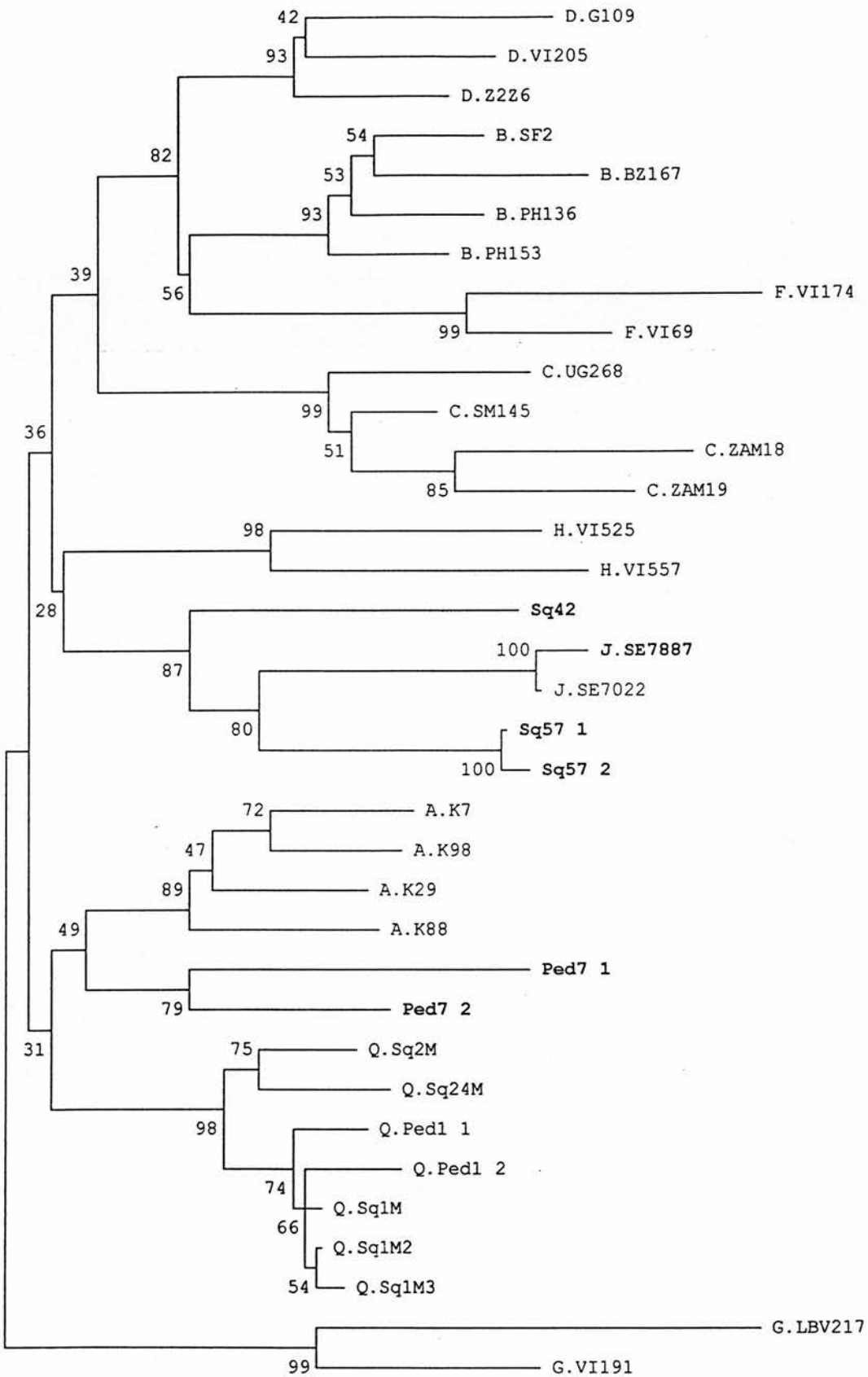
In addition, two sequences (Ped7_1 and Ped7_2) from a vertically infected child showed a separate branch topologically related to subtype A (Figure 5.8). These sequences were relatively distant from each other (12.3% nucleotide difference), although found in the same individual.

---

[1] The gag sequences of the newly characterised subtype I were not available (Kostrikis *et al.*, 1995).

FIGURE 5.8. *Phylogenetic placement of Kimpese subtype 'J'.*

Unrooted neighbour-joining tree based on 225bp long sequences from Kimpese (in **bold**) aligned with previously published sequences (Myers *et al.,* 1996). Bootstrap (in %) of 500 resampling shown.



Scale: each ———— is approximately equal to the distance of 0.02413

5.2.5. GENETIC RECOMBINATION BETWEEN SUBTYPE A AND SUBTYPE H

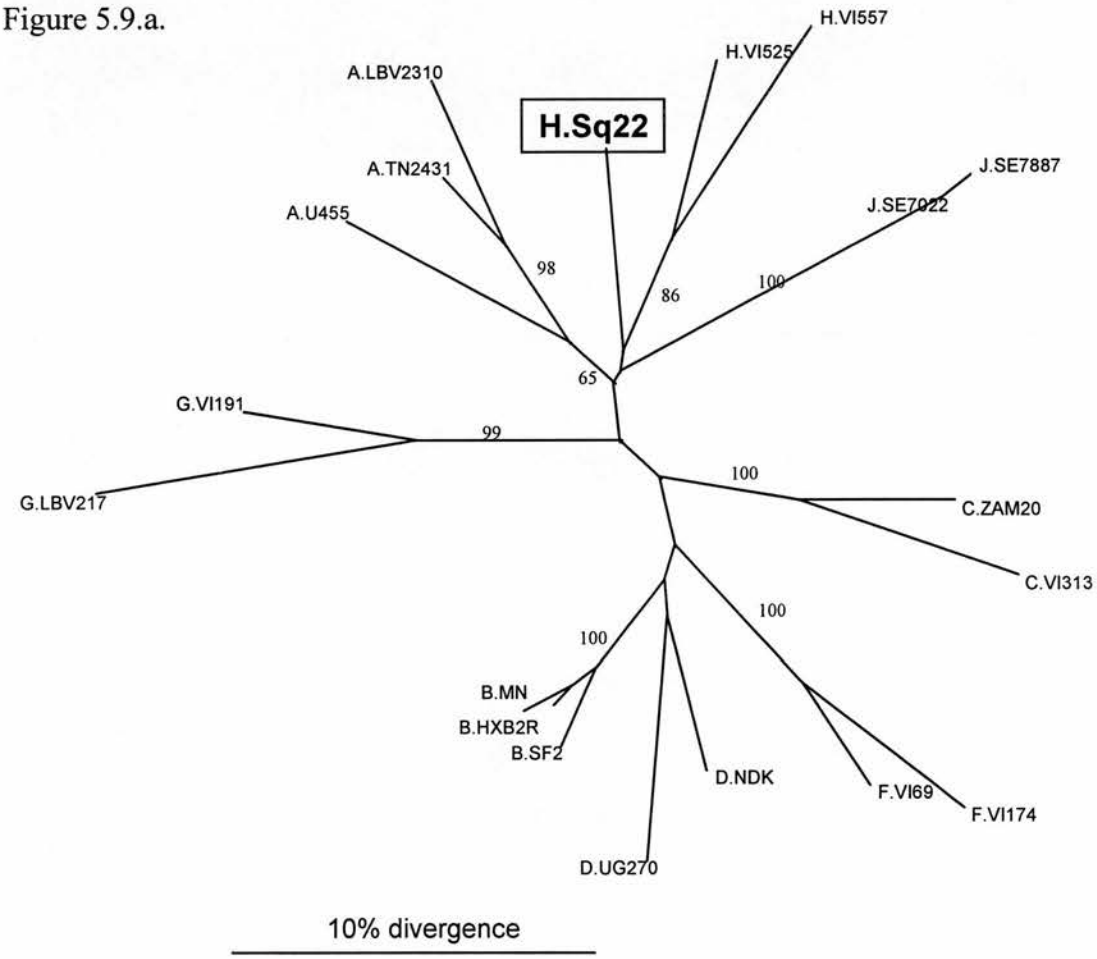
Analysis performed using sequences obtained from a 29 year old pregnant woman (Sq22M) resident in Kimpese revealed that the virus she was infected with, phylogenetically, was an A/H recombinant. Two regions of the genome, (a) region of 225bp (position[1]: 906-1131) and (b) 242bp (position 970-1212) within the *gag* gene obtained by sequencing of PCR product, were analysed as described earlier (see materials and methods). As depicted in figure 5.9., the sequence from Sq22M clusters with subtype A when the section (b) was analysed with reference sequences from the Los Alamos database (Myers *et al.,* 1995). The bootstrap support for the 'A' branch which holds together Sq22 and the reference sequences was 73% (figure 5.9.a). Interestingly, the Sq22M sequence clustered together with subtype H when the (a) region was analysed (figure 5.9.b).

---

[1] relative to HIV_{HXB2} genome

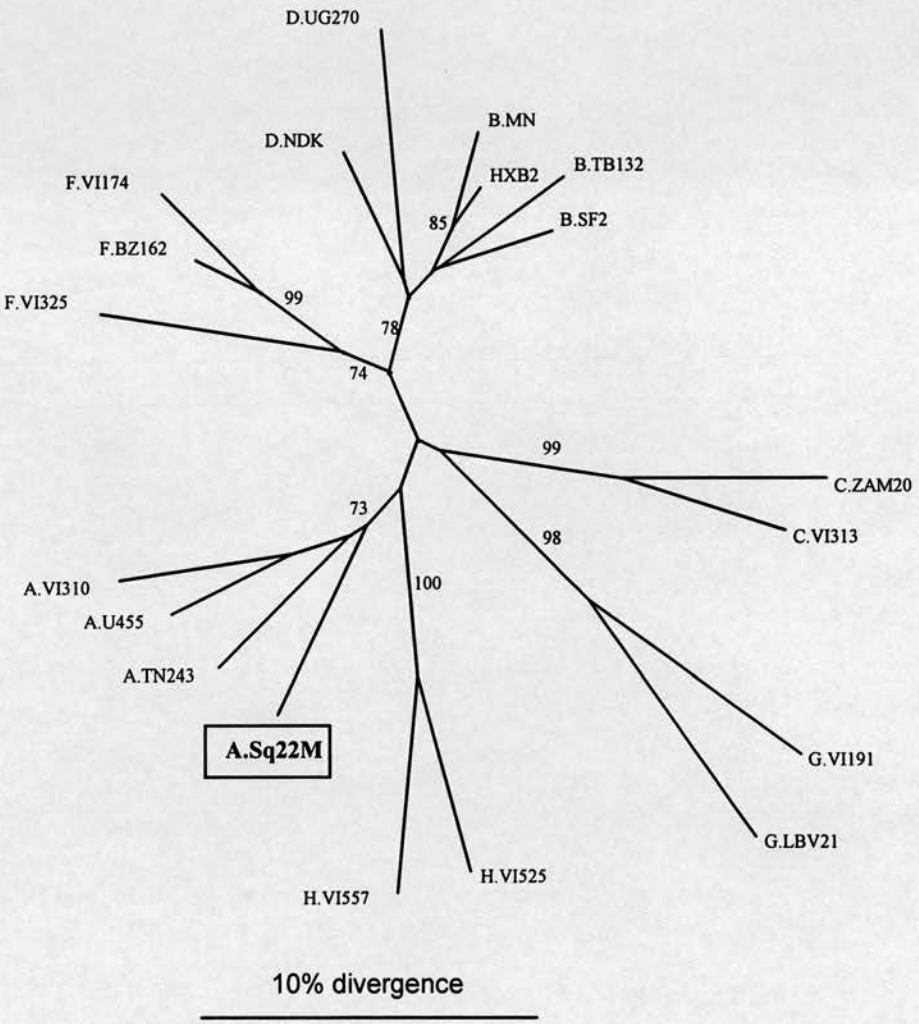
FIGURE 5.9. *Maximum likelihood trees showing the placement of a recombinant sequence Sq22M*[1]

Figure 5.9.a.



[1] The trees constructed from two alignments: 225 base-pair spanning between 906-1131 (Figure 5.12.a) and 242 base-pairs (positions: 970-1212 relative to HIV_{HXB2}) (figure 5.12.b). The trees were constructed with the PHYLIP programme using the maximum likelihood and the Neighbour-joining methods. Only bootstrap support values over 70% (of 100 re-samplings) are shown.

figure 5.9.b



For figure legend, see footnote in figure 5.9.a.

5.2.6. FUNCTIONAL SIGNIFICANCE OF SUBTYPES AND p17 DOMAINS IN MOTHER-TO-CHILD TRANSMISSION OF HIV-1

Are some subtypes more likely to be transmitted from mother-to-child than others? To date there is still no clear answer to this question. To provide further information, it was intended to sequence all the HIV-1 variants of the transmitting and non-transmitting mothers and apply statistical tests for likelihood of transmission of HIV-1 subtypes. The subtypes identified in Kimpese were grouped by the MCT status of the mother (or child) (Table 5.3). Because of the small numbers of sequences in each group and sampling error, it was not possible to make direct comparison between the groups. Overall, subtype A was the predominant subtype in Kimpese and was found in 28 (50%) out of 56 epidemiologically unlinked individuals. Of 18 transmitting mothers, 9 (50%) were infected with subtype A. The sample size was too small for a similar comparison with other subtypes.

TABLE 5.3. HIV-1 subtypes and MCT status

Subtype	Transmitters	Non-transmitters	Not known	**Total**
A	9	14	5	**28**
C	0	1	0	**1**
D	3	1	1	**5**
F	1	1	0	**2**
G	1	2	1	**4**
H	2	7	3	**12**
J	0	2	0	**2**
Q	2	1	0	**3**
Recombinant	0	0	2	**2**
Total	**18**	**29**	**12**	**59**

An alternative approach to investigate viral selection during MCT of HIV-1 was to examine the deduced amino acids corresponding to the nucleotide sequences in the p17 *gag* region of HIV-1 variants from transmitting (n=20) and non-transmitting mothers (n=31).

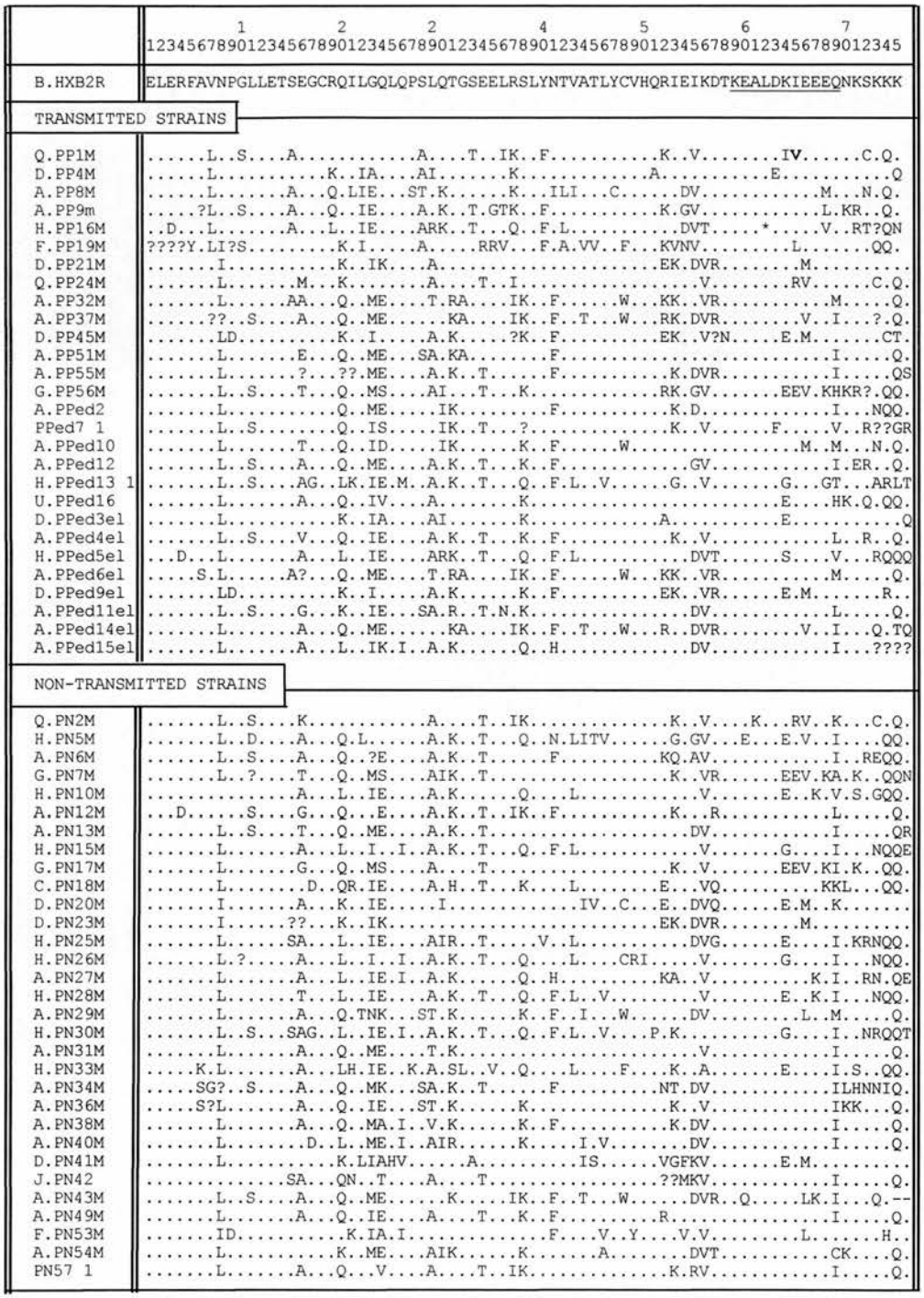
The alignment of the amino acid sequences of HIV-1 matrix protein p17 deduced from the nucleotide sequences of HIV-1 variants from transmitting and non-transmitting mothers is shown in figure 5.10. In a previous study, Narwa *et al.* (1996) identified 2 amino acid residues (glutamic acid and valine) which seemed to be sufficient to distinguish transmitting from non-transmitting mothers. The equivalent positions for these amino acids are 54 and 65, respectively, in figure 5.10. In the same study (Narwa *et al.,* 1996), within the major antibody site [(KE)ALDKIEE(EQ)] (position 59-70), the KIEEEQN motif (figure 5.10, position 64-70) was also associated with MCT of HIV-1.

The overall comparison of the nucleic and amino acid data did not show clear evidence of sequences grouping by transmitting and non-transmitting status of the mothers. Some motifs were conserved and others were more related to some particular subtypes. The N-terminal region was generally conserved. At the putative p17 polymerisation site (Chazal *et al.,* 1995), the ELERFA motif was conserved in 43 (81.1%) of 51 HIV-1 variants (Figure 5.10.). There was substantial variability at the C-terminal and was subject to statistical analysis. The KIEEEQN motif (residue

64-70), which was previously associated with transmission phenotype (Narwa *et al.,* 1996), did not show the same effect in this study. This motif was found only in 1 transmitting mother (PP4M) infected with subtype D virus (figure 5.10). Furthermore, the presence of glutamic acid and valine at positions 54 and 65, respectively (Narwa *et al.,* 1996) showed no association with infection phenotype in children born to HIV infected mothers. Ten out of 20 (50%) transmitted viruses compared with 16 out 31 non-transmitted HIV-1 strains had glutamic acid at position 54 with no statistical difference between the two groups (p=0.9). Similarly, 4 viruses in transmitting and 4 in non-transmitting mothers had valine at the 65th residue (figure 5.10.).

However, within p17 major antibody binding site [(KE)ALDKIEE(EQ)] (position 59-70) (Boucher *et al.,* 1990), the isoleucine at position 69 was associated with protection from mother-to-child transmission of HIV-1. Five of 20 transmitting mothers in comparison with 20 out of 31 non-transmitting mothers had isoleucine residue at this position (Mantel-Haenszel's Chi^2: 7.45; p=0.006).

Figure 5.10. Gag p17 deduced amino acid sequences of HIV-1 strains in Kimpese[1]



	1 2 2 4 5 6 7 12345678901234567890123456789012345678901234567890123456789012345
B.HXB2R	ELERFAVNPGLLETSEGCRQILGQLQPSLQTGSEELRSLYNTVATLYCVHQRIEIKDTKEALDKIEEEQNKSKKK
TRANSMITTED STRAINS	
Q.PP1M	......L..S....A.....A....T..IK..F.....K..V.....IV.....C.Q.
D.PP4M	......L.....K..IA....AI.....K.....A.....E.....Q
A.PP8M	......L.....A...Q.LIE...ST.K.....K...ILI...C.....DV.....M...N.Q.
A.PP9m	.....?L..S....A...Q..IE....A.K..T.GTK..F.....K.GV.....L.KR..Q.
H.PP16M	..D...L.....A...L..IE....ARK..T...Q..F.L.....DVT.....*.....V..RT?QN
F.PP19M	????Y.LI?S.....K.I.....A.....RRV...F.A.VV..F...KVNV.....L.....QQ.
D.PP21M	.....I.....K..IK....A.....EK.DVR.....M.....
Q.PP24M	.....L.....M...K.....A....T..I.....V.....RV.....C.Q.
A.PP32M	.....L.....AA...Q..ME....T.RA....IK..F.....W...KK..VR.....M.....Q.
A.PP37M	.....??..S....A...Q..ME.....KA....IK..F..T...W...RK.DVR.....V..I...?.Q.
D.PP45M	.....LD.....K..I.....A.K.....?K..F.....EK..V?N.....E.M.....CT.
A.PP51M	.....L.....E...Q..ME...SA.KA.....F.....I.....Q.
A.PP55M	.....L.....?...??.ME....A.K..T.....F.....K.DVR.....I.....QS
G.PP56M	.....L..S....T...Q..MS....AI...T...K.....RK.GV.....EEV.KHKR?.QQ.
A.PPed2	.....L.....Q..ME.....IK.....F.....K.D.....I...NQQ.
PPed7 1	.....L..S.....Q..IS.....IK..T...?.....K..V.....F.....V..R??GR
A.PPed10	.....L.....T...Q..ID.....IK.....K..F.....W.....M..M...N.Q.
A.PPed12	.....L..S....A...Q..ME....A.K..T...K..F.....GV.....I.ER..Q.
H.PPed13 1	.....L..S....AG..LK.IE.M..A.K..T...Q..F.L..V.....G..V.....G...GT...ARLT
U.PPed16	.....L.....A...Q..IV....A.....K.....E....HK.Q.QQ.
D.PPed3el	.....L.....K..IA....AI.....K.....A.....E.....Q
A.PPed4el	.....L..S....V...Q..IE....A.K..T...K..F.....K..V.....L..R..Q.
H.PPed5el	...D...L.....A...L..IE....ARK..T...Q..F.L.....DVT.....S....V...RQQQ
A.PPed6el	.....S.L.....A?...Q..ME....T.RA....IK..F.....W...KK..VR.....M.....Q.
D.PPed9el	.....LD.....K..I.....A.K.....K..F.....EK..VR.....E.M.....R..
A.PPed11el	.....L..S....G...K..IE...SA.R..T.N.K.....DV.....L.....Q.
A.PPed14el	.....L.....A...Q..ME.....KA....IK..F..T...W...R..DVR.....V..I...Q.TQ
A.PPed15el	.....L.....A...L..IK.I..A.K.....Q..H.....DV.....I...????
NON-TRANSMITTED STRAINS	
Q.PN2M	.....L..S....K.....A....T..IK.....K..V....K...RV..K...C.Q.
H.PN5M	.....L..D....A...Q.L.....A.K..T...Q..N.LITV.....G.GV...E...E.V..I....QQ.
A.PN6M	.....L..S....A...Q..?E....A.K..T.....F.....KQ.AV.....I..REQQ.
G.PN7M	.....L..?....T...Q..MS....AIK..T.....K..VR.....EEV.KA.K..QQN
H.PN10M	.....A...L..IE....A.K.....Q....L.....V.....E..K.V.S.GQQ.
A.PN12M	...D.....S....G...Q...E....A.K..T..IK..F.....K...R.....L.....Q.
A.PN13M	.....L..S....T...Q..ME....A.K..T.....DV.....I.....QR
H.PN15M	.....L.....A...L..I..I..A.K..T...Q..F.L.....V.....G....I...NQQE
G.PN17M	.....L.....G...Q..MS....A....T.....K..V.....EEV.KI.K..QQ.
C.PN18M	.....L.....D..QR.IE....A.H..T...K....L.....E...VQ.....KKL...QQ.
D.PN20M	.....I.....A...K..IE.....I.....IV..C...E..DVQ.....E.M..K.....
D.PN23M	.....I.....??...K..IK.....EK.DVR.....M.....
H.PN25M	.....L.....SA...L..IE....AIR..T.....V..L.....DVG.....E....I.KRNQQ.
H.PN26M	.....L.?.....A...L..I..I..A.K..T...Q....L....CRI.....V.....G....I...NQQ.
A.PN27M	.....L.....A...L..IE.I..A.K.....Q..H.....KA..V.....K.I..RN.QE
H.PN28M	.....L.....T...L..IE....A.K..T...Q..F.L..V.....V.....E..K.I...NQQ.
A.PN29M	.....L.....A...Q.TNK...ST.K.....K..F..I...W.....DV.....L..M.....Q.
H.PN30M	.....L..S...SAG..L..IE.I..A.K..T...Q..F.L..V....P.K.....G....I..NRQQT
A.PN31M	.....L.....A...Q..ME....T.K.....V.....I.....Q.
H.PN33M	.....K.L.....A...LH.IE..K.A.SL..V..Q....L....F....K..A.....E....I.S..QQ.
A.PN34M	.....SG?..S....A...Q..MK...SA.K..T.....F.....NT.DV.....ILHNNIQ.
A.PN36M	.....S?L.....A...Q..IE...ST.K.....K.....K..V.....IKK...Q.
A.PN38M	.....L.....A...Q..MA.I..V.K.....K..F.....K.DV.....I.....Q.
A.PN40M	.....L.....D..L..ME.I..AIR.....K.....I.V.....DV.....I.....Q.
D.PN41M	.....L.....K.LIAHV.....A.....IS.....VGFKV.....E.M.....
J.PN42	.....SA...QN..T....A....T.....??MKV.....I.....Q.
A.PN43M	.....L..S....A...Q..ME.....K.....IK..F..T...W.....DVR..Q.....LK.I...Q.--
A.PN49M	.....L.....A...Q..IE....A....T...K..F.....R.....I.....Q.
F.PN53M	.....ID.....K.IA.I.....F....V..Y....V.V.....L.....H..
A.PN54M	.....L.....K..ME....AIK.....K.....A.....DVT.....CK....Q.
PN57 1	.....L.....A...Q...V....A....T..IK.....K.RV.....I.....Q.

[1] The KIEEEQN motif (position 64-70) (Narwa *et al.*, 1996) was not associated with transmission of HIV-1 from mothers to their children.

5.3. DISCUSSION

5.3.1. VALIDITY OF THE P17 REGION FOR SUBTYPING HIV-1

Reliable phylogenetic analysis for subtype inference of HIV-1 strains depends on the lengths and the region of the genome analysed. Most studies of the molecular characterisation of HIV-1 subtypes have been using the *gag* and/or *env* section of the genome. With the discovery of genetic recombination of different HIV-1 subtypes, it was suggested that at least the full *gag* and full *env* genes should be sequenced for accurate analysis (Sabino *et al.,* 1994; Leitner *et al.,* 1995; Diaz *et al.,* 1995; Sharp *et al.,* 1994). However, recent studies by McCutchan *et al.* (1996) using the bootscanning approach, demonstrated multiple recombination points in isolates which were allocated to a single subtype based on the analysis of partial *gag* and *env* sequences. It was suggested that the only way to obtain a firm subtype allocation is by sequencing a full HIV-1 genome and then to search for recombination break points. To obtain the full length genome for even one individual sequence is both time-consuming and technically demanding. This makes it difficult, if not impossible, to make any attempt to perform large-scale molecular epidemiology studies of HIV1.

If the aim of the research is merely to determine the circulating subtypes, regardless whether they are recombinant or not, then a partial length of the genome

could be used. Indeed, it was demonstrated in this study that a *gag* region as short as 225bp was enough to determine the circulating subtypes in our study population. In this study, the subtypes identified based on analysis of a partial genome of HIV-1 are not definitive, although the results are comparable with published reports based on a similar approach. Nevertheless, if a subtype is found based on the analysis of a full or partial genome, this establishes that the subtype is present in that population, whether in a pure or recombinant form.

5.3.2. HIV-1 SUBTYPES AND TRANSMISSION

The epidemiology of HIV-1, as for many communicable diseases, is not totally dependent upon behaviour patterns of the population. For example, the biological features of the infectious agent can be very important in determining the spread of the epidemic. However, there are numerous factors that could influence transmission, some related to the host and others to the agent.

In this chapter, the aim was to investigate the molecular characteristics of the p17 *gag* region of HIV-1 isolates in Kimpese and to determine if there was any association between particular features and mother-to-child transmission. The p17 region was used because (i) it plays a very important role during the replication of the virus, (ii) is the source of cell tropism and (iii) determines viral phenotype. Direct sequencing was performed on p17 region of HIV-1 isolates from mothers and children. This provided new information on the HIV-1 subtypes present in the region

of Kimpese. In addition, the alignment of deduced amino acids was used in a comparison of transmitting and non-transmitting mothers.

The background against which the present study of the molecular characteristics of HIV-1 in Kimpese was carried out consisted of (i) this is a rural population in a sub-Saharan country, with (ii) a low and stable seroprevalence (Green *et al.,* 1994) and (iii) a relatively low mother-to-child vertical transmission rate. Against this background, it could be postulated that the predominant virus in Kimpese might a less virulent subtype. It was possible that, HIV-2 or similar virus, could be present in this population. However, HIV-2 was not found in mothers and children studied here. In some West African countries where both viruses co-circulate, it has been observed that while the prevalence of HIV-1 is increasing steadily, HIV-2 incidence was falling dramatically (de Cock *et al.,* 1993). Although these two viruses are genetically similar, they have remarkable differences in their global distribution, their transmissibility and pathogenic potential (Poulsen *et al.,* 1989; Ancelle *et al.,* 1987; Kanki *et al.,* 1992; Pepin *et al.,* 1991). There is also evidence about the lower heterosexual infectivity of HIV-2 compared to HIV-1 (Kanki *et al.,* 1994). The degree of infectivity has also been studied among HIV-1 subtypes, particularly a comparison of subtype B and subtype E. Based on *in vivo* studies, it has been been proposed that subtype E has increased ability, compared to subtype B, to infect Langerhans' cells, the possible target of HIV-1 in the mucosa of the vagina, the

cervix and the penile foreskin (Cohen, 1996a,b; SotoRamirez *et al.,* 1996). However, more recently, two independent reports by Pope *et al.* (1997) and Dittmar *et al.* (1997) have not supported the preferential tropism of subtype E for epithelial dendritic cells. This questions the early reports that the rapid heterosexual spread of subtype E in Thailand was the result of its higher infectivity compared to that of subtype B.

Phylogenetic analysis showed that the sequences from Kimpese isolates are distributed among several clades, which correspond to their respective subtypes. At least seven subtypes of HIV-1 and another possible new subtype were found in the Kimpese population. Subtype A, the predominant subtype was found in about half of the sequences obtained in this study. In Kenya (Zachar *et al.,* 1996) and in Côte d'Ivoire (Janssens *et al.,* 1994) where subtype A is also predominant, the epidemic has been rising rapidly in the last decade. If this indicates that subtype A is more transmissible than the other subtypes, it would be expected that in locales with a low and stable seroprevalence, such as Kimpese, this subtype should be rare. This was not found in the study reported here.

Can the high heterogeneity of HIV-1 subtypes we have observed in this population suggest that the epidemic has reached its plateau level? Does the heterogeneity of HIV-1 subtypes suggest that the virus has been present in Kimpese for a long time? Kimpese is situated on a main road and many people travel through every

day ; thus it is possible that the subtypes found have been gathered here from different sources including Kinshasa, and other major towns in the region. Our own studies have shown that the seroprevalence was higher among individuals who lived in bigger cities than those from rural regions (Green *et al.,* 1992). As in Gabon where a high diversity of HIV-1 subtypes and a low and stable seroprevalence has been observed over nearly 10 years (Delaporte *et al.,* 1996), the heterogeneity of HIV-1 subtype does not provide clear explanation of the trend of HIV-1 epidemic in Kimpese.

Twenty-three subtype A sequences were identified, of which 9 derived from transmitting and 14 from non-transmitting mothers. Due to the small numbers of other subtypes found it was not possible to make a direct comparison of their role in MCT.

5.3.3. GENETIC RECOMBINATION

Intersubtype recombination occurs when an individual is infected with at least two subtypes. The rate of intersubtype recombination has been estimated to be about 2% per kilobase per replication cycle (Hu & Temin, 1990). With the high heterogeneity of HIV-1 subtypes observed in this study, the identification of intersubtype recombinants was not surprising. As it was not possible to analyse more isolates, it is possible that there may be many more recombinant subtypes circulating in the Kimpese region. The allocation of an isolate to a subtype should not be considered as definitive, if based on the sequencing of a subgenomic region, as was carried out in the present study. Any subtype assignment based on a partial sequence

only relates to the nucleotide sequence of the region analysed. For example, a sample classified as subtype A based on a partial region of the *gag* gene may be another subtype in another section of the genome if the virus happens to be a recombinant. It is possible that the proportions of HIV-1 subtypes observed in Kimpese do not reflect their real distribution. Although 50% of the isolates were subtype A and the rest was falling into several other subtypes, it is not known which isolates were recombinants since the sequencing was carried out only on a partial length of the genome. In addition, the presence of recombinant subtypes can constitute a confounding factor for any study which investigates the relative role of different subtypes in transmission. In the previously published studies (SotoRamirez *et al.,* 1996; Ou *et al.,* 1993; Kalish *et al.,* 1995) which measured the relative ability of HIV-1 subtypes to infect Langerhans cells, there was no consideration about the advantages that recombinant subtypes may have over non-recombinant strains. In fact, it has been shown that specific inter-subtype recombination can lead to SI phenotype (more virulent than NSI) compared to non-recombinant virus (Nyambi *et al.,* 1996).

5.3.4. A NEW SUBTYPE OF HIV-1?

In this study, in addition to 7 previously described subtypes, the phylogenetic analysis allowed the identification of another clade of sequences which appear to be highly divergent, yet similar to each other. The topological presentation of the trees were consistent both in the *gag* region and the envelope (754bp and 409bp of the *gag*

and *env* regions, respectively). With the current knowledge of recombination in HIV-1 (Sabino *et al.,* 1994; Leitner *et al.,* 1995; Diaz *et al.,* 1995; Sharp *et al.,* 1994), every report suggesting the identification of a new subtype must be interpreted with circumspection. As suggested by McCutchan *et al.* (1996), unless the sequence of the full genome of the virus is available, it is not possible to decide conclusively if the isolates identified in this study belong to a new subtype, or whether they possess mosaic structures. Additional investigations are needed to establish the full range of subtypes present in Kimpese, DRC.

5.3.4. p17 MATRIX PROTEIN MOTIFS AND MCT OF HIV-1

Most of the previous attempts to study the molecular characteristics of transmitted and non-transmitted HIV-1 have focused on the hypervariable region of the *env* gene, the gp120 (Briant *et al.,* 1995; Wolinsky *et al.,* 1992; Scarlatti *et al.,* 1993; Mulderkampinga *et al.,* 1993; Broliden *et al.,* 1989; Rossi *et al.,* 1989, Parekh *et al.,* 1991; Halsey *et al.,* 1992; Wang *et al.,* 1995; Robertson *et al.,* 1992). However, the results are conflicting. Not much work has been done using the matrix p17 region to determine if there are any molecular factors associated with mother-to-child transmission. The importance of studying the p17 region has been outlined in a previous section (5.3.2.).

In this study, 51 amino acid sequences deduced from nucleotide sequences were grouped by their transmitting and non-transmitting phenotypes. The alignment

included two important functional domains, mainly the polymerisation site (mer 8 to 19) at the N-terminal (Chazal *et al.*, 1995) and the antibody binding site (mer 59-70) at the C-terminal (Boucher *et al.*, 1990) (figure 5.9). The ELERFA motif at the N-terminal of the polymerisation site was more conserved, irrespective of the MCT phenotype. In addition, detailed statistical analysis of all residues between the polymerisation site and the antibody-binding site showed no specific amino acids nor motifs which were associated with transmission or protection. Detailed analysis was carried out on the C-terminal of p17 region, which showed a high degree of variation. A previous study observed an association between some amino acid residues and motifs with mother-to-child transmission phenotype of HIV-1 isolates (Narwa *et al.*, 1996). In this study, there was a significant difference between the transmitted and non-transmitted HIV-1 strains for the presence of glutamic acid and valine at positions 54 and 65, respectively, in the same partial *gag* region analysed by Narwa *et al.* (1996). It was not evident that the KIEEEQN motif was sufficient to distinguish the transmitting and non-transmitting mother, as shown by Narwa *et al.* (1996). Instead, within the KIEEEQN motif, there was a statistically significant difference between transmitted and non-transmitted isolates for isoleucine at position 69. The mechanism by which isoleucine at position 69 may prevent the transmission of HIV-1 from mothers to children remains unclear. It is possible that anti-p17 antibodies may bind more effectively to the principal p17 antibody binding site which have

isoleucine instead of other residues at position 69. The effective binding of antibodies to this epitope could prevent the transmission of HIV-1 from mothers to their children. Indeed, it has been suggested that antibodies to p17 matrix can be protective and their decline has been associated with rapid disease progression (Ljunggren, *et al.,* 1990). In addition, an induced mutation (deletion) within the antibody-binding site of p17 has a significant effect on viral entry during the replication cycle (Yu *et al.,* 1992). It is important that this early observation is confirmed as it could lead to the development of simple and quick peptide-based assays to detect antibodies to p17 peptides.

In summary, this study has revealed a remarkable heterogeneity of HIV-1 subtypes in a relatively small population of isolates sequenced. Although subtype A seemed to be the predominant subtype in this region, it was not possible to make direct comparison between subtypes to determine their relative transmissibility. There were no specific nucleotide motifs which could be associated with mother-to-child transmission of HIV-1. However, the analysis of amino acid sequences showed a statistically significant difference between isolates from transmitting and non-transmitting mothers for isoleucine at position 69. The mechanism by which isoleucine within the antibody binding domain of the p17 matrix affects transmissibility remains unclear. Regarding the low and stable seroprevalence, several factors could have played a direct role in maintaining the the epidemiology of HIV-1 unchanged for several years. The role played by different subtypes could not be demonstrated in this study. However, the most important finding described in this chapter is the description of a high heterogeneity of different HIV-1 subtypes in a rural district of Africa. This study

has contributed in the global research of the mapping of HIV-1 subtypes and laid important foundations for future studies in the region including the development of any proposed vaccine to protect against HIV-1 in this region. Thus, a vaccine may need to include antigens from more than one subtype to be effective against multiple co-circulating HIV-1 variants (Mascola *et al.,* 1994). Because subtype B has thus far not been found in Kimpese, it is theoretically possible that current candidate vaccines, based on virus strains from North America and Europe (subtype B), may have little or no protective effect if used in an area such as Kimpese. An HIV-1 vaccine may therefore need to take the form of a 'subtype cocktail' which takes representative antigenic patterns of all antigenic variants of HIV-1 into account. However, although cross-clade neutralisation may broaden the protective effect of such a vaccine, there is no guarantee that it would protect against the challenge of every subtype. The continual mutational changes and common occurrence of recombination (Velandia *et al.,* 1995) within the HIV-1 genome (Velandia *et al.,* 1995) constitute two major obstacles. Because some genomes have mosaic structure (Velandia *et al.,* 1995), it is not possible to make a direct comparison of the role of different subtypes in the spread of HIV-1, if the analysis is based on the sequencing of only a partial region of the genome.

CHAPTER 6

DETECTION OF IMMUNODOMINANT ANTI-V3 ANTIBODIES BY ELISA

6.1. INTRODUCTION

The use of molecular techniques such as nucleic acid sequencing (Chapters 2 and 5) and the heteroduplex mobility assay (HMA) (Delwart et al., 1993) are so far the only accurate means of classifying HIV-1 into different subtypes. However, sequencing is currently restricted to specialised laboratories, due to its complexity and cost. Although HMA has been used as an alternative method to sequencing, highly divergent subtypes like subtype A may be difficult to subtype (Delwart *et al.,* 1993; Delwart *et al.,* 1994). Most, if not all previously published studies on molecular subtyping of HIV-1 are biased due to the small number of samples tested. To overcome sampling bias there is a need for a simple, cheap and accurate technique suitable for large scale epidemiological mapping of HIV subtypes. Therefore, the World Health Organisation Network for HIV Isolation and Characterisation proposed the use of serological methods to determine subtypes of HIV-1, using a consensus of V3 loop amino acid sequences of each subtype as the antigenic source for the detection of subtype specific antibodies (Cheingsong-Popov *et al.,* 1994). However, V3 loop consensus peptides are prone to high cross-reactivity with antibody when they are used in indirect ELISA for subtyping of HIV-1 (Pau *et al.,* 1993; Ubolyam *et al.,* 1994; Pau *et al.,* 1994; Cheingsong-Popov *et al.,* 1994).

Cheingsong-Popov *et al.* (1994) used a panel of subtype A to E monomeric peptides in an enzyme immunoassay (MPEIA) and were able to predict the viral subtype in some samples, but encountered several cases of cross-reactivity. In order to solve this problem, it was necessary to retest samples using a peptide limiting ELISA. With this, up to 90% correlation was achieved between serotype and genotype (Cheingsong-Popov *et al.,* 1994). These results need to be confirmed and applied in a much bigger population. Kimpese is such an ideal setting to evaluate the use of this method (Cheingsong-Popov *et al.,* 1994), particularly in view of the high heterogeneity of HIV-1 subtypes present in this population (see chapter 5).

In a pilot study on samples from Kimpese, using the method as described by Cheingsong-Popov *et al.* (1994), over 95 percent of samples were reactive with more than one peptide. If all cross-reactive samples had to be retested with an additional subtype limiting ELISA as suggested by Cheingsong-Popov (1994), this would be too expensive and time consuming. Alternatively, a branched peptide competitive ELISA (BPC-ELISA) was developed. The assay was based on a panel of peptides containing consensus amino acid sequence of subtypes A, B, C, D, E and F. The BPC-ELISA uses the principle of blocking cross-reactive antibodies to allow subtype specific antibodies binding to subtype specific V3 loop peptides to be detected in plasma of HIV-1 infected individuals. The BPC-ELISA was based on the method previously developed by Simmonds *et al.* (1993), which achieved the subtyping of

hepatitis C virus by creating a similar competition between high concentrations of peptides with type specific antigen.

The BPC-ELISA developed in this study has several advantages over the previously described methods which use monomeric peptides without competition. The advantages of BPC-ELISA include a high sensitivity (the test needs only 0.5µl of plasma) and the ability to detect immunodominant antibodies by the inhibition of cross-reactivity.

6.2. PATIENTS, MATERIALS AND METHODS

A total of 367 plasma samples were collected from 205 mothers during pregnancy (158 samples) and soon after delivery (299 samples). Of 205 mothers, the outcome of vertical transmission was known in 157 pregnancies: 34 children were infected (MPP, mother positive and child positive, 61 samples) and 123 uninfected (MPN, mother positive and baby negative, 214 samples) with HIV-1 as monitored by follow up of antibody in sequential paediatric samples. Forty-seven HIV-1 positive women gave birth to babies who were not followed up long enough to determine whether they were infected or not (MPI, mother positive and indeterminate child, 77 samples). In addition, there were 5 stillbirths (9 samples). The control group consisted of 22 HIV-1 uninfected mothers (38 samples).

Samples were tested initially by a monomeric peptide-based indirect ELISA then with two branched peptide ELISAs (indirect and competitive ELISA). The

indirect ELISA using monomeric peptides was the same as that described by Cheingsong-Popov *et al.* (1994), except that the conjugate was diluted 1 in 10,000 in stead of 1:100. The branched peptide based indirect ELISA was employed using the same reagents as in the monomeric V3 loop peptide ELISA, except that the amount of the peptide needed on the solid phase was 10 fold less than that used in monomeric peptide based ELISA (10μg/ml) (see chapter 2).

The hypothesis was that, samples from an individual infected with an 'X' genetic subtype should have detectable antibodies to the consensus peptide of the same peptide, an autologous peptide. Here, the concept 'autologous peptide' designates consensus amino acid sequences deduced from nucleotide sequence of the same subtype. After testing a sample with a panel of different subtype peptides, the highest optical density over the cut off (OD/CO) was recorded and used to assign the subtype of that peptide. The results of the serotype, which were determined by indirect and competitive ELISAs, were compared with genetic subtypes (chapter 5).

6.3. RESULTS

6.3.1. SPECIFICITY AND SENSITIVITY OF INDIRECT AND COMPETITIVE ELISAs

Of 39 samples collected from HIV-1 negative individuals (n=39) tested by the commercially available Abbott Recombinant ELISA, 38 were non-reactive with any of the monomeric and branched peptides (A-E) used in the indirect ELISA and competitive ELISAs. The sample which was reactive with the branched peptides (A-F, 1ng/well) was non-reactive with all the monomeric peptides in the A-E panel. RT-PCR performed on this sample was positive suggesting that this sample was a false negative by the screening assays (Abbott 2nd and 3rd generation ELISAs) and also by the monomeric (A-E) V3 indirect ELISA. This suggests that the branched peptide based ELISAs developed here are highly specific.

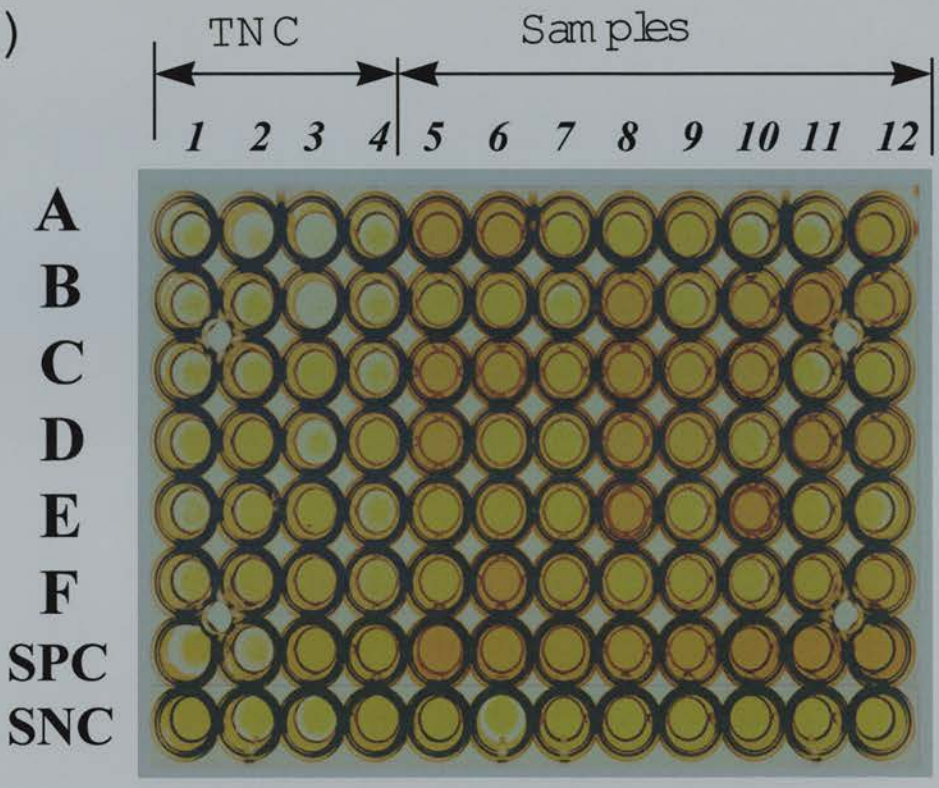
The sensitivities of the ELISAs using monomeric (A-E) (92%) and branched-peptides (A-F) in indirect (92.7%) and competitive assays (95.6%) did not differ significantly ($p>0.05$). Substantial cross-reactivity was observed in indirect V3 ELISAs with branched or monomeric peptides in 289 of 305 (94.8%) and 260 of 332 (78.3%) reactive samples, respectively. Only 9 (4.4%) of 204 reactive samples remained cross-reactive after inhibition of non-specific antibodies in competitive

ELISA. An example of cross-reactivity as was often observed in indirect ELISA is shown in figure 6.1 [note inhibition of cross reactivity in 6.1 (2)].

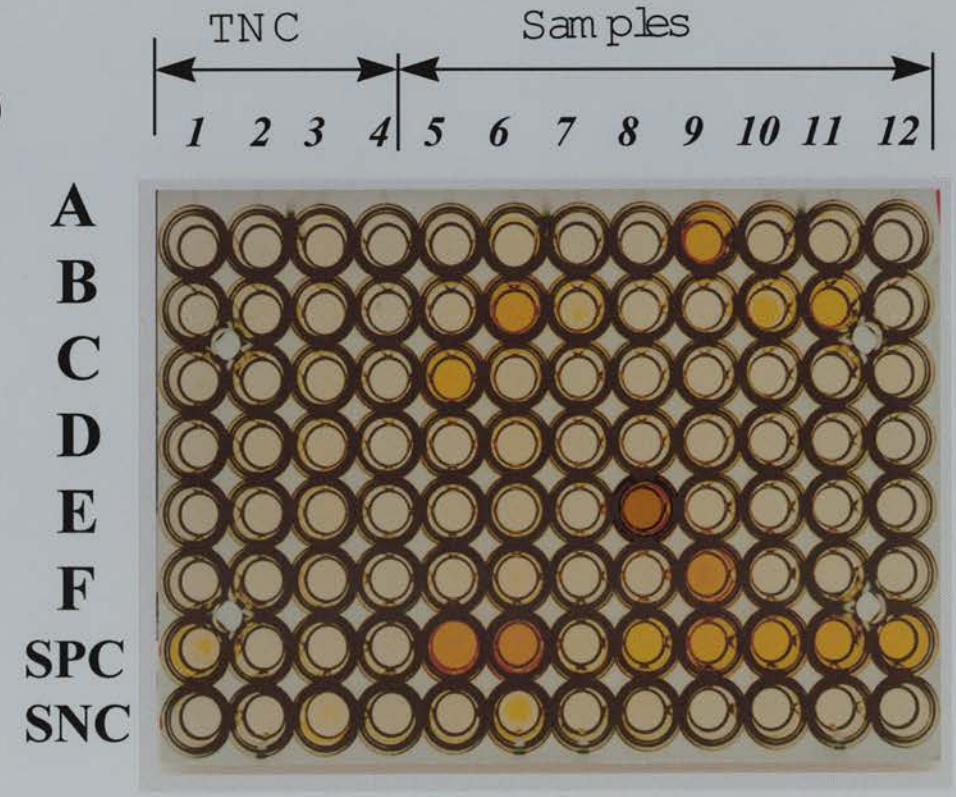
FIGURE 6.1. *Difference between indirect (1) and competitive (2) ELISAs for HIV-1 serotyping*

The same samples were tested twice by indirect ELISA and by competitive ELISA. The solid phase of both assays were identical: Each well of the rows A, B, C, D, E and F were coated with 1ng of peptides A, B, C, D, E and F, respectively, in carbonate coating buffer. The wells in SPC (sample's positive control) and SNC rows (sample's negative control) were coated with 1ng of a cocktail of equal volume of A-F peptides. The liquid phase of the indirect ELISA (1) contained 0.5µl of test negative control (TNC, columns 1-4) patient samples (columns 5-12) in 100µl of sample diluent. Only diluent was put in the SNC wells to act as negative control for the sample in indirect ELISA. For the competitive ELISA (2), the sample mix (0.5µl sample + 100µl diluent) was preincubated with 10ng of equal volume of all the peptides minus the one found in the solid phase. The SPC wells of the competitive ELISA did not have the competitors added. HIV-1 positive samples were expected to show reactivity in the SPC. In the SNC well, a cocktail of all the peptides (A-F) was added to achieve maximum competition with the sample. The ELISA procedure was carried out the same way as described in Chapter 2: Materials and metods. Note cross-reactivity in the indirect ELISA significantly reduced in the competitive ELISA.

(1)


TNC
Samples
1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
SPC
SNC

(2)


TNC
Samples
1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
SPC
SNC

6.3.2. AGREEMENT BETWEEN GENETIC SUBTYPE AND V3 SEROTYPE

Plasma samples from individuals infected with HIV-1 variants of known genotype (n=58) as determined by the sequencing of the HIV-1 proviral DNA within the p17 *gag* region were tested by ELISA to determine the subtype serologically. The serotype of each sample was determined as previously described (Cheingsong-Popov *et al.,* 1994): by assigning to the sample the subtype of the peptide which shows the highest reactivity in comparison with other peptides on the panel. The reactivity of the sample (serotype) was noted and compared with the genetic characteristics determined by sequencing (genetic subtype). The results of this comparison are depicted in table 6.1. Overall, there was a low degree of agreement between the sample genetic subtype and the serotype. In testing samples with the monomeric peptide-based ELISA, only 5 (13.8%) of 36 samples gave a subtype which matched the genetic subtype. Twenty-two further samples obtained from individuals infected with subtype F, G, H, J, Q and recombinant A/H and F/H showed reactivity to heterologous (A-E) peptides present on the panel. If the the genotype of these samples was not known, they could be assigned to the wrong subtype by serological ELISA.

TABLE 6.1.a. HIV-1 serotype (indirect ELISA) and genotype (sequencing)

	SEROTYPE (determined by **monomeric** peptide-based-V3 loop ELISA)								
Genot	A	A/C	B	B/D	C	D	E	NEG	Total
A	2	1	3	0	20	2	0	2	30
D	1	0	1	0	1	3	0	0	6
F	0	1	0	0	0	0	0	0	1
G*	1	0	0	0	0	1	0	0	2
H*	3	1	4	1	1	2	0	1	13
J*	0	0	0	0	1	0	0	1	2
Q*	1	0	0	0	0	0	1	0	2
A/H*	1	0	0	0	0	0	0	0	1
F/H*	0	0	0	0	0	1	0	0	1
Total	9	3	8	1	23	9	1	4	58

genot: genotype; * peptide of these genetic subtypes not available.

TABLE 6.1.b. Distribution HIV-1 serotype (competitive ELISA) and genotype (sequencing)

Genotype	**Serotype** (determined by **branched peptide**-based-V3 loop ELISA)								
	A	A/C	B	C	CF	F	NEG	NT	Total
A	1	0	3	10	2	4	0	0	20
D	0	0	0	0	0	1	0	5	6
F	0	0	0	0	0	1	0	0	1
G*	0	0	1	0	0	2	0	0	3
H*	2	0	1	1	0	0	1	5	10
J*	0	0	0	0	0	0	0	2	2
Q*	0	2	0	0	0	0	0	2	4
A/H	0	0	0	0	0	0	0	1	1
F/H*	0	0	0	2	0	0	0	0	2
Total	3	2	5	13	2	8	1	15	49

genot: genotype; * peptide of these genetic subtypes not available. NEG: negative;

NT: not typable

6.3.3. V3 (A-F) SEROTYPE AMONG HIV-1 TRANSMITTERS AND NON-TRANSMITTERS

Despite the poor agreement between the genetic subtype and the serotype observed in this study and elsewhere (Pau *et al.,* 1994), the reactivity to V3 loop produced the grouping of samples in what has been recognised as 'V3 loop serotype'. The V3 loop serotype was therefore considered independently from the genetic subtype. Using both the indirect and/or the competitive ELISAs with monomeric or branched peptides, samples were classified into several HIV-1 V3 loop serotypes. The distribution of HIV-1 V3 loop serotypes was compared between mothers who transmitted (n=34) and those who did not transmit (n=117) HIV-1 to their offspring (Table 6.2). There was no statistical difference between the transmission status of the mothers and the V3 loop HIV-1 serotype as determined by the serological assays. There were 137 mothers whose samples were serotyped as subtype A, B, C, D and E using the monomeric peptides but there was no statistically significant preferential transmission of one serotype over the others (p=0.48). Similary, the competitive ELISA for more precise detection of the immunodominant V3 serotype confirmed this observation with a p value of 0.42 for the distribution of different subtypes among transmitting and non-transmitting mothers (table 6.2).

6.3.4. LEVEL OF ANTI-V3 LOOP ANTIBODY AND TRANSMISSION OF HIV-1

The reactivity of the sample to a given subtype consensus peptide is expressed as the highest optical density over the cut off to a peptide. This was the criterion for assigning the serotype of the patient. The reactivities of antibodies to V3 loop peptides were identified in maternal blood samples, as shown in Table 6.3 and figure 6.2.

Overall, the level of antibody, as expressed by the highest optical density value over the cut-off, in samples collected from transmitting mothers and non-transmitting mothers did not differ significantly. The means of the highest OD/CO values (against V3 loop peptides) in 62 samples from transmitting mothers and 205 non-transmitting mothers were 7.8 (range 0.3-21.9) and 8.4 (range 0.26-21.75). When samples were grouped by the time of collection (i.e. during pregnancy or soon after the birth of the child, there was no statistically significant difference between transmitting and non-transmitting mothers, with regard to their reactivity to V3 loop peptides. These values did not differ from those recorded from mothers with indeterminate children. Even if the indeterminate mothers could be classified, it is unlikely that the difference in the reactivity to V3 loop peptide between transmitting and non-transmitting mothers will become significant.

FIGURE 6.2. *Reactivity to V3 loop peptides in samples from HIV-1 infected mothers (PP, PN and PI) and controls (NN).*

Samples obtained from HIV-1 infected (PP, n=34; PN, n=112 and PI, n=48) and unifected control mothers (NN, n=32) were tested against a panel of V3 loop peptides (A-F). The highest optical density over the cut off (ODMAX) was recorded. The ODMAX values in samples from PP, PN, PI and NN children are summarised. The horizontal line inside the box represents the median and the horizontal ends of the box represent the lower and upper hinges (the 25th and 75th percentiles). The difference between infected mothers who transmitted HIV-1 to their children and non-transmitting mothers was not statistically significant ($p>0.5$).

FIGURE 6.2. **Reactivity to V3 loop peptides in samples from HIV-1 infected mothers (PP, PN and PI) and controls (NN)**

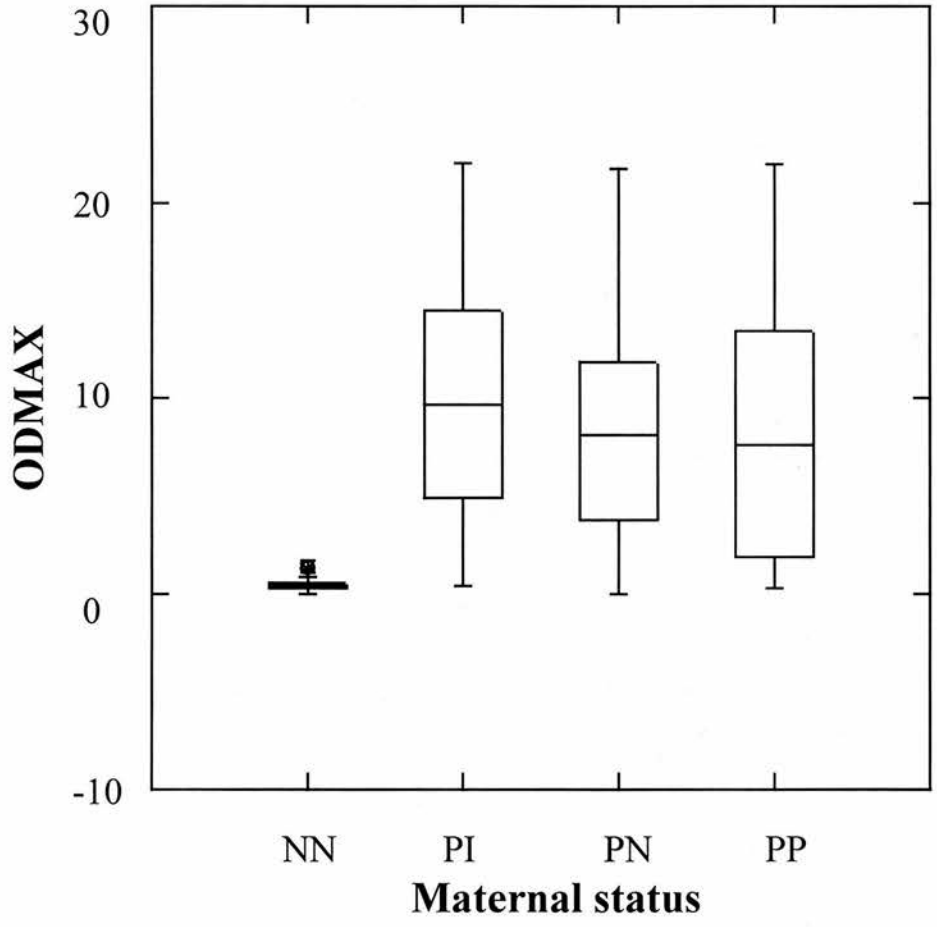


TABLE 6.2. Reactivity of maternal antibodies to V3 loop peptides

	Transmitting mothers				**Non-transmitting mothers**				**Indeterminates**			
Time	n	Mean	Min.	Max.	n	Mean	Min.	Max.	n	Mean	Min.	Max.
Pregnancy	28	7.5	0.3	19.2	93	8.1	0.26	21.7	30	9.5	0.4	22.0
Delivery	34	8.3	0.4	21.9	112	8.5	0.53	21.1	48	10.4	0.5	10.4

n: number of samples tested; Mean: average of highest optical density over the cut-off value; Min (minimum) and Max (maximum) are minimum and maximum values of the distribution of the highest optical density values over the cut-off.

TABLE 6.3. Distribution of HIV-1 V3 serotype among transmitting and non-transmittng mothers.

	MONOMERIC PEPTIDES							BRANCHED PEPTIDES								
	A	B	C	D	E	N	TOT.	A	B	C	D	E*	F	N	NT	TOT.
MPP	4	2	17	7	2	2	34	1	3	9	0	-	1	5	4	23
MPN	27	9	44	18	11	9	118	7	4	33	1	-	11	11	23	90
	21	11	61	25	19	11	152	8	7	42	1	-	12	16	27	113

[__ p=0.48 _____] [_____ p=0.42 _____.....____]

MPP: transmitting mother; MPN: non-transmitting mothers; TOT: Total; N: negative (OD/CO <1); NT: non-typable (OD/CO>1 with substantial cross-reactivity).

6.4. DISCUSSION

The use of serological methods for the identification of antibodies to the V3 loop has been of interest for the measurement of the level of antibodies available to hamper transmission of HIV-1 from mother-to-child. These methods are also useful for the mapping of HIV-1 subtypes in different populations. Unfortunately, the data so far remain controversial between different research groups. The data presented in this study provide important background information for future in Kimpese or similar settings in Africa.

6.4.1. LACK OF CORRELATION BETWEEN GENETIC SUBTYPE AND SEROTYPE

Because the tools used for genetic characterisation of different subtypes remain restricted to specialised laboratories, the widespread mapping of HIV-1 subtypes remains a goal still to be reached. It was important therefore to evaluate a simple serological assay based on well-defined principles for characterisation of immunodominant antibodies which bind to synthetic peptides. Peptides derived from the V3 loop consensus sequences have been proposed as antigens for indirect ELISA to detect subtype specific antibodies (Cheingsong-Popov *et al.,* 1993; Pau *et al.,* 1993; Pau *et al.,* 1994; Cheingsong-Popov *et al.,* 1994; Baillou *et al.,* 1993). In all these studies, the detection by indirect ELISA of subtype specific antibodies in the

plasma of HIV-1 infected individuals was masked by the presence of cross-reactive antibodies (which reacted with different V3 loop peptides). In a previous study, using an indirect V3 peptide ELISA and another assay to discriminate cross-reactive antibodies, Cheingsong-Popov and coworkers (1994) were able to predict the correct genotype in approximately 90% of cases of genotype A, B, C and E. This method was applied in this study (Cheingsong-Popov *et al.,* 1994), using the A-E consensus peptides. Recently, the genetic sequencing of HIV-1 strains in Kimpese suggests that there are highly divergent subtypes circulating within our study population. There were additional subtypes such as F, G, H, J and another as yet unpublished new subtype. The peptides for these subtypes were not included in the Cheingsong-Popov study. Ignoring the samples of which the peptides were not available (subtype F, G, H, J and Q; see chapter 5), only 5 (13%) out of 37 plasma were correctly subtyped using the Cheingsong-Popov method. The remaining samples showed a high degree of cross-reactivity and the use of peptide limiting ELISA (Cheingsong-Popov *et al.,* 1994) did not improve the test. As high as 70% tested by indirect V3 loop monomeric ELISA and 90% by branched peptide ELISA needed additional peptide limiting ELISA, another time consuming step for serotyping of cross-reactive samples. A one-step method, a competitive V3 loop ELISA, was therefore devised to solve this problem.

In a previous study, Simmonds *et al.* (1993) achieved the subtyping of hepatitis C using a competitive assay (Simmonds *et al.,* 1993). A similar peptide competitive ELISA was developed to detect immunodominant antibodies to one particular subtype-specific V3 loop consensus peptide, by blocking cross-reactive antibodies with an excess of other subtype-specific peptides. In theory, complete inhibition of cross-reactive antibody would allow serotyping of samples and the results could be correlated with genotyping data. In contrast, while we have blocked non-subtype specific antibodies, in most cases, there was a lack of correlation between serotype and genetic subtype of HIV-1. This means that the immunodominant antibodies detected after inhibition of cross-reactive antibodies using the currently proposed panel of peptides are not subtype-specific. Why is there such a mismatch between the results by serotyping and genotyping in this study?

Several factors could explain this mismatch. Firstly, subtyping by sequencing depends on the analysis of several hundred nucleotides of at least the *gag* and the *env* genes of HIV-1 whereas the serotyping assay uses only a small region of the genome at the tip of the V3 loop. With the emergence of many recombinant strains, subtyping with a small section of the viral nucleic acid provides only a partial classification. Only the analysis of the full genome of HIV can determine a pure subtype and rule out the possibility of genetic recombination. Therefore, it is

unlikely that the serotyping assay, based on a restricted region of the V3 loop subtype consensus peptide, would produce accurate results.

Secondly, there may be a lack of subtype-associated-blocking activity. HIV-1 neutralisation depends not only on the association of antibodies with linear epitopes but also with conformational epitopes within the monomeric and oligomeric forms of the envelope glycoprotein (Earl *et al.,* 1994; Ho *et al.,* 1991; Moore *et al.,* 1994; Stamatatos and Chengmayer, 1995). The icosahedral-symmetry of the envelope glycoprotein (Gelderblom *et al.,* 1987) may give rise to complex epitopes which may give unpredictable associations between antibody activity and the genetic subtype. If there is such a thing as a subtype-specific epitope, it is still unclear how the intra- and inter-clade variation would affect its structure and its presentation to antibodies.

Thirdly, the intra-clade cross-reactivity and the lack of correlation between genetic subtypes and serotype could be caused by the similarity of the peptides currently being used in the serotyping of HIV-1. For instance, the consensus peptides for subtype A and subtype C have the same tetramers GPGQ and differ only at 2 sites of 14 amino acids found at the crown of the loop. The GPGQ is the main feature for subtype A, C, D, E and is supposed to be important in the neutralisation of HIV-1. The drive to use the V3 loop region for subtyping derives from early observations of constraints on sequence variation among isolates in the United States (LaRosa *et al.,* 1990). The conservation within the tip of the V3 region, as suggested by LaRosa et

al (1990), is probably more relevant to vaccine development than for serological subtyping of HIV-1 as demonstrated in the present study.

Finally, could the length of the peptide explain the discrepency between genotype and V3 loop serotype? Previously, it was suggested by Barin *et al.* (1996) that the low cross-reactivity observed by Cheingsong-Popov *et al.,* and others (Cheingsong-Popov *et al.,* 1993; Pau *et al.,* 1994; Cheingsong-Popov *et al.,* 1994) could be due to the use of short V3 sequences. The lesser extent of cross-reactivity between subtype specific V3 loop peptides observed in these studies may explain why some studies have observed a correlation between serotype and genotype. The same peptides were used in the present study but with different results[1]. It is therefore concluded that the length of the branched peptide was irrelevant in the accuracy of the V3 peptide based ELISA for serotyping of HIV-1.

6.4.2. POTENTIAL FOR INTRA-CLADE CROSS-REACTIVITY

It is recognised that the third hypervariable domain, the V3 loop of the *env* glycoprotein, is biologically important during HIV-1 infection. It induces an antibody response with restricted neutralization activity *in vitro* (Harrowe and Chengmayer, 1995; Vancott *et al.,* 1995; Poignard *et al.,* 1996; Pinter *et al.,* 1993; Kliks *et al.,* 1993). A broad reactivity of peptides made from consensus sequences of

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[1] The testing was carried out by myself at Dr RC Cheingsong-Popov laboratory at St. Mary's Hospital, in London. I received technical help from Mr Simmon Lister, a co-author of the studies which used the monomeric peptide ELISA (Cheingsongpopov *et al.,* 1993; Pau *et al.,* 1994; Cheingsongpopov *et al.,* 1994).

different subtypes was observed in this study. For example (Table 6.4), with a single consensus peptide representing subtype A, it was possible to detect about 69.6% and 80% (with monomeric peptide and branched peptide, respectively) HIV-infected individuals. Even with a peptide derived from the consensus amino acid of subtype B sequences 88 (61.5%) of 143 patients tested appeared to have antibodies against it. Subtype B is the predominant clade in Europe and North America, but thus far not found in Kimpese (Chapter 5). The reactivity was increased to 86% when a cocktail of A-F peptides was employed in the solid phase of the ELISA plate (Table 6.4). A similar observation was reported by Baillou *et al.* (1994), who found that V3 consensus sequences representing North American/European and African isolates were also highly cross- reactive, binding 94% and 77%, respectively, of sera collected from HIV-1 individuals originating from various parts of the world.

The broad reactivity of sera to heterologous peptides of the same and different clades may have relevance to the future development of an HIV-1 vaccine. If cross-clade reactivity also reflects cross-clade neutralisation, a vaccine against HIV-1 based on one particular clade should be able to induce protection against the challenge of any other HIV-1 clades. However, as is the case of Influenza virus, if every single clade or variant has a specific neutralising epitope, an HIV-1 vaccine would have to take into account all the variants circulating in a region. From the data presented here, it can be postulated that V3 consensus sequences able to induce broadly

reactive antibodies to neutralisation region, irrespective of the clade of the virus, represent the best candidates for vaccines.

TABLE 6.4. Reactivity of monomeric and branched peptides-based ELISA

A. BRANCHED PEPTIDES

	ELISA RESULTS			
PEPTIDE	POSITIVE (%)		NEGATIVE (%)	
A	114	(79.7)	29	(20.3)
B	88	(61.5)	55	(38.5)
C	112	(78.3)	31	(21.7)
D	73	(51.0)	70	(49.0)
E	19	(13.3)	124	(86.7)
F	116	(81.1)	27	(18.9)
ABCDEFZR	123	(86.0)	20	(14.0)

B. MONOMERIC PEPTIDES

	ELISA RESULTS			
PEPTIDE	POSITIVE (%)		NEGATIVE (%)	
A	110	(69.6)	48	(30.4)
B	76	(48.1)	82	(51.9)
C	112	(77.2)	36	(22.8)
D	79	(50)	79	(50)
E	86	(54.4)	72	(45.6)

6.4.3. LACK OF ASSOCIATION BETWEEN LEVEL OF MATERNAL ANTIBODY TO V3 LOOP AND THE PROTECTION OR ENHANCEMENT OF VERTICAL TRANSMISSION

Early *in vitro* studies suggested that antibodies to the envelope gp120 protein may have some effect in preventing the binding of the virus to cellular receptors (Skinner *et al.,* 1988; Linsley *et al.,* 1988). This observation was extrapolated to *in vivo* situations to suggest that antibodies might be effective in preventing infection in adults and in children. However, interpretation of the results of studies remains controversial, particularly about the role played by maternal antibodies to the envelope V3 loop in the prevention of mother-to-child transmission of HIV-1. Whereas some reports have suggested that anti-V3 loop antibodies have a role in reducing transmission (Broliden *et al.,* 1989; Rossi *et al.,* 1989), others (Parekh *et al.,* 1991; Halsey *et al.,* 1992; Wang *et al.,* 1995; Robertson *et al.,* 1992), do not support this observation. All the previous studies differ in the methods used to detect maternal antibodies to the V3 loop. This is particularly the case for the peptide used on the solid phase in ELISAs as often only one peptide derived from one strain was used as the basis for comparison of reactivity. It is possible that patients with low or no antibodies to the peptide on the solid phase may be infected with another highly divergent strain. Also, the use of different peptides could possibly explain the conflict between different studies.

A different approach is, however, to use peptides whose structure suggests they would be more likely to detect antibodies to V3 loop irrespective of the number of different HIV-1 subtypes circulating in the studied population. In the present study, the structure of the peptides had two specificities to provide an alternative way to assess the relative association between the level of maternal antibodies and the transmission of HIV-1 from mothers to their children. Firstly, the peptides were made from consensus sequences of amino acids derived from HIV-1 subtypes. Previous studies suggested that the use of consensus sequences rather than isolated specific sequences improves the sensitivity of V3 peptides assays (Baillou *et al.,* 1993; Cheingsong-Popov *et al.,* 1994). Secondly, the peptides were constructed in a branched format. Peptides constructed in such way have an advantage over monomeric peptides in being able to detect traces of antibodies in biological fluids (Marsden *et al.,* 1992). Despite the attempt to increase the sensitivity and specificity to detect the antibodies in the plasma, there was no association between the presence and level of maternal antibodies to V3 loop to either protect children from, or make them more susceptible of acquiring HIV-1 from their mothers. In addition, *de novo* peptides based on indigenous sequences were used, but the level of antibodies to V3 loop was similar in transmitting and non-transmitting mothers (figure 6.2.). This study produced no evidence to support the proposal that pregnant HIV-1 infected

mothers should be passively immunized with antibodies to V3 loop in order to prevent the transmission of the virus to their offspring.

6.4.4. EFFECTIVENESS OF COMPETITIVE ASSAY FOR THE DETECTION OF IMMUNODOMINANT ANTIBODIES AGAINST V3 LOOP CONSENSUS PEPTIDES

Cross-reactivity is common in assays to detect antibodies to peptides of the V3 loop of HIV-1. This was observed elsewhere (Baillou *et al.,* 1993; Barin *et al.,* 1996) and in this study. It has been shown also that cross-reactivity impairs accurate serological subtyping of HIV-1 (Barin *et al.,* 1996; Pau *et al.,* 1994; Cheingsong-Popov *et al.,* 1994). Therefore, a method to inhibit cross-reactive antibodies was considered as an alternative means of serotyping HIV-1. In this study, a significant reduction of cross-reactivity from 78% and 90% (in indirect ELISA with monomeric and branched peptides, respectively) to 4.4% was achieved when samples were tested by a *de novo* competitive method. Barin *et al.* (1996) have used a similar cross-reactive antibody inhibition method but cross reactivity was still a problem in 26% of samples tested. The persistence of cross-reactivity in some samples could be due to infection with recombinant subtypes or with two or more different subtypes. It was evident that the immunodominant antibody to the principal neutralising domain of HIV-1 is not subtype specific and these findings are in accord with previous studies

(Moore *et al.,* 1996; Kostrikis *et al.,* 1996) but conflict with those of Cheingsong-Popov *et al*. (1994).

CHAPTER 7:

GENERAL DISCUSSION AND CONCLUSION

Risk factors are important both in forecasting the the course of the epidemic and also in defining possible types of intervention. At the start of the study, Kimpese presented two unique epidemiological features. Firstly, the town of Kimpese is located in a rural part of an African country, where no other HIV studies have been conducted. Secondly, we have previously observed a low and stable seroprevalence over a 5-year period (Green *et al.*, 1994) and a low MCT rate, for which the reasons have been unclear. It was planned to carry out a multidisciplinary study of risk factors associated with HIV transmission in Kimpese. On the one hand, it was important to study the behavioural factors of pregnant women in Kimpese in order to define potentially relevant host factors[1]. On the other hand, it is necessary to study in parallel the factors associated with the virus and to link them with host-associated factors.

The aims of this project were to: (i) develop a simple and inexpensive method for early diagnosis of HIV-1 infection in children (Mokili *et al.,* 1996), (ii) to investigate the distribution of HIV-1 subtypes in Kimpese, and to establish their biological significance in mother-to-child transmission. Because of the large number of cases studied in this project, the determination of HIV-1 subtypes by sequencing was too expensive and time consuming. A serological method to subtype large

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[1] Dr Louise de la Gorgendière, of the Department of Sociology, University of Edinburgh, visited Kimpese to carry out a pilot study on social behaviour. No funding was available for the continuation of the study.

numbers of samples containing antibodies to the principal neutralising domain of HIV-1 was developed.

Serological methods for the identification of HIV-1 infected individuals (adults) are readily available and relatively simple to perform. The sensitivity and specificity for most of these techniques are often very high, approaching 100%. These methods, however, particularly those based on the detection of IgG antibodies in biological secretions have little diagnostic significance in children born to HIV infected mothers. This is due to the presence of passively acquired maternal IgG in children which can be detected at 12, or even 15 months of age, and so delay the diagnosis of HIV-1 infection until 18 months (Newell *et al.,* 1995). Highly sensitive techniques for the diagnosis of HIV-1 in children such as the polymerase chain reaction (PCR) and virus culture are not always available in most African countries. As mentioned previously in this study (chapters 1 and 4), there are other highly sensitive techniques which can achieve early diagnosis of HIV in children. However, they require a high level of technical support and expertise, which are a limiting factor for most laboratories in developing countries. The development of a simpler diagnostic tool was a prerequisite for the identification of HIV-1 in children in this population (Mokili *et al.,* 1996).

The physical and functional properties of immunoglobulins were taken into account for the development of diagnostic assays to detect HIV infection in children.

The generally accepted principle, that IgA and IgM 'do not cross' the placental barrier, suggested that the detection of these antibodies in children born to HIV-1 positive mothers indicates infection with HIV-1. In testing children born to HIV infected mothers, it was not expected that IgM and IgA anti-HIV would be detected in neonatal samples. One of the most important findings in this study was the detection of IgM and IgA in neonatal samples of children born to seropositive mothers beyond 3 months of age. Below this age some uninfected children did give positive results for specific IgA and IgM. Is maternal contamination of the babies' samples the likely explanation? Simple cross-contamination at collection of cord blood is not relevant as there was no significant statistical difference between cord blood and other neonatal samples. The analysis of sequential samples showed a decline and complete loss of IgA and IgM by 3 months of age, despite the continuing detection of IgG antibodies. This indicates that the IgA and the IgM results were not due to cross-reactivity with IgG. Leakage of maternal antibodies into the fetal circulation is a possibility and may be due to the placental infections noted in both transmitting and non-transmitting pregnancies. Therefore it was concluded, based on the evidence from this study, that IgA and IgM class anti-HIV-1 do cross the placenta (Mokili *et al.,* 1996). The observation of IgA and IgM in neonates was also found in another HIV MCT study of a small number of European children (Connell *et al.,* 1992). Other studies that have not found IgA and IgM in neonatal samples may have used less sensitive methods than the capture assays

used in the present study. In the light of the findings, it is not surprising that the results of most assays for detection of IgA and IgM, which are often used for the diagnosis of various congenital infections (including Rubella, Syphilis, toxoplasmosis, CMV, EBV etc.) are often confusing. Although not demonstrated in this study, it is possible that maternal IgA and IgM (probably also IgE) raised against any other infectious agents in mothers may cross the placenta. Results of tests where IgA antibodies and more often IgM are routinely used for diagnosis of congenital infection should be interpreted with caution.

Although IgA and IgM assays were not very useful for diagnosis with neonatal samples, their detection in the blood of children of 3 months and older ages is highly predictive of infection. By contrast, other anti-HIV reactivity in screening tests, predominantly IgG, is often present in uninfected children even beyond 12 months of age, making it difficult to distinguish infected from uninfected children. The method employed in this thesis was shown to be not only robust, but more importantly, simpler and cheaper than most tests for IgA anti-HIV which require preliminary removal of IgG and an immunoblot assay. At present, the cost for an IgA test should not be more than 2 to 3 dollars (US): immunoblot reagents cost approximately 10-fold more.

In addition, the fact that it was not possible to determine accurately when children became infected affected the overall sensitivity of the IgA capture assay.

Indeed, PCR positivity was also delayed in children suspected to have become infected postnatally. Therefore, it was not surprising to find that the sensitivity of the IgA capture assay in breast-fed infants was imperfect. In circumstances where other diagnostic tests (PCR, culture, etc.) are not available, the IgA capture method, by 6 months of age, would identify three-quarters of babies who have been infected *in utero*, perinatally or postnatally.

After identification of infected patients, the characterisation of HIV-1 in mothers and children was studied. Two approaches (molecular and serological) were employed for this purpose. The molecular work included the phylogenetic analysis of the p17 region of HIV variants found in mothers and children. The main objectives were to identify which HIV-1 subtypes were present in the population and to examine in infected children, transmitting and non-transmitting mothers and to determine whether there were intrinsic differences in transmission between different HIV-1 subtypes. The analysis of the genetic sequences from this population was compared with the Los Alamos database (Myers et al, 1996)[1]. Interestingly, a high degree of genetic heterogeneity of HIV-1 was observed with 7 subtypes and another outlier clade. No clear differences between transmitting and non-transmitting mothers

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[1] Some of these sequences have now been removed as there is now an emphasis on the use of full length reference sequences for subtype allocation. The most recently published reference sequences were used to re-analyse the data from Kimpese and the results were consistent with those included in the thesis (figure 7).

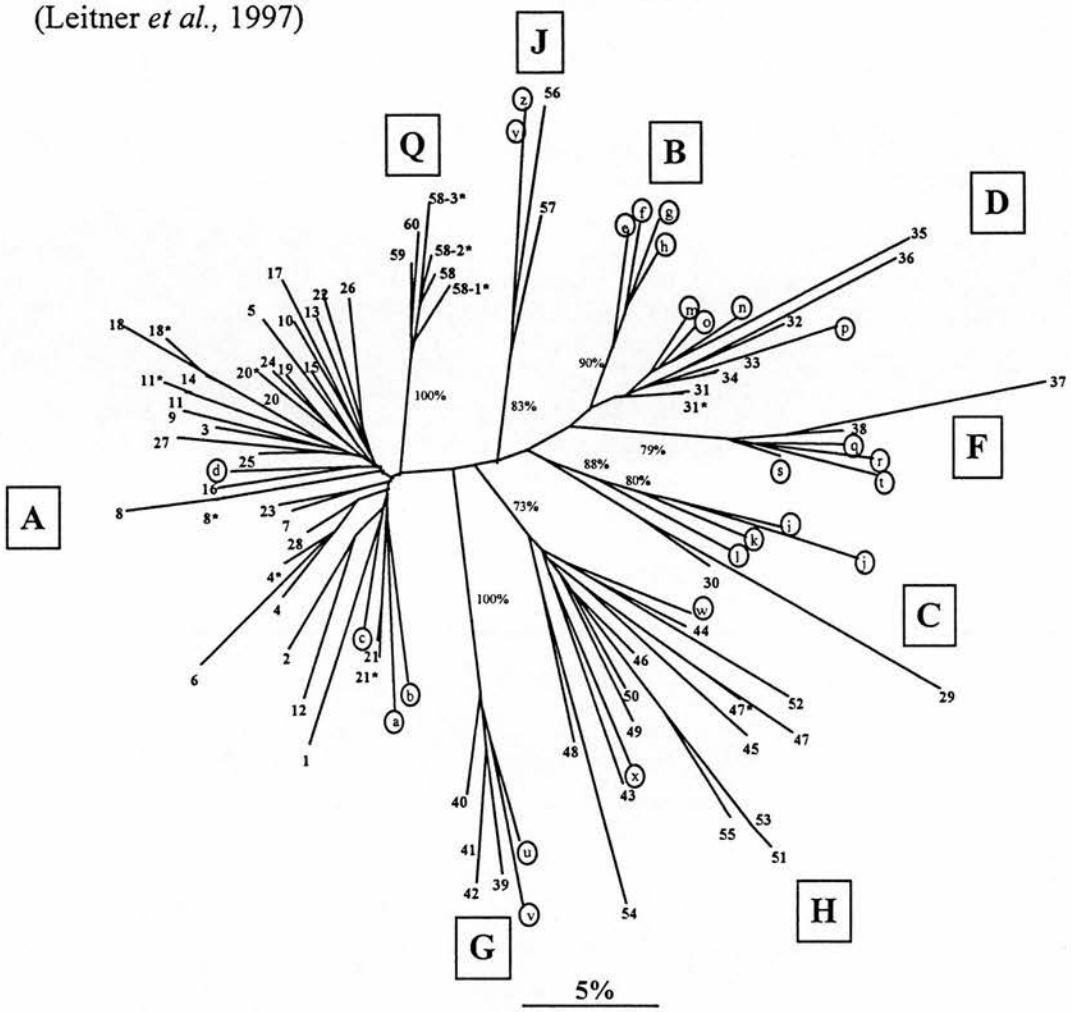
were observed within the p17 *gag* region, although a single isoleucine was found to be associated with protection against MCT of HIV-1. Although a high heterogeneity of HIV-1 subtypes was observed in the study group, it would require a large sample size to make a direct comparison between the different subtypes and MCT. Sequencing using a large number of samples is not only time consuming, but expensive and requires specialised expertise.

A serological method, developed by Cheingsong-Popov *et al.* (1994) for detection of V3 subtype specific antibodies, was applied in this study. The method was modified by the use of highly sensitive branched peptides (Marsden *et al.,* 1992), in a newly developed competitive ELISA based on a well characterised approach of inhibition of cross-reactive antibodies (Simmonds *et al.*, 1994). It was shown that the immunodominant antibodies to the principal neutralising domain of theV3 loop of HIV-1, are not subtype specific. In addition the level of maternal antibody to PND was not associated with either transmission or protection of the babies.

The lack of correlation between genetic subtype and serotype could be due to various factors, including the similarity of V3 loops from different subtypes (Cheingsong-Popov *et al.,* 1994, Myers et al, 1996). It is also possible that the lack of correlation between serotype and genetic subtype may be due to genetic recombination.

In the future, the full-length sequencing of HIV-1 subtypes present in this population is required. Such a study will allow the identification of 'pure' HIV-1 subtypes (as opposed to recombinants) and determine their relative risk of transmission from mother-to-children. A serological approach may still be relevant if alternative immunodominant domains within the HIV-1 genome are identified. However,due to the high level of recombination, the same difficulties encountered in this study may arise.

FIGURE 7: **Re-analysis of sequence data with the newly published reference sequences** (Leitner *et al.*, 1997)



Neighbour-joining p17 *gag* gene phylogeny showing the distribution of this study sequences relative to A-H and J reference sequences . The phylogenetic tree is based on an unambiguously aligned 330bp region of the *gag* gene (positions 907 to 1236 in the HIV_{HXB2} genome) and distance estimated using the maximum likelihood method with transition/transversion ratio of 3. The major clades are designated by capital letters and are boxed. Reference sequences are represented by lower case letters as follows : **subtype A** : a, VI32 ; b, K89 ; c, 92UG037 ; d, U455. **subtype B :** e, RF ; f, OYI ; g, JRFL ; h, HXB2. **subtype C :** i, 92BR025 ; j, ZAM18 ; k, UG268 ; l, ETH2220. **Subtype D :** m, ELI ; n, NDK ; o, Z2Z6 ; p, 94UG114. **subtype F :** q, VI69 ; r, 93BR02 ; s, BZ162 ; t, VI174. **subtype G** : u, 92NG003 ; v, 92NG083 ; **subtype H :** w, 90CF056 ; x, VI557 ; **subtype J :** y, SE7022 ; z, SE7887. Sequences from patients in this study are lebelled with numerical numbers in the tree and were as follows : **subtype A** :Mothers : 1, Sq3M ; 2, Sq6M ; 3, Sq8M ; 4, Sq9M ; 4*, Ped4el ; 5, Sq11M ; 6, Sq12M ; 7, Sq13M ; 8, Sq27M ; 8*, Ped15el ; 9, Sq29M ;10, Sq31M ; 11, Sq32M ; 11*, Ped6el ; 12, Sq34M ; 13, Sq36M ; 14, Sq37M ; 15, Sq38M ; 16, Sq39M ; 17, Sq40M ; 18, Sq43M ; 18*, Ped14el ; 19, Sq49M ; 20, Sq51M ; 20*, Ped8el ; 21, Sq52M ; 21*, Ped11el ; 22, Sq54M ; 23, Sq55M ; 24, Sq59M ; 25, Ped2 ; 26, Ped7 ; 27, Ped10 ; 28, Ped12; **subtype C** : 29, S18M ; 30, Ped16; **subtype D** : 31, Sq4M ; 31*, Ped3el ; 32, Sq20M ; 33, Sq21M ; 34, Sq23M ; 35, Sq41M ; 36, Sq58M; **subtype F** : 37, Sq19M ; 38, Sq53M; **subtype G**: 39, Sq7M ; 40, Sq17M ; 41, Sq47M ; 42, Sq56M ; **subtype H** : 43, Sq5M ; 44, Sq10M ; 45, Sq14M ; 46, Sq15M ; 47, Sq16M ; 48, Sq25M ; 49, Sq26M ; 50, Sq28M ; 51, Sq30M ; 52, Sq33M ; 53, Sq35M ; 54, Sq60M ; 55, Ped13; **subtype J** : 56, Sq42 ; 57, Sq57M. The samples (58, Sq1M ; 58-*, Ped1 ; 58-2*, Ped1b-el ; 58-3*, Ped1c-el ; 59, Sq2M ; 60, Sq24M) from patients which did not accurately cluster with any of the published sequences were grouped in the **'Q'** (query) clade. The scale bar corresponds to 5 changes per 100 nucleotide positions. Bootstrap values (shown if above 70%) are based on 100 bootstrap replications.

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APPENDIX 1: ETHICAL PERMISSION

MOUVEMENT POPULAIRE DE LA REVOLUTION

REPUBLIQUE DU ZAIRE

Conseil exécutif
Département de la Santé Publique
Cabinet du Commissaire d'Etat

LE COMMISSAIRE D'ETAT

Réf. :

Objet : Etude de la transmission périnatale du HIV à Kimpese.

Kinshasa, le 17 SEP. 1988

N°BUR/CE/SP/ 3014 /88

TRANSMIS copie pour information aux :

- Médecin Inspecteur Régional
  BAS - ZAIRE
- Médecin Directeur de l'Hôpital de Kimpese
  Kimpese / Bas - Zaire
- Médecin Sous-Régional
  Sous-Région de Cataractes
  Bas - Zaire.

Au DR STEPHEN GREEN
Chef de Service de Pédiatrie
Membre du Comité Régional de Lutte contre le SIDA / KIMPESE
KIMPESE / BAS - ZAIRE

Docteur,

M'ayant assuré que les préalables suivants ont été ou seront remplis à savoir :

- Associer des Médecins Zaïrois à l'étude
- Réserver le primeur des résultats de l'étude au Comité National de Lutte contre le SIDA par le trichement de son Bureau Central de Coordination (BCC/SIDA),

j'ai le plaisir de vous notifier mon accord pour le démarage de l'étude sur la transmission périnatale du HIV à Kimpese, dont le protocole m'a été soumis.

Par conséquent, je vous autorise à utiliser dans le cadre de cette étude les deux autres médecins expatriés ci-après :

- DR IAI HARDY, Pédiatre, pour une durée de 2 ans.
- DR WILLIAM CUTTING pour des visites périodiques du Projet, une ou deux fois par an.

J'insiste à nouveau sur la participation effective et continue des Médecins Zaïrois qui seront désignés dans ce but.

En outre, je vous demande d'envoyer des rapports trimestriels au Comité National de Lutte contre le SIDA via le Bureau Central de Coordination du Programme de Lutte contre le SIDA (BCC/SIDA) afin de nous permettre de suivre les résultats de ces études.

Veuillez agréer, Docteur, l'expression de mes sentiments très distingués.

Lothian Health Board

Simpson Memorial Maternity Pavilion,
Lauriston Place,
Edinburgh.
EH3 9EF.
Tel: 031-229-2477 Ext. 2670

Our ref : EW/JHNL
Your ref :
Date : 10th November 1987.

Dr. W.A.M. Cutting,
Department of Child Life and Health,
17 Hatton Place,
Edinburgh,
EH9 1UW.

Dear Dr. Cutting,

Paediatric/Reproductive Medicine Ethics of Medical Research Sub-Committee
Protocol 33/87: The Natural History of Human Immunodeficiency (HIV) Virus
Infection in Rural Zaire

The Standard Form of Application, Protocol, Information Sheet and Consent Form, submitted by you in respect of the above project have been considered by the Ethics of Medical Research Sub-Committee, and I am pleased to advise you that the Sub-Committee have agreed to grant ethical approval to this project.

Please note that if new ethical issues arise you should consult with the Chairman or myself to ascertain if a reapplication requires to be made.

APPENDIX 2: Consent form for entry in the study

(Translated from Lingala and Kikongo)

It is very good children are in good health. However, children can catch disease and even die as a result of it. There are several diseases, which can cause damage to children. These include measles, malaria, tuberculosis and AIDS.

At Institut Médical Evangélique, there is a project, which provides service for children to help them have to fight various diseases. The projects carried out research on these diseases, including AIDS, and information obtained will help the researchers to understand these diseases and to provide policy for better care for Zairian children.

The health of the mothers is also very important for their children. If they carry some infectious agents when they are still pregnant, they can pass the disease to their children during the pregnancy, at delivery or later through breast-feeding (the breast milk is the BEST food for the baby).

To be invited to join the project is very important for you and your baby. The project team members will provide immunisation for your baby. Immunisation will protect your child against measles, tuberculosis, poliomyelitis, tetanos and diphteria.

The project will cover the cost of the delivery at IME hospital, CIZA, Cité and Lamba.

The project medical team will examine the baby at birth, at 3, 6, 9, 12, 15, 18 and 24 months and every time when the baby is unwell. No money will be asked for the care provided. The project will also pay for the transport to and from the hospital

Consent: The project was clearly explained to me. I understand that the project will provide care for my child and carry out research during the first 2 years from birth. I will bring the baby to the scheduled clinic for immunisation and routine examination and blood (me and the baby) and breast-milk samples will be taken for laboratory analysis.

Signature: ..... Date:..../..../199....

Witness: .....

APPENDIX 3: Classification for HIV infection in children (CDC, 1992).

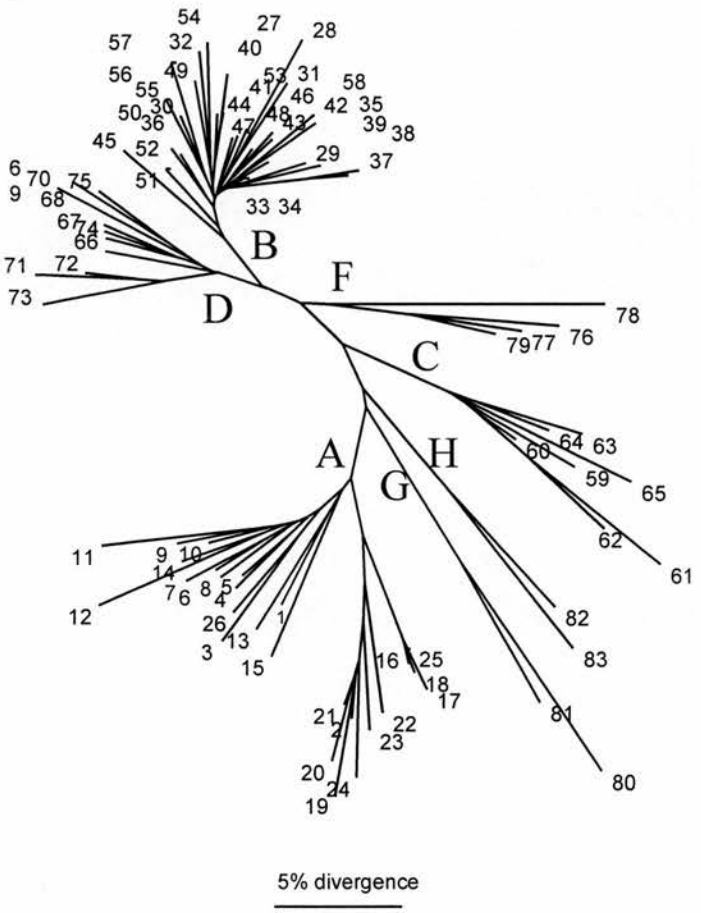
CLASS P-0. INDETERMINATE INFECTION
Infants <15 months born to infected mothers but without definitive evidence of HIV infection or AIDS
CLASS P-1. ASYMPTOMATIC INFECTION
Subclass A. Normal immune function
Subclass B. Abnormal immune function
Hypergammaglobulinemia, CD4 lymphopenia, decreased CD4-to-CD8 ratio, or absolute lymphopenia
Subclass C. Immune function not tested
CLASS P-2. SYMPTOMATIC INFECTION
Subclass A. Nonspecific findings (at least two for ≥2 months)
Fever, failure to thrive, generalised lymphadenopathy, hepatomegaly, splenomegaly, enlarged parotid glands, persistent or recurrent diarrhoea
Subclass B. Progressive neurologic disease
Loss of developmental milestones or intellectual ability, impaired brain growth, or progressive symmetrical motor deficits
Subclass C. Lymphoid interstitial pneumonitis (LIP)
Subclass D. Secondary infectious diseases
Category D-1. Opportunistic infections in CDC case definition
Bacterial: mycobacterial infection (noncutaneous, extrapulmonary, or disseminated); nocardiosis
Fungal: candidiasis (oesophageal, bronchial, or pulmonary), coccidiomycosis, disseminated histoplasmosis, extrapulmonary cryptococcosis
Parasitic: P. carinii pneumonia, disseminated toxoplasmosis with onset ≥1 month of age, chronic cryptosporidiosis or isosporiasis, extraintestinal strongyloidiasis
Viral: cytomegalovirus disease (onset ≥1 month of age), chronic mucocutaneous/disseminated herpes (onset ≥1 month age), progressive multifocal leukoencephalopathy
Category D-2. Unexplained, recurrent, serious bacterial infections (two or more in 2 years), Sepsis, meningitis, pneumonia, abscess of an internal organ, bone/joint infections
Category D-3. Other infectious diseases including persistent oral candidiasis, recurrent herpes stomatitis (at least two episodes in 1 year), multidermatomal or disseminated herpes zoster
Subclass E. Secondary cancers
Category E-1: Kaposi's sarcoma, B cell non-Hodgkin's lymphoma, or primary lymphoma of brain
Category E-2. Other malignancies possibly associated with HIV

Subclass F. Other conditions possibly caused by HIV: including hepatitis, cardiopathy, nephropathy, hematologic disorders, dermatologic diseases.

APPENDIX 4: Phylogenetic tree showing the placement of previously documented sequences

A reconstructed maximum likelihood tree of a partial length of p17 *gag* gene phylogeny showing the distribution of 83 representatives *gag* subtypes. The phylogenetic tree is based on an unambiguously aligned 225bp region of the *gag* gene (position: 907-1131 in the HIV_{HXB2} genome). The scale bar corresponds to 5 changes per 100 nucleotide positions. The reference sequences were as follows: **Subtypes A**: 1.U455, 2.IBNG, 3.VI59, 4.VI310, 5.VI57, 6.K112, 7.K88, 8.K29, 9.K7, 10.K98, 11.K89, 12.VI32, 13.VI415, 14.CI4, 15.LBV23, 16.TN2431, 17.TN245, 18.TN240, 19.CI20, 20.CI59, 21.LBV2310, 22.CI51, 23.IC144, 24.DJ258, 25.TN238, 26.G266; **Subtype: B**: 27.SF2, 28.BZ167, 29.PH153, 30.PH136, 31.TB132, 32.BZ190, 33.LAI, 4.HXB2R, 35.MN, 36.JH31, 37.JRCSF, 38.JRFL, 39.OYI, 40. NY5CG, 41.NL43, 42.CDC41, 43.HAN, 44.CAM1, 45.RF, 46.D31, 47.UG280, 48.YU2, 49.BCSG3C, 50.P896, 51.3202A12, 52.3202A21, 53.GAG46, 54.MANC, 55.GAG314, 56.GAG22, 57.GAG15, 58.WEAU160; **Subtype C**: 59.UG268, 60.SM145, 61.ZAM18, 62.ZAM19, 63.ZAM20, 64.DJ259, 65.VI313; **Subtype D:** 66.ELI, 67.Z2Z6, 68.NDK, 69.VI205, 70.G109, 71.K31, 72.UG274, 73.UG270, 74.SE365, 75.VI203; **Subtype F:** 76.VI174, 77.VI69, 78.BZ163B; **Subtype G:** 79.LBV217, 80.VI191; **Subtype H:** 82.VI525, 83.VI557.

APPENDIX 4: Phylogenetic tree showing the placement of previously documented sequences



APPENDIX 5: Evolutionary distance between sequences collected in Kimpese, Democratic Republic of Congo.

The distance matrix was obtained using the two-parameter Kimura method contained in the MEGA software (Kumar *et al.,* 1993). The distance between two sequences is situated at the intersection point inside the rectangle of the main diagram. The sequences are represented by numbers (1-62) shown in **bold** on the far left column and the far bottom row and were as follows: 1. A.Sq3M, 2. A.Sq6M, 3. A.Sq8M, 4. A.Sq9M, 5. A.Sq11M, 6. A.Sq12M, 7. A.Sq13M, 8. A.Sq27M, 9. A.Sq29M, 10. A.Sq31M, 11. A.Sq32M, 12. A.Sq34M, 13. A.Sq36M, 14. A.Sq37M, 15. A.Sq38M, 16. A.Sq39M, 17. A.Sq40M, 18. A.Sq43M, 19. A.Sq46M, 20. A.Sq48M, 21. A.Sq49M, 22. A.Sq51M, 23. A.Sq52M, 24. A.Sq54M, 25. A.Sq55M, 26. A.Ped2, 27. A.Ped10, 28. A.Ped12, 29. C.Sq18M, 30. C.Ped16, 31. D.Sq4M, 32. D.Sq20M, 33. D.Sq21M, 34. D.Sq23M, 35. D.Sq41M, 36. D.Sq45M, 37. F.Sq19M, 38. F.Sq53M, 39. G.Sq7M, 40. G.Sq17M, 41. G.Sq47M, 42. G.Sq56M, 43. H.Sq5M, 44. H.Sq10M, 45. H.Sq14M, 46. H.Sq15M, 47. H.Sq16M, 48. H.Sq22M, 49. H.Sq25M, 50. H.Sq26M, 51. H.Sq28M, 52. H.Sq30M, 53. H.Sq33M, 54. H.Sq35M, 55. H.Sq50M, 56. H.Ped13, 57. Q.Sq1M, 58. Q.Sq2M, 59. Q.Sq24M, 60. Ped7, 61. Sq42M, 62. Sq57M.

1
2	13.4
3	14.9	15.0
4	16.2	11.2	15.0
5	20.1	14.9	15.4	17.1
6	16.0	15.5	18.3	9.6	16.4
7	15.6	9.1	10.7	10.7	13.2	13.8
8	18.3	14.3	16.6	15.5	17.6	21.9	13.8
9	17.7	14.9	9.0	15.5	15.3	18.2	11.6	17.0
10	16.2	13.9	10.1	12.9	13.7	14.4	8.1	16.8	13.2
11	19.2	15.1	12.2	14.6	14.2	15.6	12.2	17.3	11.1	11.7
12	16.5	10.1	14.3	15.5	16.4	16.5	11.1	17.7	14.2	14.3	16.6
13	15.7	13.9	9.6	11.3	13.8	14.4	10.7	14.4	11.1	8.7	12.3	12.7
14	17.3	12.0	12.0	13.2	13.6	13.7	12.1	18.9	10.9	14.4	10.0	15.3	13.8
15	17.8	10.1	11.7	11.2	10.0	12.7	8.6	13.8	10.6	10.1	10.6	12.7	9.1	9.9
16	16.5	13.7	11.1	11.1	11.5	13.7	8.0	14.2	12.1	10.6	13.7	14.2	10.1	12.0	8.5
17	18.9	17.8	12.7	16.7	15.3	18.2	11.1	17.3	14.8	9.0	14.9	17.7	12.2	14.2	10.6	12.1
18	17.1	15.9	11.9	16.6	13.6	16.4	13.0	19.9	12.5	14.7	13.7	17.0	15.9	4.9	14.2	14.1	15.2
19	11.8	9.0	14.0	9.0	12.7	13.4	6.3	16.2	15.1	10.1	14.1	13.4	13.5	13.7	10.6	10.5	13.3	14.4
20	25.0	20.9	20.3	22.3	19.4	22.8	13.3	23.9	18.8	17.9	21.5	20.7	19.8	20.3	15.6	16.7	18.9	21.4	14.6
21	13.2	10.6	11.1	11.2	13.1	12.7	8.5	13.7	10.5	11.7	12.2	11.1	10.1	12.0	8.5	10.0	13.7	15.2	11.2	16.6
22	14.9	10.1	11.1	12.3	12.6	12.8	8.0	14.9	10.0	8.6	9.6	10.5	9.1	10.9	7.5	9.5	13.2	14.7	10.1	18.4	6.5
23	13.2	11.7	10.0	11.7	14.7	14.3	7.1	16.5	12.6	11.7	13.8	13.7	11.2	13.1	7.5	10.0	13.2	15.2	10.5	18.0	10.0	10.1
24	19.9	14.3	14.2	15.4	13.6	15.8	10.0	18.1	14.2	12.1	15.3	17.5	11.1	14.6	9.5	10.0	11.6	18.0	14.3	17.5	11.5	11.5	12.1
25	18.4	10.4	12.5	10.4	13.5	13.0	5.7	15.8	13.0	10.3	12.0	11.9	11.5	11.2	8.8	8.7	13.0	12.8	7.6	15.4	8.7	8.2	10.9	11.3
26	14.9	9.0	11.1	12.2	12.1	13.2	8.0	14.2	11.0	10.1	11.1	11.6	9.6	9.3	7.5	10.0	10.6	11.9	9.0	17.1	8.0	6.5	12.1	10.5	8.2
27	16.7	14.9	10.5	14.5	15.3	15.4	11.6	17.1	10.0	12.3	11.7	16.5	12.3	13.2	11.1	13.2	15.5	13.6	14.0	20.1	9.0	10.6	14.3	12.6	13.0	8.0
28	14.4	8.6	11.6	6.1	12.6	11.1	5.6	12.6	11.6	9.6	12.3	11.6	9.7	10.9	8.0	8.0	12.1	13.0	5.3	16.6	8.0	8.0	8.0	11.0	7.7	9.0	12.2
29	23.2	21.4	18.8	20.1	23.4	20.4	17.0	25.0	23.5	17.0	20.6	22.4	17.7	26.5	17.5	20.5	18.2	23.6	18.6	25.6	17.6	20.0	18.7	21.0	18.7	18.8	21.7	18.8
30	18.8	20.7	18.8	18.3	21.0	18.7	18.8	24.3	21.8	16.0	21.3	23.5	15.5	23.9	17.1	16.5	19.9	22.9	17.4	25.0	14.3	17.0	17.7	18.8	19.2	18.2	20.6	18.2	14.7
31	19.3	20.6	17.0	18.2	18.6	19.8	16.4	21.8	21.0	15.4	19.4	22.3	15.4	22.5	14.8	16.5	17.6	22.3	16.7	21.2	14.7	16.4	16.4	15.9	18.7	15.3	18.2	17.6	14.2	12.6
32	26.3	21.8	17.6	22.4	21.1	26.2	18.2	25.0	21.6	16.4	21.8	23.0	19.4	22.7	19.3	20.0	19.3	21.8	20.3	27.0	19.3	19.9	21.7	21.1	19.3	19.4	21.1	21.1	18.1	17.0	10.5
33	22.0	19.5	18.3	19.5	18.2	19.3	16.6	23.3	21.2	16.0	19.6	19.4	16.6	21.5	15.4	19.0	20.2	23.1	18.6	22.6	17.7	18.9	18.2	17.7	16.5	17.1	21.3	20.1	15.9	15.9	8.0	8.0
34	23.0	19.2	17.4	19.2	17.9	19.7	16.3	23.1	20.3	15.1	18.6	19.7	15.7	20.0	15.0	18.7	20.4	21.6	18.3	22.3	18.0	18.0	17.9	17.9	16.6	16.3	20.3	19.8	16.1	15.0	8.1	6.6	0.9
35	28.7	29.4	24.1	28.1	28.4	27.8	25.3	27.5	28.4	22.8	30.3	31.3	23.6	31.2	24.8	27.3	26.7	30.9	26.8	31.5	23.4	26.7	27.3	24.6	28.2	25.4	26.6	26.6	24.7	20.4	15.4	15.3	14.2	13.9
36	23.8	21.9	23.0	20.0	21.6	22.3	21.1	25.1	24.9	17.5	21.3	23.7	18.8	25.3	17.5	22.3	23.0	24.5	19.3	23.7	21.1	21.2	21.6	19.8	19.3	19.4	21.8	21.2	18.1	17.4	10.8	13.5	9.2	9.3	19.2
37	31.9	25.4	29.7	24.8	30.8	28.8	26.1	33.5	31.3	24.6	30.7	29.4	24.7	29.5	23.3	28.8	24.6	33.3	26.2	34.9	26.8	26.1	28.1	25.9	25.0	24.2	32.0	27.4	26.1	26.6	21.2	23.8	20.1	20.4	26.3	21.9
38	26.4	22.4	24.2	23.1	22.9	25.6	21.7	26.3	23.6	20.6	26.4	25.5	21.9	25.4	18.9	22.5	20.5	27.2	20.5	27.6	20.0	21.2	22.3	22.2	21.8	18.3	23.6	22.4	22.5	22.5	15.3	17.0	14.9	14.0	19.9	15.8	14.5
39	23.1	20.4	24.9	21.3	25.0	21.4	17.4	28.2	24.6	18.0	24.2	22.0	21.4	24.3	19.7	22.0	22.9	25.3	18.5	25.6	19.7	19.7	20.9	21.9	17.8	17.9	19.9	21.2	23.3	21.5	22.1	26.0	20.1	19.8	29.5	24.0	32.0	27.8
40	22.9	17.8	20.2	19.2	22.3	19.6	15.5	22.5	20.6	14.9	19.6	18.8	16.2	21.4	15.4	18.8	20.1	22.4	17.0	22.6	14.9	15.5	18.3	21.7	16.4	16.0	18.4	17.4	19.5	17.2	20.1	21.8	12.2	17.4	27.4	22.5	28.9	27.1	7.1
41	24.1	22.9	25.5	21.0	27.6	21.8	19.1	30.0	25.8	20.9	26.7	24.6	21.7	24.1	20.8	23.2	24.1	24.4	18.9	24.6	20.9	24.0	20.2	21.1	21.5	18.4	19.6	20.3	23.2	23.3	22.6	28.9	22.6	22.3	30.8	24.4	32.9	29.8	8.1	9.1
42	28.8	24.0	28.1	24.3	28.3	24.4	22.1	32.8	28.4	24.0	29.3	26.4	23.0	26.0	23.9	26.6	27.4	27.8	21.9	25.8	20.2	25.9	22.7	22.1	23.3	21.4	22.0	22.1	25.9	21.8	25.3	30.3	26.0	25.7	33.1	27.9	37.1	33.6	11.2	11.2	4.1
43	23.4	22.1	15.5	19.0	22.9	22.6	17.8	20.1	21.7	17.8	22.6	25.0	17.8	22.6	17.6	18.2	17.6	23.0	16.9	22.6	18.9	20.1	17.0	21.0	20.7	20.1	23.9	16.0	17.6	19.4	19.3	22.4	21.2	20.9	24.7	21.8	27.2	23.6	25.8	20.3	26.2	29.5
44	19.0	18.4	14.9	17.8	16.5	19.5	16.6	17.8	20.6	15.0	18.4	22.5	15.0	17.7	14.3	16.0	14.9	18.7	15.7	21.9	17.1	16.6	15.4	18.1	18.2	16.0	20.8	16.0	14.8	14.8	14.2	18.8	16.5	16.2	24.7	18.1	25.2	21.8	25.2	20.4	24.9	27.5	12.8
45	18.9	18.4	14.3	16.6	18.1	20.6	16.0	19.4	18.8	17.7	17.7	20.0	16.6	18.3	16.0	16.4	18.8	18.1	16.3	25.8	17.1	18.9	15.9	20.4	17.0	17.1	18.3	15.4	17.0	18.2	18.1	21.7	20.6	20.3	30.7	22.5	27.4	25.6	28.5	22.8	27.6	31.3	13.8	11.7
46	19.6	16.7	13.7	16.1	17.0	18.9	15.5	16.1	19.4	14.9	19.0	19.4	14.9	18.9	12.1	15.8	16.6	19.3	14.0	22.5	14.9	15.5	14.2	18.6	15.9	14.9	18.3	13.8	15.3	17.0	15.3	22.3	19.9	19.6	27.3	19.3	25.9	22.5	24.5	19.1	25.6	28.9	10.6	8.6	6.5
47	21.8	18.3	20.5	19.4	21.6	21.1	21.8	21.0	25.4	18.7	24.9	24.8	20.5	23.2	18.7	22.8	20.4	25.0	19.9	29.1	21.1	22.4	20.4	22.7	20.6	21.8	25.5	19.3	21.0	21.6	19.7	24.6	22.1	21.9	30.3	21.6	26.3	25.2	28.2	25.0	30.8	34.4	18.8	12.6	12.1	11.0
48	19.0	16.6	15.4	14.4	18.2	15.4	14.4	17.2	18.2	13.8	15.4	18.8	11.7	15.9	11.0	14.2	13.7	19.3	14.5	20.1	12.7	14.3	13.7	16.4	14.1	14.8	18.3	12.7	15.3	17.0	14.7	20.5	17.7	17.4	26.7	19.2	23.8	23.0	23.0	17.3	24.2	27.5	14.3	11.6	14.3	10.0	14.8
49	22.0	16.6	16.0	19.8	18.1	23.0	15.4	18.8	21.7	17.7	18.7	19.3	16.6	22.6	17.0	19.3	17.7	21.7	16.8	25.8	16.4	17.0	16.5	19.2	15.2	15.4	20.0	16.5	17.0	19.9	17.5	19.3	18.7	18.4	29.8	22.9	29.3	26.7	22.6	19.1	27.4	27.8	15.5	13.3	11.1	10.1	14.8	16.0
50	19.8	17.3	13.3	17.4	17.6	20.2	14.4	16.2	17.7	16.2	17.2	20.7	15.0	19.0	12.7	16.5	17.9	20.6	15.9	22.7	14.9	15.6	14.4	18.2	17.1	14.9	16.6	15.0	16.0	18.4	17.1	21.2	20.1	19.8	27.6	20.6	28.0	22.5	23.4	19.3	25.8	29.2	12.3	10.8	8.6	5.6	15.5	12.8	11.8
51	19.7	16.7	13.8	16.2	17.6	18.9	14.4	16.6	19.4	14.9	18.4	19.4	16.1	19.0	13.2	17.0	14.3	18.7	13.5	22.6	14.9	15.5	14.8	16.9	15.3	14.3	17.2	13.8	15.4	18.3	16.4	23.0	20.0	19.7	28.7	20.7	24.6	21.8	24.6	19.8	25.7	29.0	10.1	8.1	8.1	5.1	12.1	11.7	10.6	9.2
52	21.4	17.2	20.0	17.1	21.8	19.9	17.6	19.4	23.6	20.7	21.3	23.1	19.5	20.0	16.5	21.0	20.6	23.8	17.8	29.1	17.6	18.2	17.5	22.7	18.1	17.6	22.5	17.1	22.3	22.9	21.5	29.3	25.3	25.1	35.7	25.5	29.1	25.4	28.9	23.9	28.0	32.1	16.5	14.9	13.2	10.1	16.5	17.0	16.0	13.4	10.6
53	21.8	20.5	17.5	19.9	21.5	22.8	18.7	22.4	22.8	15.8	18.8	24.7	16.5	21.1	15.8	19.8	18.6	25.4	18.5	25.3	19.8	18.7	17.5	20.9	19.1	18.6	22.3	18.6	18.0	20.4	19.2	24.6	21.5	21.3	33.2	22.8	28.4	27.9	27.3	23.1	29.1	31.1	17.6	12.1	14.7	12.1	18.0	13.6	15.9	14.4	12.8	16.9
54	21.6	16.1	18.9	16.1	20.7	20.1	16.6	18.4	22.5	19.6	20.2	21.4	18.4	18.8	15.5	19.9	19.5	22.6	16.5	27.9	17.8	18.4	16.5	21.6	17.0	17.7	22.7	16.0	21.2	23.1	21.7	28.1	24.2	23.9	34.4	24.4	27.9	24.3	27.7	22.7	26.8	30.9	15.5	13.9	12.2	9.1	15.4	16.0	15.0	12.4	9.6	0.9	15.9
55	17.8	17.2	16.0	20.0	16.8	23.4	17.2	19.7	21.8	17.9	22.0	19.2	17.4	16.8	15.8	15.9	17.1	19.7	19.2	23.7	15.8	19.2	15.8	17.7	19.1	19.9	21.2	16.5	18.3	16.6	17.7	21.2	19.2	18.9	25.8	19.0	24.8	22.5	21.6	17.9	21.5	20.8	12.7	9.2	14.0	11.5	16.5	14.5	16.0	14.8	11.5	15.1	17.0	14.0
56	22.4	19.4	23.8	21.8	26.1	26.1	21.7	23.0	27.3	25.0	27.8	26.1	25.0	24.4	21.8	24.6	24.9	26.4	19.4	31.1	22.5	23.6	21.6	25.2	22.5	23.6	26.9	20.5	23.5	26.2	24.6	31.3	28.6	28.4	34.7	26.4	31.4	24.8	32.0	30.0	33.6	37.7	18.8	17.1	15.4	12.7	16.0	21.2	20.6	16.2	13.2	10.0	21.0	9.0	13.3
57	17.1	14.3	17.7	12.2	17.6	12.7	13.2	19.3	17.0	13.8	16.1	15.9	12.7	14.2	11.6	14.3	17.0	16.4	12.8	20.8	12.7	13.8	14.8	15.3	11.4	13.7	18.3	11.6	17.6	18.2	14.7	21.1	16.5	16.2	23.4	16.8	19.4	19.9	19.5	17.7	18.2	21.3	18.2	17.0	17.6	15.3	20.4	12.6	19.8	16.5	17.7	19.9	20.9	18.8	16.3	23.4
58	19.5	15.4	18.9	13.8	18.7	13.8	12.1	19.3	18.1	14.9	17.2	18.2	14.9	15.3	12.6	14.3	17.6	18.8	12.7	19.5	13.8	13.8	15.4	15.3	11.9	14.2	18.2	12.6	19.3	21.2	17.8	23.7	19.5	18.6	26.7	21.0	23.9	22.9	20.7	18.4	19.4	21.9	20.6	20.6	21.1	18.7	25.3	14.8	22.3	17.7	18.8	22.3	22.7	21.2	19.6	25.9	4.1
59	19.0	16.0	18.4	15.1	18.2	14.9	11.6	20.1	17.6	13.3	17.9	18.2	15.6	17.2	13.2	13.8	17.7	19.5	12.2	19.0	13.3	12.7	15.4	14.2	12.5	13.7	17.7	13.2	18.8	20.1	15.9	21.9	17.8	16.8	23.5	20.5	24.8	21.1	19.0	17.8	20.1	23.3	20.1	19.4	22.5	18.8	24.1	16.1	21.1	17.8	17.7	25.0	24.1	23.8	19.7	27.4	5.6	4.6
60	18.1	13.4	20.4	15.7	20.3	19.7	14.5	19.7	20.2	16.3	21.1	19.6	16.9	18.5	15.0	17.9	19.9	19.8	13.0	18.7	17.4	18.0	15.0	16.1	16.2	14.1	17.5	15.1	24.1	24.0	17.4	25.5	20.3	19.4	28.5	18.3	29.1	22.8	18.3	20.5	18.0	21.0	24.2	18.5	24.0	19.1	21.9	18.5	22.8	19.2	20.4	20.8	23.1	19.7	17.7	22.6	15.5	16.1	16.8
61	22.4	19.9	18.6	18.1	20.3	17.4	16.3	23.6	21.5	14.5	20.4	19.8	15.7	21.8	15.7	16.9	21.0	24.1	16.5	19.9	15.2	18.6	15.7	19.6	16.1	19.1	22.3	16.9	15.6	16.2	14.4	19.0	13.9	14.1	19.5	19.5	24.9	21.7	23.2	17.4	21.7	23.0	16.8	15.1	19.0	16.2	21.3	15.1	18.5	17.5	17.4	21.5	19.6	20.4	17.4	22.7	16.2	18.6	17.5	20.6
62	22.0	18.3	18.3	14.4	23.5	16.6	17.8	23.1	22.4	14.9	19.0	21.2	14.9	20.3	16.6	17.7	17.6	22.5	18.1	21.5	15.0	18.3	17.8	21.7	17.6	18.2	21.5	16.0	18.2	13.7	17.0	19.8	16.4	17.3	24.7	19.1	25.2	25.0	25.0	17.9	24.2	25.5	17.7	17.0	19.9	17.0	21.6	13.8	20.5	18.4	18.3	22.9	20.4	21.8	21.8	26.2	15.5	17.8	18.6	21.0	13.0
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62

APPENDIX 6: Distance between individual sequences from rural Democratic Republic of Congo and previously established subtypes (Myers *et al.*, 1995).

(Distance estimated by the Kimura two-parameter model).

		REFERENCE SUBTYPES						
SAMPLES		**A**	**B**	**C**	**D**	**F**	**G**	**H**
A. EPIDEMIOLOGICALLY UNLINKED								
SUBTYPE A								
1	A.Sq3M	**15.91**	22.6	24.4	23.1	26.8	23.2	21.0
2	A.Sq6M	**12.88**	21.7	20.8	21.9	25.2	22.7	19.0
3	A.Sq8M	**12.55**	20.0	24.2	20.3	24.0	23.2	17.4
4	A.Sq9m	**13.72**	20.5	22.4	20.2	24.6	22.4	18.6
5	A.Sq11M	**15.44**	19.6	22.8	21.6	26.4	26.1	19.7
6	A.Sq12M	**15.15**	19.5	20.3	21.9	26.8	21.7	20.8
7	A.Sq13M	**7.66**	17.5	19.7	17.8	23.2	20.2	17.4
8	A.Sq27M	**17.85**	23.4	26.1	23.1	29.2	29.2	21.5
9	A.Sq29M	**13.53**	21.7	23.8	24.1	26.0	22.4	21.5
10	A.Sq31M	**12.20**	17.1	20.7	16.8	19.8	20.8	17.3
11	A.Sq32M	**13.35**	20.0	20.9	21.5	27.5	23.8	22.5
12	A.Sq34M	**14.28**	23.5	23.5	23.4	27.3	24.1	21.8
13	A.Sq36M	**11.81**	17.9	19.3	18.3	20.6	22.7	17.7
14	A.Sq37M	**12.36**	22.2	24.4	23.9	27.9	23.5	22.0
15	A.Sq38M	**10.93**	15.7	19.1	17.7	21.9	21.0	16.2
16	A.Sq39M	**11.27**	17.1	23.0	19.7	23.8	22.2	19.6
17	A.Sq40M	**13.56**	20.1	22.5	19.8	22.3	23.7	17.0
18	A.Sq43M	**15.05**	24.6	23.6	24.2	29.1	23.1	21.7
19	A.Sq46M	**11.22**	18.6	20.4	19.4	23.4	20.0	17.1
20	A.Sq48M	**16.74**	21.3	25.0	23.1	30.8	27.3	22.2
21	A.Sq49M	**11.03**	17.7	19.7	18.6	22.6	17.5	16.5
22	A.Sq51M	**10.13**	18.5	22.6	20.5	22.2	20.8	17.9
23	A.Sq52M	**9.79**	20.1	21.6	19.9	23.5	22.6	18.2
24	A.Sq54M	**12.45**	17.7	19.5	17.8	23.7	23.2	18.9
25	A.Sq55M	**10.57**	17.7	20.1	19.8	22.7	22.0	16.7
26	A.Ped2	**10.91**	17.0	19.9	19.0	21.1	20.8	17.8
27	A.Ped10	**13.61**	21.4	23.5	21.4	27.7	20.4	20.9
28	A.Ped12	**9.39**	18.9	20.7	19.5	24.8	20.3	17.7

SUBTYPE C

29	C.Sq18M	21.45	19.6	**14.0**	17.4	21.2	21.0	16.9

SUBTYPE D

30	D.Sq4M	19.03	9.8	14.5	**7.5**	15.0	22.9	16.4
31	D.Sq20M	23.01	14.1	16.9	**10.8**	16.5	25.8	21.3
32	D.Sq21M	21.55	10.7	13.4	**8.7**	15.9	24.9	18.4
33	D.Sq23M	20.96	10.0	13.0	**8.3**	14.8	24.0	18.4
34	D.Sq41M	28.60	17.0	20.2	**14.2**	20.3	29.2	26.5
35	D.Sq45M	23.50	15.3	18.1	**12.4**	17.5	28.0	19.8

SUBTYPE F

36	F.Sq19M	28.95	20.0	24.6	21.6	**14.1**	33.1	23.3
37	F.Sq53M	24.30	13.2	22.6	16.6	**7.3**	26.8	21.1

SUBTYPE G

38	G.Sq7M	22.29	24.3	21.0	20.8	24.7	**11.7**	25.1
39	G.Sq17M	19.05	21.2	20.0	20.0	24.6	**11.7**	22.6
40	G.Sq47M	21.04	25.7	22.6	23.2	28.1	**12.2**	26.4
41	G.Sq56M	23.56	28.5	25.7	25.9	31.3	**14.9**	30.4

SUBTYPE H

42	H.Sq5M	20.38	21.1	21.8	19.7	21.5	23.4	**13.2**
43	H.Sq10M	18.56	17.4	18.2	17.2	20.8	25.5	**11.1**
44	H.Sq14M1	18.52	21.9	21.0	21.7	24.9	25.8	**11.4**
45	H.Sq15M	18.14	19.6	20.2	19.1	22.1	24.2	**10.1**
46	H.Sq16M	25.09	24.3	24.7	22.7	26.9	30.2	**13.7**
47	H.Sq22M	15.97	18.1	17.7	17.4	22.1	22.8	**14.8**
48	H.Sq25M	20.51	22.2	19.9	20.5	25.2	26.5	**13.8**
49	H.Sq26M	17.79	19.1	19.9	19.8	22.7	23.3	**13.1**
50	H.Sq28M	17.12	20.7	20.9	20.3	21.3	22.3	**9.3**
51	H.Sq30M	19.45	24.8	27.4	26.8	26.8	27.9	**13.8**
52	H.Sq33M	21.02	22.8	25.3	23.1	27.2	29.0	**16.4**
53	H.Sq35M	18.68	24.3	26.3	25.7	26.1	27.5	**13.3**
54	H.Sq50M	18.85	20.3	23.3	19.3	22.8	21.1	**13.6**
55	H.Ped13	23.56	26.5	28.4	27.2	28.5	30.3	**16.5**

B. EPIDEMIOLOGICALLY LINKED CHILDREN

(associated-maternal sequences are shown between brakets)

		A	B	C	D	F	G	H
56	D.Ped3el (D.Sq4M)	19.03	9.8	14.5	**7.5**	15.0	22.9	16.4
57	A.Ped4el (A.Sq9M)	**11.74**	18.6	19.3	18.8	23.1	20.3	16.6
58	H.Ped5el (H.Sq16M)	23.35	24.8	25.0	23.8	27.2	28.5	**12.1**
59	A.Ped6el (A.Sq32M)	**13.56**	20.8	21.9	22.1	28.5	24.6	23.0
60	D.Ped9el (D.Sq45M)	21.19	12.8	16.0	**10.4**	16.0	25.4	17.9
61	A.Ped11el (A.Sq52M)	**10.39**	20.2	21.2	19.9	24.5	24.1	18.7
62	A.Ped14el (A.Sq37M)	**14.88**	23.7	26.2	24.4	30.1	24.6	22.9
63	A.Ped15el (A.Sq27M)	**14.16**	19.4	22.0	20.0	26.0	24.2	16.6

APPENDIX 7: PUBLICATIONS

ELSEVIER

Clinical and Diagnostic Virology 5 (1996) 3–12

Clinical and Diagnostic Virology

How valuable are IgA and IgM anti-HIV tests for the diagnosis of mother–child transmission of HIV in an African setting?

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Abstract

Background: Babies born to HIV-infected mothers retain anti-HIV of maternal origin until 15–18 months of age. Because of this, HIV proviral DNA and p24 antigen measurements have become the methods of choice for timely diagnosis of HIV infection in infancy. They are, however, too expensive for widespread use in the developing world.

Objective: To evaluate a simple, inexpensive serological method for diagnosing mother–child transmission of HIV, in an African population, which takes account of the effects of placental transfer of maternal antibody and continued exposure to HIV through breast-feeding.

Study Design: Plasma specimens for a prospective study of mother-to-infant transmission of HIV in rural Zaire were collected at birth, 3, 6, 9, 12, 18 and 24 months from 21 infected infants (PP group), 21 uninfected infants (PN group) born to seropositive mothers and 21 control infants (NN group) born to uninfected mothers. The specimens were retrospectively tested for IgG, IgM and IgA anti-HIV by immunoglobulin class-specific capture EIAs, and by a commercial anti-HIV EIA.

Results: In neonatal specimens, IgA and IgM anti-HIV were present, respectively, in 13 of 14 (97%) and 8 of 14 (57%) of the PP group and in 6 of 11 (55%) and 2 of 11 (18%) of the PN group. Later, at 3 months and older, IgA and IgM anti-HIV were only detected in the PP group. They peaked at 18 months (93%) and 24 months (67%) respectively. Of the 21 PP group children, 8 (38%) were transiently IgG anti-HIV-negative in the first year, indicating that infection had probably taken place after birth; four of the 8 had no detectable IgA anti-HIV during the first year. None of the specimens collected from the NN group babies were reactive for IgA, IgM or IgG anti-HIV.

Conclusions: IgA and IgM anti-HIV may be passively transferred across the placenta. Where breast-feeding is prevalent, about half of the transmissions may occur after birth, thus delaying the diagnosis of mother–child transmission. Nevertheless, this simple, cheap IgA anti-HIV, EIA identified 65% of transmissions by 9 months of age, and 93% at 18 months of age. It is a more useful marker than IgM anti-HIV, and gave a much more rapid answer than did tests for IgG anti-HIV seroreversion.

Keywords: HIV vertical transmission; IgA anti-HIV; IgM anti-HIV; Breast-feeding; HIV diagnosis

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1. Introduction

The timing and mechanism of mother–child transmission of HIV are not clear. There is evidence for infection in utero (Courgnaud et al., 1991; Mano and Chermann, 1991), just prior to or during delivery (Ehrnst et al., 1991; Krivine et al., 1992) and by breast-feeding (Stiehem and Vink, 1991; Van de Perre et al., 1992; Datta et al., 1992; Ziegler, 1993). In the developing world, breast-feeding is recommended by the WHO (World Health Organisation, 1992), but it nevertheless increases the risk of HIV transmission from mother to child (Dunn et al., 1992). A number of laboratory tests, reviewed by Sison and Campos, 1992, may detect transmission which has occurred before or during the neonatal period, including polymerase chain reaction (PCR), HIV culture, and in vitro antibody and in vitro antigen production. However, most are expensive, technically demanding and unsuitable for use in laboratories in developing countries. A possible exception is the detection of HIV p24 antigen. While Andiman et al., 1992 found only 20% of samples collected from infected babies in the first month of life to be HIV antigen-positive, Miles et al., 1993, employing a preliminary acid dissociation step (Ascher et al., 1992), reported p24 antigen in 80% of HIV-infected neonates. However, when Fauvel et al., 1993 further assessed this modification they concluded that, although a small improvement in sensitivity was possible in the first two months of life, acid treatment of specimens made confirmation of reactive samples essential. This confirmation increases the volume of specimen needed and the cost.

The presence of IgA anti-HIV in at-risk infants has been shown to be predictive of HIV infection in the child. Immunoblots have been employed to identify IgA and IgM anti-HIV in samples collected at and following birth, but removal of competing IgG from the samples by absorption with protein G prior to testing was necessary (Wieblen et al., 1990b). Many of the published data have been obtained using Western Blot or immunoblots which incorporate recombinant HIV antigens, and the reported findings are similar (Wieblen et al., 1990a; Martin et al., 1991; Landesmann et al., 1991; Quinn et al., 1991; Re et al., 1992). In particular, when samples collected between birth and three months have been tested, the sensitivity for IgA detection has been low (range 16–18%). In one study, however, IgA anti-HIV was detected in all ten HIV-infected children tested at birth and in a further two infected children tested at 2 months of age, but not in any of five uninfected children at risk (Portincasa et al., 1992). This study assumes that maternal IgA anti-HIV does not cross the placenta in which case transmission must have occurred, in all cases, long enough before birth for the babies to mount an IgA response. This conflicts with the current consensus that a substantial proportion of transmissions occur perinatally (Ehrnst et al., 1991; Krivine et al., 1992). In another study, however, IgA anti-HIV was detected in similar proportions of uninfected (12/37, 32%) and infected (4/12, 33%) neonates born to HIV-infected mothers (Connell et al., 1992). Its presence was associated with strong IgA anti-HIV reactivity in the mothers' sera, suggesting that transfer of maternal IgA had occurred. With the exception of one study (Portincasa et al., 1992), these studies indicate that, in the infected infants, IgA anti-HIV production commences between 3 and 6 months (22–66% IgA anti-HIV-positive), and that most are producing IgA anti-HIV by 9 months (range 54–99%). IgA anti-HIV has rarely been found in uninfected infants of 3 months and older.

We evaluated the suitability of immunoglobulin class-specific antibody capture EIAs for IgA, and also for IgM and IgG anti-HIV, for the diagnosis of HIV infection in Zairian children at risk of infection from their mothers. Our assays are simple, cheap and require only small volumes of untreated serum or plasma specimens. They can be readily used by laboratory services in developing countries where, because of breast-feeding, children continue to be at risk of HIV infection into the second year of life and beyond (Datta et al., 1992).

2. Patients and methods

2.1. Patients

In a longitudinal prospective study of mother–child transmission of HIV in rural Zaire, 21 children born to seropositive mothers were diagnosed as infected (PP group) by the presence of IgG anti-HIV at 18 months of age, the detection of p24 antigen, AIDS defining illness before 18 months of age or HIV-related death (Centers for Disease Control, 1987). There were two matched control groups of children: 21 who did not develop the infection during the period of follow up but whose mothers were HIV-positive (PN group), and 21 children born to anti-HIV-negative mothers (NN group). Signed consent was obtained from all mothers prior to admission to the study. Plasma samples were collected from the three groups of children in the neonatal period (cord blood and/or peripheral blood) and at 3, 6, 9, 12, 18 and 24 months of age. For some children, both cord blood and peripheral blood specimens collected in the neonatal period (usually < 14 days) were available. In these cases the findings were identical and were thus treated as if they were a single specimen. Because no alternative was available, the mothers were not advised to stop breast-feeding.

2.2. Methods

Samples from the PP group ($n = 123$), PN group ($n = 128$) and the NN group ($n = 135$) were tested for IgG anti-HIV by an IgG antibody-capture enzyme linked immunosorbent assay (Connell et al., 1993), Wellcozyme HIV $1 + 2$ GACELISA (Code VK61, Murex Diagnostics, Dartford, UK). Specimens were also investigated for the presence of HIV-specific IgA (AACELISA) and IgM (MACELISA) by similar class-specific antibody capture ELISAs. In AACELISA, anti-human IgA (Dako code No. AO92, Dako, High Wycombe, UK), and in MACELISA anti-human IgM (Dako code No. A425), replaced anti-human IgG on the solid phase (Connell et al., 1992). The procedures were similar to that of GACELISA except that human sera with known IgA and IgM anti-HIV reactivity were included as positive controls. Each sample was originally tested by the Abbott recombinant HIV1/HIV2 EIA (No. 1a80-24, Abbott Diagnostics, Maidenhead, UK).

3. Results

3.1. PP group (21 infected children born to HIV-positive mothers)

IgA anti-HIV: Of the 14 neonatal samples 13 (93%) contained IgA anti-HIV, but this proportion declined to 2 of 17 (12%) at 3 months (Fig. 1 and Table 1). After 3 months, an increasing proportion of samples contained IgA anti-HIV: 6 of 13 (46%) and 16 of 19 (84%) were positive in AACELISA at 6 and 12 months respectively and 13 of 14 (93%) were positive at 18 months. Four (19%) of the 21 children did not have detectable IgA anti-HIV in any sample collected between 3 and 12 months of life (Table 1). These four IgA anti-HIV-negative children also lost IgG anti-HIV reactivity during their first 12 months, suggesting that their infection occurred well after birth. Two of them had symptoms possibly due to HIV at 6 months, the third had non-specific findings and the fourth child was asymptomatic throughout the first year. The presence or absence of HIV-associated symptoms in the first year of life did not correlate with the presence or absence of IgA anti-HIV.

IgM anti-HIV: Of the 14 neonatal specimens eight (57%) contained IgM anti-HIV; but none of the 17 specimens collected at 3 months did (Fig. 1). After 3 months, an increase in the proportion of samples containing IgM anti-HIV was observed to a maximum of six of nine (67%) samples obtained at 24 months. Of the 21 PP group children 11 (52%) did not have detectable IgM anti-HIV at any time between 3 and 12 months; eight of these were symptomatic and three were asymptomatic.

IgG anti-HIV: All samples collected before 3 months of age were positive when tested by GACELISA and Abbott HIV 1/2 (Table 1 and Fig. 2). At 3 months IgG anti-HIV was detected

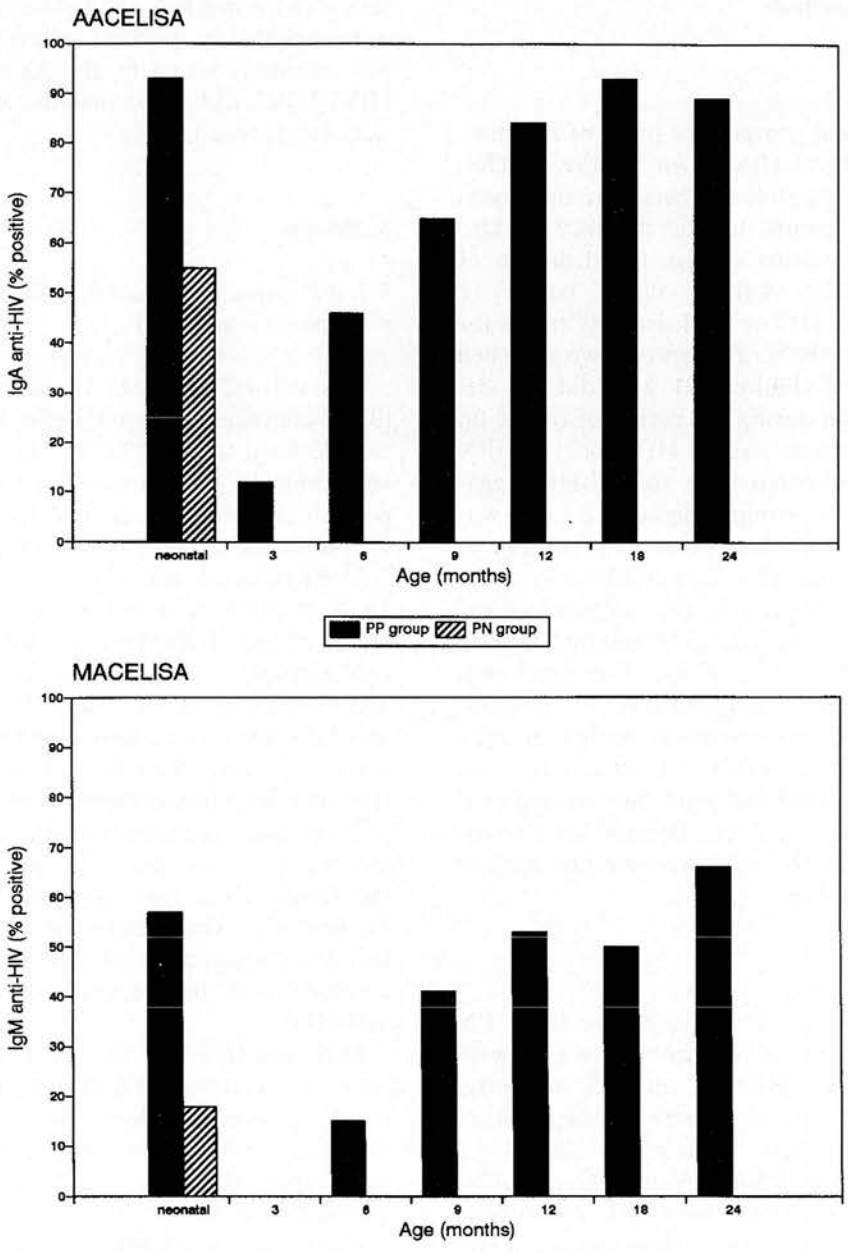

Fig. 1. IgA and IgM anti-HIV reactivity, measured by class-specific antibody capture EIAs, in sequential plasma specimens collected from 42 children born to HIV-infected mothers; 21 children were HIV-infected (PP group) and 21 were not (PN group).

in 15 of 17 (88%) samples by GACELISA and 16 of 17 (94%) by Abbott HIV 1/2. Of samples collected at intervals after 3 months of age, similar proportions were reactive in GACELISA and Abbott HIV 1/2. Of the 21 PP group children eight (38%) had at least one sample unreactive in GACELISA HIV 1/2 and/or Abbott HIV 1/2 during the first year (Table 1); four of the eight

Table 1
IgA (A) and IgG (G) anti-HIV reactivity (optical density/cut-off) of sequential plasma specimens collected from 21 HIV-infected children born to HIV seropositive mothers

Child ID	Neonatal specimen		Age (months)												CDC stage[a]	
			3		6		9		12		18		24			
	A	G	A	G	A	G	A	G	A	G	A	G	A	G	6m	12m
Infants persistently IgG anti-HIV-positive																
1	Neg[b]	++	Neg	++					+	++	+	++	+	++	P1	P1
2	+	++	Neg	++	++	++	++	++	++				++	++	P2A	P2D2
4			Neg	+	Neg	++	+	++	+	++	+	++	+	++	P2F	P2F
5			Neg	++			++	++	++	++			++	++	P2A	P2A
6	++	++					+	++	+	++	Died				P2A	P2D2
8	+	++	Neg	+	++	++	+	++	++	++	++	++			P2A	P2F
10	+	++	Neg	++					++	++	++	++			P2A	P2A
12	+	++	++	++			++	++	++	++	++	++	++	++	P2F	P2F
13					Neg	++	+	++	+	++	Neg	++			P2A	P2A
14	+	++	Neg	+	+	++	+	++	+	++	+	++	+	++	P2A	P2F
16			+	++	++	++	++	++	++	++	++	++	++	++	P2F	P2F
18					Neg	+	Neg	+	+	++			Died		P1	P2A
20	+	++	Neg	++	++	++	++	++	++	++	++	++			P1	P2A
Infants not persistently IgG anti-HIV-positive (GACELISA and/or Abbott): putatively infected by breastfeeding																
3	+	++	Neg	++	Neg	Neg	Neg	++			+	++			P1	P1
7			Neg	Neg	Neg	+	Neg	++	Neg	++	+	++			P2F	P2F
9	+	++	Neg	+[c]					++	++	+	++	+	++	P2D2	P2D2
11	+	++	Neg	+			Neg	Neg[c]	Neg	Neg[c]	+	+	Neg	+	P1	P1
15	+	++	Neg	+	Neg	Neg	Neg	+	Died						P2A	
17			Neg	+			Neg	Neg[c]	+	++					P1	P2A
19	+	++	Neg	Neg	++	++	++	++	++	++	++	++			P1	P2A
21	+	++			Neg	+			Neg	Neg	Died				P2F	

[a] CDC stage: P1: asymptomatic; P2: symptomatic; P2A: non-specifc findings; P2D2: recurrent serious bacterial infection; P2F: other diseases possibly due to HIV infection.
[b] Neg: OD/CO <1.0; +: OD/CO $>1<3$; ++: OD/CO ≥ 3.
[c] Abbott HIV1/HIV2 EIA-negative.

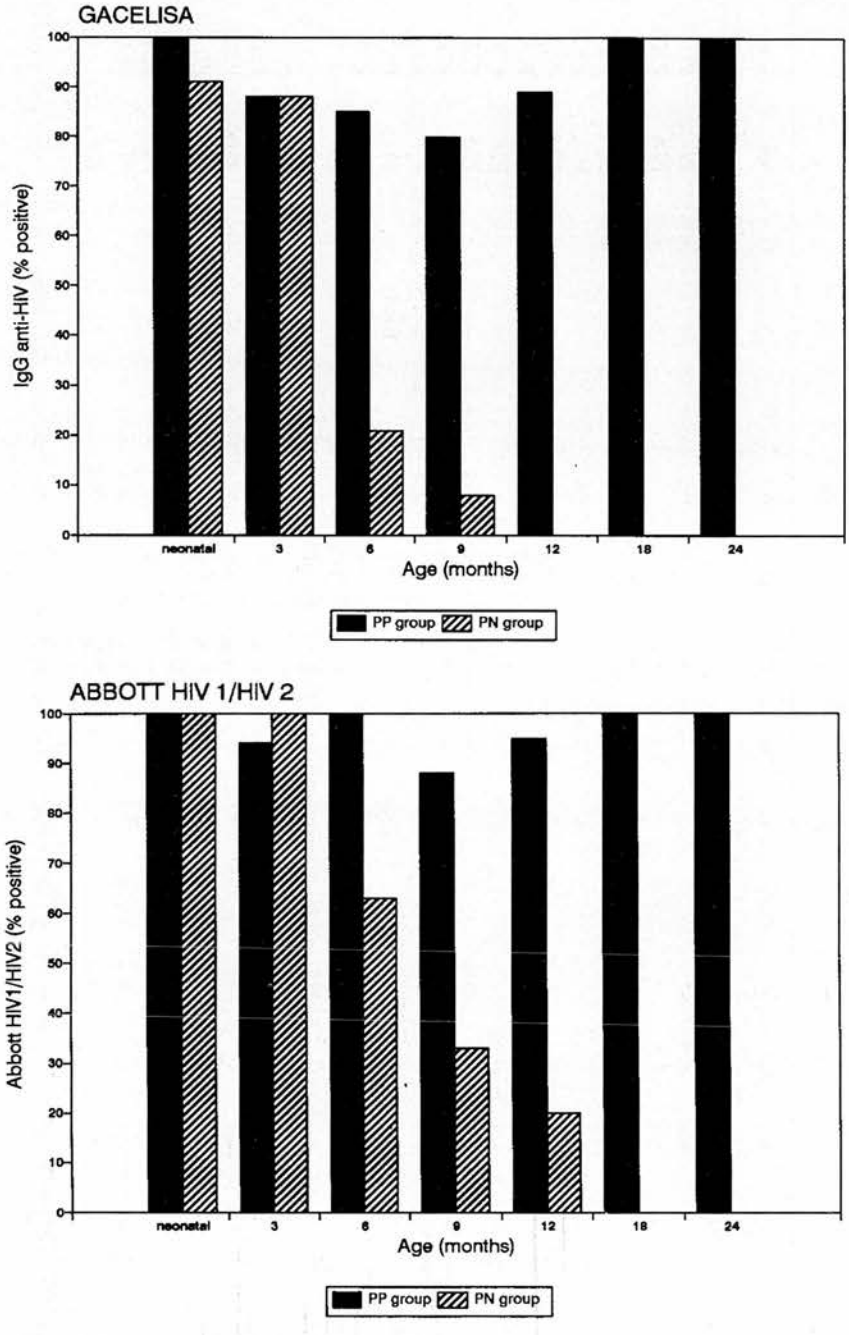

Fig. 2. IgG anti-HIV reactivity, measured by IgG antibody capture EIA and the Abbott HIV 1/2 EIA (second generation), in sequential plasma specimens collected from 42 children born to HIV-infected mothers; 21 children were HIV-infected (PP group) and 21 were not (PN group).

had no detectable IgA anti-HIV throughout this period. Between 6 and 9 months of age only one of seven infants who lost IgG anti-HIV had detectable IgA anti-HIV whereas ten of 11 infants with persistent IgG anti-HIV were IgA anti-HIV-positive (Table 1).

3.2. PN group (21 uninfected children born to HIV-infected mothers)

IgA anti-HIV: Of 11 neonatal samples, six (55%) contained IgA anti-HIV. This was lost by 3 months of age (Fig. 1), after which time all specimens were unreactive in AACELISA.

IgM anti-HIV: Two (18%) of 11 neonatal specimens contained IgM anti-HIV, but none of the specimens collected later were reactive in MACELISA (Fig. 1).

IgG anti-HIV: All ten neonatal samples that were tested in both GACELISA and Abbott HIV 1/2 were reactive (Fig. 2). Another neonatal specimen, unreactive in GACELISA, was not tested in Abbott HIV 1/2; tests on the mother's plasma indicated that she was probably seroconverting around the time of delivery. GACELISA reactivity decreased more rapidly than Abbott HIV 1/2 reactivity: at 6 months only four of 19 (21%) specimens were reactive in GACELISA compared to 12 of 19 (63%) in Abbott HIV 1/2. At 12 months, no sample contained IgG anti-HIV when tested by GACELISA but 20% were still positive by Abbott HIV 1/2.

3.3. NN group (21 uninfected children born to HIV-negative mothers)

All 135 plasma samples collected from 21 anti-HIV-negative children born to uninfected mothers, at ages between 0 and 24 months, were unreactive in AACELISA, MACELISA, GACELISA and Abbott HIV 1/2 assays.

4. Discussion

An earlier study of European babies has demonstrated that the simple and inexpensive class-specific antibody capture assays employed in this study were sensitive and specific (Connell et al., 1992). In that study, 20 (83%) of 24 HIV-infected babies of between 3 and 12 months of age were IgA anti-HIV-positive by AACELISA. Substantial proportions of uninfected infants between birth and 3 months of age, but not older, also had IgA anti-HIV in their blood and it was suggested that this antibody had been passively transferred from the mother. The presence of IgA and IgM anti-HIV in the neonatal blood of some uninfected infants born to HIV-positive mothers was confirmed in this study of Zairian children. In view of the earlier findings, however, we were initially surprised to find an apparently lower rate of positivity for IgA and IgM anti-HIV in those Zairian children who were subsequently confirmed to be HIV-infected. Overall, only 35 of 66 (53%) bloods collected from them between 3 and 12 months of age were IgA anti-HIV-positive, many of them weakly so. Tests for the presence of IgM anti-HIV were also less often positive and they seemed not to augment the IgA findings. The IgA findings were also at variance with those of Quinn et al. (1991) and Wieblen et al. (1990a) who used immunoblot procedures to detect IgA anti-HIV in HIV-infected infants.

Why should this have been? We believe that two factors accounted for this discrepancy. Firstly, all of the babies in this study, unlike others, were breast-fed by their mothers. Whereas breast-feeding had been thought, until recently, to carry only a small risk of mother–infant transmission of HIV (Ryder et al., 1991), it has now become apparent that up to half of mother–child transmissions in breast-fed children occur by this route (Dunn et al., 1992). Such transmissions are delayed, compared with those in utero and at birth, so that IgA anti-HIV may appear later. Secondly, it has been our experience, and that of others (Wieblen et al., 1990a; Landesmann et al., 1991; Re et al., 1992), that an IgA anti-HIV response is absent in many, but not all, children who experience rapid HIV-disease progression. This could account for a poor IgA response in three of the 21 HIV-infected children studied by us.

In testing European children, for whom breast-feeding is discouraged, we have so far not seen

any child, who was subsequently diagnosed as HIV-infected, lose reactivity for IgG anti-HIV and then regain it. We had, therefore, concluded that this was an infrequent occurrence. In the series of HIV-infected Zairian children reported here, however, eight of 21 (38%) children lost anti-HIV, as measured by the GACELISA and/or Abbott EIA, at some stage in the first year of life; several others became only weakly reactive (Table 1). With the exception of two children who died, all later became unequivocally seropositive. This is evidence that perhaps a half of the HIV-positive infants studied by us were infected well after birth, presumably through breast-feeding, and is in accord with others' findings (Dunn et al., 1992). In the four infants in whom an IgA anti-HIV response was not detected between 3 and 12 months, all showed transient loss of IgG anti-HIV reactivity.

For the PN group, IgG anti-HIV reactivity was lost earlier in the GACELISA assay than in the indirect (Abbott) assay. This confirms similar findings in another study (Parekh et al., 1993) and may prove to be a diagnostically useful feature. Particularly in babies who have not been breast-fed, it may permit earlier identification of babies who have escaped HIV infection than when employing a conventional anti-HIV screening test.

Unfortunately, there is no information on the frequency or duration of breast-feeding in the earlier published work on IgA anti-HIV detection in at-risk children. If, as seems likely, many children in these other studies were wholly or largely bottle-fed, infection might only rarely have occurred post-natally. By contrast, in this study, transmission might have occurred at any time from birth until the children who escaped intrauterine or perinatal infection were completely weaned (Stiehem and Vink, 1991; Van de Perre et al., 1992; Datta et al., 1992; Ziegler, 1993). Risk of transmission by breast-feeding may accrue with time, due to the duration of exposure of the child (de Martino et al., 1992), the possible decline of the mother's immune competency—leading to higher levels of viral replication and lower levels of partially protective maternal antibody—and the emergence of more transmissible strains of HIV in the mother. Moreover, it might be that the dynamics of the immune response, in a child infected orally under 'cover' of maternal antibodies, are suppressed compared to that of adults or of children infected at or before birth.

If it is the case that an IgA response is delayed or absent in breast-fed babies infected post-natally, is there any value in attempting early diagnosis by IgA antibody capture anti-HIV testing? We believe there is, for the following reasons. First, we and others have demonstrated that, when present, IgA anti-HIV (and IgM anti-HIV) in the blood of children of 3 months and older is highly predictive of HIV infection in that child. By contrast, other anti-HIV reactivity in screening tests, predominantly IgG, is often present in uninfected children even beyond 12 months of age, making it difficult to distinguish infected from uninfected children. Second, the method we have employed is robust, simpler and cheaper than tests for IgA anti-HIV which require preliminary removal of IgG and immunoblot. The IgA antibody capture ELISA employs conventional ELISA methods and its reagent cost should be no more than US$ 2–3—immunoblot reagents cost approximately ten-fold more. Third, although the precise time of transmission was not known for our 21 HIV-infected children, it is clear that post-natal transmission had frequently occurred. In such circumstances it would not be possible to determine at what point other tests, e.g. for HIV p24 Ag or proviral DNA might become positive, relative to IgA anti-HIV seroconversion. It can be assumed that p24 antigen detection and polymerase chain reaction (PCR) would also have shown an apparently low sensitivity in the first few weeks or months of life as, in some cases, transmission would not yet have occurred. Fourth, even in situations where PCR and HIV p24 antigen testing can be afforded and done to a high standard of accuracy, we have found it valuable to have a serological method, such as detection of IgA anti-HIV, to confirm HIV infection in children who are positive for these other markers of HIV infection. Fifth, we have experienced failures both of HIV p24 Ag testing and PCR to diagnose HIV infection in HIV-infected babies of over 3 months of age whose serum was shown to contain IgA anti-HIV (Tosswill et al., 1994).

Lastly, it is often difficult to obtain sufficient blood from an infant or young child to perform HIV p24 Ag testing and/or PCR. The IgA capture method requires only tiny volumes (≤ 5 μl) and can be performed on specimens which are insufficient for a p24 Ag test. Although not formally demonstrated here, the IgA capture assay, like the IgG capture assay, would probably function equally well on eluates prepared from dried blood spots (Tappin et al., 1991; Thongcharoen et al., 1992).

A simple, inexpensive assay for IgA anti-HIV, such as we have described, would be ideally suited to laboratories that are unable to perform complex and expensive tests to diagnose mother–child transmission of HIV. Although the sensitivity of the method in breast-fed infants is imperfect, it is highly specific. In circumstances where other approaches are not available the method will, by 6 months of age, permit the identification of the majority of babies who had become infected in utero or perinatally. Especially if breast-fed, other babies will need to be followed up for longer, perhaps until at least 6 months after their last exposure. For many families, the stress to parents of not knowing their child's HIV status could be removed, and appropriate prophylactic therapy and monitoring of the infected child initiated.

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A Preliminary Evaluation of the Cognitive and Motor Effects of Pediatric HIV Infection in Zairian Children

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Fourteen asymptomatic HIV-infected Zairian children under 2 years of age displayed social and motor developmental deficits on the Denver Developmental Screening Test when compared with 20 HIV-negative cohorts born to HIV-infected mothers and 16 control children. In a second study, 11 infected children over 2 years of age had sequential motor and visual-spatial memory deficits on the Kaufman Assessment Battery for Children and motor development deficits on the Early Childhood Screening Profiles. HIV infection affects central nervous system structures mediating motor and spatial memory development, even in seemingly asymptomatic children. Furthermore, maternal HIV infection compromises the labor-intensive provision of care in the African milieu and undermines global cognitive development in even uninfected children.

Key words: HIV, pediatrics, cognitive ability, intelligence, motor development, social development, neuropsychology, Africa, cross-cultural

When one considers what specific domains of development are affected by pediatric HIV infection, language and motor skill deficits have been noted for children tested with the Stanford–Binet (Ultmann et al., 1985). With the McCarthy Scales of Children's Abilities, quantitative, verbal, and memory ability deficits have also been documented with infected children and are particularly significant for those children with accompanying neurological impairment from the virus (Levenson, Mellins, Zawadzki, Kairam, & Stein, 1992). Condini et al. (1991) observed that HIV-1-positive children showed significant delays in language development in comparison with HIV-negative cohorts, as indicated by mean length of utterance obtained by observation of natural child–parent interactions. In addition, deficits of visual–spatial integrative ability and memory have been identified with the Kaufman Assessment Battery for Children (K-ABC; Belman et al., 1988; Diamond, 1989; Diamond et al., 1987).

With respect to the developmental effects of pediatric HIV infection, investigators have noted the environmental influences of the risk factors associated with the infection in parents, particularly among the inner-city poor, impoverished and minority populations among which HIV infection is concentrated (Diamond et al., 1990; Levenson et al., 1992; Ultmann et al., 1985; Ultmann et al., 1987). Such risk factors can include parental intravenous drug use and associated deficient or nonexistent antenatal medical care and poor nutrition. Even in the absence of HIV infection, such characteristics of the home environment can significantly compromise the cognitive and behavioral development of a child over the long term (Mellins, Levenson, Zawadzki, & Kairam, 1991). Irrespective, HIV-positive children infected by blood transfusion neonatally still demonstrate significant deficits in school achievement and in neuropsychological tasks that emphasize

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motor speed, visual scanning, and cognitive flexibility (Cohen et al., 1991). The children in Cohen et al.'s study were not disproportionately more likely to be prenatally subjected to illicit drug or alcohol exposure or nurtured in impoverished home environments than were their noninfected counterparts. Thus, it is likely that the direct effects of the virus on the brain and central nervous system produce neuropsychological deficits independent of the quality of the perinatal developmental milieu, but such deficits are likely compounded by the developmental risk factors pre- and postnatally that are conducive to parental HIV infection in the American urban setting.

There is an additional means of developmental impairment less often considered in the context of the neuropsychological effects of pediatric HIV infection, that exists apart from the lifestyle issues that place a parent at risk for infection. These involve the direct effects on the child of having key family members, especially the mother, chronically ill from the disease. These effects can be especially severe among more impoverished socioeconomic classes in developing countries. For example, in rural areas of much of sub-Sahara Africa, where the family must rely on labor-intensive subsistence agriculture to provide for its nutritional needs, maternal HIV disease can severely disrupt the nutritional resources of the family and undermine this vital aspect of the early development of the children.

Furthermore, chronic illness on the part of any family member in the absence of a national or universal health-care insurance system or social welfare safety net can quickly drain the meager financial resources of the family as it seeks to provide for the expenses of treatment. This is an important consideration in light of the significant relation between overall economic well-being for the family and the long-term development of cognitive ability and performance that has been documented in such African environments (Boivin & Giordani, 1993; Boivin et al., 1993). Hence, when AIDS or any serious long-term illness ravages a family in a developing country, it has the potential of profoundly influencing the neuropsychological development and well-being of both infected and noninfected children within the family.

As a means of differentiating among these effects, HIV-1-positive and -negative children born to infected African mothers were compared directly for cognitive and motor developmental differences. Both groups were subsequently compared to a third group of HIV-negative children born to noninfected mothers in order to better assess some of the second-order effects of the epidemic on the development of children who are not themselves infected, but who bear the consequences of the disease in the form of illness of the primary caregiver (mother), and the economic hardship that this imposes on the entire family. Such factors are expected to be especially severe for nonaffluent families in developing countries, such as in the major urban centers of sub-Sahara Africa where the disease is epidemic (Goodgame, 1990; Novicki, 1988).

An additional important aspect of this study is that the analysis of primary and second-order effects of pediatric HIV infection can take place apart from the effects of antiretroviral drug treatments (e.g., AZT). Unlike American and European children, the vast majority of pediatric HIV cases in Africa are never given these drugs because of inaccessibility and expense. Although tragic, this fact allows for the effective study of the natural course of the infection in children unaffected by the medical treatment protocols—something that is no longer possible for such children in most industrialized nations.

Study 1: Comparing HIV-Positive and HIV-Negative Children on the Denver Developmental Screening Test During the First 18 Months

Method

Respondents. Women undergoing prenatal evaluation at a 300-bed hospital near Kimpese, a commercial center in lower Zaire, were screened for HIV-1 and HIV-2 infection with the ELISA recombinant DNA enzyme-linked immunoassay test (Abbott Laboratories, Delkenheim, Germany). Confirmation of a positive test was obtained with the Wellcozyme (enhanced antigen sandwiched enzyme-linked immunoassay) assay (Murex Diagnostics, Dartford, United Kingdom) on the same specimen. Positive cases were subsequently retested for HIV-1 p24 antigen and core antibody in the Regional Virus Laboratory (University of Edinburgh, Edinburgh, United Kingdom). This series has an estimated predictive positive value of over 99% in the identification of HIV-1 or HIV-2 infection. All of the infection cases identified in this study were of the HIV-1 variety.

Confirmed HIV-positive women were recruited for participation in a 2-year longitudinal study beginning at the time of birth of the child. The health, immunological, and developmental status of the child born to the HIV-positive mother were evaluated on a monthly basis. About a third of the women identified as HIV-positive during the prenatal examination chose to participate in the project, and about half of those women had completed the entire 2-year study with their child at the time of this research.

A second group of women was recruited for the 2-year study through the prenatal clinic at the hospital. These women were HIV-negative and were matched to the HIV-positive mothers in terms of maternal age, residential neighborhood, socioeconomic status, and parity. The children born to these mothers constituted the control group in this analysis.

All of the infected women in the project who had completed the 2 years of evaluation with their child on a reliable basis were included in this analysis. Of the cohort of children born to HIV-positive mothers, 14 children maintained the HIV-positive status throughout the 2 years, and 20 were confirmed HIV-negative (sero-reverters). Sixteen children were in the control group. There were no significant complications reported at birth for the children included in this analysis, and the HIV-positive children remained pre-AIDS (Stage P1) according to World Health Organization (WHO) criteria (Newell, Peckham, & Lepage, 1990) for pediatric HIV infection.

Measures and materials. In this study a physician assessed psychomotor development as part of the comprehensive health examination during one of the monthly project sessions. The mother was present throughout the evaluation to respond to questions from the nurse or pediatrician. The psychomotor development measures at 3, 6, 9, 12, and 18 months of age are included in the repeated measures analysis.

The developmental measures for the children were obtained with the most recent version of the Denver Developmental Screening Test (DDST; Frankenberg, Fandal, Sciarillo, & Burgess, 1981) in the four main areas of development (personal–social, language, fine motor–adaptive, and gross motor). To enhance the statistical analysis of these measures, the ordinal rankings typically used in this screening battery were replaced by an interval (quantitative) measure that uses a scoring system developed for the Woodside Developmental Assessment Battery (Eu, 1986).

If a Zairian child demonstrated a developmental item on the DDST that is performed by less than 25% of the normative American sample for that age group, the child received four points added to his or her total for that developmental domain. Demonstrating an ability performed by 25%–50% of the normative sample resulted in a score of three, one performed by 50%–75%, in a score of two, and one performed by over 75%, in only a score of 1. Likewise, failing to demonstrate an ability that over 90% of the American normative sample could perform at that age resulted in four points subtracted from the total in that developmental domain for a Zairian child. Failing an item demonstrated by 75%–90% of the normative sample resulted in a score of −3; failing an item demonstrated by 50%–75% of the normative sample resulted in a score of −2; failing an item performed by 25%–50% of the sample resulted in a score of −1; and failing an item that less than 25% of the normative sample could do resulted in a score of zero. The total pass/fail score for each of the four developmental domains of the DDST is then divided by the total number of tasks or items that are applicable from birth to that age of the child in order to arrive at a final score.

Results

When the three groups of children were compared in a one-factor repeated measures analysis of variance (ANOVA) for the developmental assessment scores at 3, 6, 9, 12, and 18 months of age, there were significant between-group (main effect) differences for the domains of personal–social, fine motor–adaptive, and gross motor development (see Table 1). For the personal–social and gross motor summary measures, the between-group differences remained significant at $p < .01$ even when a Bonferroni correction was applied for the number of simultaneous statistical comparisons within a single experimental design. Subsequent pairwise comparisons revealed that in each of these areas, the HIV-positive children scored significantly lower than both the HIV-negative and control children, and there were no differences between the HIV-negative and control children.

The Main Factor (HIV status) × Repeated Measure interaction effect for the ANOVA was statistically significant for the domains of personal–social, language, and gross motor (Table 1). These effects remained significant even when the significance values were adjusted according to the Greenhouse-Geisser method of profile analysis in order to account for the possibility of compound symmetry in the repeated measure. The Greenhouse-Geisser epsilon values were 0.62, 0.80, and 0.66 for the repeated measures in the developmental domains of personal–social, language, and gross motor, respectively. The interaction effect plots of the cell means for this analysis clearly indicate that for gross motor development, this effect is largely due to a significant lag in development for the HIV-positive children that progresses across the periodic 3- and 6-month assessments, as compared with the HIV-negative and control children (see Figure 1).

Table 1
Comparison of HIV-Positive, HIV-Negative, and Control Children on the Denver Developmental Screening Test

Measure	HIV group[a]	Repeated measure[b]	Interaction[c]
Personal–social	7.61***	7.76***	3.70***
Language	3.00	22.76***	8.24***
Fine motor–adaptive	4.47*	1.10	0.82
Gross motor	7.12***	6.96***	2.79**

[a] *dfs* = 2, 236. [b] *dfs* = 4, 236. [c] *dfs* = 12, 236.
*p < .05. **p < .01. ***p < .001.

Study 2: Comparing HIV-Positive and HIV-Negative Preschool Children on the Kaufman Assessment Battery for Children and the Early Childhood Screening Profiles

Method

Respondents. From the information database for the mothers and infected and noninfected children who had been enrolled in the assessment project, irrespective of how reliably they had completed the 2 years of assessment, a list of those mothers who had a home address in the Kimpese area near the hospital center with project children at least 2 years of age was generated. From this list of about 200 infected mothers, 26 children who had been confirmed HIV positive and 26 children who had been confirmed HIV negative at the end of the assessment were identified.

These 52 children represented almost all of the children in the project who lived in the vicinity.

Of the 26 HIV-positive children, we were able to complete the assessment protocol with only 4 of the children. The remaining children had died, were too sick to test, were not sufficiently responsive for valid assessment, or had moved to elsewhere to live with members of the extended family after the death of one or both parents. Of the 26 HIV-negative children in the original sampling frame, 15 eventually completed the assessment protocol. Clearly, the HIV-positive children were disproportionately more likely to have died or to be too ill or not sufficiently responsive for cognitive and motor assessment than the HIV-negative children, $\chi^2(4, N = 52) = 23.77, p < .01$.

In order to obtain additional cases of pre-AIDS HIV-positive children perinatally infected, another list of 26 eligible children was compiled from an information base for pediatric HIV-positive cases at the hospital; these children had not been enrolled in the 2-year postnatal evaluation study. Of these 26 children, 7 completed the assessment protocol. Therefore, we had a total of 11 perinatally infected HIV-positive children over 2 years of age. These 11 children did not present any of the major signs and no more than three of the minor signs of AIDS (according to WHO criteria; Newell et al., 1990) at the time of testing. The major signs consist of significant weight loss and failure to thrive, persistent fever, and persistent diarrhea. The minor signs consist of oropharyngeal candidiasis, repeated common infections, generalized lymphadenopathy, persistent cough for more than a month, generalized dermatitis, and confirmed maternal HIV infection.

In addition to the HIV-positive and HIV-negative children born to infected mothers, we sought a control group comparison of HIV-negative children born to noninfected mothers. Mothers with children from a village area that had participated in a previous study (Boivin et al., 1993) were recruited for assessment. We selected from this sampling frame 15 children who matched the HIV-positive children on the indicators of age, gender, educational level of the mother, and general economic status of the home environment as assessed from a home evaluation (described by Boivin et al., 1993). Both children and their mothers were screened for HIV infection with the HIVchek blot test (Dupont Pharmaceuticals, Wilmington, DE) and all 15 children and their mothers tested negative. This group of HIV-negative children born to HIV-negative mothers constituted the control group.

Instruments and assessment. Usually the children who were evaluated were brought to the project clinic at the hospital by the mother, who remained throughout the assessment. The children were tested

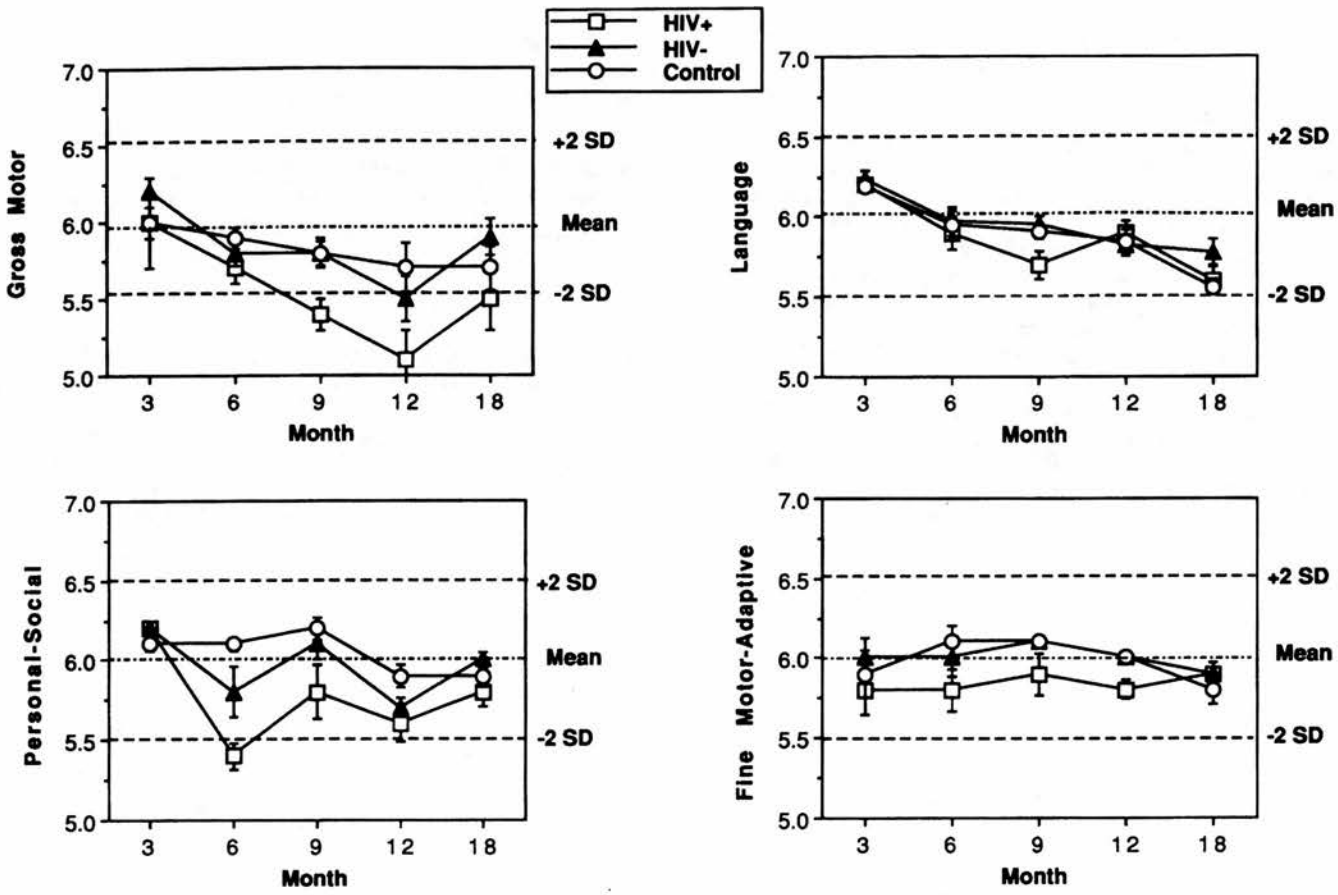

Figure 1. Group means and standard errors on the four domains of the Denver Developmental Screening Test (scored according to the Woodside system) for the HIV-positive, HIV-negative, and control children in Study 1. Data are plotted against the normative mean.

individually by one of four Zairian teachers trained in the administration of the instrument and enlisted as members of the research team. The training and supervision of the Zairian teachers for the administration of the K-ABC is described in Boivin et al. (1993). A principal-component factor analysis completed on K-ABC data from Zairian children in previous studies (Boivin & Giordani, 1993; Boivin et al., 1993) revealed that although the instrument had been administered in Kituba (the regional trade language), the internal factor structure of the subtests remained the same and supported the validity of the instrument when administered in this cultural setting. In this study the examiners readily communicated with the parents in the local language of Kikongo (a dialect related to Kituba) or the urban language of Lingala. For the HIV-positive and -negative groups born to infected mothers, the examiners did not know the infection status of the children.

After the child was examined and the mother was interviewed about the presence of major and minor AIDS symptoms (Newell et al., 1990), weight, height, head circumference, and arm circumference were measured. The overall nutritional and developmental status of the children was scored according to the nutritional surveillance scheme presented by Brown (1987) for Zaire. The single criterion denotes whether a child's upper-arm circumference is at least 80% of standard arm circumference for the child's height (based on standards originating in Nigeria) and is referred to as the *Quaker arm circumference* (QUAC). For statistical comparisons between groups in this study, the QUAC measure is equal to Upper Arm Circumference ÷ Height × 100. For the physical development measures, the percentile ranks based on standardized normative values for age were also evaluated. Weight, height, and weight standardized according to height percentiles were obtained from normative values provided by the WHO (1983), arm circumference values were obtained from norms provided by Jelliffe and Jelliffe (1989), and head circumference values were provided by McCammon (1970).

Early Childhood Screening Profiles (ECSP). We adopted portions of the ECSP (Harrison et al., 1990). From the Cognitive/Language Profile, the children were scored in the areas of verbal concepts, visual discrimination, logical relations, and basic school skills. Instructions and test items were administered in Kikongo, the major dialect in this region of Zaire and the primary language of fluency for the children. The motor profile portion of the ECSP was also administered, which consists of the subtests of gross motor skill and fine motor skill.

Kaufman Assessment Battery for Children. The K-ABC (Kaufman & Kaufman, 1983a, 1983b) has been proposed as the test of choice for researchers who wish to measure specific domains of cognitive ability within a cross-cultural context (Moon, 1988; Utairatanakit, 1987) and has already been used in other health-related assessment research with Zairian children (Boivin & Giordani, 1993; Boivin et al., 1993). The mental processing portions of the K-ABC (sequential and simultaneous processing) were administered (Easels 1 and 2), and all of the subtests were attempted with all of the children in the study

Table 2
Between-Groups Comparisons of the HIV-Positive (n = 11), HIV-Negative (n = 15), and Control (n = 15) Children in Study 2 on the Standardized Measures from the Early Children Screening Profiles

Measure	HIV-positive		HIV-negative		Control		
	M	*SD*	*M*	*SD*	*M*	*SD*	*F*(2, 38)
Cognition/Language	81.8	12.6	91.8	14.9	90.4	18.2	1.11
Cognition	96.0	10.3	102.3	13.9	97.5	18.6	0.54
Language	79.1	9.3	82.8	7.4	84.6	14.0	0.54
Motor	83.0	14.2	104.6	24.7	115.2	29.4	4.69*

*p < .05.

except for the photo series subtest. This subtest proved too difficult for most of the children because of their age and the cultural specificity of the depicted activities and events.

Results

Early Childhood Screening Profiles. For the global ECSP indicators of cognitive or language development, no significant differences emerged among the three groups except on motor development (see Table 2). Pairwise comparisons for the significant between-group differences on motor development revealed that both the HIV-negative and control groups scored higher than the HIV-positive group (Fisher's protected least significant differences [PLSDs] = 21.14 and 21.42, $p < .001$).

One factor that may have contributed to the poorer motor development by the HIV-positive children was that their general physical development was impaired, as indicated by the QUAC (see Table 3). The infected children were also significantly below their noninfected counterparts born to infected mothers on their age-standardized percentile rank measures for weight (Fisher's PLSD = 17.32, $p < .01$), arm circumference (Fisher's PLSD = 9.51, $p < .05$), and weight standardized by height (Fisher's PLSD = 19.31, $p < .01$).

For both HIV-positive and -negative children born to infected mothers, the QUAC measures were significantly correlated to the number of immunity deficiency symptoms reported over the final 6 months of the 2-year assessment project. For HIV-positive children who were not enrolled in the project, this measure was compiled from their medical charts if it was available. This medical information was not available for 3 of the HIV-positive and 3 of the HIV-negative children. Although the HIV-negative children did display some of the symptoms of immunity deficiency, presumably because of other health-risk factors and infectious pathogens that they were exposed to, they tended to be fewer in number and corresponded to higher QUAC development measures than for the HIV-positive children, $r(21) = -.54, p < .01$ (see Figure 2).

Kaufman Assessment Battery for Children. The HIV-negative children for both the infected (HIV-negative group) and noninfected mothers (control group) scored significantly higher than the HIV-positive group on all of the K-ABC global mental processing measures (see Table 4 and Figure 3). These included sequential processing, simultaneous processing, mental processing composite, and nonverbal performance total.

For the sequential processing, simultaneous processing, and mental processing composite, the between-group differences remained significant at $p < .01$ even when a Bonferroni correction was applied for the number of simultaneous statisti-

Table 3
Physical Development Descriptive Statistics and Between-Groups Comparisons for the HIV-Positive (n = 11), HIV-Negative (n = 15), and Control (n = 15) Children

Measure	HIV-positive		HIV-negative		Control		
	M	*SD*	*M*	*SD*	*M*	*SD*	*F*(2, 38)
Age (in months)	54.8	8.6	34.8	14.2	46.2	12.0	2.81
Weight (in kilograms)	13.0	3.9	12.5	2.1	14.5	3.4	3.69*
Height (in centimeters)	94.6	14.7	86.1	10.2	103.7	9.4	15.53***
Head circumference (in centimeters)	47.8	2.5	45.3	8.5	49.3	1.9	1.98
Arm circumference (in centimeters)	13.7	2.1	16.8	8.4	14.8	1.5	1.04
Quaker Arm Circumference (Arm Circumference ÷ Height)	14.7	1.8	19.7	10.4	14.4	1.5	3.02*
Age-standardized percentile rank scores							
Weight	6.1	8.6	18.5	17.7	32.5	29.8	4.89*
Height	14.6	29.5	12.6	22.9	63.2	34.0	13.88***
Head circumference	11.5	24.4	10.6	13.3	39.7	38.2	5.17*
Arm circumference	78.2	11.5	88.4	6.1	84.1	15.8	2.37
Weight × Height	16.8	17.1	50.6	29.0	16.5	22.7	9.52***

Note. Percentile ranks are based on data from the World Health Organization (1983).
*$p < .05$. **$p < .01$. ***$p < .001$.

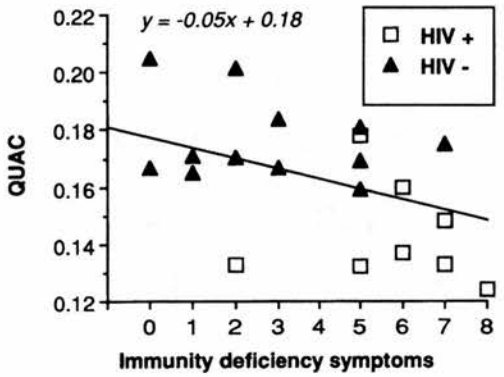

Figure 2. Relation between the number of immunity deficiency symptoms reported during the final 6 months of the 2-year assessment and the Quaker Arm Circumference measure (Arm Circumference ÷ Height), an indicator of physical development and nutritional well-being. The data are from the HIV-positive and HIV-negative children in Study 2 and have a Pearson product–moment correlation coefficient of $-.54$ ($df = 25$), $p < .01$.

cal comparisons within a single experimental design. Among the various subtests of the K-ABC, the two groups of children differed significantly on all the visual recognition tasks in the simultaneous processing domain as well as on the sequential processing (immediate recall) tasks of number recall and hand movements. Finally, the groups differed significantly on spatial memory, which is a simultaneous processing subtest.

Multifactor covariate analysis for the Kaufman Assessment Battery for Children. An additional analysis was completed for the K-ABC raw score values rather than the standardized values based on a normative sample of American children. This was a one-factor blocked analysis of covariance with the Zairian children blocked into four subgroups on the basis of gender and age (boys 3 years of age and younger, boys older than 3 years, girls 3 years and younger, and girls older than 3 years). HIV status of the child was the main effect, and weight standardized according to height (WHO, 1983) served as the covariate. Significant between-group differences remained for all of the K-ABC global measures and the ECSP motor measure (Table 5). However, the subsequent pairwise comparisons for the covariate ANOVA was particularly instructive in attempting to statistically separate the direct and indirect developmental effects of HIV infection in children and their mothers. For this analysis, the control group born to noninfected mothers performed significantly better than the HIV groups born to infected mothers on the ECSP motor development total and all of the global K-ABC scales.

For the children born to infected mothers, the HIV-negative group performed significantly better than the HIV-positive group, specifically on the sequential processing global score and mental processing composite ($p < .001$). These preliminary findings indicate that the infected children demonstrated global cognitive impairment that apparently extended beyond the indirect developmental effects of the mother's being ill with HIV disease and remained even when nutritional and developmental well-being was statistically controlled for by using weight standardized according to height as a covariate.

Discussion

In the initial study, motor and social development were implicated as the major domains in which significant differences began to emerge between HIV-negative and HIV-positive perinatally infected children during the first 2 years of life. The developmental screening measures that these conclusions were based on, however, are most useful in the context of the general screening and identification of developmentally at-risk children for further intervention. They were not in-

Table 4
Between-Groups Comparisons of the Standardized Scores from the Kaufman Assessment Battery for Children (K-ABC) in Study 2

Measure	HIV-positive		HIV-negative		Control		
	M	*SD*	*M*	*SD*	*M*	*SD*	*F*(2, 38)
Simultaneous Processing–Visual Recognition							
Magic Window	3.1	1.2	4.0	1.6	8.1	2.4	20.77***
Face Recognition	8.0	2.4	9.4	2.1	11.1	2.7	4.00*
Gestalt Closure	5.6	3.1	7.1	2.1	9.7	3.7	6.06**
Simultaneous Processing–Visual-Spatial Organization							
Triangles	6.0	2.8	8.7	3.1	8.0	2.7	1.34
Spatial Memory	5.7	2.9	8.0	1.2	11.4	3.4	5.75*
Matrix Analogies	6.0	2.5	6.5	0.7	7.4	1.4	0.94
Sequential Processing (Immediate Recall Memory)							
Number Recall	7.8	2.2	9.1	2.9	13.2	1.9	18.70***
Hand Movements	8.3	2.4	11.9	2.9	14.3	4.6	9.46***
Word Order	6.1	2.1	7.7	2.5	8.1	1.2	2.12
Global performance							
Sequential Processing	86.9	12.3	105.2	14.3	122.7	21.9	14.03***
Simultaneous Processing	71.1	10.4	81.0	6.6	96.5	17.5	13.76***
Mental Processing	75.1	10.9	89.6	9.6	109.0	21.2	16.32***
Nonverbal total	70.4	9.3	86.0	7.1	91.6	12.9	4.93*

*$p < .05$. **$p < .01$. ***$p < .001$.

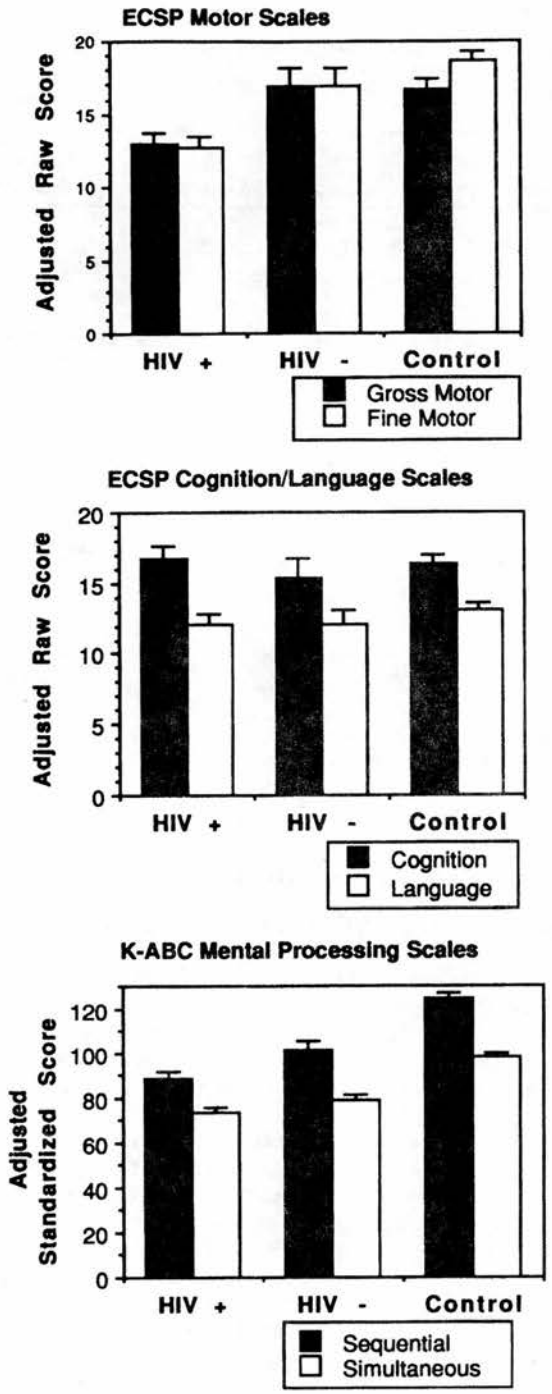

Figure 3. Group means and standard errors on the Early Childhood Screening Profiles (ESCP) and on the Kaufman Assessment Battery for Children (K-ABC) for HIV-positive, HIV-negative, and control children in Study 2. The plotted values represent the raw score totals adjusted for age and the Quaker Arm Circumference (Arm Circumference ÷ Height) for physical development.

tended to monitor very specific developmental effects of subacute infections, such as in the very initial stages of HIV infection (Goldman, Stein, & Guerry, 1983). The assessment protocol in the second study, however, was intended to provide for a more comprehensive and detailed measurement of both cognitive and motor processes that might be affected by pediatric HIV infection in children who were still asymptomatic.

In comparing the HIV-positive with HIV-negative children, consistent deficits were noted for the HIV-infected children with respect to the sequential and simultaneous processing abilities assessed with the K-ABC. Based on the use of the K-ABC with Zairian children in other health intervention settings and on reports by Diamond et al. (1987) and Belman et al. (1988), who used the K-ABC with HIV-infected children, spatial memory seems to be the subtest most sensitive to the direct impact of the virus on the neurological integrity of the developing child. Significant differences in motor development also emerged in the second study, which confirms some of the major findings in the first study even though entirely different tests and samples were used. However, these findings cannot be considered definitive because of the small size of the sample of HIV-positive children. Also, our sample was selective in that these were African children who were asymptomatic with respect to the immunodeficiency associated with the initial stages of AIDS and who were socially responsive enough to complete the assessment. Such a sample may represent only those children with a static subacute course of pediatric HIV infection, rather than those with an acute progressive encephalopathic course for the disease (Belman et al., 1988).

The control group of HIV-negative children born to negative mothers differed from the HIV-negative Kimpese group born to infected mothers on K-ABC global cognitive performance measures. This finding suggests that at least a portion of the deficits seen in the HIV-positive children was due to the effects of HIV infection on the health of the mother and the subsequent developmental integrity of the home environment. On the other hand, the fact that the control group did not differ from the Kimpese group with respect to motor development and performance suggests that the motor deficits that have been consistently observed in other studies (Belman et al., 1988; Diamond et al., 1987) are probably the direct result of HIV infection in children's early development.

In cases of pediatric AIDS, basal ganglia calcification has been documented (Price et al., 1988). Even in cases where calcification is barely detectable, T2-weighted magnetic resonance imaging showed increased signal in this region, which indicates that even in the pre-AIDS stage, the virus is attacking important motor regions of the brain directly (Belman et al., 1988; Epstein, Sharer, & Goudsmit, 1988). Thus, central nervous system motor centers are being affected in the early stages of the disease and perhaps contributing to the neurodevelopmental delays consistently seen in HIV-infected children even in the absence of substantial immunodeficiency. This dynamic of the disease process is likely a contributing factor in the motor developmental deficits witnessed for the HIV-positive children in our studies.

Language and global cognitive deficits on the part of the HIV-positive children in this study were possibly due to both the direct effects of the virus on neuropsychological development and the impact of maternal infection on the favorability of the home environment for the child. However, Pizzo et al. (1988) achieved significant improvement in global cognitive ability and social development level for children with AIDS in

Table 5
Between-Groups Comparisons for the Raw Scores for the Global Measures from the Kaufman Assessment Battery for Children (K-ABC) and Motor Development from the Early Childhood Screening Profiles (ECSP) for HIV-Positive (n = 11), HIV-Negative (n = 15), and Control (n = 15) Children in Study 2

Measure	HIV-positive		HIV-negative		Control		HIV group[a]	Age × Sex[b]	Covariate[c]	Pairwise comparisons
	M	*SD*	*M*	*SD*	*M*	*SD*				
K-ABC										
Sequential Processing							12.11***	1.35	3.50	ab*, ac***, bc***
Raw score	18.6	3.5	22.4	3.2	30.3	4.8				
Covariate-adjusted score	18.5	5.3	21.1	6.1	32.0	4.9				
Simultaneous Processing							20.54***	2.58	2.09	ac***, bc***
Raw score	19.7	4.9	22.3	2.8	31.0	5.8				
Covariate-adjusted score	22.0	5.5	22.4	6.2	30.6	5.0				
Mental Processing Comprehension							20.36**	2.22	3.24	ab*, ac***, bc***
Raw score	38.4	6.7	44.9	4.9	61.3	9.4				
Covariate-adjusted score	40.3	9.5	43.9	10.8	62.7	8.7				
Nonverbal total							7.31**	1.06	6.24*	ac***, bc**
Raw score	20.3	5.6	23.4	3.1	29.2	6.2				
Covariate-adjusted score	22.6	7.2	24.6	8.2	30.4	6.6				
ECSP Motor Development							6.90**	2.50	2.35	ac**, bc**
Raw score	28.9	9.4	27.9	7.2	35.5	4.6				
Covariate-adjusted score	27.8	8.0	28.5	9.1	36.1	7.4				

Note. For pairwise comparisons, a = HIV-positive group, b = HIV-negative group, and c = control group.
[a]Main effect (*df*s = 2, 23). [b]Blocking variables (boys more than 3 years of age, boys 3 or fewer years, girls more than 3 years, and girls 3 or fewer years; *df*s = 3, 23). [c]Weight standardized to height (*df*s = 1, 23).
*$p < .05$. **$p < .01$. ***$p < .001$.

response to continuous intravenous infusion of AZT. Because the home environment for these children was unlikely to have dramatically changed during the treatment, the effects of the drug on the virus as well as on the improved immunological and health status of the children may have been largely responsible for the improvements in global cognitive ability that were documented. These findings also emphasize the importance of evaluative pediatric HIV research with children who are not being treated with antiretroviral drugs if one is to understand the natural course of the infection on neuropsychological development.

For families that struggle to maintain their standard of living in rural subsistence agricultural settings, AIDS can have a devastating socioeconomic impact. HIV infection and illness on the part of the mother of an HIV-infected child can seriously undermine the social and nutritional favorability of that home environment, given that so much of it depends on the mother in sub-Sahara Africa. The fact that verbal ability and development for a child depends largely on the interactions that the mother provides and that a verbal deficit is a major feature of pediatric HIV infection (e.g., Condini et al., 1991) is consistent with our findings. Subsequently, such second-order effects may ultimately be as much at fault for the verbal, social, and global intellectual deficiencies of the HIV-infected child as the direct effects of the virus itself. To summarize, within the context of the overwhelming human and economic need associated with life for African children in the interior of Zaire, the additional burden of AIDS on family members can have devastating effects on the development of those children irrespective of their infection status.

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Stable seroprevalence of HIV-1 in antenatal women in rural Bas-Zaïre, 1988–1993

For many countries the HIV-1 seroprevalence rate in pregnant women is the most representative picture available of HIV infection in the general population. It is a valuable measure because it relates to the infection risk in both mother and baby and because it is an index of heterosexual transmission in the most sexually active age-group, without focusing on special risk groups.

In a longitudinal study of vertical transmission, plasma samples from 8733 pregnant women attending two maternity units in Kimpese, rural Zaïre, were tested for HIV antibody (Ab) during a 5-year period between September 1988 and July 1993. The study protocol was approved by both the regional paediatric ethical committee in Edinburgh and the National AIDS Committee in Zaïre. Kimpese is a town of about 30 000 people situated on the main road between Kinshasa (225 km) and the port of Matadi (140 km). The economy of this savannah region is based on agriculture with some commerce and two cement factories. Approximately 70% of all deliveries in the area were performed in the two maternity departments. A number of women from larger cities returned to Kimpese (their family home) for delivery but most were living in the town and and surrounding rural area. At their first visit, all women had a venesection for haemoglobin which was to be measured as part of the antenatal service. Samples were tested for HIV Ab using either the Abbott recombinant enzyme-linked immunosorbent assay (ELISA) HIV-1 + 2 or the Wellcome competitive ELISA (latterly Murex, Dartford, England, UK), and positive results were confirmed by the alternative ELISA test and Western blot (Organon Teknika, Cambridge, England, UK).

The overall prevalence of HIV Ab was 3.8% [95% confidence limits (CL), 3.4–4.2]. Ab-positive specimens were tested for HIV-2 during the first 3 years, but in no case was antibody to HIV-2 found. During the 5-year period the seroprevalence varied little, with a maximum of 4.2% in the first year and a minimum of 3.4% in the third year (Table 1). The difference between years was not significant (χ^2, 2.5; $P = 0.5$).

Table 1. HIV-antibody prevalence in women attending two antenatal clinics in Kimpese, Bas-Zaïre.

Date of first attendance	Number	% HIV-positive (95% CL)
1 Aug 88–31 Jul 89	2364	4.2 (3.4–5.0)
1 Aug 89–31 Jul 90	2530	3.9 (3.1–4.7)
1 Aug 90–31 Jul 91	2591	3.4 (2.7–4.1)
1 Feb 93–31 Jul 93	1248	4.0 (2.9–5.1)

CL, confidence limits.

Since 1985, seroprevalence studies of pregnant women have been conducted in a number of African countries. Direct comparisons between this study and others are difficult because of the different ways in which data have been collected. In general, surveys of HIV infection in antenatal women in Uganda, Zambia and Malawi have shown a rise to levels of 20–30%. In Kenya (Nairobi) and the Central African Republic (Bangui) the rise has been slower [1]. In a few countries with high levels there has been a tendency for rates to plateau. In antenatal women in the capital of Zaïre, Kinshasa, a more stable level at 5–6% has been reported [1]. Kaseka *et al.* [2] reported that 4611 spouses of factory workers in Kinshasa had rates of 2.7% in 1988, 3.7% in 1989 and 2.8% in 1990. Magazani *et al.* [3] have recently reported a marked contrast in seroprevalence rates between the Shaba region of Zaïre and the adjacent province of Zambia. In Shaba, 13 studies on 4285 antenatal women between December 1989 and April 1991 indicated a steady seroprevalence of 3% (range, 2.8–3.4%). In contrast in Zambia, a rate of 12% in Lusaka in 1987 [4] had risen to 21% by 1990 [5], and rural rates increased to 14% [6]. The stable rate in our study in Zaïre is, like that of Magazani *et al.* [3], in clear contrast to the results of most surveys in sub-Saharan Africa.

Our results, obtained by the same team in the same maternity units and covering the same population during a 5-year period, show that the rate was relatively low as well as being stable. Although an HIV seroprevalence of 3.8% still constitutes a major health problem, it is significantly less than that in several other African countries. The causes of this stability, whether biological or behavioural in origin, need to be investigated.

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