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GENOTYPIC CHARACTERIZATION OF HIV-1 ISOLATED FROM SEMEN AND BLOOD OF EARLY SEROCONVERT IDUs

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HIV-1 transmission rates among intravenous drug users (IDUs) in Thailand have remained high since 1988, followed by stable seroprevalence at approximately 30-40 % with continued high incidence. Estimated incidence from IDUs during 1995-1998 was 6.3 per 100 person-years at risk. Beginning in 1988, HIV-1 infections among IDUs consisted mainly of subtype B' virus, but recently the predominant cause of infection by subtype E virus. This study was conducted to determine the quantity, quality, and the relationship of proviral DNA and RNA genome in semen and blood of 46 early seroconvert IDUs. The shedding of HIV-1 DNA was detected by nested PCR. The quantity of proviral DNA and RNA was detected by competitive PCR and NUCLISENS QT TEST (Organon), respectively. Direct sequencing of *gag* and *env* gene was performed by automated DNA sequencing system.

HIV-1 proviral DNA was detected in 100 % of PBMC and 78.3 % of seminal cells by nested PCR *gag* gene. HIV-1 proviral *env* DNA were found 52.8 % of seminal cells samples. HIV-1 RNA was detected in 100 % of seminal fluid. V3 nucleotide sequences were 21.7 % of the IDUs samples were classified as subtype B', and 78.3 % classified as subtype E. There was no correlation between CD4+ cell numbers and HIV-1 RNA level in both plasma and seminal fluid.

Mean of proviral DNA level in PBMC and semen were 75.82 (±86.29) (range 5.86-321.24) copies/10^5 PBMC and 17.81 (±25.19) (range 2.11-89.13) copies/10^5 mononuclear cell, respectively. There was no correlation between HIV-1 proviral DNA level in PBMC and seminal cells, but the concentration of HIV-1 proviral DNA was significantly higher in blood than semen. The concentration of HIV-1 DNA subtype E was no higher than subtype B in blood and semen. The relationship between HIV-1 proviral DNA level of both PBMC and seminal cells was significantly correlated with HIV-1RNA quantity in plasma and seminal fluid.

The range of HIV-1 RNA level of seminal fluid from 2.48 to 5.27 log copies/ml and mean (±SD) was 3.92 (±4.44) log copies/ml. Mean of HIV-1 RNA subtype B was 3.40 (±3.46) log copies/ml and subtype E was 4.02 (±4.49) log copies/ml. HIV-1 RNA level subtype E was not higher than subtype B in semen.

Mean of *gag* gene in intrasubtype E divergence (interperson) and B' divergence was 4.06 % and 2.72 %, respectively. Mean of V3 region subtype E divergence (interperson) and B' divergence was 8.29 % and 16.25 % (±4.7 %, n=5), respectively. The phylogenic tree of *gag* gene showed a tight cluster in the blood and semen from the same person. The phylogenic tree of *env* gene showed a tight cluster within each subtype in semen. The most V3 crown motif predominant in subtype E was GPGQ, and next GPGR, but did not differ in subtype B. The compartmentalization of HIV-1 V3 and *gag* nucleotide divergence between the blood and semen showed a mean of (± SD) 3.34% (±3.85%), (0-6.67%, n=5), 0.88% (±0.54%), (0-2.24%, n=14). There is a compartment of HIV-1 in semen and blood (by V3 nucleotide divergence). The V3 protein sequences were not divergent between direct sequencing and cloning. This study might be one explanation in molecular epidemiology of HIV-1 for the need to develop an HIV-1 vaccine in Thailand

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ธวัช เสนาะสำเนียง : ลักษณะจีโนทัยป์ของเชื้อ เอช.ไอ วี 1 ที่แยกได้จากน้ำอสุจิและเลือดของผู้ฉีดสารเสพติดที่ติดเชื้อเริ่มแรก (GENOTYPIC CHARACTERIZATION OF HIV-1 ISOLATED FROM SEMEN AND BLOOD OF EARLY SEROCONVERT IDUs) คณะกรรมการควบคุมสอบ : รวงผึ้ง สุทเธนทร์, M.D., Ph.D.; ประเสริฐ เอื้อวรากุล M.D., Dr.Med.; วรรณี กัณฐกมาลากุล, Ph.D. 134 หน้า ISBN 974-664-592-7.

อัตราการติดต่อของเชื้อ เอช ไอ วี, ผ่านทางผู้ฉีดสารเสพติดในประเทศไทยยังคงสูงอยู่ตั้งแต่ พ.ศ.2531. อัตราการระบาดยังคงอยู่ที่ 30-40% ด้วยความสูงของอัตราเสี่ยงทั้งคงดำเนินต่อไป การประมาณอัตราการเสี่ยงของผู้ฉีดสารเสพติดตั้งแต่ปี พ.ศ.2538-2541 ประมาณ 6.3 ต่อ 100 คนต่อปี จุดเริ่มต้นในปีพ.ศ.2531 เชื้อ เอช ไอ วี1 ที่ติดต่อผ่านทางผู้ฉีดสารเสพยังคงเป็นสับทัยป์ บี แต่ปัจจุบันได้พบการระบาดจากสับทัยป์ อี เพิ่มขึ้น การศึกษาครั้งนี้เป็นการตรวจสอบหาทั้งปริมาณ, คุณภาพ และความสัมพันธ์ของ DNA และ RNA ในน้ำอสุจิและเลือดของผู้ฉีดสารเสพติดที่ติดเชื้อเริ่มแรกจำนวน 46 คน

จากการศึกษาสามารถตรวจสอบ DNA ของเชื้อ เอช ไอ วี 1 โดยวิธีปฏิกิริยาลูกโซ่โพลีเมอเรสขยายส่วนยีน *gag* ได้ 100% ในเลือด และ 78.3% ในน้ำอสุจิ และได้ใช้วิธีเดียวกันขยายส่วนยีน *env* ได้ 52.8% ในน้ำอสุจิ ตรวจพบ RNA ของเชื้อ เอช ไอ วี ได้ 100% ในน้ำอสุจิ โดยการหาลำดับเบสของส่วน V3 สามารถแยกสับทัยป์ได้ 21.7% เป็นสับทัยป์ บี (B') และ 78.3% เป็นสับทัยป์ อี (E) และพบว่าไม่มีความสัมพันธ์ระหว่างจำนวนเซลล์ CD_4^+ และปริมาณ RNA ทั้งในเลือดและน้ำอสุจิ

ตรวจสอบหาปริมาณ DNA ด้วยวิธี competitive PCR พบค่าเฉลี่ยในเลือดและในน้ำอสุจิเท่ากับ 78.52(± 86.29) (ตั้งแต่ 5.86-321.24) และ 17.81 (± 25.19) (ตั้งแต่ 2.11-89.13) ต่อ10^5เซลเม็ดเลือดขาว ตามลำดับและไม่พบความสัมพันธ์ระหว่างปริมาณ DNA ทั้งในเลือดและน้ำอสุจิ แต่พบว่าปริมาณ DNA ในเลือดสูงกว่าในน้ำอสุจิอย่างมีนัยสำคัญ ปริมาณ DNA ของเชื้อ เอช ไอ วี สับทัยป์ อี (E) ไม่สูงไปกว่าสับทัยป์ บี ทั้งในเลือดและในน้ำอสุจิ เมื่อเปรียบเทียบความสัมพันธ์ระหว่างปริมาณ DNA และ RNA ของเชื้อ เอช ไอ วี พบมีความสัมพันธ์กันทั้งในเลือดและน้ำอสุจิ การตรวจสอบปริมาณ RNA ของเชื้อ เอช ไอ วี ในน้ำอสุจิพบค่าเฉลี่ยเท่ากับ 3.92(± 4.44) (ตั้งแต่ 2.48-5.27) log copiesต่อมล. และปริมาณ RNA ของเชื้อ เอช ไอ วี สับทัยป์อี (E) พบค่าเฉลี่ยเท่ากับ 4.02(± 4.49) และสับทัยป์ บี (B') พบค่าเฉลี่ยเท่ากับ 3.40(± 3.46) log copiesต่อมล. ปริมาณ RNA ของเชื้อ เอช ไอ วี สับทัยป์อี (E) ไม่สูงไปกว่าสับทัยป์ บี (B')

ค่าแปรผันเฉลี่ยของยีน *gag* ภายในสับทัยป์อี (E) และ บี (B') ของกลุ่มฉีดสารเสพติดเป็น 4.06% และ 2.72% ตามลำดับ ค่าแปรผันเฉลี่ยของยีน *env* ภายในสับทัยป์ อี (E) และ บี (B') ของกลุ่มเดียวกันเป็น 8.29% และ 16.25% ตามลำดับ เมื่อหาความแตกต่างและวิวัฒนาการด้วยการสร้าง phylogenetic tree พบว่าในส่วนยีน *gag* มีความเกาะกลุ่มใกล้ชิดกันทั้งในเลือดและน้ำอสุจิภายในบุคคลเดียวกัน ส่วนของยีน env มีความเกาะกลุ่มกันภายในสับทัยป์เดียวกัน

ลำดับกรดอะมิโนในส่วนยอดของ V3 พบว่าส่วนมากทั้งสับทัยป์ อี (E) และ บี (B') เป็น ไกลซีน โปรลีน, ไกลซีน กลูตามีน (GPGQ) และลำดับกรดอะมิโนที่พบรองลงไปคือ ไกลซีน โปรลีน ไกลซีน อาจินีน (GPGR) ค่าแปรผันเฉลี่ยของยีน *gag* และยีน *env* ภายในในคู่ของเลือดและน้ำอสุจิของบุคคลเดียวกันมีค่าเท่ากับ 0.88% (± 0.54) และ 3.34% (± 3.85%) ตามลำดับ จากข้อมูลยีน *env* แสดงว่าเชื้อ เอช ไอ วี มีแหล่งกำเนิดต่างกัน(มีcompartment) ในขณะเดียวกันก็เปรียบเทียบลำดับเบสจากการหาโดยตรงกับการโคลนนิ่งพบว่าไม่มีความแตกต่างกัน การศึกษานี้เป็นส่วนหนึ่งในการศึกษาการระบาดของเชื้อ เอช ไอ วี ในระดับโมเลกุลได้เป็นอย่างดีเพื่อนำไปสู่การพัฒนาวิจัยวัคซีนต่อไป

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LIST OF ABBREVIATIONS

Abbreviation or symbol	
AMV-RT	avian myeloblastosis virus-reverse transcriptase
Bp	base pair
BSA	bovine serum albumin
°C	degree celsius
DMSO	dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
ECL	Electrochemiluminescence
EDTA	Ethylene diamine tetraacetic acid
ELISA	Enzymej-linked immunosorbent assay
GuSCN	Guanidine thaiocyanate
hr.	hour
IDUs	Intravenous Drug Users
IL-2	Interleukin-2
Kb	kilobase (1,000 base)
Kd	kilodalton
l	litre
M	molar
mg	milligram
min	minute

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LIST OF ABBREVIATIONS (Cont.)

ml	millititre
mM	milimolar
NASBA	Nucleic acid sequence-based amplification
ng	nanogram
nm	nanometer
nt	nucleotide
OD	optical density
PBMC	Peripheral blood mononuclear cell
PBS	phosphate buffer saline
PCR	Polymerase chain reaction
PHA	Phytohemagglutinin
pmol	picomolar
QL	qualitative
RNA	Ribonucleic acid
rpm	revolutions per minute
RT	reverse transcriptase
sec	second
TBE	Tris-borate-EDTA
TEMED	N,N,N',N'-tetramethyl ethylenediamine
TMB	Tetramethyl benzidine
TPA	Tripropylamine
U	Unit
ug	microgram

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LIST OF ABBREVIATIONS (Cont.)

ul	microlitre
UV	Ultraviolet
v/v	volume by volume
w/v	weight by volume
ZDV	Zidovudine

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CHAPTER I

INTRODUCTION

The explosive initial phase of the epidemic of human immunodeficiency virus type 1 (HIV-1) infection in Thailand occurred in 1988 among injection drug users (IDUs) in Bangkok, the capital, presumably as a result of transmission via the sharing of injection equipment (1,3). No infection was detected among IDUs during 1985 to mid-1987. The first HIV infections in IDU were found at prevalence rates of about 1 % (2) during the latter half of 1987 when routine HIV testing of all patients began in the Ministry of Public Health's (MOPH) Thanyarak Hospital. In early 1988, a sudden rapid increase in HIV prevalence was noted among drug users in Bangkok, rising from about 1 % at the start of the year to 32-43 % by August-September 1988 (3). From 1989 through 1993, seroprevalence among persons in opiate treatment programs stabilized at approximately 35 % to 40 % (3). Existing data indicated that heroin is the principal drug used by 97 % of persons seeking drug treatment in Bangkok and that 89 % of such persons consume drug by injection.

By May 1999, there were an estimated 114,837 AIDS cases reported, and 80.70 percents were attributed to heterosexual HIV transmission, 5.18 % to IDUs, 4.96 % to perinatal transmission, 1.21 % to male homo/bisexual transmission, 0.04 % blood transmission and 7.09 % of other or unknown. Males accounted for 83 % of AIDS cases and females, 17 % (Ministry of Public Health, Thailand May 31, 1999). Approximately

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95 % serotype of heterosexually acquired cases in Thailand were subtype E and 5 % was serotype B′ (4). Although HIV1 subtype B was first found to be the common subtype among IDU group, recently were infected cases among IDU group in Thailand was reported to be infected by HIV-1 subtype E for 75 % (5).

Sexual transmission has a major role in the spread of human immunodeficiency virus type1 (HIV-1). The semen of infected men may contain high level of HIV-1, and infectious viruses can be recovered from seminal cells or seminal fluid of these men. Seminal cells are mixture of spermatozoa, precursor of germ cells, T lymphocytes, macrophages, and epithelial cells. HIV-1 proviral DNA has been detected in several types of these cells, it was never detected in motile spermatozoa or immature germ cell population (6). The level of HIV-1 RNA in seminal fluid can also be correlated with the level in plasma (7) but, Coombs et al. found that there were weakly correlation (8). Virus can be isolated from the cellular fraction of semen (9) but more rarely from the seminal plasma (10). HIV variants found in acute seroconverters infected by sexual contact predominantly match those from the seminal cell fraction of the transmitter and detection of HIV in semen is strongly correlated with seminal leukocytosis (>$1x10^6$ leukocytes/ml of semen) (11). Both virus infected $CD4^+$ lymphocytes and macrophage, but not germ cells, are play in major sources of HIV transmission in semen (6).

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CHAPTER II

OBJECTIVES

This study was conducted according to these following objectives

1. To detect HIV-1 shedding in quantitative and qualitative of proviral DNA and RNA genome in blood and semen of intravenous drug users (IDUs) seroconverter.
2. To study the correlation between peripheral CD_4^+ cell count and HIV-1 proviral DNA/RNA copy numbers in semen.
3. To compare *gag* and *env* V3 nucleotide sequence of HIV-1 isolated from blood and semen of IDUs seroconverter.

Information of HIV-1 proviral DNA in blood and semen, HIV-1 RNA level in seminal plasma, peripheral CD4 cell count and the variation of *gag/env* gene in blood and semen from this study will provide a better understanding of the transmission, epidemic and diseases progression of HIV-1 in the IDU groups.

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CHAPTER III

LITERATURE REVIEW

Human immunodeficiency virus (HIV), the causative agent of acquired immunodeficiency syndrome (AIDS) was first reported in 1983 under the name human T-lymphotropic retroviruses-III (HTLV-III) (11). Later, the virus was given the name human immunodeficiency virus by the International Committee on Taxonomy of Virus in the year 1986 and was recognized as a number of Lentivirinae a subfamily of the Retroviridae (12). Two major types of AIDS viruses, HIV-1 and HIV-2, can be distinguished and have been described (13). HIV-1 is associated with the AIDS epidemic in central Africa, Haiti, western Europe, Asia and the United State while HIV-2 is endemic in western Africa. A classification scheme, best on the viral C2-V3 envelope sequences, 11 sequence subtypes (or clades) of HIV-1 (A to J and O) have been identified in the world, as a major group (group M), outlineres groups (group O) and non group M non group O (group N). A biologic different involving the capsid (CA) protein has been described between the HIV-1 group M and group O viruses. For group N, the structural gene and *tat*, *vpr* and *nef* were approximately equidistant from group M and SIV cpz-gab but, *vif* and *rev* gene were closer to group M, and vpu gene was highly divergent (14).

Subtype A is found primary in central Africa, subtype B in North America, Japan, Australia, the Caribbean and Europe, subtype C in South Africa and India, subtype D in central Africa, and subtype E in Thailand and Southeast Asia countries, subtype F include a few isolates from Brazil (15). Each subtype differ from the others in amino acid

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composition by at least 20 % in the envelope region and 15 % in the Gag region. Within each subtype, the differences in Env can be up to 10 % and those in Gag can be up to 8 % (16).

Viral genome structure and organization

Infectious virions of HIV contain two identical copies of single stranded RNA, about 9.2 Kb long. In the early stages of infection, the virion RNA genome is converted into double-stranded linear DNA by reverse transcriptase (RT), which involves two strand-transfer steps to synthesize linear viral DNA with long terminal repeats (LTRs) and integrated into the host cell genome to produce the provirus. HIV encode several gene for precursor polypeptide of viral proteins (Fig.1; Table1).

Table 1 Genes and proteins of primate lentiviruses

Gene[a]	Dispensable for Replication	Protein	Function	Localization
gag	No	$Pr55^{gag}$	Polyprotein precursor for virion core proteins MA (p17), CA (p24), NC (p9), p7	Virion nucleocapsid
pol	No	$Pr160^{gag\text{-}pol}$	Polyprotein precursor for virion enzymes PR: p10, RT and RNAse-H: p51/66, IN: p32	Virion (nucleocapsid?)
vif	Yes[b]	p23	Viral infectivity factor, function unresolved	Cell cytoplasm
vpx[c]	Yes	p16	Virion protein, function unresolved	Virion
vpr	Yes	p15	Virion protein, function unresolved	Virion
tat	No	p14	Transcriptional transactivator, binds TAR and cell factor(s) (initiation and elongation of viral transcripts)	Primarily in cell nucleus
rev	No	p19	Posttranscriptional transactivator, binds RRE and cell factor(s) (splicing and/or transport and translation of viral mRNA)	Primarily in cell nucleus
vpu[d]	Yes	p16	Influences virus release, augments turnover of CD4 antigen	Integral cell membrane protein
env	No	gp160	Precursor for envelope glycoprotein: SU (gp120): CD4 receptor binding, TM (gp41): membrane fusion	Virion envelope, plasma membrane
nef	Yes	p27	Negative effector?, downregulates CD4 receptor, influences T-cell activation, enhances virion infectivity	Cell cytoplasm, plasma membrane

(Luciw PA, Fields Virology Third Edition: p1885, 1996)

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Virion structure

Virions have a spherical shape, are about 110 nm in diameter, and consist of a lipid bilayers membrane or envelope that surrounds the cone-shaped nucleocapsid (Fig.1). The virion is composed of two molecules of the viral single-stranded RNA genome encapsulated by proteins proteolytically processed from the gag precursor polypeptide. The *gag* gene products are the matrix protein (MA), which is presumably located between the nucleocapsid and the virion envelope; the major capsid protein (CA), which forms the capsid shell; and the nucleocapsid protein (NC), which binds tightly to the viral RNA genome. Viral enzymes derived from the *pol* gene precursor polypeptide are also packaged into virions; these enzymes are PR, RT and IN. Reverse transcriptase is a heterodimer composed of p51 and p66 subunits. Reverse transcriptase (and RNaseH) and IN are contained within the nucleocapsid in mature virions. The *env* gene encodes the precursor for envelope glycoprotein (Env gp), which is a heterodimer consisting of the gp120 and gp41 subunits held together by noncovalent bonds (Fig.2) (17). Gp120, also designated the SU subunit, is a highly glycosylated, hydrophilic protein positioned on the external surface of virion membranes as well as plasma membranes of infected cells. Gp 41 (TM), is classified as a type 1 integral membrane protein and adopt a coiled coil conformation which facilitates insertion of the gp41 fusion peptide into the target cell membrane (18).

The transcriptional transactivator (*tat*) and regulator of viral expression (*rev*) genes are each encoded by two overlapping exons and produce small non-virion proteins, which are essential for viral replication. Non essential genes also designated " accessory" or

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"auxiliary" genes encoded by HIV-1 are *vif, vpr, vpu,* and *nef*; HIV-2 and SIV encode *vif, vpx* and /or *vpr* and *nef*. The proviral genomes of primate lentiviruses display a high content of adenosine deoxyribonucleotide (A) residues (38%-39%) and low frequency of cystidine deoxyribonucleotide (C) residues (16 %-19 %). The preference for A residues is a feature of all lentiviruses (19).

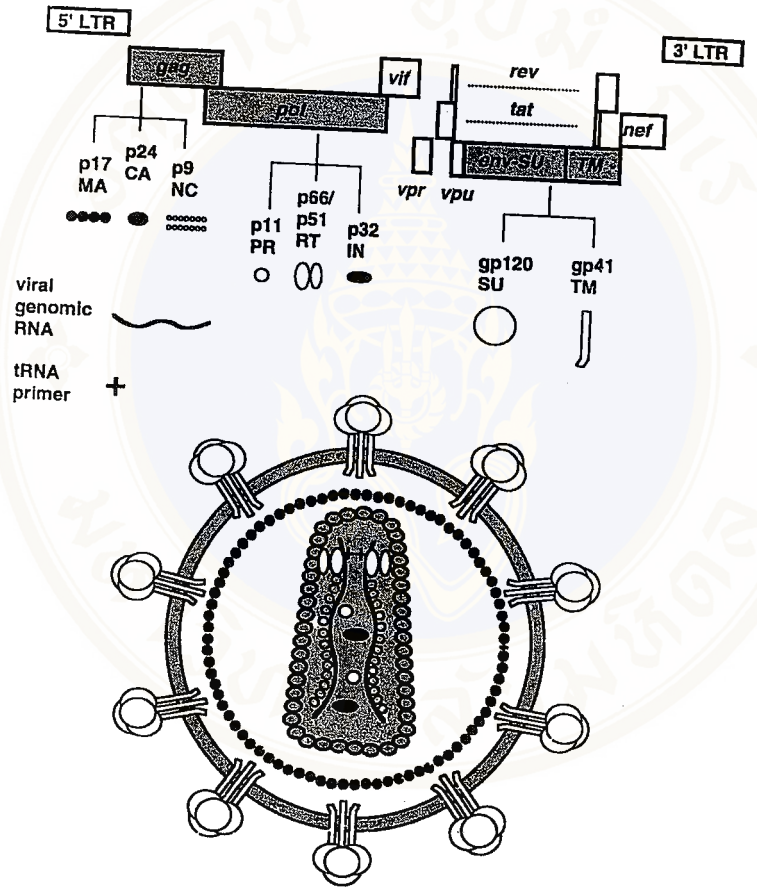

Figure 1 Virion structure of HIV (Luciw PA, Fields Vilology Third Edition: p1885, 1996)

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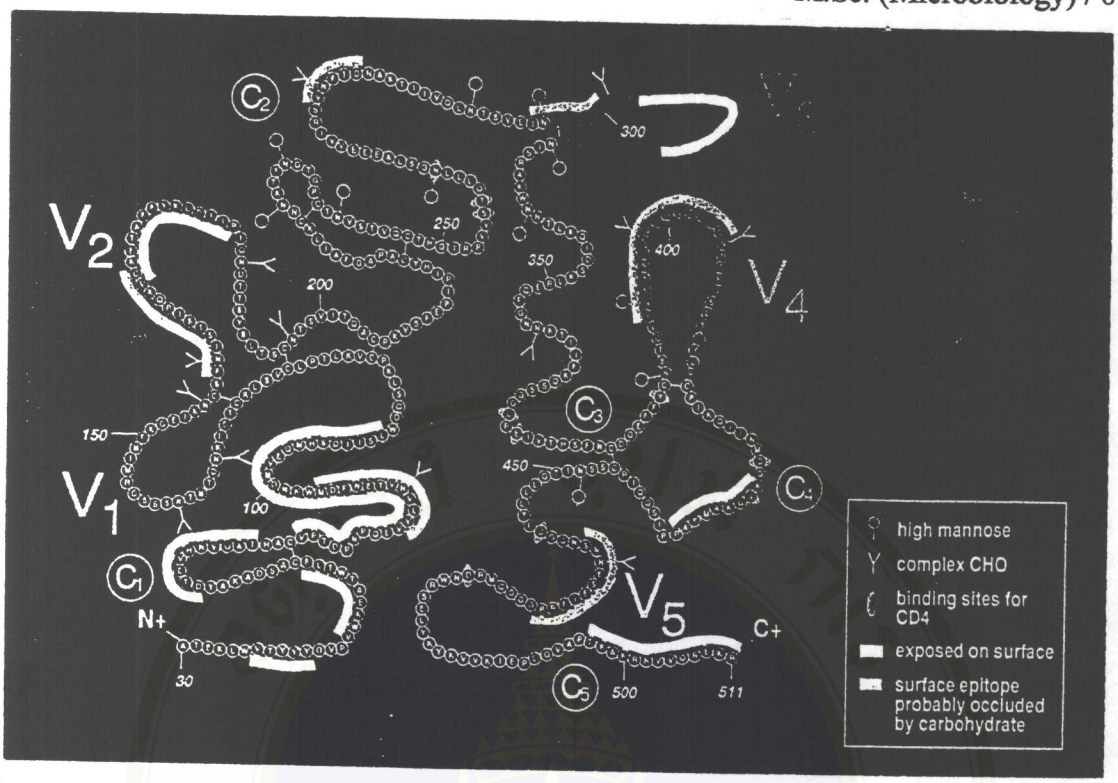

Figure 2. Schematic pattern of HIV-1 Env gp. Nine disulfide bonds have been identified.

(Levy JA, HIV and the pathogenesis of AIDS Second Edition: p189, 1998)

Sequence Variation in the *env* Gene

Comparison of the sequences of *env* genes from numerous HIV-1 isolates reveals a pattern of five variable regions interspersed with conserved regions for the gp120 subunit; in contrast, the gp41 subunit shows less heterogeneity and is thus more highly conserved (Fig.3) (20). This sequence variation consists of nucleotide changes, which produce amino acid substitutions, as well as small deletions and insertions. Up to 25% of the amino acids encoded by *env* may vary in HIV-1 strains from geographically separated locations (interpatient variation). Currently, molecular epidemiology surveys, based on *env* sequences (C2-V3) of numerous HIV-1 isolates, reveal a minimum of 11 distinct

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viral subtypes (or clades) of HIV-1 (A to J and O) in the AIDS pandemic (20). Neutralizing antibodies may either block binding of gp 120 to the CD4 receptor or may inhibit virion entry at a step after attachment.

The V3 Domain of Env gp

The V3 sequence, a variable domain in the gp102 subunit of HIV-1, contains 35 amino acids arranged in a disulfide loop involving Cys301 and Cys336 (Fig.3) (21). This domain plays an important role in governing several biologic properties of the virus (i.e., cell tropism, cytopathicity, and fusogenicity), and deletions in the V3 loop abrogate viral infectivity. In infected individuals, B-cell as well as T-cell responses are directed to the V3 loop (22); this region has been designated the principal neutralizing determinant because virus neutralizing antibodies are elicited by peptides encompassing the V3 sequence. Because synthetic peptides representing V3 sequences block HIV-1 infection of lymphocytes and macrophages.

The Gly317- Pro318- Gly319-Arg320-Ala321-Phe322 motif, located at the top (or crown) of the loop, is highly conserved among HIV-1 isolate, and sequences near the Cys300 and Cys336 at the bottom of the loop also show relatively little variability (Fig.5). Secondary structure predictions that the HIV-1 V3 loop folds into a β-hairpin structure (19), and x-ray diffraction studies on crystals of V3 peptides complexes with Fab fragments of virus neutralizing antibodies reveal that the Gly317- Pro318- Gly319-Arg320- Ala321- Phe322 motif adopts an S-shaped double turn (23). Analysis of several HIV-1 isolates in clade B shows that the majority of sequence changes are in the 10 to 12 amino acids on either side of the conserved crown of the loop; these changes are not

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random because substitutions are conservative and usually involve amino acids with similar chemical properties (21).

Figure 3 V3 loop of HIV-1 Env. $G^{15}P^{16}G^{17}R^{18}A^{19}F^{20}$ at the crown of the loop is a stretch of relatively conserved amino acids.

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VIRAL ACCESSORY GENES

The accessory genes of HIV (*vif, vpr, vpu,* and *nef*) and HIV-2/SIV (*vif, vpx* and/or *vpr*, and *nef*) are exclusive of the *gag, pol*, and *env* genes and the viral tranactivators *tat* and *rev* (Fig.1 and Table 1)(24).

vif

Vif has not been detected in virions, but encodes a protein necessary for production of infectious virions.

vpr

Vpr is assembled into virions, this protein may play a role in an early step in the viral life cycle such as nuclear localization of the viral preintegration complex.

vpu

Vpu has functional helps in permitting efficient budding; virus releases and disrupts gp 160-CD4 complex (25).

nef

Nef is multifunctional and exerts pleiotropic effects (e.g., enhancement of virion infectivity, activation of T-cells, CD4 downregulation, and association with a cellular serine kinase). However, several studies on *nef*-deficient viruses in other HIV-1 strains as well as in SIV_{mac} demonstrated that this gene either had no effect or had a positive effect on viral replication

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Viral Tropism

The CD4 receptor, present on most T-helper cells, many cells of monocyte-macrophage lineage, and certain other cell types, appears to be the principal receptor for attachment of both HIV-1 and HIV-2.

The virus an individual carries broadens in tropism and biologic variability. Small changes in the envelope glycoprotein amino acid composition can lead to large differences in phenotype (26). Although initial infection may be limited to macrophage-tropic virus incapable of causing syncytia *in vitro* (nonsyncytium- inducing, NSI), sequence variation occurs rapidly (27). Although a predominant HIV species is maintained over time, swarms or quasispecies of subtly altered viruses emerge with broadened tropism (lymphocytes and macrophages) and increased cytopathic capacity (syncytium-inducing, SI) (28). The appearance of SI viruses often heralds worsening disease and heightened replication rates, with decreases in the numbers of circulating CD4 lymphocytes. The rates at which these events occur vary among individuals, but increased viral burden and increased viral cytopathicity appear closely correlated with CD4 cell decline (Table 2) and poor prognosis (28) and, Hart CE et al. reported that the amount of cell-free HIV-1 RNA in blood plasma was significant invert correlation with CD4+ T lymphocyte count (29), with similar to the effect observed in blood plasma, initiation of antiretroviral therapy significantly reduced the amount of HIV-1 RNA in vaginal secretion (29) and semen (30)

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The factors that relate to cytopathicity, cell regulatory factors are also critical. Cell factors such as nuclear factor kB, NFAT, AP-1, and tat-binding proteins interact with regulatory HIV genes and influence virus replication (31).

Table 2 Titer of infectious HIV-1 recovered from plasma in relation to $CD4^+$ cell count

$CD4^+$ cells/mm^3	No. of individuals	Range of HIV titer (TCID/ml)	Mean of HIV titer (TCID/ml)
≥500	13	1-500	114
300-499	8	1-500	205
200-299	4	25-500	381
<200	8	25-5,000	1,466

(Levy JA, HIV and the pathogenesis of AIDS Second edition: p18, 1998)

CD4 cell count in IDUs

Evidence for CD4 cell count was suggested by Coombs et al. (8) that noted a weak correlation between viral RNA level in semen and CD4 cell count in blood, difference in the biologic variability of viral RNA level, and difference in the viral load response to antiretroviral therapy. The early post-infection viral levels were somewhat higher for subtype E than for B', but CD4 cell counts were similar all time points (32). The clinical implication that markers of systemic HIV infection, such as CD4 cell count or HIV-1 RNA level in the blood plasma, cannot be used alone to accurately predict HIV-1 RNA levels in the semen. Peripheral CD4 cell count showed a significant inverse correlation

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with HIV-1 DNA in blood and semen (33). Govitrapong P et al. suggested that heroin addicts and short period of heroin withdrawal have decreases in their immune system functioning and that the heroin withdrawal subjects seem to gradually reverse their immunological parameters to normal levels when withdrawal was sustained ≥ 2 years (34). While Carr DJ et al. showed that both infrequent and daily morphine exposed monkeys were decreases (10%) in the percentage of $CD4^+$ circulating lymphocytes and an increases (19%) in the percentage of $CD8^+$ cell however, monkeys exposed daily to morphine showed 30% increase in the helper-inducer $CD4^+$ lymphocyte, $CD4^+CD29^+$, compared to untreated controls (35). $CD4^+$ T-cell counts invariably falls with progression of HIV infection.

Therefore, any treatment, which results in an increase in CD4+ T-cell count should, in theory, be associated with clinical improvement. Indeed, changes in CD4+T-cell count in asymptomatic patients during treatment with zidovudine (ZDV) have been shown to correlate significantly with disease progression and survival. However, CD4+T-cell count appears to be an incomplete marker of response to therapy and clinical outcome (36).

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Routes of transmission

Sexual transmission, parenteral inoculation or transfusion of blood and blood products, and perinatal transmission remain the principal routes of viral spread. The relative concentrations of HIV in various body fluids were shown in table 3.

In the United States, anal intercourse between men has been the most common mode of transmission, whereas in Africa, the Caribbean, and Asia, heterosexual vaginal intercourse appears the dominant mode of transmission. HIV-1 can be isolated from both semen and female genital secretions (36), and in both it appears to be largely cell-associated.

Table 3 Isolation of infectious HIV from body fluid

Table 2.1 Isolation of infectious HIV from body fluids[a]

Fluid	Virus isolation	Estimated quantity of HIV
Cell-free fluid		
Plasma	33/33	1–5,000[b]
Tears	2/5	<1
Ear secretions	1/8	5–10
Saliva	3/55	<1
Sweat	0/2	—[c]
Feces	0/2	—
Urine	1/5	<1
Vaginal-cervical	5/16	<1
Semen	5/15	10–50
Milk	1/5	<1
Cerebrospinal fluid	21/40	10–10,000
Infected cells		
PBMC	89/92	0.001–1%[b]
Saliva	4/11	<0.01%
Bronchial fluid	3/24	ND
Vaginal-cervical fluid	7/16	ND
Semen	11/28	0.01–5%

(Levy JA, HIV and the pathogenesis of AIDS Second Edition: p17,1998)

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In cervical biopsies, HIV antigens are detectable in inflammatory cells (37). In both homosexual and heterosexual intercourse, the insertive partner appears less at risk than the recipient. Whole blood, cellular blood components, plasma, and clotting factors have all transmitted HIV-1 infection HIV-1 infection, whereas other blood products (e.g., immunnoglobulin, albumin, and hepatitis B vaccine) have not been implicated (38). Among The first group of individuals showing evidence of HIV infection were intravenous drug users (IDUs) in the United States and Europe. The seroprevalence in this group is now estimated to be about 50 to 60 % (35,39)

The epidemiology of HIV infection IDUs in Europe and Southeast Asia

Human immunodeficiency virus is transmitted among injecting drug users through use

of contaminated needles and other equipment. Risk factors include frequency of needle sharing, frequency of injections, use of "shooting galleries," In 1999, 114,837 people in Thailand living with HIV infection, 5.18 % were attributed to injection drug use (Ministry of Public Health, Thailand May 1999).

Epidemic in Europe

Homosexual men and IDUs form two major risk groups for HIV-1 infection and AIDS in the United States and in most of the European countries (40). During 1990-1995, incidence increased an average annual rate of 11% overall and >23% in central and Eastern Europe (41). Kuiken et al. Show on the basis of the analysis of *vpr*, *vpu* and gp

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120 V3 sequence that HIV-1 strains circulating among IDUs in Northern Europe differ from those in homosexual (42), and suggested that HIV-1 epidemics in homosexual men and IDUs in Northern Europe were established from difference source. About half of U.S. IDUs HIV-1 sequence share these signature patterns and cluster together with Northern European IDU sequence. The V3 sequence from both Dutch homosexual men and IDU (40).

Since 1995, there is evidence of rapid HIV spread in Eastern Europe with estimates suggesting between 50 % and 90 % of new HIV infections among IDUs (43). The former Soviet Union republics, 83 %, 17 % of IDUs were subtype A and B (wide spread in U.S.), sequence data showed a marked intrasubtype homogeneity of HIV-1 (the average means of interpatient genetic distance were 1.1 and 1.7 % (in the *gag* gene) or 1.8 and 2.3 % (in *env* gene) for subtype A and B, respectively (44).

Epidemic in Southeast Asia

The earliest HIV infections in Asia in the early 1980s were related to sexual or needle-sharing contacts with persons from outside the region. For a few years, there was limited transmission, but in the late 1980s extensive HIV transmission occurred in a number of settings, most notably in Thailand. The "Golden Triangle" region of northern Thailand, Myanmar, and Laos. This region has long been one of the world's leading opium and heroin producing areas (46). HIV transmission among IDUs has emerged as a major problem in around of this region. Another mobile population at risk of HIV infection of IDUs are fishermen (from Thailand, Myanmar and Cambodia) or truck drivers, within their own countries and when crossing borders.

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In Vietnam, The prevalence of HIV among injecting drug users rose dramatically from 1 % in 1992 to 39 % in 1996, compared with 1.2 % among sex workers, 0.3 % among blood donors and 1.3 % among tuberculosis patients in 1996 (47). The population of IDUs in Ho Chi Minh City are estimated to be 30,000, HIV-1 subtype E sequences identified in 9 of the 12 IDUs from northern provinces were closely related phylogenetically to those in IDUs in nearby Guangxi Province of China (48) genetic subtyping based on the env C2/V3 sequence of HIV-1 subtype E predominated throughout Vietnam in all risk population (49). The inter strain genetic variation among the Vietnam HIV-1 env sequence ranged from 0.3 % to 9.0 % (mean, 4.6 %). Phylogenetic analysis verified that some of the Vietnam HIV-1 strains from discrete clusters and were indistingquishable from other southeast Asian strain (48). In Malaysia, report in 1994 showed that HIV infected in Malaysia predominantly occurred in IDU accounting for 78 % of total infected cases, which mainly, identified as subtype B′ (50), Brown et al. found HIV-1 subtype B′, C and E in Malaysian IDUs between 1992-1993 but subtype E, which were similar to Thai subtype E, were major subtype of in Malaysian IDU in 1996. The genetic diversity of B' nucleotide sequence ranged from 1.5 to 5.8 % (mean 2.8 %) compared to 1.5 to 8.2 % (mean 4.6 %) for Thailand sequence (50). The Malaysian sequence showed >90 % identity to the Thai sequence, which are known to be gag subtype A/env subtype E (51).

In Myanmar, extremely high prevalence rates among IDUs in northern Myanmar may be related to the HIV spread in southwestern China (52), along the trafficking route "Golden triangle" near the borders of Thailand, Myanmar and Laos, HIV-1 subtype B′ was found both in IDUs and heterosexuals in the capital city Yangon, the interperson

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nucleotide sequence variations in env C2/V3 regions of B' and E, prevailing in Yangon were 6.7 ± 2.1 % and 7.1 ± 0.7 % respectively, which was similar to those level observed in Thailand (53).

Thailand

The prevalence of HIV infections among IDUs has started in early 1988 at Bangkok (primary iv heroin users), which receiving opiate detoxification treatment at the Thanyarak Hospital for Drug Treatment, near Bangkok and at Bangkok Metropolitan Administration drug treatment clinics (54). HIV seroprevalance among drug users has been reported to be 30 to 40% throughout Thailand since the first sentinel surveillance study in June 1989 (55). Despite this stable prevalence the incidence of HIV among drug users seen repeatedly at drug treatment centers in Bangkok has been high; incidence varied from 20 per 100 persons/year in late 1987, to a peak of 57 per 100 persons/year in 1988 to stable incidence of 11 per 100 person/year in 1991 and 1992 (56).

In northern Thailand, during 1993-1995, with patient drug treatment program, 60 patients presenting for detoxification acquired HIV-1 infection, for a crude incidence rate of 18.6 per 100 person-year. HIV-1 incidence varied by the current route of drug administration 31.3 per 100 person-year for injectors and 2.8 per 100 person-year for noninjectors (smoking and ingestion) (57). Significant differences were found by ethnicity: HIV-1 incidence was 29.3 per 100 person year for Thai lowlanders and 8.5 per 100 person-years for hill tribes (57). A prospective cohort study of HIV-negative IDUs in Bangkok measured an HIV-1 incidence rate of 7.9 persons/year of follow-up, despite methadone treatment and other interventions, during 1995 to 1997 (58).

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The HIV-1 strain that infected approximately 75 % of IDUs clustered phylogenetically with env subtype B viruses typically found in the Americas and Europe, than African isolates but was genetically distinct from other subtype B strains. This strain was originally named Thai genotype B "referred to as subtype B′ " (59). In 1994 and 1995, after 7 years of extensive HIV-1 transmission in Thailand, only subtype E and B, with very limited intrasubtype variation, among a geographically diverse collection of viruses from IDUs and persons who were infected via sexual transmission. Among 65 IDUs were due to a mix of subtype E (45%) and subtype B (55%) of 39 total subtype B infections, 37 (95%) were B′ strains (Thai B) (60).

In 1991, 76% of infections among IDUs were due to subtype B, but later have reported increasing proportions of subtype E infection among newly infected IDUs in the Bangkok area (61) and among IDUs in other regions of Thailand (northern and southern Thailand) from 1992 to 1995 (59). It was possible that the northern Thai epidemic of subtype E first started among IDUs and then quickly spread to female sex workers (FSWs), while subtype B′ accounted for most strains in central and northeastern Thailand (60). Subtype E viruses are increasing among the HIV-infected IDU population. Factors contributing to this predominance are incompletely explained and Don C et al. Reported that "The high percentage of subjects reporting no sexual partners, the small number of subjects with casual partners, and the increasing consistent condom use with casual partners all indicate that IDUs in Bangkok are not likely to become infected through sexual activities (62).

During 1995-1997, estimated incidence from a prospective cohort of IDUs attending 15 BMA methadone clinics was 8.1 per 100 per years at risk (63). Through

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December 1998, for samples from IDUs were 79% subtype E and 21% subtype B' (64), with an estimated seroincidence rate of 5-8 per 100 persons year of follow up. Factors associated with HIV seroconversion in a proportional hazard model were: daily injection of heroin, daily injection of heroin plus sharing of equipment, being incarcerated since last visit and drug injection while incarcerated since last visit (64). Comparisons of the amino acid sequence of the V3 1oop of HIV-1 in Thailand in 1992 demonstrated that HIV-1 strains within a subtype were closely related to each other. The average intraperson variation for subtype A/E and B were 2.0% and 2.7%, respectively and average interperson variation within subtype were 3.8% and 3.7%, respectively (65). The average interperson variation between subtype A/E and B from difference person was 18.1% (65).

The inter-strain nucleotide distance (C2-V4) within subtype E strains was low (mean = 6.8%), and pair-wise comparisons to a prototype subtype E strain CM244 showed limited divergence (mean = 5.6%), with a narrow spectrum of genetic diversity (66). The subtype B strains showed greater inter-strain divergence (mean = 9.2%) and were significantly divergent from the prototype B strain HIV-MN (mean = 13.0%), the subtype E strains had significantly lower mean V3 loop charge than did subtype B strains and, based on analysis of amino acid sequence, were predicted to be predominantly (91%) non-syncytia inducing (NSI), chemokine co-receptor CCR5-using (CCR5+) virus (66). The subtype B strains had a higher mean V3 loop charge, and a smaller proportion (23 %) were predicted to be NSI/CCR5 + virus (66). The GPGQ sequence is that most common motif in African HIV-1 strain but rarely is found in HIV-1 strains in North

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America. In contrast, most (>85%) of the HIV-1 strains isolated from North America, Europe and Japan process the GPGR motif

HIV in Genital Fluids

Seminal and vaginal fluids have shown the presence of free infectious virus and/or virus-infected cells in 10 to 30% of specimens (67) (Fig.9). The quantity of virus found can differ considerably (Table3). Recently, measurement of viral RNA levels have shown a greater frequency of free virus in these fluids, but the risk of transmission will depend on the number of infectious virions. The biologic and epidemiologic factors that can influence virus shedding in the genital track are listed in Table 4 (68).

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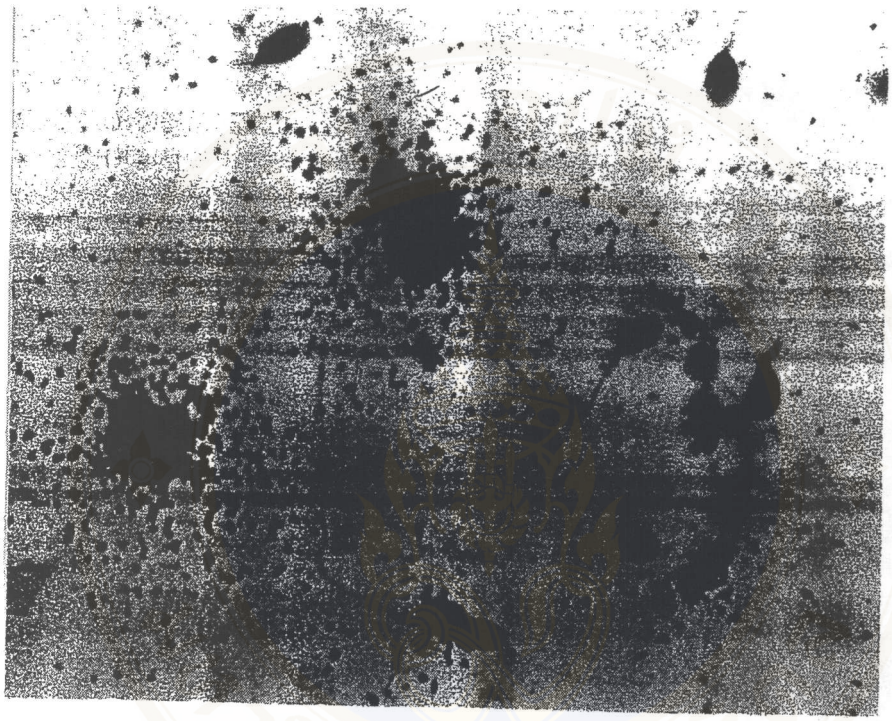

Figure 4 HIV-infection cells detected in seminal fluid by in situ hybridization.

(Levy JA, HIV and the pathogenesis of AIDS Second edition p 22, 1998)

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Table 4 Factors affecting HIV-1 shedding in the genital tract

Status	Factors in: Females	Males
Confirmed correlates[b]	Pregnancy Oral contraceptives Cervical ectopy Cervicitis	HIV disease stage $CD4^+$ lymphocyte count $CD8^+$ lymphocyte count Antiretroviral therapy Leukocytospermia Gonorrhea Urethritis
Potential correlates	HIV disease stage $CD4^+$ lymphocyte count Plasma viremia Viral phenotype or subtype Antiretroviral therapy Injectable contraceptives Nutritional deficiency states Specific cervical or vaginal STD Lactobacillus and H_2O_2 production Mucosal HIV antibodies Douching or drying agents	Plasma viremia Viral phenotype or subtype Vasectomy Circumcision status Nutritional deficiency states Nongonoccocal urethritis Mucosal HIV antibodies

(Levy JA, HIV and the pathogenesis of AIDS Second edition p 23, 1998)

HIV in seminal cells and fluids.

Sexual contact is the major route of human immunodeficiency virus (HIV) transmission. The composition of human semen was shown as table 5. Virus can be cultured from the cellular fraction of semen but more rarely from the seminal plasma (10). The cellular fraction of semen contains spermatozoa, immature germ cell, leukocyte and epithelial cell (6). Leukocyte subpopulations in human are granulocytes, monocyte/macrophages, helper&surpressor/cytotoxic T lymphocyte and B lymphocyte (69). Normally, leukocyte numbers in semen are approximately 10^6 WBC/ml, WBC

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population in semen were shown as table 6&7 and most WBC appear to originate from the epididymis (70), but Pudney J et al. suggested that the localizing HIV infected leukocyte located the prostate as well as to the seminiferous tubules, the interstition of the testis, and the epididymal epithelium and stroma which was supported by in site hybridization (71).

Both T cells and macrophages were major cellular factors of HIV transmission in semen but viral DNA was never detected in motile spermatozoa or immature germ cell population (6). HIV-1 proviral DNA was found in 75 % of viable semen cell sample by PCR and in 88 % of paired blood cell sample from HIV seropositive men were found to be most commonly HIV infection (75 % of sample) followed macrophage (35 % of sample) (6). Phylogenetic analysis revealed a distributed pattern of variant representation, with little evidence of substantial colonal out growths and no clustering in one or the other tissue evident and the deduced amino acid sequences of the V3 loop of NSI phenotype, no distinct differences were found between proviral populations in pair blood and semen (72).

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Table 5 Composition of human semen.

Color: White, opalescent		
Specific gravity:1.028		
pH: 7.35-7.50		
Sperm count: Average about 100 million/mL, with fewer than 20% abnormal forms		
Other componants:		
Fructose (1.5-6.5mg/mL)	From seminal vesicles (contribute 60% of total volume)	
Phosphorylcholine		
Ergothioneine		
Ascobic acid		
Flavins		
Prostaglandins		
Spermine	From prostate (contributes 20% of total volume)	
Citric acid		
Cholesterol, Phospholipids		
Fibrinolysin, fibrinogenase		
Zinc		
Acid phosphate	Buffers	
Phosphate		
Bicarbonate		
Hyaluronidase		
WBC/ml	10^6	from Epididymis and rete testis (70)

(Ganong WF, Review of medical physiology: p402, 1989)

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Table 6 WBC population in semen

Leukocytes	Range (%)
Granulocytes	50-60%
Macrophages	20-30%
T Lymphocytes	2-5%
Plasma cells & B Lymphocytes	rare

(Wolff H et al. Fertil Sterl, 1995)

Table 7 comparing the distribution of leukocyte subsets in semen and blood (74)

Source	Monocytes/Macrophage	Lymphocytes	Granulocytes
In blood	4%	34%	62%
In semen	20-30%	4%	50-60%

HIV in Seminal Fluid

Most studies suggested that the presence of infectious virus in seminal fluid did not correlate with clinical state (73). Both syncytium-inducing (SI) and nonsyncytium-inducing (NSI) strains can be isolated from semen. The quantity of HIV-1 viral RNA in semen also does not necessarily reflect the level of plasma viremia. In addition, the viruses found in semen does not always show the same biologic phenotype as these in the blood (NSI versus SI) (74). The prevalence of virus-infected cells appeared to be an

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important variable in genital fluids (Table 3). In seminal fluid, the number of infected cell can range from 0.01 to 5% (Fig. 4). Semen usually contains 1 million leukocytes/ejaculate, but levels and subsets of cells can vary widely from day to day in the same individual (75). Nevertheless, the HIV-infected cells ($>10^4$ in some cases) seem to be a greater source of transmission than free infectious virus. T cells appear to be most commonly infected, followed by macrophages.

The cellular source of HIV in seminal fluid is not well defined. By culture and in situ PCR hybridization procedures, HIV has been detected in large quantities in the testes of infected individuals. It has been found in urethral cells, spermatogonia, spermatocytes, and occasionally in spermatids, but not in Sertoli cells (76). The presence of HIV in spermatogonia could explain the puzzling finding of HIV DNA in the midportion of some spermatozoa, as noted by in situ PCR hybridization (77). Since only the head enters the egg during fertilization, a relevance of this finding to possible germ line transmission of HIV is unlikely. Nevertheless, a glycolipid resembling galactosyl ceramide on the middle portion of the sperm tail (78) could serve as an attachment site for HIV, as it does on some brain and bowel cells.

The virus and virus-infected cells must come from the urethra, prostate, and other secretory glands, as well as the testes (79). HIV has not been detected in the epithelia of the prostate, epididymis, seminal vesicles, or penis of men with AIDS by in situ PCR hybridization procedures (76).

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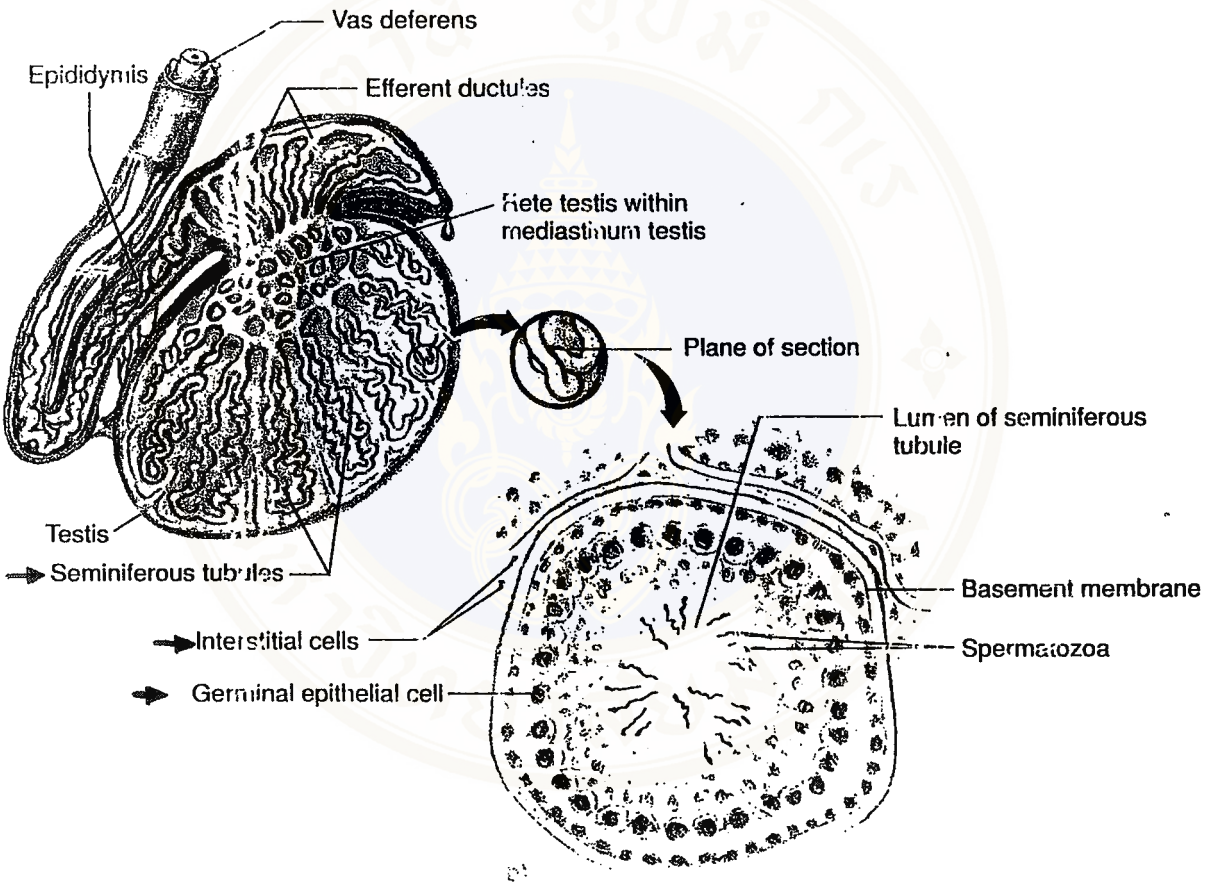

Figure 5 Spermatogenesis and male reproductive system (arrow indicate HIV-1 infected cell) (Marier EN: Human Anatomy & Physiology, 1992)

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HIV in Vaginal Fluid

Vaginal fluid has been found to contain free infectious virus only rarely (80). Even by PCR analysis, HIV was found in cervicovaginal secretions of only 28% of infected women (81). As with seminal fluid, plasma viremia does not correlate with virus shedding (81). Moreover, the prevalence of free virus in cervicovaginal secretions does not reflect the clinical state, zidovudine (AZT) therapy, or risk group (80). The prevalence of the virus, however, has been found to be significantly higher in pregnant women (80). It is also higher in women with cervical ectopy, abnormal vaginal discharge, or severe vitamin A deficiency and in those taking oral contraceptive pills (82).

The source of HIV in vaginal fluid is not known but is most probably the secretory glands in the vagina or cervix, leukocytes in the uterine cavity and, in some cases, menstrual blood. HIV has been detected in the cervix where, by in situ PCR hybridization procedures, it is found primarily in monocytes and macrophages and in the glandular epithelium in the zone of transformation between columnar and squamous epithelial cell (83). The cervix is a more frequent source of virus than the vagina (82).

CLINICAL FEATURES

Acute Primary Infection syndromes

Some HIV-1 seroconversations are asymptomatic or subclinical, whereas other are associated with a self-limited mononucleosis or influenza-like syndrome characterized by fever, rigors, arthralgias, myalgias, malaise, lethargy, anorexia, nausea,

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diarrhea, sore throat, and a truncal maculopapular, urticarial, or vesicular rash. Neurologic signs and symptoms often predominate, including headaches, stiff neck, retro-orbital pain, neuritis, myelopathy, photophobia, irritability, depression, or frank encaphalopathy. The illness may last 2 to 3 weeks, but it usually results in clinical recovery.

In adults, mean incubation periods before development of AIDS have been estimated at 10 years in the absence of therapy (84). Laboratory testing is useful to predict risk, such as percent of $CD4^{+}$lymphocytes in peripheral blood and the ratio of CD4 to CD8 cells.

A quantitative and qualitative virology has increased the markers available to monitor prognosis as well as response to therapy. Amplification techniques, employing PCR or branch chain DNA technologies, allow quantitative measurements of HIV-1 RNA, Table (8) even in early infection (85). The sequelae of virologic and immunologic events associated with primary infection in peripheral blood and lymph nodes (Fig.7), initially localizes in lymphoid organs as determined by the detection of virus-expressing. The appearance of virus-specific cytotoxic T lymphocytes (CTL) coincides with a rapid clearance of virus-expressing cells in the lymph nodes. Production of complement (C') binding antibodies (Abs) facilitates trapping of virions in germinal centers of lymph node. Neutralizing Abs are detectable at the transition from the acute to the chronic stage of infection

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Table 8 Plasma viremia and clinical stage

Clinical stage	Viremia (virions/ml)
Acute (primary) infection	$5x10^6$
Asymptomatic	$8x10^4$
Early symptomatic	$35x10^4$
AIDS	$2.5x10^6$

(Levy JA, HIV and the pathogenesis of AIDS Second edition: p19, 1998)

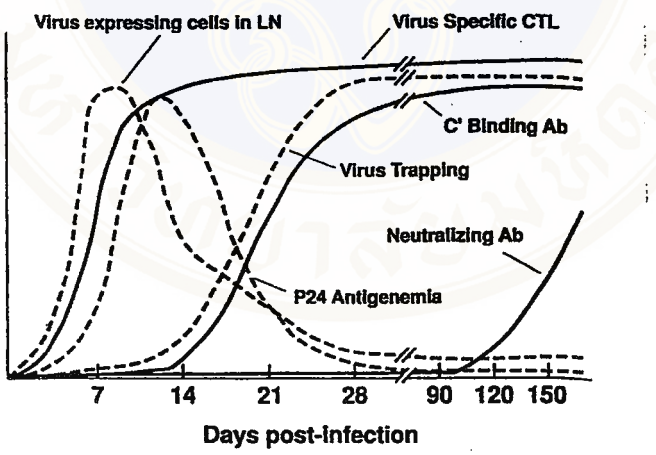

Figure 6 Virologic and immunologic events associated with primary HIV infection

(Pantaleo G, AIDS Etiology, Diagnosis, Treatment and Prevention Fourth Edition p78, 1997)

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Genetic Sequence Differences

At least 6 to 10% of the total viral genome can differ among strains from different individuals (86). Some isolates vary widely in both synnonymous sequence changes (mutations that do not affect amino acid representation) and nonsynonymous sequence changes (mutations that affect the amino acid expressed). The greatest nonsynonymous sequence heterogeneity is observed in the genes of the regulatory and envelope proteins (87); with some strains, nearly 40% differences in amino acid sequences in some viral genes can be appreciated (Fig.7).

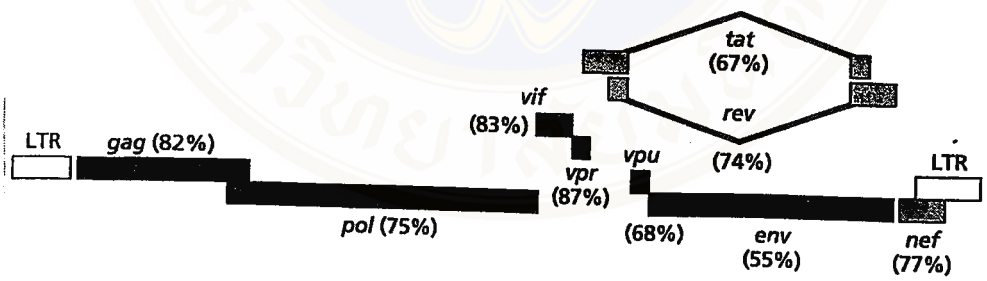

Figure 7 Estimated similarities in amino acids for the different HIV-1 gene products.

(Levy JA, HIV and the pathogenesis of AIDS Second edition p152, 1998)

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Recombinant A/E (*gag/env*) and A/I strains have been identified in Thailand and Cyprus, respectively (88). Moreover, the viruses in clade F from Brazil have GPGR in their crowns whereas the clade F isolates from Romania have GPGQ (89). Similarly, the clade B virus in Thailand differs from clade B viruses in North America by the amino acids in its V3 loop (GPGR) (87). Clades A and C have similar genetic sequences; in contrast, clade D has very divergent V3 loop sequences (90). These and other data suggest that the extent of replication influences the interpatient variation that develops during the spread of virus subtypes through populations in the world. Some researchers have estimated that the level of variability among viruses within a clade in a country can predict how long that virus has been present in the country (Fig.8) (91). Great diversity would suggest a longer that viruses have existed, spread, and diversified in the population.

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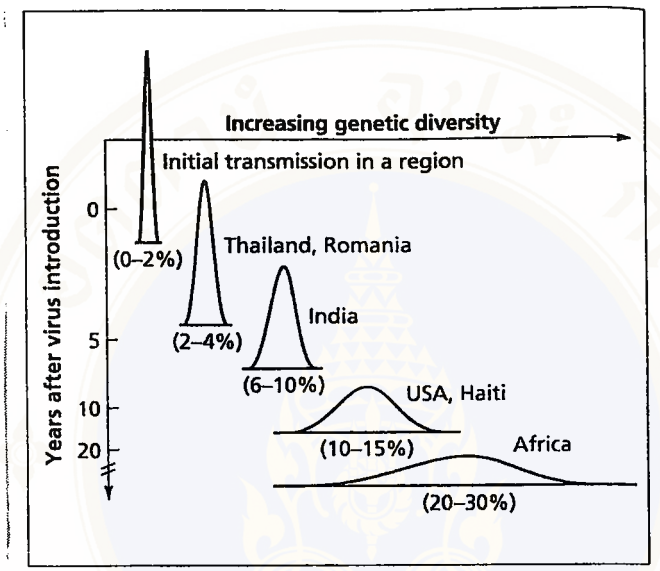

Figure 8 Relationship between the degree of genetic diversity of a genotype and the duration of the epidemic in a region. (Weniger BG., AIDS, 1994)

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CHAPTER IV

MATERIALS AND METHODS

Subjects

Unclotted EDTA blood and semen were collected with informed consents from HIV-Infected intravenous drug users(IDUs). Forty six HIV-1 infected intravenous drug users from Bangkok Metropolitan Authority (BMA) IDU seroconvert cohort Bangkok in collaboration with HIV/AIDS collaboration (HAC) who were had the time of seroconvert from 0 to 28 month. These individuals had been interviewed, physical examined, without sexually transmitted disease (STD), asymtomatically, CD_4^+ & CD_8^+ lymphocyte count and no sexual activity before donate semen within 48 hours. Semen samples were collected in sterile cup by masturbation and must be process within 4 hours after ejaculation. Ten milliliters of pair blood were collected in EDTA vacutainer tube.

Subjects were divided according to level of CD4+ T-cell into 3 groups [<200 CD^+_4 WBC/mm^3 (group 1), 200-499 CD^+_4 WBC/mm^3 (group 2), and ≥ 500 CD^+_4 WBC/mm^3 (group 3]), which was followed as CD4+ T-lymphocyte categories diagnosis of AIDS (CDC revised 1993). SC was indicated as seminal cell and PB indicated as PBMC.

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Specimen collection.

For PBMC (peripheral blood mononuclear cells)

Unclotted EDTA blood was collected from subject in vacutainer tube. The tube was centrifuged at 800xg for 10 min to remove plasma from blood cells. The plasma was collected into sarstete tube and stored at -70°c for viral RNA quantification. The remaining EDTA blood was diluted with equal volume of 1x phosphate buffer saline (PBS). The diluted blood cells was transferred onto the top of one-third volume of Isoprep^{R} (Robbins Scientific, CA, USA) in 15 ml centrifuge tube, avoid mixed of blood and Isoprep^{R}.

The tube was centrifuged 800xg for 20 min at room temperature in a swing-out rotor, without brake and the PBMC layer was removed to new 15 ml centrifuge tube, which was seen as and opaque band located at the gradient interface. The PBMC was washed 2 times with 5 ml of 1x PBS by centrifuge 250 xg for 10 min and the supernatant was discarded, 10 µl of the cell suspension was diluted in 90 µl of tryphan blue solution (see appendix) the number of cells counted in a disposable hemocytometer (KOVA Glasstic slide 10).

The cell pellet was resuspended to 10^6 cells/ml with PCR lysis buffer (See Appendix) containing 10 mg/ml proteinase K (Ameresco, Ohio,USA). The Cells suspension was incubated at 56°c for 1 hour and proteinase K was inactivated by heating the suspension to 95°c for 10 min. The cell lysate was then kept for PCR amplification at –20°c until used.

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For semen

Semen volume was measured and then semen was diluted 1:1 with VTM medium (See Appendix) containing with 1000 unit antibiotic, well mixed semen was placed in a 15 ml centrifuge tube and was allowed at room temperature for 30 min. The tube was centrifuged at 600xg for 15min; seminal plasma was carefully pipetted off and passed approximately 1-1.5 ml through 0.45 μm filter and placed 0.5 ml of filtered seminal plasma in a small snap cap tube. The seminal cells were resuspended 2 times with 10 ml RPMI 1640 medium (Gibco BRL, NY, USA) and centrifuged at 600xg for 10 min. The supernatant was carefully pipetted off by single-motion pouring being careful not to disturb the cell pellet. The seminal cells were resuspened in 3 mL of culture medium (RPMI 1640) with containing 100 unit antibiotic and counted mononuclear cells with hematocytometer (same as PBMC step). Half of resuspended cells (1.5 ml) were divided for coculture.

The remaining seminal cells (1.0ml) should be mixed 1:1 with freezing medium (See Appendix) and frozen in approximately 1ml aliquots at –70°C for 24 hours and then moved to liquid nitrogen for storage. The remaining seminal cells (0.5ml) were washed 2 times with 5ml of 1x PBS by centrifuge 600xg for 10 min and resuspended cell pellet to 10^6 cells/ml with PCR lysis buffer (same PBMC lysate) containing 10mg/ml proteinase K. The cell lysate was also kept at

-20 °C.

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Nested Polymerase Chain Reaction (Nested PCR)

The nested PCR was performed in a two-steps reaction. First, with outer primer pair and then with inner primer pair. In this study, two sets of primer which is specific to *gag* gene (SK380N & SK390 as an outer and SK38KNP & 39E as an inner primers) and for *env* gene should be used 2 set of pair primers (envB & AO_2 as an outer and AI_1 & AI_2 as an inner primers, JH44 & JH35 as an outer and JH33 & JH48 as an inner primers). The regions, fragments and sequences were shown as table 10.

For the amplification, *gag* gene was using SK380N & SK390 primers in the first round and 5 µl of first round PCR product was amplified further using SK38KPN & SK39E primers. The first round PCR reaction mixture for *gag* gene consisted of 5.0 µl of 10x PCR buffer (suppli), 2.5 µl of 25mM $MgCl_2$ (suppli), 4.0 µl of 2.5 mM each of dNTP (Pharmacia, NY, USA.), 1.0 µl each of 10 pmol/µl SK 380N & SK 390 primer pair, 11.0 µl of sterile deionized water, 0.5 µl of 5 U/µl Tag Polymerase enzyme (Promega, WI, USA, GIBCO BRL, NY, USA) and 25 µl of cell lysate.

The second round PCR reaction mixture consisted of 5.0 µl of 10x PCR buffer, 2.5 µl of 25 mM $MgCl_2$, 4.0 µl of 10 mM each dNTP, 1.0 µl of each 10 pmol/µl SK38KPN & SK9E primer pairs, 31.0 µl of sterile deionized water, 0.5 µl of 5 U/µl Tag Polymerase enzyme, and 5 µl of first round PCR product.

For amplification *env* gene was using 2 set of pair primers. 1) For amplification gp120 *env* gene, the first round PCR reaction mixture consisted of 5.0 µl of 10x PCR buffer, 3.0 µl of 25 mM $MgCl_2$, 4.0 µl of 10 mM each of dNTP, 1.0 µl each of 10 pmol/µl envB & AO_2 primer pairs, 23.5 ul of sterile deionized water, 0.5 µl of 5 U/µl of Tag Polymerase enzyme, and 10 µl of cell lysate. The second round PCR reaction mixture

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consisted of 5.0 µl of 10x PCR buffer, 2.5 µl of 25mM $MgCl_2$, 4.0 µl of 2.5mM dNTP, 1.0 µl each of 10 pmol/µl AI1 & AI2 primer pairs 30.5 µl of sterile deionize water, 0.5 µl of 5U/µl of Tag Polymerase enzyme, and 5.0 µl of first round PCR product.

For amplification C2-V4 *env* gene, the first round PCR reaction mixture consisted of 10.0 ul of 10x buffer, 8.0 µl of 25 mM $MgCl_2$, 8.0 µl of 10 mM each of dNTP, 0.25 µl each of 100 pmol/µl JH44 & JH35 primer pairs, 51.75 µl of sterile deionized water, 0.5 ul of 5U/µl of Tag Polymerase enzyme, and 20.0 µl of cell lysate. The second round PCR reaction mixture consisted of 10.0 µl of 10x buffer, 10.0 µl of 25 mM $MgCl_2$, 8.0 µl of 10 mM each of dNTP, 0.25 µl each of 100 pmol/µl JH48 & JH33 primer pairs, 66.0 µl of sterile deionized water, 0.5 µl of 5U/µl Tag Polymerase enzyme, and 5.0 µl of first round PCR product.

PCR was performed by using the automated Gene Amp PCR System 9700 and 2400 DNA Thermal Cycle (Perkin Elmer Cetus, CT, USA.). It was programmed for each of primer pairs. The reaction cycle for amplification are as followed. For the *gag* gene, the first round reaction cycle are 35 cycles of 95° c for 1 min, 60° c for 1 min, 72° c for 1 min. After the last cycle, the final extension was performed at 72° c for 7 min. The second round PCR was programmed as the first round PCR cycles but annealing temperature was changed from 60° c to 55°c.

For the C2-V4 *env* gene, the first and second round reaction cycles are 94°c for 0.30 min, 55°c for 0.30 min, 72°c for 1 min. After the last cycle, the final extension was performed at 72°c for 7 min. The PCR product was examined by agarose gel electrophoresis.

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Table 9 Primers sequence and location for amplify *gag* and *env*-gene

Gene	Primers	Sequence 5′→3′	location	PCR product (kb)
gag	SK380N	CAG GGA CAA ATG GAT CAT CAG CCT	1236-1259	
	SK390	GGG AAA TCT CTG ATA CAT CTG CCC AAG AT	1689-1717	
	SK38KPN	GGG TAC CGA ACT AAT ACC CAT GTT TTC A	1311-1338	0.375
	SK39E	CTG TAT TCT GTT CCT GGA TTA CTG	1665-1686	
	SK380	GAG AAC CAA GGG GAA GTG ACA TAG GAG	1507-1533	
	SK38	ATA ATC CAC CTA TCC CAG TAG GAG AAA T	1573-1600	0.115
	SK39	TTT GGT CCT TGT CTT ATG TCC AGA ATG C	1662-1688	
env	ENV-B	AGA AAG AGA AGA AGA CAG TGG AAA TAG	6193-6219	
	AO_2	GAA TTC AAA GGT GAG TAT CCC TG	8337-8359	
	AI_1	GCA TCC TTA TTA TGG GGT TCC TGT GTG G	6326-6345	1.301
	AI_2	GAA TTC TTT CCT CCT CCA GGT CTG AA	7611-7627	
	JH44	ACA GTA GCA AGT GCT ACA CAT GG	983-7002	
	JH35	CAC TTC TCC AAT TGT CCI TCA	7668-7688	
	JH33	CTG TTI AAT GGC AGI CTA GC	7031-7050	0.524
	JH48	AGA TGG GAG GAG GCT ATA CAT	7538-7555	

SK sequence derived from HIV-1 isolate SF2, GenBank Accession number K02007

AI, AO, ENV and JH Sequence correspond to those from the IIIB isolate BH 10 sequence

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Examination of Amplified DNA by agarose gel electrophoresis

The amplified product was normally verified on agarose gel electrophoresis to see whether the certain DNA fragment was amplified and obtained as expected. The 2% and 1% agarose gel (GIBCO BRL, NY, USA) was used to detect the amplified DNA fragment of *gag* and *env* gene respectively (except JH48 & JH33 primers used as 2% agarose). The gel was prepared by boiling in 0.5X TBE buffer (see appendix) until it was completely dissolved and allowed to cool at about 60-80°c. Then the gel was poured in a gel-casting platform with a well former.

The gel was left for hardening at room temperature for 30 min and the well former was then carefully taken off. The gel and casting platform were transferred into an electrophoresis chamber which consisting of sufficient 0.5x TBE buffer. The solution of 100 base pair DNA marker (New England Biolabs, MA, USA) was mixed with 2 μL gel loading dye (see appendix) and applied into the first well and 5 μL of PCR product was mixed with 1μL of loading dye and applied into each well. The electrophoresis was carried out at constant voltage at about 100 volts. The running gel was stopped when the dye migrated to the bottom of gel. The gel was stained in ethidium bromide solution (see Appendix) for 5-10 min and destained in distilled water. The amplified DNA in the gel was observed on an UV-transluminator and photographed by using Polaroid camera fitted with a red-orange fillter. The *env*, *gag* fragment and location were shown as table 9.

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Quantification of HIV-1 RNA by using NUCLISENS HIV-1 QT

The NUCLISENS HIV-1 QT (Organon Teknika, Boxtel, NL) was a NASBA based nucleic acid amplification assay which requires no strand serration, amplification was isothermal and continuos for quantitative of HIV-1 RNA. It comprises four separate stage: nucleic acid release from viral particle, nucleic acid isolation from the solid phase, nucleic acid amplification to get HIV-1 RNA and nucleic acid detection by electrochemiluminescense method.

The quantification of HIV-1 viral RNA was performed by using a Quatitation Standard (QS) RNA which is a non-infectious RNA transcript that contains the identical primer binding site as the HIV-1 target and a unique probe binding region. HIV-1 viral RNA was extracted from blood plasma and seminal plasma by using a Nuclisen lysis buffer containing guanidine thiocyanate (GuSCN) and TritonX-100. Any viral particles and cells present in the sample were disintegrated; any RNases and DNases present in the sample were inactivated. Nucleic acid was released and then was added with 3 synthetic RNAs (Qa, Qb, Qc) of known high, medium and low concentration, respectively. These RNAs serve as internal calibrators, each differing from the HIV-1 wild type (WT) RNA by only a small sequence.

Under high salty condition, all nucleic acid in the buffer including the calibrators, was bound to silicondioxide particles. These particles were washed and eluted from the solid phase by elution buffer. Amplificaion (*gag* gene) was based on repeated transcription, multiple copies of each WT and calibrators RNA target sequence are synthesized by T7-RNA polymerase by means of an intermediate DNA molecule which contains the double stranded T7-RNA polymerase promotor and each transcribed RNA

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copy enter a new amplification cycle. The DNA intermediate is generated by binding a primer to the RNA template, extending the primer by AMV-RT (Avian Myeloblastosis Virus Reverse Trascriptase) to form an RNA-DNA duplex, degrading the RNA strand of the duplex by RNase H, binding a second primer to the remaining DNA strand and extending the second primer to form the double-stranded T7-RNA-polymerase promotor needed for transcription and transcription has started, the RNA transcript, which were " negatives " of the original RNA present in the sample would be subject to the same process, only in this case extension was not restricted to the second primer, since the extension product of the first primer would be extended, too. The primers, which contains the sequence of the T7-RNA-polymerase promotor were complimentary to two difference parts of the HIV-1 RNA. Detection of HIV-1 RNA in a sample is based on the Nuclisens Reader electrochemiluminescence principle.

To differentiated between the amplificates (WT, QA, QB and QC), aliquots of the amplified sample were added to four hibridization solutions, each specific for one of the amplificates. Here, the respective amplificates were hibridized with a bead-oligo (Streptavidine coated paramagnetic beads) and a ruthenium-labeled probe, they were form complex on the surface of an electrode by means of a magnet. Voltage applied to this electrode triggers the electrochemiluminecence (ECL) reaction. The light emitted by the hybridized ruthenium labled probes is propotional to the amount of amplicate. Calculation based on the relative amounts of the four amplificated the original amount of WT HIV-1 RNA in the sample.

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Competitive Nested Polymerase Chain Reaction (cPCR)

Thirteen of pair PBMC and seminal cells were quantitated by a competitive nested PCR-based assay. This method consists of the subsequent amplification *gag* gene in the same tube of two similar DNA templates, the wild-type template to be quantified and a known amount of the internal deleted synthetic template (competitor), both with identical primer recognition sequence, and after PCR amplification product must be clearly distinguishable by gel electrophoresis analysis, in order to allow desitometric comparison of the relative intensities of the bands for both species.

For competitor DNA (pSKAN), was synthesized by cloning the 18-base deletion *gag* fragment (position 1579 to 1596) of the HIV-1 genome into the plasmid vector pBS (formerly Bluescribe. Stratagene, La Jolla, Calif.) and were diluted with serial dilution. Clinical sample were subdivided into three parallel reaction of known copy number competitor (25, 50 and 100 copies) and were added with a constant amount of wild type DNA (per $1.00x10^5$ PBMC or mononuclear cells) in each dilution.

Briefly, the nested PCR was performed in a two-steps reaction. First, with outer primer pair (SK380 & SK390) and then with inner primer pair (SK38 & SK39). The first round PCR reaction mixture for *gag* gene consisted of 5.0 µl of 10x PCR buffer (suppli), 3.0 µl of 25 mM $MgCl_2$ (suppli.), 4.0 µl of 2.5 mM each of dNTP (Pharmacia, NY, USA), 2.0 µl each of 10 pmol/ul SK380 & SK390 primer pairs, 0.5 µl of 5 U/µl Tag Polymerase enzyme (Promaga, WI, USA), volume of cell lysate per $1.00x10^5$ PBMC or mononuclear cells, 5.0 µl of each serial dilution competitor and maked up with sterile deionized water to 50 µl of total volume.

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The second round PCR reaction mixture consisted of 5.0 µl of 10x buffer, 3.0 µl of 25 mM $MgCl_2$, 4.0 µl of 10 mM each dNTP, 2.0 µl of each 10 pmol/µl SK38 & SK39 primer pairs, 28.5µl of sterile deionized water, 0.5 µl of 5 U/µl Tag Polymerase, and 5 µl of first round PCR product.

PCR was performed by using the automated Gene Amp PCR System 9700 and 2400 DNA Thermal cycle (Perkin Elmer Cetus, CT, USA). The reaction cycle for amplificatoin are as followed, the first round reaction cycle are 35 cycle of 95°c for 1 min, 60°c for 1 min, 72°c for 1 min. After the last cycle, the final extension was performed at 72°c for 7 min. The second round PCR was programmed as the first round PCR cycles but annealing temperature was changed from 60°c to 55°c. The PCR product was examined by 10% polyacrylamide gel (see appendix) at 150V for 70 min in order to obtain complete separation of the 115-bp wild-type fragments from the 97-bp deleted fragments. The final step, 10 % polyacrylamide gel was stained by ethidium bromide (same previously) and was scanned by using a video densitometer (Ultra violet Products Ltd.,Cambridge, United Kingdom) by positive fluoresent emission on the transilluminator and were calculated by the machine soft ware (Gel Analysis Program; Ultra violet Products) in each land. DA was corrected (DAc) for its lower molar ethidium bromide incorporation as follows:

$$DAc = DA \times (\text{wild-type length/deleted length})$$

$$= DA \times 115/97\ (1.1855)$$

The DAc/WA ratio was calculated for each sample and plotted on the Y axis against the copy number of the deleted competitor (D). A simple regression curve was fitted for

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positive controls and for each sample. The copy number of the wild-type template (W) could be calculated from the curve expression for DAc/WA = 1.

DNA Sequencing

Proviral DNA extraction

The DNA extraction procedure was using QIA quick Gel Extraction Kit (QUIGEN Gmbh, Hilden, Germany) in a microcentrifuge tube, according to protocol.

Briefly, add 3 volumes of buffer QG (supplied) containing chaotropic salts to 1 volume of DNA fragment from the agarose gel in 0.5x TBE (Tris-borate/EDTA buffer). Incubated at 50°c for 10 min (or until the gel slice has completely dissolved) and added 1 gel volume of isopropanol to the sample and mix. Then the mixture was transferred to QIA quick spin column in a provided 2 ml collection tube for bind DNA, and centrifuged at ≥10,000xg for 1 min. The flow-through was discarded and placed QIA quick column back in the same collection tube and added 0.5 ml of buffer QG to QIA quick column and then same centrifuged, 1 min for remove all traces of agarose. Then 0.75 ml of PE (Supplied) was added to QIA quick column and centrifuged at ≥ 10,000xg for 1 min. The flow-through was discard and centrifuged the QIA quick column for additional 1 min at ≥ 10,000xg and placed QIA quick column into a clean 1.5ml microfuge tube. Then 30µl elution buffer was added to the center of the QIA quick column, leted stand for 1 min, then centrifuged for 1 min, and store DNA at –20°c until used.

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Automated Sequencing

The partial p24 *gag* gene and *env* V3 gene were sequenced directly from PCR products of PBMC and seminal cells by Big Dye Terminator (ABI, Foster City, CA), which contains fluorescent-dye terminators, deoxynucleoside triphosphates, AmpliTaq DNA Polymerase, FS, with thermally stable pyrophosphate, $MgCl_2$ and Tris-HCL buffer pH 9.0.

Briefly, the purification of PCR product, 30-90 ng concentration was mixed with 4.0 µl terminator reaction, 4.0 µl of 0.8 pmole primers (BI_2 for *env* gene and SK39E for *gag* gene

(Table 10), and maked up with deionized water to 20µl in thin wall tube. Mixed well, spinned and was performed by using automated Gene Amp PCR system 9700 and 2400 (Perkin Elmer Cetus ,CT, USA).

The sequence reaction cycles are 25 cycle of 96°c for 10 sec., 50°c for 5 sec., 60° c for 4 min. After that DNA was precipitated by ethanol precipitation, followed as added 64µl of 95% ethanol, 16µl deionized water and 20µl of sequence product in appendrof tube. Mixed well, left at room temperature 15 min to precipitate the extension product and centrifuged 12,000 rpm for 20 min at room temperature. The supernatant was discarded very carefully by gentle pipetting. 250ul of 70% ethanol was added to wash the precipitant, mixed well and centrifuged 12,000rpm for 10 min at room temperature. The supernatant was discarded very carefully by gentle pipetting and dried at room temperature or 20 min at 37°c. Then 25ul of Template Suppression Reagent (TSR)

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(suppli.) was added for resuspened each sample pellet, vortex, spinned the sample and transferred to sequencing tube.

The samples were done by heat at 95°c for 2 min to denature, then chilled on ice, mixed well and spinned the sample again. Placed on ice until loading sample in capillary electrophoresis ABI Prism 310 Genetic Analyzer (ABI Prism, USA).

Analysis of *gag* and *env*V3 DNA sequence.

Nucleotide sequences were analyzed by DNA Sequencing and Data Analysis Sorfware for the Apple MacIntosh II computer.

DNA sequence and amino acid alignments were performed with DNASIS for Windows sequence analysis sorfware version 2.1 (Hitachi, Tokyo, Japan). The phylogenetic analysis was performed using the ESEE and MEGA (Molecular Evolutionary Genetics Analysis) program. The distance matrix for the neighbor-joining tree was using the Kimura two-parameter method.

Statistical Analyses

Virologic and Immunologic markers of disease activity were evaluated by non parametrix methods. The quantitation of both HIV-1 RNA in the cell-free blood plasma, seminal plasma were transformed to their 10 logarithms to normalize data. The quantitation of both HIV-1 RNA in the cell-free blood plasma, seminal plasma and both HIV-1 DNA in PBMC, seminal cell were analyzed by the Kolmogorov-Smirnov test for test the normally distribution; the Spearman rank order test, T-test for test and Mann-Whitney U test was used to assess potential associations of HIV-1 DNA and RNA level in blood and semen.

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CHAPTER V

RESULT

1. Clinical statuses of early infected IDUs.

All 46 HIV-1 infected IDUs from Bangkok Metropolitan Authority (BMA) IDU seroconvert cohort Bangkok in collaboration with BMA and HIV/AIDS collaboration (HAC) were asymtomatic and healthy at the time of sample collection (1998-1999). There had the time of seroconvert from 0 to 28 month and no signs or symptoms of sexually transmitted diseases. Blood CD^{+}_{4} cell count of these individuals could be classified into 3 groups;

Group 1) 6/46 (13.64%) of CD^{+}_{4} count less than 200 cells/mm^3, with subject No 12, 14, 24, 34, 36 and 41.

Group 2) 27/46 (58.70%) of CD^{+}_{4} count between 499 cells/mm^3 to 200 cells/ml, with subject No1, 2, 3, 4, 5, 6, 7, 9, 11, 15, 16, 17, 21, 22, 23, 26, 27, 31, 32, 35, 38, 39, 40, 42, 44, 45 and 46.

Group 3) 13/46 (28.26%) of CD^{+}_{4} count $\geq$ 500 cells/mm^3, with subject No 10, 13, 18, 19, 20, 25, 28, 29, 30, 33, 37, 43 and 47.

The range of mononuclear cells in semen was 1.4×10^5 to 5.0×10^6 cell/ml. Of the 36/46 subjects with mononuclear cells range from 1.4×10^5 to 3.0×10^6 cell/ml were positive for HIV *gag* DNA in semen and 10/46 subjects with mononuclear cells range from 3.3×10^5 to 5.0×10^6 cell/ml were negative for HIV *gag* DNA in semen.

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2. Subjects determine characteristic.

V3 nucleotide sequences were used to classify HIV-1 subtype into 2 major subtypes (determined by HIV/AIDS collaboration (HAC), 1999).

There were 10/46 (21.7%) classified as subtype B', and 36/46 (78.3%) classified as subtype E. The demographic characteristics were represented as table 10.

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Table: 11 Demographic data of HIV-1 isolated from semen and blood of early seroconvert IDUs.

No	Subject number SC	HAC IDU No 033	Amount of MNC (cell/ml)	Time of seroconvert (month)	T lymphocyte count (Cell/mm^3)		HIV-1 RNA (copies/ml)		HIV-1 DNA /10^5PBMC		PCR Result *gag* - gene		PCR Result *env*-V3	Coculture /Biotype	Sequence subtype
					CD_4^+	CD_8^+	plasma*	seminal plasma**	PBMC	seminal cell	PBMC	seminal cell	seminal cell		
1	1	13-0101	ND	0	395	741	5198	550	ND	ND	+	+	+	-	E
2	3	10-0016	ND	0	299	403	72733	1000	ND	ND	+	+	+	-	E
3	14	12-0018	$1.2X10^6$	0	160	410	21767	1400	ND	ND	+	-	-	-	E
4	16	12-0056	ND	0	344	941	60659	1000	ND	ND	+	+	-	-	B
5	37	03-0065	$3.0X10^6$	0	1376	1002	7056	1000	13.92	6.38	+	+	-	-	B
6	42	16-0023	$1.4X10^5$	0	475	1526	71544	600	ND	ND	+	+	-	-	E
7	43	04-0010	$8.0X10^5$	0	740	1302	74795	5200	ND	ND	+	-	-	-	E
8	45	12-0067	$2.0X10^6$	1	427	1601	16451	1940	30.48	8.49	+	+	-	-	E
9	46	16-0048	$2.3X10^6$	1	317	1069	31818	4000	58.63	17.52	+	+	-	-	E
10	47	11-0015	$6.7X10^5$	2	627	2062	19498	600	38.41	6.63	+	+	+	-	B
11	5	03-0005	ND	4	407	1069	11327	500	ND	ND	+	+	-	-	E
12	7	10-0109	ND	5	413	787	81119	30000	169.8	89.13	+	+	+	-	E
13	6	07-0111	ND	8	431	983	46935	450	ND	ND	+	+	+	-	E
14	11	09-0100	ND	8	421	1217	71316	800	ND	ND	+	+	+	-	E
15	21	13-0063	ND	8	468	756	158090	500	321.2	3.05	+	+	+	+	E
16	29	06-0035	$1.1X10^6$	8	546	1711	17373	18600	ND	ND	+	+	+	-	E
17	35	09-0061	$3.3X10^5$	8	311	970	30795	1960	ND	ND	+	-	-	+	E
18	39	06-0001	$5.0X10^6$	8	314	1085	45588	7600	ND	ND	+	-	-	-	E
19	44	11-0062	$2.0X10^5$	11	350	1603	20427	2600	ND	ND	+	+	+	-	B
20	19	04-0089	ND	12	894	1126	2200	700	ND	ND	+	-	-	-	E

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No	Subject number SC	HAC IDU No 033	Amount of MNC (cell/ml)	Time of seroconvert (month)	T lymphocyte count (Cell/mm^3)		HIV-1 RNA (copies/ml)		HIV-1 DNA /10^{5}PBMC		PCR Result			Coculture /Biotype	Sequence subtype
											gag - gene		*env*-V3		
					CD_4^-	CD_8^+	plasma*	seminal plasma**	PBMC	seminal cell	PBMC	seminal cell	seminal cell		
21	20	09-0098	ND	12	572	1190	2810	1920	ND	ND	+	-	-	-	E
22	25	06-0074	ND	12	1102	1028	11005	600	ND	ND	+	+	+	-	B
23	27	11-0008	$2.3X10^6$	12	471	1017	2004	1580	ND	ND	+	+	-	-	B
24	38	02-0083	$1.7X10^6$	12	329	1318	13657	1420	ND	ND	+	+	-	+	E
25	2	10-0080	ND	16	354	815	95912	1920	ND	ND	+	-	+	-	E
26	4	10-0026	ND	16	410	980	78126	5000	ND	ND	+	-	-	-	E
27	9	09-0053	ND	16	298	1093	91104	22000	ND	ND	+	+	-	-	E
28	10	09-0095	ND	16	709	1247	9069	550	ND	ND	+	-	-	-	E
29	12	12-0081	ND	16	171	403	54059	700	108.5	5.07	+	+	+	-	E
30	13	11-0011	ND	16	651	1920	71289	2000	ND	ND	+	-	-	-	B
31	22	12-0008	ND	16	423	788	12443	600	25.56	4.62	+	+	-	-	E
32	28	12-0080	$1.8X10^6$	16	562	1466	58130	700	ND	ND	+	+	-	-	E
33	31	03-0024	$2.1X10^6$	16	295	862	7768	4200	18.35	20.39	-	+	+	+	E
34	32	13-0008	$1.3X10^6$	16	423	627	1148	9200	ND	ND	+	-	-	+	E
35	34	03-0014	$4.0X10^5$	16	186	893	24026	300	ND	ND	+	+	-	-	E
36	36	03-0030	$8.3X10^5$	16	37	279	137465	5600	ND	ND	+	+	-	-	B
37	41	07-0005	$1.2X10^6$	16	106	218	4551	26000	ND	ND	+	+	-	+	E
38	40	02-0087	$1.6X10^6$	17	283	1093	43197	186000	ND	ND	+	+	+	-	E
39	15	12-0073	ND	18	357	867	62751	14200	ND	ND	+	+	+	+	E
40	23	11-0072	ND	18	381	830	34694	780	78.39	5.43	+	+	+	-	B
41	18	01-0034	ND	20	835	1159	2982	700	5.86	2.11	+	+	+	-	E
42	26	09-0031	ND	20	252	1260	32734	1000	ND	ND	+	+	-	-	E

No	Subject number	HAC IDU	Amount of MNC	Time of seroconvert	T lymphocyte count (Cell/mm^3)		HIV-1 RNA (copies/ml)		HIV-1 DNA /10^{6}PBMC		PCR Result *gag* - gene		*env*-V3	Coculture /Biotype	Sequence subtype
	SC	No 033	(cell/ml)	(month)	CD_4^+	CD_8^+	plasma*	seminal plasma**	PBMC	seminal cell	PBMC	seminal cell	seminal cell		
43	33	13-0042	2.7X10^6	20	539	1334	22708	9600	45.77	52.04	+	+	+	-	B
44	17	08-0015	ND	24	390	698	32794	1340	ND	ND	+	-	-	+	E
45	24	13-0010	ND	24	176	643	35967	1760	70.83	10.71	+	+	-	-	E
46	30	12-0007	7.0X10^5	28	710	1460	48896	1000	ND	ND	-	-	-	-	E

C=Peripheral blood mononuclear cell * , ** at complementary time of semen collection. MNC= mononuclear cell

Determined by The HIV/AIDS Collaboration, Thailand. ** Determined by Virology department, Siriraj hospital.

,+ positive ,- negative

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3. Shedding of HIV-1 proviral DNA in blood and semen.

HIV-1 proviral DNA in PBMC and seminal cells of IDUs was detected by nested PCR to amplified *gag* and *env* V3 gene. For amplification *gag* gene was using SK380N & SK390 primers in the first round and was amplified further using SK38KPN & SK39E primers. The *gag* fragment was 375 bp (Table 9)

In forty-six IDUs samples, HIV-1 proviral *gag* DNA were found in 46/46 (100%) of PBMC samples with CD4 cell counts <200 to 1376 cell/mm^3 and found in 36/46 (78.3%) seminal cell samples with mononuclear cells range from 1.4 x 10^5 to 3.0 x 10^6 cell/ml (Table 10, 11) (Fig.9). HIV-1 RNA level in these 45 samples was range from 300 to 1.9 x 10^5 copies/ml.

The *env* gp120 fragment, first was amplified from PBMC and seminal cells by using envB & AO_2 primers and AI_1 & AI_2 primers as outer and inner primers, respectively. The amplified product was 1,301 bp. (Table 9, Fig.10).

Also HIV-1 *env* (C2-V4 region) was amplified from PBMC and seminal cells by using JH44 & JH35 primers and JH33 & JH48 primers as outer and inner primers, respectively. The amplified product was 524 bp. (Table 9, Fig.11). HIV-1 proviral *env* DNA were found in 19/36 (52.8%) of seminal cell samples with mononuclear cells range from 2.0 x 10^5 to 2.7 x 10^6 cell/ml. HIV-1 RNA in blood plasma and seminal fluid were found 46/46 (100 %). The culturing HIV-1 from blood and semen were found 23/46 (50%) and 8/46 (17.4%), respectively (Table 11).

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Table 11 Shedding of HIV-1 DNA/RNA in semen and blood of IDUs

Shedding of HIV-1	Semen	Blood
Cell associated virus*	36/46 (78.3%)	46/46 (100%)
Cell free virus**	46/46 (100%)	46/46 (100%)
culture	8/46 (17.4%)	23/46 (50%)

* DNA (*gag* gene) detected by nested PCR ** RNA detected by Nuclisens,

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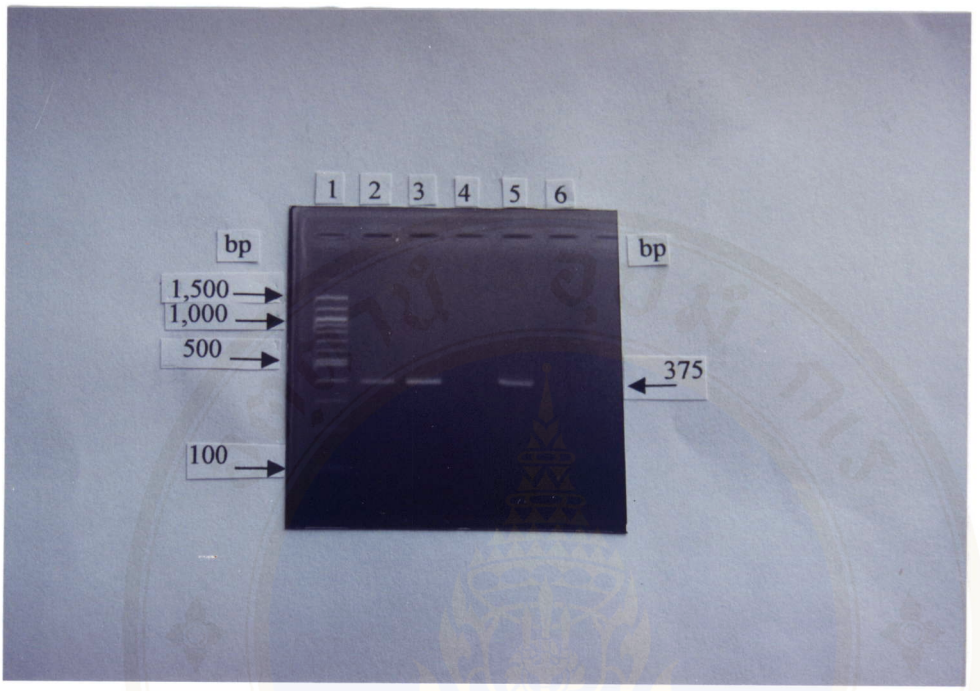

Figure 9 Agarose gel electrophoresis of PCR amplified HIV-1 *gag* (partial p24) gene. Amplified HIV-1 gag (partial p24) gene fragment of 375 bp from PBMC and Seminal cell of IDUs sample by using SK380N/SK390 as outer primers and SK38KPN/SK39E as inner primers.

Lane 1 100 base pair marker

Lane 2,3 amplified DNA from positive sample

Lane 4 negative control

Lane 5 positive control

Lane 6 reagent control

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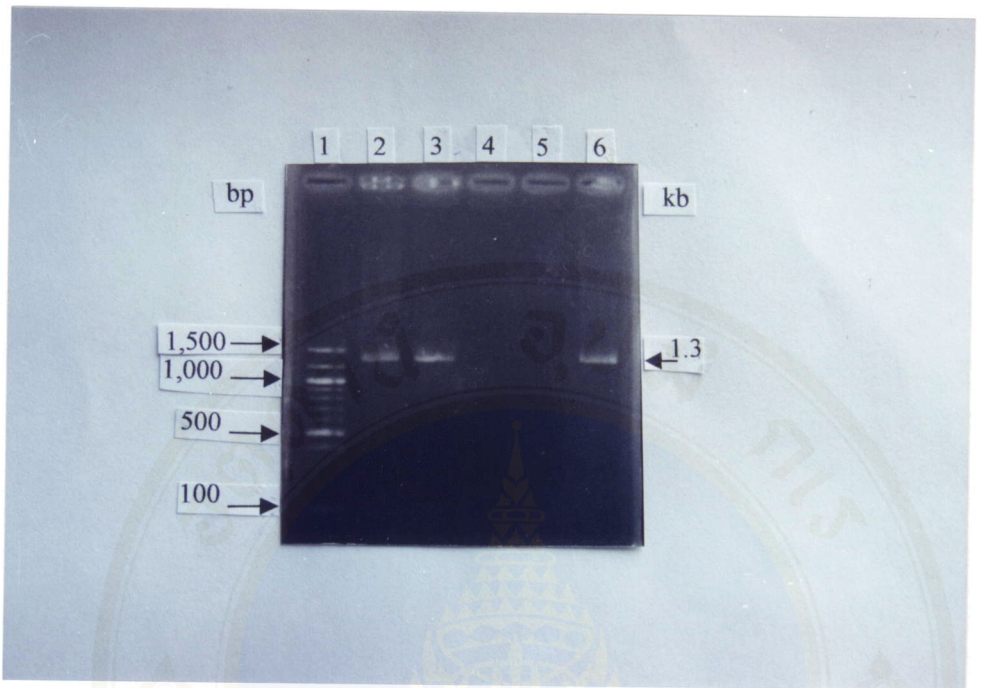

Figure 10 Agarose gel electrophoresis of PCR amplified HIV-1 *env* (gp 120). Amplified HIV-1 *env* gene fragment of 1.3 kb from Seminal cell of IDUs sample by using ENVB/AO2 as outer primers and AI1/AI2 as inner primers.

Lane 1 100 base pair marker

Lane 2,3 amplified DNA from positive sample

Lane 4 negative control

Lane 5 reagent control

Lane 6 positive control

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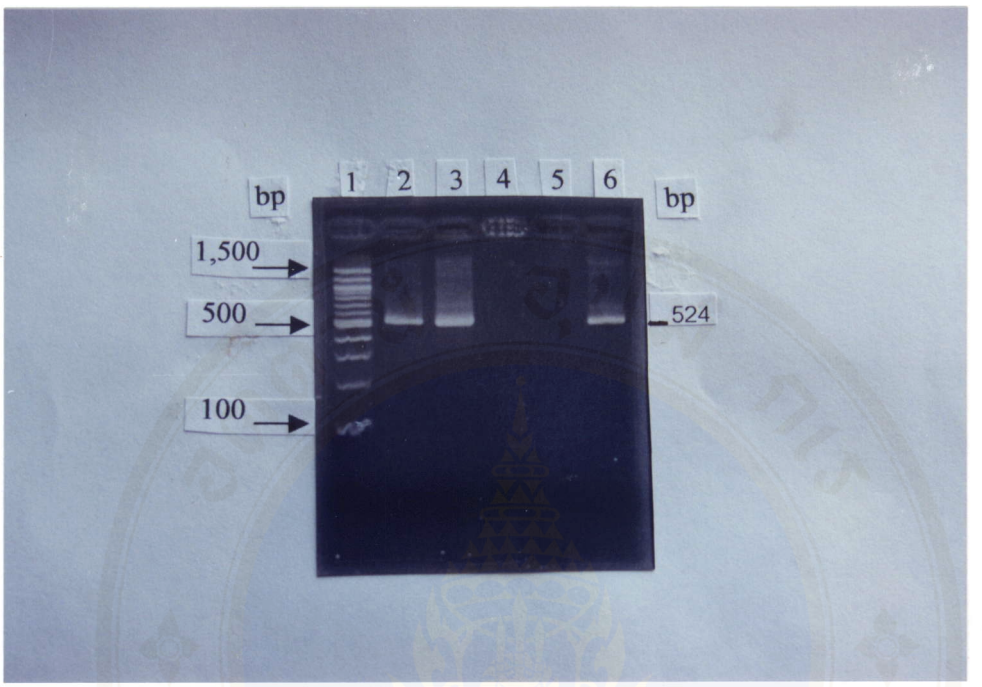

Figure 11 Agarose gel electrophoresis of PCR amplified HIV-1 *env* (C2-V4) gene . Amplified HIV-1 *env* (C2-V4) gene fragment of 524 bp from Seminal cell of IDUs sample by using JH35/JH44 as outer primer and JH33/JH48 as inner primer.

Lane 1 100 base pair marker.

Lane 2,3 amplified DNA from positive sample

Land 4 negative control

Land 5 reagent control

Land 6 positive control

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4 The relationship between peripheral CD^+_4 cell count and HIV-1 RNA level in seminal fluid.

The subjects were divided in according to blood CD^+_4 T cell number into 3 groups; group 1: CD^+_4 <200 WBC/mm^3, group 2: CD^+_4 200-499 WBC/mm^3 and group 3: $CD^+_4 \geq$ 500 WBC/mm^3.

HIV-1 viral RNA in seminal fluid from HIV-1 IDUs were quantified by using NUCLISENS HIV-1 QT (Organon Teknika, Boxtel, NL). The test was done according to the manufacturer instruction. The limitations of the test was approximately 50 copies per input volume.

The mean of HIV-1 RNA level in forty-six male seminal plasma samples from seroconvert IDUs were 3.78 (±4.00), 4.05 (±4.55), 3.52 (±3.72) log copies/ml in group 1, 2, 3, respectively (Table12, Fig.12). There was no correlation between level of CD^+_4 cell numbers and HIV-1 RNA level in seminal fluid (rho-.291, p = 0.05).

Table 12 HIV-1 RNA level in semen of infected IDUs that were divided into 3 groups; group 1, 2, and 3

$CD4^+$ T Cell count ($cell/mm^3$)	**Mean (±SD) of HIV-1 RNA Level (cell/ml)** seminal plasma
Group 1 <200 (n=6)	$6.0x10^3(\pm1.0x10^4)$
Group 2 200-500 (n=27)	$1.1x10^4(\pm3.6x10^4)$
Group 3 >500 (n=13)	$3.3x10^3(\pm5.3x10^3)$

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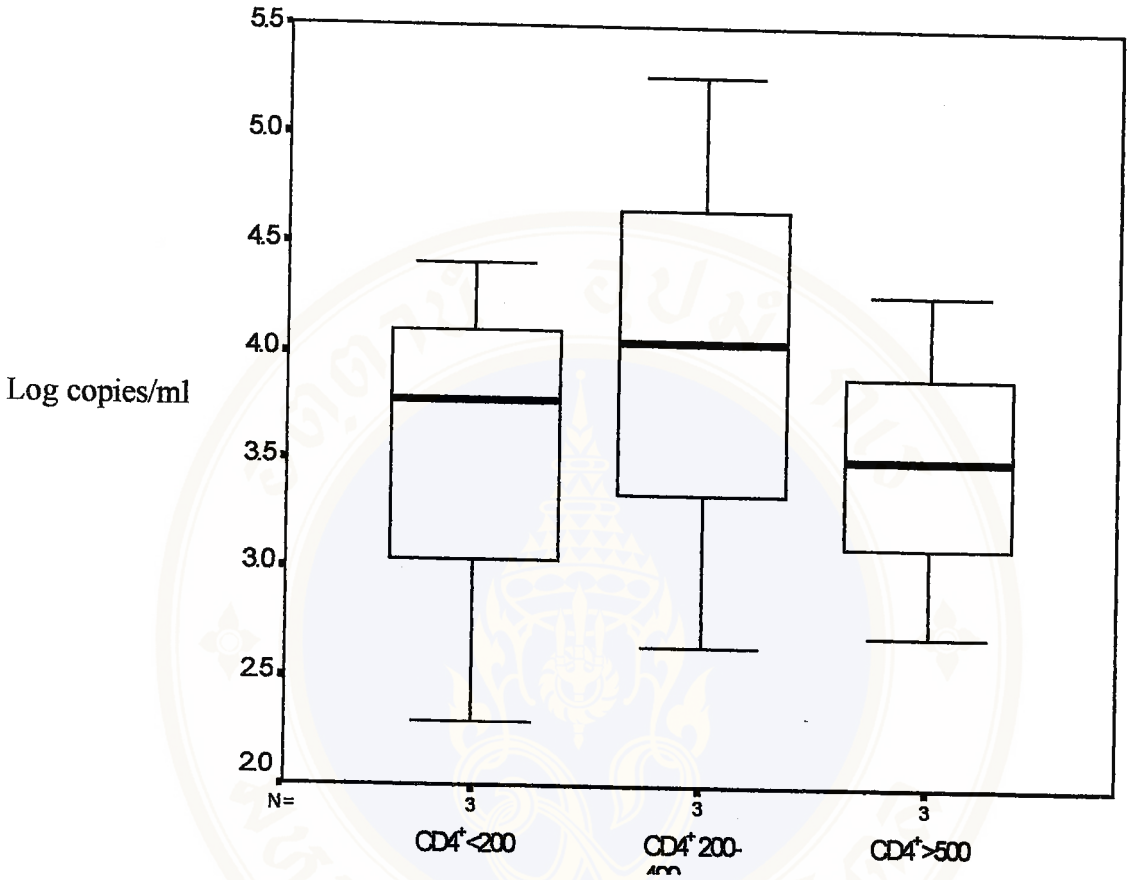

Figure 12 HIV-1 RNA level in seminal fluid compared with CD_4^+ cell numbers of 46 seroconvert IDUs. Median (from left to right) HIV-1 RNA level in seminal fluid was 3.78, 4.05, and 3.52 log copies/ml in according to CD_4^+ cell numbers <200, 200-499, and >500 WBC/mm^3, respectively. There was no correlation between CD_4^+ cell numbers and HIV-1 RNA level in seminal fluid (rho-0.291, p=0.05).

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5 Quantification of HIV-1 proviral DNA in cells isolated from blood and semen by competitive PCR (cPCR)

HIV-1 proviral DNA in PBMC and seminal cells were quantitated by a competitive nested PCR-based assay, which consisted of the subsequent amplification *gag* gene (115 pb) in the same tube of two DNA templates, proviral DNA template to be quantified and known amount of the internal deleted synthetic template (97 bp competiter), both with identical primer recognition sequence and after PCR amplification product must be clearly distinguishable by gel electrophoresis analysis. The relatives intensities of the band from both species were scanned by video densitometer and calculated amount of DNA by Gel Pro Analysis program.

HIV-1 DNA level was determined in PBMC and seminal cells of 13 HIV-1 infected seroconvert IDUs (Subject No 07, 12, 18, 21, 22, 23, 24, 31, 33, 37, 45, 46 and 47) (Table 13) (Fig 13, 14, 15). There is no relationship between HIV-1 proviral DNA level in PBMC and seminal cells (rho 0.132, p=0.668). Mean of proviral DNA level in PBMC and seminal cell were 75.82 (± 86.29) copies/10^5 PBMC and 17.81 (± 25.19) copies/10^5 mononuclear cell, respectively. The concentration of HIV-1 proviral DNA in blood was significantly higher than semen (t=2.327, p=0.029)

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Table 13 Quantitation of HIV-1 DNA and RNA in blood and semen

Subject No.	[1]No. of RNA (copies/ml) of plasma [1]	[2]No. of DNA/10^5 PBMC of PBMC	[3]No. of RNA (copies/ml) of seminal fluid	[2]No. of DNA /10^5 PBMC of seminal cell
07	81119	169.75	30000	89.13
12	54059	108.48	700	5.07
18	2982	5.86	700	2.11
21	158090	321.24	500	3.05
22	12443	25.56	600	4.62
23	34694	78.39	780	5.43
24	35967	70.83	1760	10.71
31	7768	18.35	4200	20.39
33	22708	45.77	9600	52.04
37	7056	13.92	1000	6.38
45	16451	30.48	1940	8.49
46	31818	58.63	4000	17.52
47	19498	38.41	600	6.63

[1] RNA detected by Amplicor (performance at HAC)
[2] DNA detected by cPCR
[3] RNA detected by Nuclisens

5.1 Quantitation of HIV-1 DNA level in blood

HIV-1 proviral DNA level in PBMC was range from 5.86 to 321.24 copies/10^5 PBMC. Mean of proviral DNA level in PBMC was 75.82 (±86.29) copies/10^5 PBMC. The relationship between HIV-1 proviral DNA level in PBMC and HIV-1 RNA quantitative in plasma was significant correlation (rho 0.995, p=.01) (Fig.14) and regression equation was Y = 488.705X + 226.986 (Y=HIV-1 RNA, X=HIV-1DNA in copies/ml).

Mean HIV-1 proviral DNA level of subtype B (No 23, 33, 37 and 47) was 44.19 (± 26.59) copies/10^5 PBMC and subtype E (No 07, 12, 18, 21, 22, 24, 31, 45 and 46) was

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89.9 (±100.89) copies/10^5 PBMC (Table 14) (Fig.13). The concentration of HIV-1 DNA subtype E was not significantly higher than subtype B in blood (T=-0.874, p=0.40).

5.2 Quantitation of HIV-1 DNA level in semen

HIV-1 proviral DNA level in seminal cells was range from 2.11 to 89.13 copies/10^5 mononuclear cell and mean (±SD) was 17.81 (±25.19) copies/10^5 mononuclear cell. The relationship between HIV-1 proviral DNA level in seminal cell and HIV-1 RNA in seminal fluid were significant correlation (rho 0.895, p= .01) (Fig.14), and the regression equation was Y = 313.217X – 1242.431 (Y=HIV-1 RNA, X=HIV-1 DNA in copies/ml).

Mean HIV-1 proviral DNA level of subtype B was 17.6 (±22.9) copies/10^5 mononuclear cell and subtype E was 17.9 (±27.5) copies/10^5 mononuclear cell (Table 14) (Fig.13). The concentration of HIV-1 DNA subtype E was not significantly higher than subtype B in semen (T=-0.018, p=0.986)

Table 14 Range of proviral DNA level (copies/10^5 PBMC or mononuclear cell) in PBMC and seminal cells.

Subtype	Source	Range	Mean	±SD
B (n=4)	Blood	13.92-78.39	44.19	26.59
	Semen	5.43-52.04	17.6	22.9
E (n=9)	Blood	5.86-321.24	89.9	100.89
	Semen	2.11-89.13	17.9	27.5

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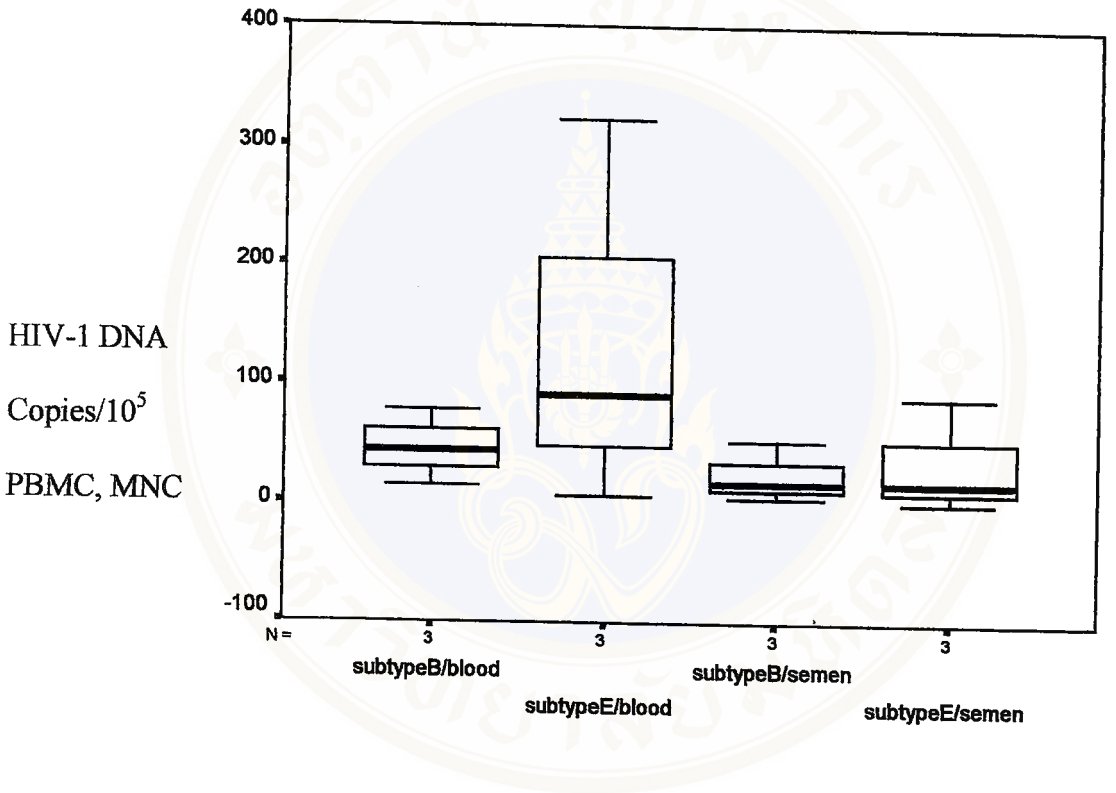

Figure 13 HIV-1 proviral DNA subtype B/E from blood and semen isolated of 13 IDUs. Median (from left to right) HIV-1 DNA was 44.1, 89.9, 17.6, and 17.9 copies/10^5 PBMC, mononuclear cell. The concentration of HIV-1 DNA subtype E was not significantly higher than subtype B in blood and semen (T=-0.874, p=0.40), (T=-0.018, p=0.986), respectively.

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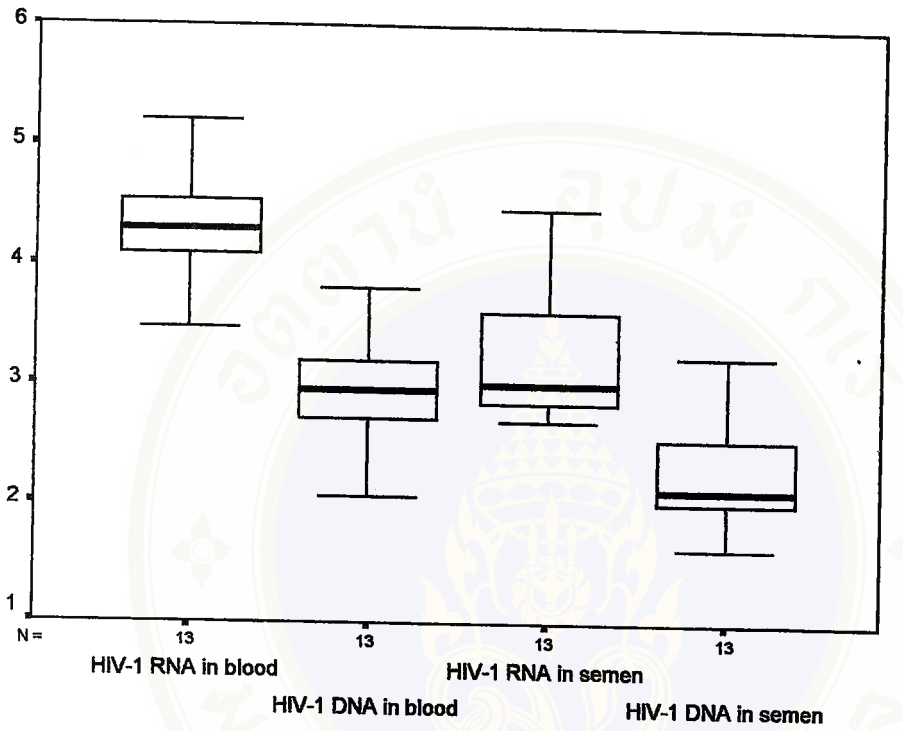

Figure 14 HIV-1 RNA level compared with HIV-1 DNA in 13 IDUs pair blood and semen. Median (from left to right) HIV-1 RNA/DNA was 4.29, 2.96, 3.00, and 2.12 log copies/ml. The relationship between HIV-1 RNA level and HIV-1 DNA level in both blood and semen was significant correlation (rho 0.995, p=0.01) and (rho 0.895, p=0.01), respectively.

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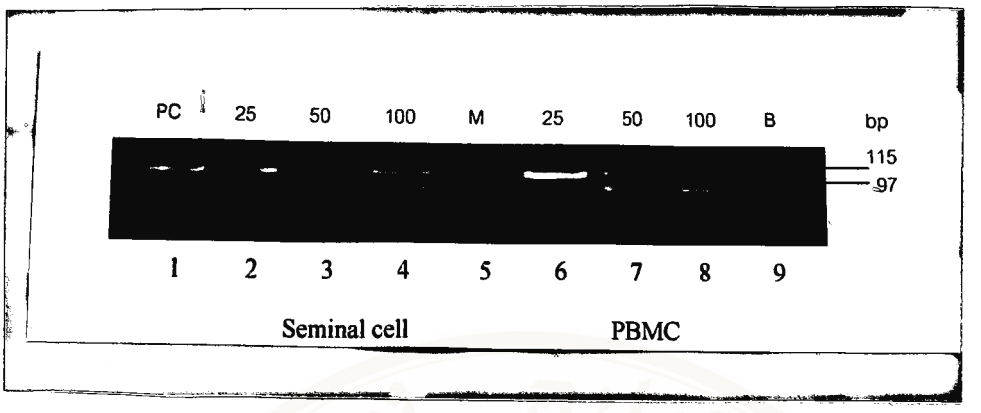

Figure 15 10 % polyacrylamine gel electrophoresis of cPCR amplified HIV-1 *gag* gene . Amplified HIV-1 *gag* gene fragment of 115 bp wide-type, 97 bp competitor from PBMC and seminal cell of IDUs samples by using SK380/SK390 as outer primers and SK38/SK39 as inner primers. Coamplification between amont copies (25, 50, and 100 copies/10^5 PBMC or MNC*) competitor and wide-type.

*Mononuclear cell in seminal cell

Lane 1 positive control

Lane 2 positive seminal cell sample 1 + 25 copies competitor

Lane 3 positive seminal cell sample 1 + 50 copies competitor

Lane 4 positive seminal cell sample 1 + 100 copies competitor

Lane 5 marker

Lane 6 positive PBMC sample 1 + 25 copies competitor

Lane 7 positive PBMC sample 1 + 50 copies competitor

Lane 8 positive PBMC sample 1 + 100 copies competitor

Lane 9 reagent control

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6 Quantification of HIV-1 viral RNA genome in seminal fluid by NUCLISENS HIV-1 QT.

HIV-1 viral RNA in seminal plasma of IDUs was quantified by using NUCLISENS HIV-1 QT (Organon Teknika, Boxtel, NL).

HIV-1 RNA level was detected in all 46 (100%) blood plasma and seminal plasma samples and HIV-1 viral RNA copy in each sample was shown in table 10, 15, and fig. 15. Mean HIV-1 RNA level in plasma and seminal plasma were 4.61 (±4.56) and 3.92 (± 4.44) log copies/ml, respectively. Blood plasma samples contained HIV-1 RNA level 0.69 log higher than seminal plasma samples. The relationship between HIV-1 RNA level in blood plasma and seminal fluid was no correlation (rho 0.116, p=0.436). When quantification of HIV-1 RNA level was classified in according to subtype, found that subtype E was not significantly higher than subtype B in seminal fluid isolated (t-0.516, p=0.607) (Fig.16).

The range HIV-1 RNA level of seminal fluid from 2.48 to 5.27 log copies/ml and mean (±SD) was 3.92 (±4.44) log copies/ml. Mean HIV-1 RNA subtype B was 3.40 (± 3.46) log copies/ml and subtype E was 4.02 (±4.49) log copies/ml (Table 15) (Fig.16).

Table 15 Range of viral load (copies/ml) in blood and semen

Subtype	Source	Range	Mean	±SD
B (n=10)	Blood	2,004-137,465	38,680.5	41,349.63
	Semen	600-9,600	2,536.0	2,901.95
E (n=37)	Blood	2,200-158,090	40,057.22	34,760.89
	Semen	300-186,000	10,386.79	30,847.07

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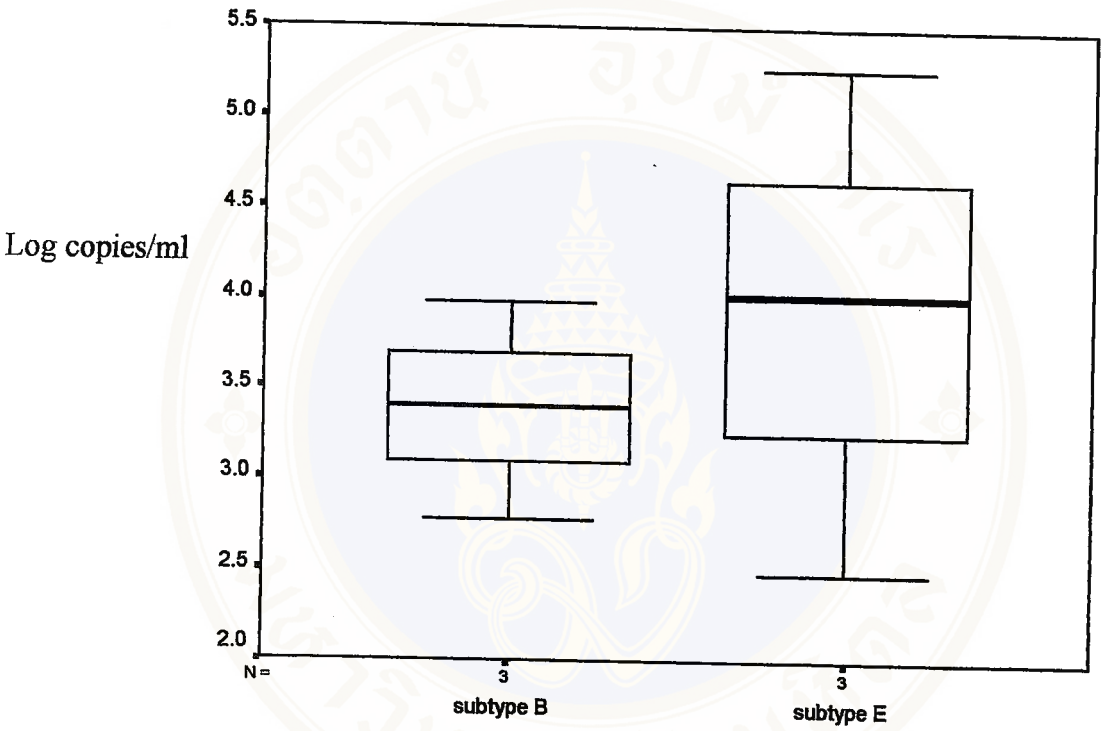

Figure 16 HIV-1 RNA level subtype B/E in semen of 46 IDUs samples. Mean of HIV-1 RNA level (log copies/ml) subtype B, E in semen was 3.40, and 4.02. HIV-1 RNA level subtype E was not higher than subtype B (t-0.516, p=0.607).

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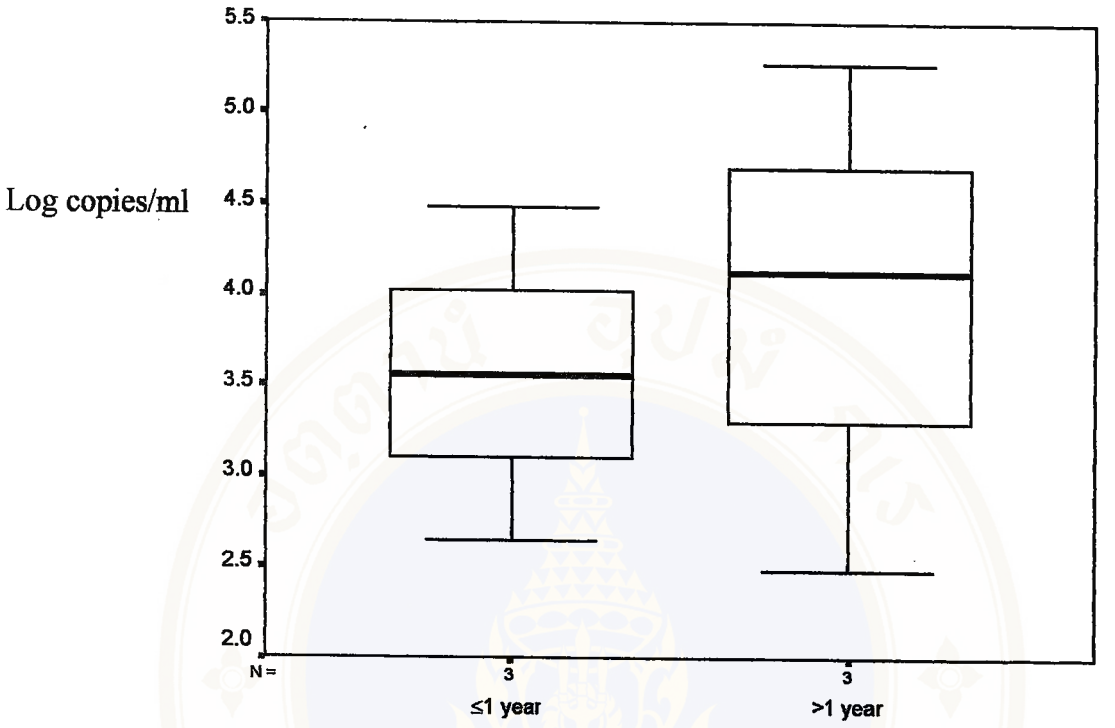

Figure 17 HIV-1 RNA level in semen of 46 IDUs samples at time of seroconvert. Mean of HIV-1 RNA level (log copies/ml) at ≤ 1 year, > 1 year were 3.56 (±3.38) (n=24), and 4.13 (±4.59) (n=22), respectively. HIV-1 RNA level at time of seroconvert > 1 year was not higher than at time of seroconvert ≤ 1 year (t-0.198, p=0.234).

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7. Nucleotide divergence of *gag* and *env* gene

Amplified PCR products fragment of *gag* and *env* genes were used to be template for direct sequence by using Big Dye Terminator sequence reaction. The sequence reactions were run on an automated DNA sequencer (ABI 310, Nucleotide). DNA sequence and amino acid alignments were performed with DNASIS version 2.1. The phylogenetic analysis was performed using the ESEE and MEGA program. The distance matrix for the neighbor-joining tree was performanced by the Kimura two-parameter method.

7.1 Nucleotide divergence of *gag* (p24) gene in PBMC and semen.

The nucleotide sequences analysis of a representative set of 9 pairs of HIV-1 subtype E detected from blood and semen samples (PB/SC No 1, 6, 7, 12, 15, 21, 24, 29, and 40), 5 pairs subtype B (PB/SC No 23, 25, 33, 44 and 47) and nucleotide sequences were shown in figure 18. The phylogenetic tree (Fig. 20) of all 14 pairs HIV-1 isolated from IDU seroconverter were divided into two major subtype: 9 pairs were classified as subtype E (PB/SC No 1,6,7,12,15,21,24,29 and 40) and 5 pairs were classified as subtype B (PB/SC No 23,25,33,44 and 47). All of them were shown a tight cluster within person from blood (PB) and semen (SC), and No 7 sample was more diverse cluster from the others.

The mean intrasubtype E divergence (interperson) was 3.9 % (± 2.6 %) in blood isolated, 4.7 % (± 2.3 %) genital fluid isolated, the mean intrasubtype B divergence

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(interperson) was 2.81 % (± 0.79 %) in blood isolated, 3.01 % (± 0.90 %) in genital fluid isolated (Table 16). Within person; (intraperson) compared mean divergence between PBMC and seminal cells was 0.88 % (±0.54 %) (range 0-2.24 %) (Table 19) (subtype E 0.85 %, B 0.95 %).

Table 16 Comparision of *gag* gene nucleotide distance of subtype B and E from IDUs samples.

subtype	% distance		% range	
	PBMC	seminal cell	PBMC	seminal cell
E	3.9 (n=18)	4.7 (n=18)	0.6-7.67	1.26-10.05
B	2.81 (n=10)	3.01 (n=10)	1.91-4.18	1.90-4.52

7.2. Nucleotide analysis divergence of *env* V3 gene in seminal cells.

The third hypervariable domain of envelope is the target of a vigorous humoral immune response and elicits antibodies capable of virus neutralization in vitro. The nucleotide divergence were analyzed by compared with a reference subtype HIV-1 CM240 (Thai A/E) and Thai B (TH141).

The nucleotide analysis of a representative set of 14 subtype E (SC No 1, 2, 3, 6, 7, 11, 12, 15, 18, 21, 24,29, 31, and 40), 5 subtype B′ (SC No 23, 25, 33, 44 and 47). Nucleotide sequences of subtype E and B were represented as figure 21 and 22, respectively. The overall mean intrasubtype E divergence in blood and semen was 6.83 (0.3-12.3) % and 8.29 % (± 3.77 %, n=14), respectively (Table 17); the mean

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intrasubtype B′ divergence in blood and semen was 9.2 (1.5-16.8) % and 16.25 % (± 4.7 %, n=5), respectively (Table 17). The motif nucleotide of subtype E at the position 58 was changed from *G*-base to *A*-base (Fig.20). The compartmentalization of HIV-1 V3 nucleotide divergence between the blood and semen was shown mean (± SD) 3.34% (± 3.38 %) (range 0-6.67%) (Table 19).

By phylogenetic tree, V3 nucleotide sequences of 19 HIV-1 isolated were divided into two major subtype: 14 samples were classified as subtype E (SC No 1,6,7,12,15,21,24,29 and 40) and 5 samples were classified as subtype B' (SC No 23,25,33,44 and 47) (Fig. 25).

Table 17 Nucleotide distance of V3 compared subtype B and E in blood and semen

subtype	% distance		% range	
	PBMC*	seminal cell	PBMC*	seminal cell
E	6.8 (n=106)	8.29 (n=14)	0.3-12.3	0.02-15.51
B	9.2 (n=26)	16.25(n=5)	1.5-16.8	9.25-18.54

* Determined by, The HIV/AIDS collaboration 1999, Thailand (submitted for *AIDS Res Hum Retrovirus*)

8. Amino acid sequence analysis.

8.1 Comparison of *gag* amino acid sequence.

The protein sequence subtype E of pair PBMC and seminal cells showed 95.3% to 100% homology, and subtype B′ showed 97.5% to 100% homology (Fig. 19).

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8.2 Comparison of *env* V3 protein.

Among the subtype E strains the V3 loop crown motif GPGQ was present in 9 of 14 (64.3%), GPGR in 3 of 14 (21.4%) and GPGK in 2 of 14 (14.3%) of the sequence. Among the subtype B′ strains that were sequenced, the GPGQ motif was present in 2 of 5 (40%), GPGR in 2 of 5 (40%), and GPGH in 1 of 5 (20%) of the sequence (Table 18, Fig.23, 24). The range of positive V3 charge was found from 1 to 7. A charge ≤ +4 was found 9/14 (64.3%) with samples SC No 7, 12, 15, 21, 23, 25, 29, 33 and 44), ≥ +5 was found 5/14 (35.7%) with samples SC No 1, 6, 24, 40 and 47. There was no significant divergence between same directed sequence and cloning (Mann-Whitney Test, $p>0.05$, n=4), (directed sequence 2.9-11.4 %, mean 7.18 %, ±3.68 %), (cloning 3.8-14.3 %, mean 6.78 %, ± 6.09 %). The HIV-1 V3 protein divergence between the blood and semen was shown mean 8.02 % (± 5.08 %), (2.9-14.3 %, n=5) and 6.30 % (± 6.16 %), (0-17.1 %, n=5), respectively. There was no significant divergence between pair semen and blood (Mann-Whitney Test, $p>0.05$, n=5).

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Table 18 V3 crown motif variable of subtype E, B′

Motif	V3 crown motif variable	
	Subtype E (%)	Subtype B′ (%)
GPGQ	9/14 (64.3)	2/5 (40)
GPGR	3/14 (21.4)	2/5 (40)
GPGH	Not found	1/5 (20)
GPGK	2/14 (14.3)	Not found

Table 19 Intraperson nucleotide divergence of HIV-1 V3 and *gag* (p24) isolated from blood and semen of HIV infected IDUs.

Gene	% distance (mean)	% range	± SD
Gag (n=14)	0.88	0-2.24	0.54
Env (n=5), (subtype E)	3.34	0-6.67	3.85

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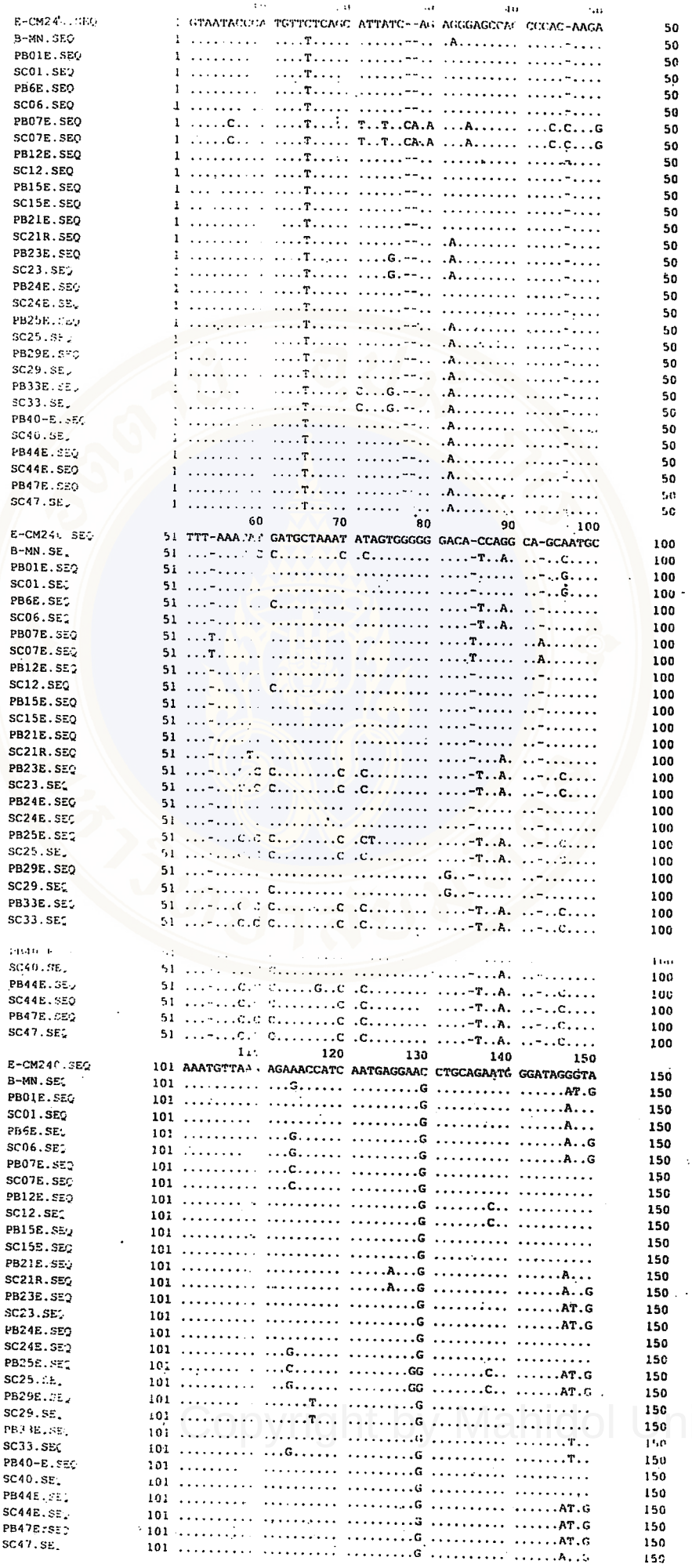

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```
                                                                  190
E-CM240.SEQ   151 GACCCAGTA. ATGCAGGGCC TATTCCACGA GGCCAGATGA GGGAACCAAG  200
B-MN.SEQ      151 ..T.....G ..........  ....A..... . ......... .A.......  200
PB01E.SEQ     151 .......... ... ...... .......... .......... ..........  200
SC01.SEQ      151 .......... .......... ....G..... .......... .A........  200
PB6E.SEQ      151 ..T..... .......... G...G..... .......... .A......G.  200
SC06.SEQ      151 ..T..... .......... G...G..... .......... .A....A.G.  200
PB07E.SEQ     151 .......... .......... .......... .......... .. .......  200
SC07E.SEQ     151 ......... . .......... .......... .......... ..........  200
PB12E.SEQ     151 .......... .......... .......... .......... ..........  200
SC12.SEQ      151 .......... .......... ....G..... .......... ..........  200
PB15E.SEQ     151 .......... .......... .......... .......... ..........  200
SC15E.SEQ     151 ......... . .......... .......... .......... ..........  200
PB21E.SEQ     151 ..... . ... .......... .......... .......... ..........  200
SC21R.SEQ     151 .......... .......... .......... .......... ..........  200
PB23E.SEQ     151 ..T.....G. .......... AG..G..... .......... .A........  200
SC23.SEQ      151 ..T.....G. .......... A.......... .......... .A........  200
PB24E.SEQ     151 ......... . .......... .......... .......... ..........  200
SC24E.SEQ     151 ..T..... .. .......... .......... .......... ..........  200

PB25E.SEQ     151 ..T.....G. .......... A...G..... .......... .A........  200
SC25.SEQ      151 ..T.....G. .......... A...G..... .......... .A........  200
PB29E.SEQ     151 ..... ... .......... .......... .......... ..........  200
SC29.SEQ      151 ..... .... .......... ....G..... .......... ..........  200
PB33E.SEQ     151 ..T.    G. .......... ACC.G..... .......... .A........  200
SC33.SEQ      151 ..T.... G. .......... ACC.G..... .......... .A........  200
PB40-E.SEQ    151 ..... .... .......... .......... .......... ..........  200
SC40.SEQ      151 .......... .......... ....G..... .......... ..........  200
PB44E.SEQ     151 ..T.... G. .......... AG..G..... .......... .A........  200
SC44E.SEQ     151 ..T.... G. .......... .G..G..... .......... .A........  200
PB47E.SEQ     151 ..T.....G. .......... AG..G..... .......... .A........  200
SC47.SEQ      151 ..T.....G. .......... AG..G..... .......... .A........  200
                        210        220        230        240        250
E-CM240.SEQ   201 GGGAACCGAC ATAGCAGGAA CTACTAGTAC CCTTCAAGAA CAAATAGGAT  250
B-MN.SEQ      201 ..... .... .......... .......... ......G... ..........  250
PB01E.SEQ     201 ..... .... .......... .......... .......... ..........  250
SC01.SEQ      201 ..... ... .......... .......... .......... ..........  250
PB6E.SEQ      201 .......... .......... .......... .....G.... ..........  250
SC06.SEQ      201 ..A.. ... .......... .......... ......G... ..........  250
PB07E.SEQ     201 .......... .......... .......... .......... ..........  250
SC07E.SEQ     201 .......... .......... .......... .......... ..........  250
PB12E.SEQ     201 ..... ... .......... .......... .......... ..........  250
SC12.SEQ      201 .......... .......... .......... ......G... ..........  250
PB15E.SEQ     201 .......... .......... .......... .......... ..........  250
SC15E.SEQ     201 ..... ... .......... .......... .......... ..........  250
PB21E.SEQ     201 ..... .... .......... .........A .......... ..........  250
SC21R.SEQ     201 ..... .... .......... .........A .......... ..........  250
PB23E.SEQ     201 ..... .... .......... .......... ......G..G ..........  250
SC23.SEQ      201 ..... .... .......... .......... ......G..G ..........  250
PB24E.SEQ     201 .......... .......... .......... .......... ..........  250
SC24E.SEQ     201 .......... .......... .......... .......... ..........  250
PB25E.SEQ     201 ..... ..... .......... .......... ......G..G ..........  250
SC25.SEQ      201 ..... ... .......... .......... ......G... ..........  250
PB29E.SEQ     201 .....  ... .......... .......... .......... ..........  250
SC29.SEQ      201 .......... .......... .......... ......G... ..........  250
PB33E.SEQ     201 .......... .......... .........A T........G ..........  250
SC33.SEQ      201 ..... ..... .......... .........A T........G ..........  250
PB40-E.SEQ    201 ..  ... .......... .......... .......... ..........  250
SC40.SEQ      201 .... . ... .......... .......... .......... ..........  250
PB44E.SEQ     201 .....  ... .......... .......... T.....G..G ..........  250
SC44E.SEQ     201 .....  .... .......... .......... T.....G... ..........  250
PB47E.SEQ     201 ..........T .......... .......... T.....G..G ..........  250
SC47.SEQ      201 ..... ... .......... .......... T.....G..G ..........  250
                        260        270        280        290        300
E-CM240.SEQ   251 GGATTAAAAA CAATCCACCC ATCCCAGTGG GAGACATCTA TAAAAGGTGG  300
B-MN.SEQ      251 .......... T.........T ........A. ....A..... ......A...  300
PB01E.SEQ     251 .......... .........T .......... ....A..... ..........  300
SC01.SEQ      251 .......... .........T .......... ....A..T.. ..........  300
PB6E.SEQ      251 ........GG .......T.T ........A. ....A..T.. ......A...  300
SC06.SEQ      251 ........GG .......T.T ........A. ....T..T.. ..........  300
PB07E.SEQ     251 ........G. .........T .......... .......... ..........  300
SC07E.SEQ     251 ..... ...G. .........T .......... .......... ..........  300
PB12E.SEQ     251 .......... ......T..T .......... ....T..... ..........  300
SC12.SEQ      251 .......... T.....T..T .......... ....T..... ..........  300
PB15E.SEQ     251 .........G .........T .......... .......... ......A...  300
SC15E.SEQ     251 .....  ... .........T .......... .......... ......A...  300
```


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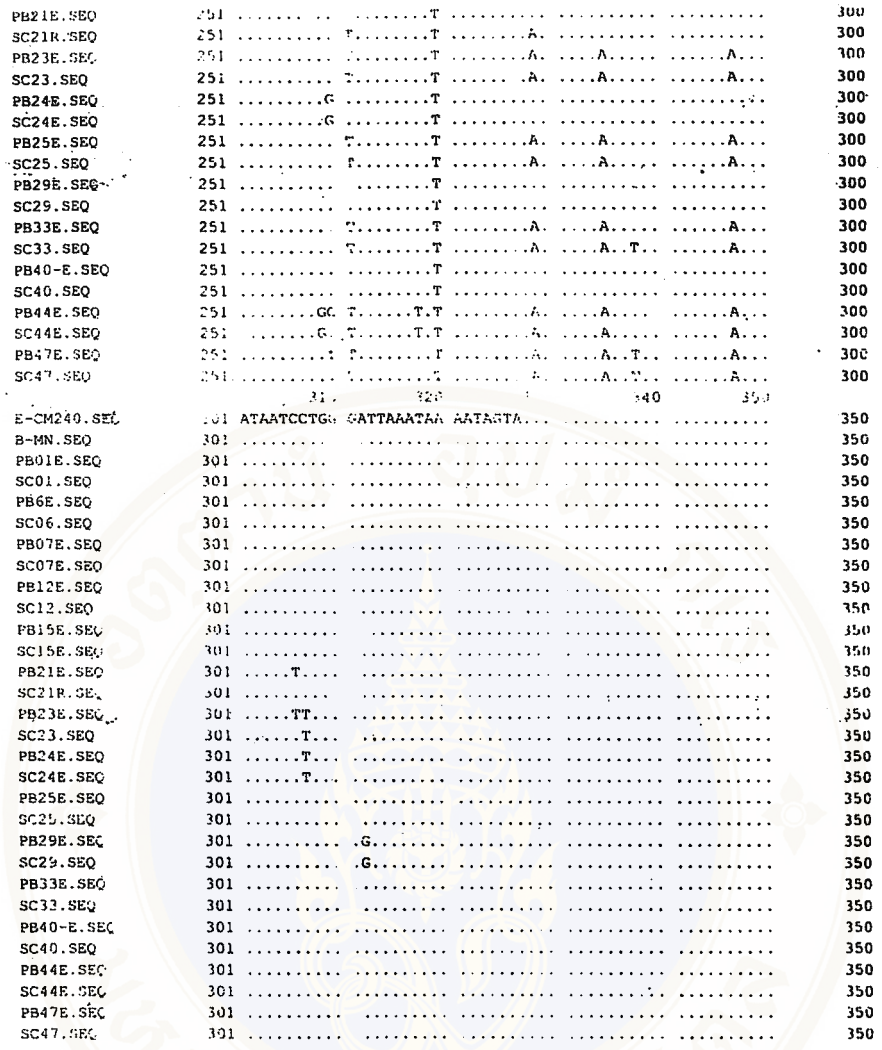

Figure 18 Nucleotide sequence alignment HIV-1 *gag* (partial p24) region of 14 pair blood (PB) and semen (SC) of early seroconvert IDUs, which were compared with a consensus sequence Thai E (CM240) and B (MN). Dots indicate nucleotide sequence identical to the consensus sequence; dashes gaps introduced to maintain the alignment.

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```
                          10         20         30         40         50
E-CM240.AMI          1 VIPMFSALSE -GATPQDLNM MLNIVGGH-Q AAMQMLKETI NEEPAEWDRV  50
B-MN.AMI             1 .......... -........T ...T....-. .......... ...A.....L  50
PB01E.AMI            1 .......... -......... .........-. .......... ...A......  50
SC01.AMI             1 .......... -......... .........-. .......... ...A......  50
PB06E.AMI            1 .......... -........T ...T....-. .......... ...A......  50
SC06.AMI             1 .......... -......... .........-. .......... ...A......  50
PB07E.AMI            1 .......F.K RKPP..GFKY DAKYS..TSR Q......D.. ...A......  50
SC07E.AMI            1 .......F.K RKPP..GFKY DAKYS..TSR Q......D.. ...A......  50
PB12E.AMI            1 .......... -......... .........-. .......... ...A.D....  50
SC12.AMI             1 .......... -........I .........-. .......... ...A.D....  50
PB15E.AMI            1 .......... -......... .........-. .......... ...A......  50
SC15E.AMI            1 .......... -......... .........-. .......... ...A......  50
PB21E.AMI            1 .......... -......... .........-. .......... ...A......  50
SC21R.AMI            1 .......... -........L .........-. .......... ...A......  50
PB23E.AMI            1 ........A. -........T ...T....-. .......... ...A.....L  50
SC23.AMI             1 ........A. -........T ...T....-. .......... ...A.....L  50
PB24E.AMI            1 .......... -......... .........-. .......... ...A......  50
SC24E.AMI            1 .......... -......... .........-. .......... ...A......  50
PB25E.AMI            1 .......... -........T ...T....-. .......D.. ...A.D...L  50
SC25.AMI             1 .......... -........T ...T....-. .......... ...A.D...L  50
PB29E.AMI            1 .......... -......... .........-. .......... ...A......  50
SC29.AMI             1 .......... -........I .........-. .......... ...A......  50
PB33E.AMI            1 ........A. -........T ...T....-. .......... ...A.....L  50
SC33.AMI             1 ........A. -........T ...T....-. .......... ...A.....L  50
PB40.AMI             1 .......... -......... .........-. .......... ...A......  50
SC40.AMI             1 .......... -........T .........-. .......... ...A......  50
PB44E.AMI            1 .......... -........T ...T....-. .......... ...A.....L  50
SC44E.AMI            1 .......... -........T ...T....-. .......... ...A.....L  50
PB47E.AMI            1 .......... -........T ...T....-. .......... ...A.....L  50
SC47.AMI             1 .......... -........T ...T....-. .......... ...A......  50
                          60         70         80         90        100
E-CM240.AMI         51 HPVHAGPIPP GQMREPRGSD IAGTTSTLQE QIGWMTNNPP IPVGDIYKRW 100
B-MN.AMI            51 ........T. .......... .......... .......... ....E..... 100
PB01E.AMI           51 .......... .......... .......... .......... ....E..... 100
SC01.AMI            51 ........A. .......... .......... .......... ....E..... 100
PB06E.AMI           51 ........A. .......... .......... .......... ....E..... 100
SC06.AMI            51 ........A. .....QGE.. .......... ......G..S .......... 100
PB07E.AMI           51 .......... .......... .......... ......D... .......... 100
SC07E.AMI           51 .......... .......... .......... ......D... .......... 100
PB12E.AMI           51 .......... .......... .......... .......... .......... 100
SC12.AMI            51 ........A. .......... .......... .......... .......... 100
PB15E.AMI           51 .......... .......... .......... ......S... .......... 100
SC15E.AMI           51 .......... .......... .......... .......... .......... 100
PB21E.AMI           51 .......... .......... ......N... .......... .......... 100
SC21R.AMI           51 .......... .......... ......N... .......... .......... 100
PB23E.AMI           51 .......VA. .......... .......... .......... ....E..... 100
SC23.AMI            51 .......... .......... .......... .......... ....E..... 100
PB24E.AMI           51 .......... .......... .......... ......S... .......... 100
SC24E.AMI           51 .......... .......... .......... ......S... .......... 100
PB25E.AMI           51 .......VA. .......... .......... .......... ....E..... 100
SC25.AMI            51 ........A. .......... .......... .......... ....E..... 100
PB29E.AMI           51 .......... .......... .......... .......... .......... 100
SC29.AMI            51 ........A. .......... .......... .......... .......... 100
PB33E.AMI           51 .......PA. .......... ......N... .......... ....E..... 100
SC33.AMI            51 .......PA. .......... ......N... .......... ....E..... 100
```

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PB40.AMI          51 .......... .......... .......... .......... ..........  100
SC40.AMI          51 .........A. .......... .......... .......... ..........  100
PB44E.AMI         51 .......VA. .......... .......... ......A..S ....E.....  100
SC44E.AMI         51 .......VA. .......... .......... ......D..S ....E.....  100
PB47E.AMI         51 .......VA. .......... .......... .......... ....E.....  100
SC47.AMI          51 .......VA. .......... .......... .......... ....E.....  100
                        110       120       130       140       150
E-CM240.AMI      101 IILGLNKIV. .......... .......... .......... ..........  150
B-MN.AMI         101 .......... .......... .......... .......... ..........  150
PB01E.AMI        101 .......... .......... .......... .......... ..........  150
SC01.AMI         101 .......... .......... .......... .......... ..........  150
PB06E.AMI        101 .......... .......... .......... .......... ..........  150
SC06.AMI         101 .......... .......... .......... .......... ..........  150
PB07E.AMI        101 .......... .......... .......... .......... ..........  150
SC07E.AMI        101 .......... .......... .......... .......... ..........  150
PB12E.AMI        101 .......... .......... .......... .......... ..........  150
SC12.AMI         101 .......... .......... .......... .......... ..........  150
PB15E.AMI        101 .......... .......... .......... .......... ..........  150
SC15E.AMI        101 .......... .......... .......... .......... ..........  150
PB21E.AMI        101 .......... .......... .......... .......... ..........  150
SC21R.AMI        101 .......... .......... .......... .......... ..........  150
PB23E.AMI        101 .......... .......... .......... .......... ..........  150
SC23.AMI         101 .......... .......... .......... .......... ..........  150
PB24E.AMI        101 .......... .......... .......... .......... ..........  150
SC24E.AMI        101 .......... .......... .......... .......... ..........  150
PB25E.AMI        101 .......... .......... .......... .......... ..........  150
SC25.AMI         101 .......... .......... .......... .......... ..........  150
PB29E.AMI        101 .......... .......... .......... .......... ..........  150
SC29.AMI         101 .......... .......... .......... .......... ..........  150
PB33E.AMI        101 .......... .......... .......... .......... ..........  150
SC33.AMI         101 .......... .......... .......... .......... ..........  150
PB40.AMI         101 .......... .......... .......... .......... ..........  150
SC40.AMI         101 .......... .......... .......... .......... ..........  150
PB44E.AMI        101 .......... .......... .......... .......... ..........  150
SC44E.AMI        101 .......... .......... .......... .......... ..........  150
PB47E.AMI        101 .......... .......... .......... .......... ..........  150
SC47.AMI         101 .......... .......... .......... .......... ..........  150
```

Figure 19. Amino acid alignment of the *gag* (p24) region present in blood and semen of early seroconvert IDUs. Dots indicate amino acids indentical to the consensus sequence; dashes indicated gaps introduced to maintain the alignment.

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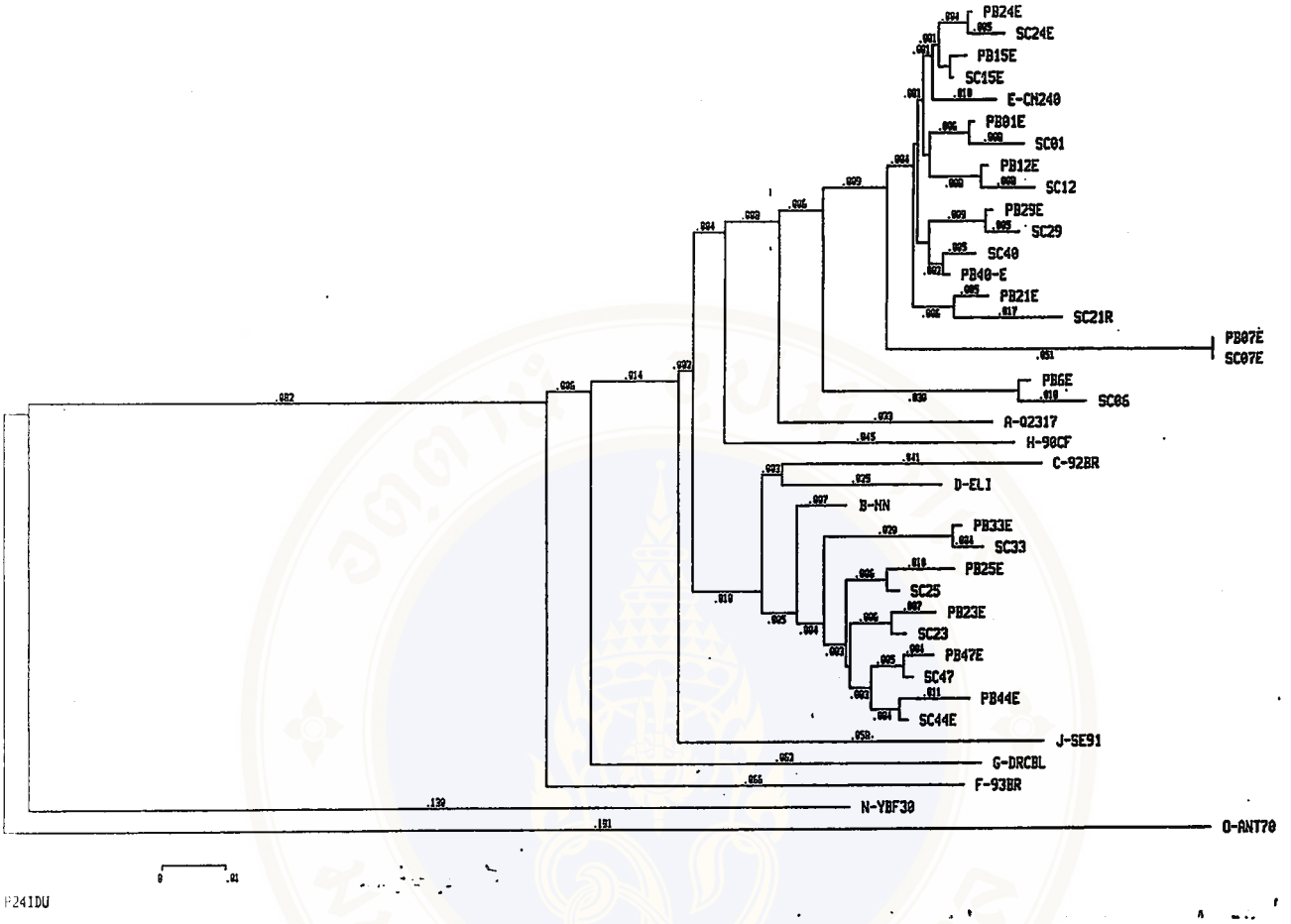

Figure 20. The phylogenetic tree of HIV-1 *gag* (p24) nucleotide sequence isolated from blood and semen of early seroconvert IDUs, constructed based on gag p24 sequence unambiguously aligned positions of 9, 5 pair samples represent subtype E and B strains, respectively along with a consensus sequence Thai E (CM240) and B (MN). Phylogenetic tree was constructed using ESEE and MEGA program.

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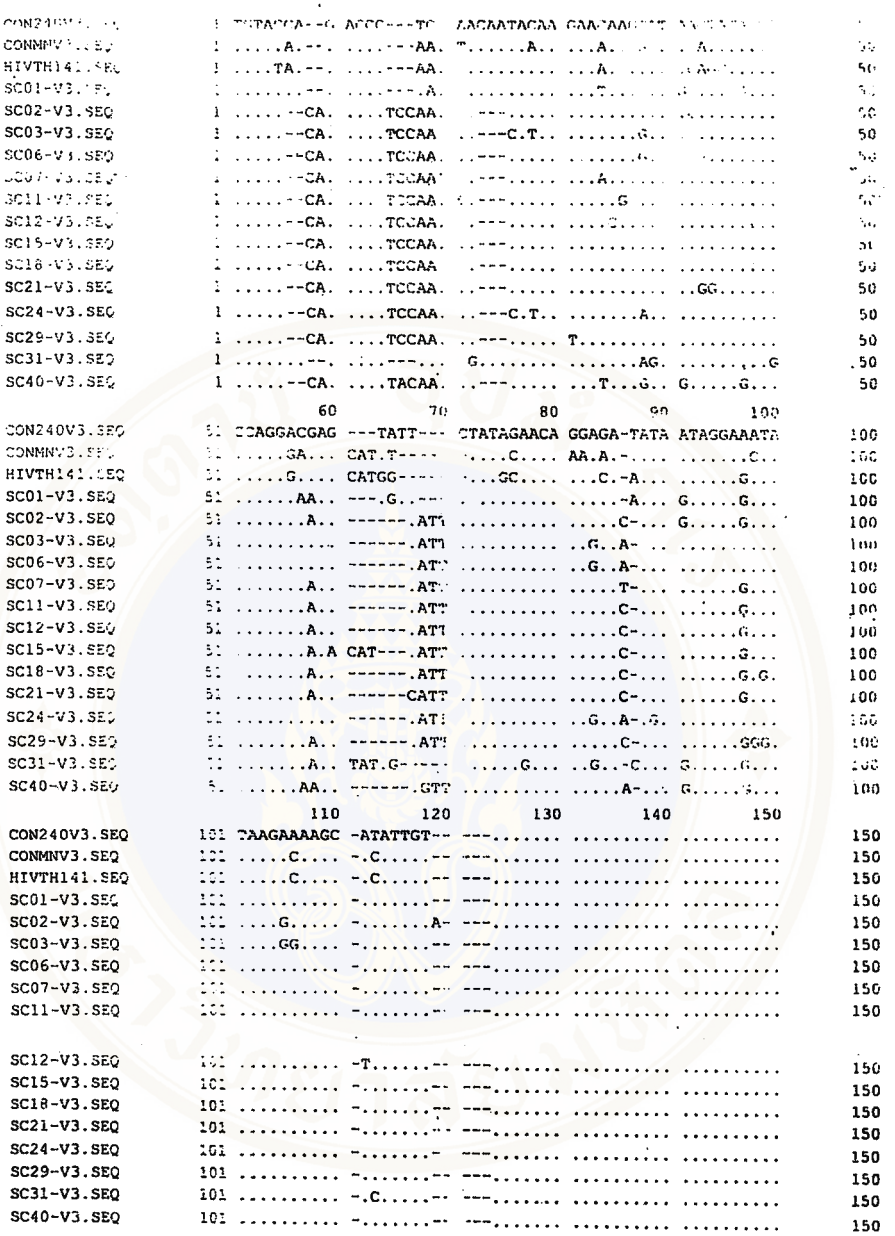

of early seroconvert IDUs, which were compared with a consensus sequence Thai E (CM240) and B′ (TH141). Dots indicate nucleotide sequence identical to the consensus sequence; dashes gaps introduced to maintain the alignment.

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```
                          10         20         30         40         50
THAIB.SEQ               1 TGTAC--AAG ACCCAAC--- AACAATACAA GAAAAGGTAT ACCTCTAGGA
CONMNV3.SEQ             1 .....--... .........--- T......A.. .....A.G.. ..A.A.....
CON240V3.SEQ            1 .....CA--. ....---TCC .......... ...C.A.... .A..A.....
SC23V3-1.SEQ            1 .....--... .........--- T...C..T.. ...C.A.G.. .AA.......
SC25V3-1.SEQ            1 .....--... .........--- .......... ...C.A.... .A..A.....
SC33V3-1.SEQ            1 .....--... .......--- ..A.GGGT.. ...GTA.... .---A.....
SC44V3-1.SEQ            1 .....--... ......T--- G.A....... A..C.A.G.. GA..A.G...
SC47V3-1.SEQ            1 .....--... .........--- T.-------- A..C.AAG.. .A..AGG...
                          60         70         80         90         100
THAIB.SEQ              51 CCAGGGCAAG CATGG----- -TATACAACA GGACAA-ATA ATAGGAGATA
CONMNV3.SEQ            51 ......AG.. ...TT----- -.......... AA.A.T-... ......AC..
CON240V3.SEQ           51 .....A.G.. ---TATT--- C....G.... ...G.-T... ......A...
SC23V3-1.SEQ           51 .....A.G.. .....----- -.......... ..GG..-... ......A...
SC25V3-1.SEQ           51 .....A.... .....----- -....G.... .......-... .-......G.
SC33V3-1.SEQ           51 .....A.... .....----- -....G.... .A.GGT-G.. GAT.......
SC44V3-1.SEQ           51 .....A..C. .....----- -.......... ...G..-... ..........
SC47V3-1.SEQ           51 .....A.G.. T.-----GT- C....GT... ...G.C-..G GC........
                         110        120        130        140        150
THAIB.SEQ             101 TAAGACAAGC -ACAGTGT-- ---------- .......... ..........
CONMNV3.SEQ           101 .......... -...T...-- ---------- .......... ..........
CON240V3.SEQ          101 .....A.... -.T.-----T TGT....... .......... ..........
SC23V3-1.SEQ          101 ....CAC... -.T.T...-- ---------- .......... ..........
SC25V3-1.SEQ          101 .T........ T.T.T...-- ---------- .......... ..........
SC33V3-1.SEQ          101 .......... -.T.T...-- ---------- .......... ..........
SC44V3-1.SEQ          101 .......... -...T...-- ---------- .......... ..........
SC47V3-1.SEQ          101 .....A.... -.T.T...-- ---------- .......... ..........
```

Figure 22 Nucleotide sequence alignment of V3 region subtype B from 5 semen (SC) of early seroconvert IDUs, which were compared with a consensus sequence Thai E (CM240) and B′ (TH141). Dots indicate nucleotide sequence identical to the consensus sequence; dashes gaps introduced to maintain the alignment.

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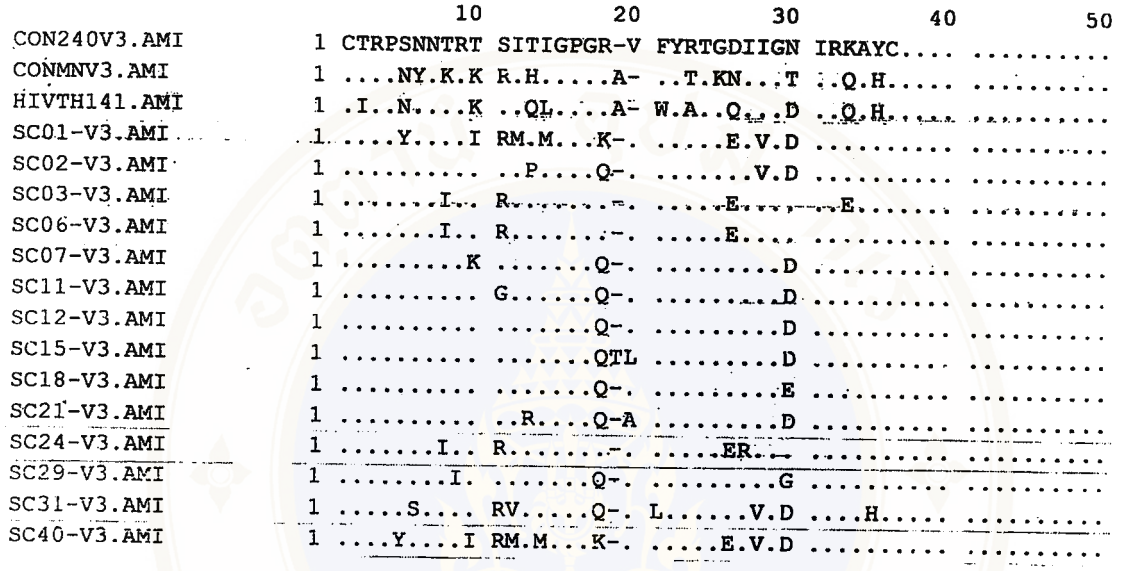

Figure 23. Amino acid alignment of the V3 region subtype E present in 14 semen of early seroconvert IDUs. Dots indicate amino acids indentical to the consensus sequence; dashes indicated gaps introduced to maintain the alignment.

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```
                      10         20         30         40         50
THAIB.AMI          1 CTRPNNNTRK GIPLGPGQAW YTTGQIIGDI RQAQC..... .....
CONMNV3.AMI        1 .....Y.K.. R.HI...R.F ...KN...T. ...H..... .....
CON240V3.AMI       1 ....S....T S.TI...RVF .R..D...N. .K.Y..... .....
SC23V3.AMI         1 .....YTI.T R.N....R.. ....E...N. ST.Y..... .....
SC25V3.AMI         1 .........T S.TI..... .R....KERL DKLY..... .....
SC33V3.AMI         1 .....KRV.S S.-I..... .R.EGVD... ...Y..... .....
SC44V3.AMI         1 .....E..KT RMTM...H.. ....E..... ...H..... .....
SC47V3.AMI         1 .....YTKT. IT-R...RVV .S..DMA... .K.Y..... .....
```

Figure 24. Amino acid alignment of the V3 region subtype B present in 5 semen of early seroconvert IDUs. Dots indicate amino acids indentical to the consensus sequence; dashes indicated gaps introduced to maintain the alignment.

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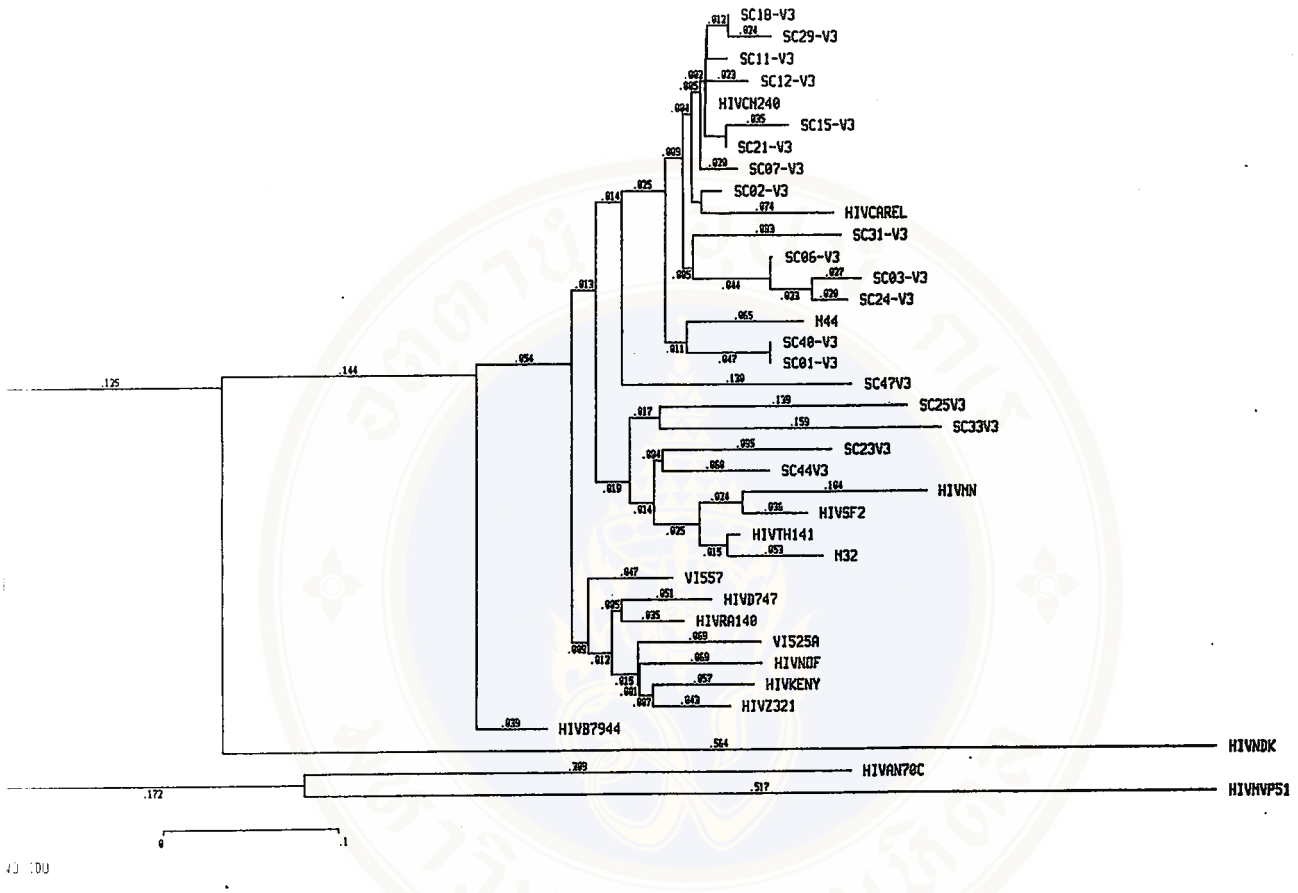

Figure 25. The phylogenetic tree of HIV-1 V3 nucleotide sequence isolated from semen of early seroconvert IDUs, constructed based on V3 region sequence unambiguously aligned positions of 14, 5 represent subtype E and B' strains, respectively along with a consensus sequence Thai E (CM240) and B' (TH141). Phylogenetic tree was constructed using ESEE and MEGA program.

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CHAPTER VI

DISCUSSION

Sexual contact is play a major role in HIV transmission. Although HIV-1 subtype E is predominately spreaded in Thailand mainly in heterosexual transmission, the initial epidemic of HIV-1 transmission documented among IDUs in and near Bangkok in central Thailand in 1988 was predominantly due to subtype B′, but recently report showed the increasing number of HIV-1 subtype E predominating among intravenous drug user group, while IDU have played a major role in the spread of HIV-1 in Europe. The reasons for rapid transmission of HIV-1 subtype E *via* IDU in Thailand, despite the presence of subtype B strains among before 1989, are incompletely explained. To understand the genotypic characterization of HIV-1 isolated from semen and blood of early seroconvert IDUs, the quantify, qualify, and molecular characterized of proviral DNA and RNA genome must be determine. Well-defined studies are required to define further the relative contributions of epidemiologic and viral phenotypic factors to the transmission dynamics of HIV-1 subtype E and B (92).

Paired blood and semen samples from 46 early seroconvert IDUs were used to study genotypic characterization of HIV-1. All of subjects were asymptomatic and their range time of seroconvert from 1 to 24 month. The number of mononuclear cells in

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semen were range from $2.0x10^5$ to $5.0x10^6$ cells/ml. HIV-1 asymptomatic men have less leukocytes in semen than do symptomatic, similarly the number of leukocytes in semen increases with the presence of other sexual transmitted disease (93). Paired bloods and semen samples were also quality, quantity and sequencing of HIV-1 genome in each sample.

The relationship between peripheral $CD4^+$ cell count and HIV-1 RNA level in blood plasma and seminal fluid

Forty-six paired blood plasma and seminal fluid were quantified for HIV-1 RNA and $CD4^+$ cell count. Subject No 8 was lost follow up. According to their $CD4^+$ cell count were classified according to their $CD4^+$ cell count into 3 groups, group 1 was <200 CD^+_4 WBC/mm^3, group 2 was 200-499 CD^+_4 WBC/mm^3, and group 3 was ≥ 500 CD^+_4 WBC/mm^3, most of subjects were have $CD4^+$ cell count from 500 to 200 cells/ml (58.70 %). The mean of HIV-1 RNA level in forty-six male blood plasma and seminal plasma samples from seroconvert IDUs were 4.67 (± 4.68), 4.66 (±4.56), 4.43 (±4.43) log copies/ml and were 3.78 (±4.00), 4.05 (±4.55), 3.52 (±3.72) log copies/ml in group 1, 2, 3, respectively. Most of them were have HIV-1 RNA level in group 2 and only 2 subjects were have CD_4^+ cell more than 1000 cells/mm^3; SC 25 and SC 37. There was no correlation between both HIV-1 RNA level in plasma, seminal plasma, and CD_4^+ cell count. This study, quite similarly with that reported Coombs RW et al; study with 114 subject were quantified by Amplicor, which reported that the CD4 cell count in

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peripheral blood show a weak inverse correlation with the seminal plasma HIV-1 RNA level and stronger inverse correlation with blood plasma (8).

Quantification of HIV-1 proviral DNA in blood and semen by competitive PCR (cPCR)

13 paired PBMC and seminal cell were quantified HIV-1 proviral DNA by competitive nested PCR.

HIV-1 DNA level was detected in 100 % PBMC and seminal cell. There was no correlation between HIV-1 proviral DNA in blood and semen, however the concentration of HIV-1 proviral DNA in blood was sinificantly higher than in semen, because of WBC counts in semen are generally lower than in blood (63, 68). Only 2 of 13 (subject No 31 and 33) subject had higher numbers of HIV-1 DNA copies/10^5 mononuclear cell in semen than in blood. HIV-1 subtype B DNA copy level in PBMC ranged from 13.92 to 78.39 copies per 10^5 PBMC with mean (± SD) 44.1 (± 26.59) copies per 10^5 PBMC, while HIV-1 subtype E DNA copy level ranged from 5.86 to 321.24 copies per 10^5 PBMC with mean (± SD) 89.9 (± 100.89). Although, amount of mean HIV-1 proviral DNA subtype E in blood was higher than subtype B′, but there was no correlation between subtype E and subtype B′. The correlation between HIV-1 DNA in PBMC was significantly lower than HIV-1 RNA in plasma.

HIV-1 subtype B DNA copy level in seminal cell ranged from 5.43 to 52.04 copies per 10^5 seminal mononuclear cell with mean (± SD) 17.6 (± 22.9) copies per 10^5

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mononuclear cell, while HIV-1 subtype E DNA copy level ranged from 2.11 to 89.13 copies per 10^5 seminal mononuclear cell with mean (± SD) 17.9 (± 27.5) copies per 10^5 seminal mononuclear cell. Although, amount of mean HIV-1 proviral DNA subtype B′ in semen was higher than subtype E, but there was no correlation between subtype E and subtype B′. The correlation between HIV-1 proviral DNA in seminal cell was significantly lower than HIV-1 RNA in seminal fluid.

There was a larger variation in PBMC and seminal cell HIV-1 DNA level between men, because of the lack of association between the number of unprotected sexual contacts and the probability of infection (94). This study was similar with Xu C, et al have reported that the relationship between HIV-1 DNA/10^6 mononuclear WBC in paired blood and semen samples revealed a significant positive correlation (33) and the concentration of HIV-1 DNA was significantly higher in blood than semen, but contrast with Xu C, et al have reported HIV-1 in peripheral blood have been inversely correlated with peripheral blood CD4 cell count.

Quantification of HIV-1 viral RNA genome in blood plasma and seminal fluid

Although 46 paired plasma and seminal fluid samples were quantified by using Amplicor and Nuclisens HIV-1 QT, respectively but there was a strong positive correlation of RNA levels measured by the two methods (95). HIV-1 DNA level was detected 100 % in plasma and seminal plasma. The range HIV-1 subtype B RNA level in plasma was 3.30 to 5.14 log copies/ml with mean (± SD) 4.59 (± 4.62) log copies/ml, while ranged in seminal fluid was 2.78 to 3.98 log copies/ml with mean (± SD) 3.40 (±

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3.46) log copies/ml. The range HIV-1 subtype E RNA level in plasma was 3.34 to 5.20 log copies/ml with mean (± SD) 4.60 (± 4.54) log copies/ml, while range in seminal fluid was 2.48 to 5.27 log copies/ml with mean (± SD) 4.02 (± 4.49) log copies/ml. Blood plasma samples contained HIV-1 RNA level 0.69 log higher than seminal plasma samples. Mean HIV-1 RNA level of both subtype B′ and E in plasma was higher 1.19 and 0.58 log copies/ml than in seminal plasma, respectively. Although, HIV-1 RNA level subtype E was a little higher than subtype B′, but HIV-1 RNA level subtype E was not significantly higher than subtype B′. There was a larger variation of both plasma and semen HIV-1 RNA between men, because of unprotect sexual contact and probability of infection. Although amount of HIV-1 RNA in plasma was significantly higher than seminal fluid, but there was not correlation between HIV-1 RNA in plasma and seminal plasma. Only 5 of 47 (10.6%) (subject No 08, 29, 33, 40 and 41) paired subject had higher numbers of HIV-1 RNA level higher in semen than in blood.

Because of a number of factor that inactivate cell free HIV-1, including anti HIV-1 antibodies, zinc, lactoferrin and peroxidase are present in genital tract secretion (96), especially zinc, that specifically inhibit some viral enzyme (97, 95). Other investigator have reported that presence of HIV-1 RNA subtype E in early seroconversion higher geometric mean than subtype B′ in IDUs, however 12 months after seroconversion, those with E and B′ has similar mean viral level (98), while Coombs et al. reported that there was a weak correlation between HIV-1 RNA level in the blood plasma measured by the bDNA assay and HIV-1 RNA levels in the seminal plasma measure by the RT-PCR assay (8), but contrast with Tachet A et al. reported that there was significant correlation between HIV-1 RNA level in blood plasma and seminal plasma measure by HIV-Monitor

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technique (Roche) (99). Ball JK et al. (100) detected HIV-1 RNA in 76 % and 63 % of blood and semen samples, Rowe T et al. (101) detected HIV-1 RNA in 9 (75%) of 12 blood plasma and 7 (58.3%) of 12 seminal plasma samples. Duliose et al. (102) detected HIV-1 RNA in 94 % blood plasma and 84 % of seminal plasma samples.

Shedding of HIV-1 proviral DNA in blood and semen

In this study, nested PCR has been shown to be sensitive for detecting HIV in blood (*gag* gene) and semen (*gag* and *env* gene). HIV-1 proviral *gag* DNA were found 46/46 (100 %) in blood and 36/46 (78.3 %) in semen samples. Only 10/46 (21.7 %) in semen could not detect HIV-1 *gag* proviral DNA, which had range of mononuclear cell from $3.3x10^5$ to $5.0x10^6$ cells/ml and ranged HIV-1 RNA level from 550 to $4.9x10^4$ copies/ml. Because of semen contains more inhibitor than blood, and a number of inhibitor antimicrobial compounds, such as exceptionally high levels of zinc, that specifically inhibit some viral enzyme (103), whereas within spermatozoa, zinc is closely associated with sulfhydryl groups and disulfide linkages and is concentrated in the tail (97). Other investigators reported that presence of proviral *gag* DNA in blood and semen were 88 and 70 %, respectively (8).

The average means genetic distance of intrasubtype homogeneity of HIV-1 *gag* gene for subtype B′ and E were 2.72 % (range 0.6-4.52 %) and 4.06 % (range 0-10.05 %), respectively and within person averaged mean divergence between PBMC and semen were 0.88% (±0.54%) (range 0-2.24%) (subtype B′ and E 0.95 % and 0.85 %, respectively. Most of the DNA can be found in blood isolated more than semen isolated,

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because of mononuclear cell count in semen are generally lower than in blood (63). Gag gene was more conserve than *env* gene, the phylogenetic tree of 14 pairs blood (PB) and semen (SC) were shown a tight cluster within person, whereas 5 pairs were classified for subtype B compare with HIV-1 MN and 9 pairs were classified for subtype E compare with HIV-1 CM240.

T-lymphocyte recognition, cell tropism and viral infectivity. In this study, HIV-1 proviral *env* DNA was found 19/36 (52.8 %) in seminal cell. The average mean genetic distance of intrasubtype (interperson) homogeneity of HIV-1 env gene for subtype B′ and E were 16.25 % (range 9.25-18.54 %, n=5) and 8.29 % (range 0.02-15.51 %) respectively. And previously have reported that IDUs of Thailand, in 1995-1998; Subbarao et al (66) reported that the inter-strains nucleotide distance (C2-V4) within subtype E strains was 6.8 % and 9.2 % for subtype B, in 1994-1995 the intrasubtype nucleotide divergence within the V3 and flanking regions of *env* gene was 5.7 % for subtype E and 3.1 % for subtype B′ (60). In 1991, the mean intrasubtype E and B′ nucleotide divergence in the same region of the gp 120 gene of Thai isolates was 3.4 and 3.5 %, respectively, whereas the intersubtype distance was estimated to be 22 % (59). However, among Thai patients with AIDS, the mean interisolate or intrasubtype distance in subtype E gp 120 (entire gene) is much larger as much as 12.1 % for sample isolated in 1993 in patients from northern Thailand (104). In this study, among the subtype E strains the V3 loop crown motif GPGQ was present in 9 of 14 (64.3%), GPGR in 3 of 14 (21.4%) and GPGK in 2 of 14 (14.3%) of the sequence whereas GPGH not found. Among the subtype B′ strains that were sequenced, the GPGQ motif was present in 2 of 5 (40 %), GPGR in 2 of 5 (40 %), GPGH in 1 of 5 (20 %) of the sequence, whereas GPGK

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next common motif was GPGR. It is noteworthy that GPGR which is commonly seen in sequences from person infected with North-American subtype B HIV-1, is now increasing in frequencey compared to GPGQ among Bangkok IDUs. Subbarao S, et al have reported that IDU in 1994-1995 had among the subtype E strains the V3 loop crown motif GPGQ 85.1 %, GPGR 7.5 %, GPGH 7.5 %, while among subtype B' strains were found the GPGQ 65.4 %, GPGR 30.8%, and GPGE 3.8 %. The motif nucleotide *(gga caa gga cga)* of subtype E at the position 58 was changed from G-base to A-base which made amino acid changed from GPGR to GPGQ. The motif property of each subtype more important to study for develop vaccine, immunoassays and others. The range of positive V3 charge was found from 1 to 7. A charge ≤ +4 was found 9/14 (64.3%), ≥ +5 was found 5/14 (35.7%). The correlation between biological phenotype of HIV-1 isolate and the clinical course of infection was demonstrated that fast-replicating, syncytium-inducing (SI) variants, positive charge ≥ 5 emerge in 50% of HIV-1 seropositive individuals preceding progression to AIDS. Slow-replicating, non-syncytium inducing (NSI) variants, positive charge ≤ 4 are predominant in the asymptomatic stage and persist throughout all stages of HIV-1 infection (105, 30). Most of IDU (64.3 %) in this study may be long-term non-progresses. The compartmentalization of HIV-1 V3 and *gag* nucleotide divergence between the blood and semen were shown mean (± SD) 3.34 % (± 3.85 %), (0-6.67 %, n=5). There was compartmentalization of HIV-1 in semen and blood by V3 nucleotide, this study same as some investigator (106). HIV-1 V3 protein divergence between the blood and semen was shown mean (± SD) 8.02 % (± 5.08 %), (2.9-14.3 %, n=5) and 6.30 % (± 6.16 %), (0-17.1 %, n=5), respectively. There was no significant divergence between pair semen and blood (pair T-test 0.421, n=5). There was

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no significant divergence between same directed sequence and cloning (pair T-test, p=0.836, n=4), (directed sequence 2.9-11.4 %, mean 7.18 %, ±3.68 %), (cloning 3.8-14.3 %, mean 6.78 %, ±6.09 %). For best study the two viral compartmentalization, directed sequence and cloning must more population than this.

There were two variants of subtype B (Thai B) identified by Ou et al; with majority of V3 motif GPGQ and McCutchan et al; with V3 motif GPGR. The arginine (R) amino acid of typical subtype B sequences from America and Europe is usually encoded by AGA codon, but the arginine in V3 motif GPGR were encode by CGA codon instead. Only one nonsynonymous nucleotide change to a codon of CAA, is needed to produce the glutamine (Q).

Callahan MC et al (106) reported evidence for viral RNA compartmentalization within blood and semen that 16/21 IDUs samples showed C2-V3 region phylogenetic relatedness (>90 % bootstrap) and minimal DNA distance (0-0.88 %), while 5/21 IDUs samples showed significant divergence in the two viral populations (2.95-6.19 %), with weak to strong phylogenetic clustering. The distinct proviral sequence variants have been reported in some patients in PBMC compared to brain tissue, cerebrospinal fluid, spleen, lymph node, lung, and semen (107), because of HIV variants may evolve to replicate more efficiency in specific target cell types through selection for particular tropisms, such as enhance or receptor specificity or other viral properties (72). Brain-derived isolates show enhanced macrophage replication competence relative to those simultaneously derived from blood (108). Difference patches of epidermal langerhans cells and adjacent splenic white pulps have also been shown to contain distinct proviral variants (109). Testicles could also be an immmunologically privileged site for HIV-1 more

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macrophages and lymphocytes are present in semen relative to blood (110). The titer of anti immmunoglobulin G in semen are on average, 1/10 of the titer present in blood including antibodies against HIV-1 proteins p55, p24, and p17, which are less prevalent in semen than in blood (111). This raises the possibility that viral evolution in semen may be different from that in blood because of the differences in immune pressure.

The inter strain genetic variation among the Vietnam HIV-1 env sequence ranged from 0.3 % to 9.0 % (mean, 4.6 %). Brown et al. found that the genetic diversity of B' nucleotide sequence ranged from 1.5 to 5.8 % (mean 2.8 %) compared to 1.5 to 8.2 % (mean 4.6 %) for Thailand sequence (50). In Myanmar, the interperson nubleotide sequence variations in env C2/V3 regions of B' and E, prevailing in Yangon were 6.7 ± 2.1 % and 7.1 ± 0.7 % respectively, which was similar to those level observed in Thailand (53).

These observation help resolve some important controversies concerning HIV-1 shedding in semen, confirm the compartmentalization of HIV-1 between the blood and the genital, and hold important implication for patient counseling and the design of clinic trials design HIV vaccine development and epidemiologic studies of HIV-1 IDUs.

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CHAPTER VII

CONCLUSION

To study the genotypic characterization of HIV-1 isolated from semen and blood of early seroconvert IDUs. This study was carried out to determine the quantify, qualify, and relationship between proviral DNA and RNA genome in semen compared the blood of 46 early seroconvert IDUs. The shedding of HIV-1 DNA was detected by nested PCR. The quantitative of proviral DNA and RNA was detected by competitive PCR and NUCLISENS QT TEST (Organon), respectively. Directivity sequencing of *gag* and *env* gene was performed by automated DNA sequencing system.

All 46 HIV-1 infected IDUs from BMA were asymtomatic and healthy at the time collection. V3 nucleotide sequences were classified into 2 major subtypes; 21.7% classified as subtype B′, and 78.3% classified as subtype E. The most of CD4 cell counts in 46 IDUs samples were 58.7 %, with CD4 cell counts 200-500 cell/mm^3. There was no correlation between CD^+_4 cell numbers and both HIV-1 RNA level in plasma (rho-0.243, p=0.104), and in seminal fluid (rho-.291, p = 0.05).

HIV-1 DNA level was determined in all PBMC and seminal cells from 13 HIV-1 infected seroconvert IDUs. There was no relationship between HIV-1 proviral DNA level PBMC and seminal cells (rho 0.132, p=0.668). The concentration of HIV-1 DNA was significantly higher in blood than semen (t=2.327, p=0.029). The relationship between HIV-1 proviral DNA level in PBMC and HIV-1 RNA quantitative in plasma was

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significant correlation (rho 0.995, p=.01). The concentration of HIV-1 DNA subtype E was no higher than subtype B in blood (T-Test, p>0.05). The relationship between HIV-1 proviral DNA level in seminal cell and HIV-1 RNA in seminal fluid were significant correlation (rho 0.895, p= .01). The concentration of HIV-1 DNA subtype E was no higher than subtype B in semen (T-Test, p>0.05).

HIV-1 RNA level was detected in all 46 (100%) blood plasma and seminal plasma samples. Blood plasma samples contained HIV-1 RNA level 0.69 log higher than seminal plasma samples, and amount of HIV-1 RNA level in plasma was significantly higher than seminal plasma (t=4.796, p<0.01). The relationship between blood plasma and seminal fluid was no correlation HIV-1 RNA level (rho 0.116, p=0.436). HIV-1 RNA level subtype E was not higher than subtype B in blood and semen (t-0.516, p=0.607).

In forty-six IDUs samples, HIV-1 proviral *gag* DNA were found 100 % PBMC samples and found 78.3% seminal cell samples. HIV-1 proviral *env* DNA were found 52.8 % seminal cells samples. The mean intrasubtype E divergence (interperson) was 3.9 % (± 2.6 %) in blood isolated, 4.7 % (± 2.3 %) genital fluid isolated, the mean intrasubtype B divergence (interperson) was 2.81 % (± 0.79 %) in blood isolated, 3.01 % (± 0.90 %) in genital fluid isolated. Within person; compaired mean *gag* gene divergence between PBMC and seminal cells of subtype E and B were 0.85% and 0.95%, respectively. The phylogenic tree of *gag* gene was shown a tight cluster within person from blood (PB) and semen (SC), and only No 7 sample was more diverse cluster from the others. The overall mean *env* gene intrasubtype E divergence in blood and semen was 6.83 (0.3-12.3 %, n=106) % and 8.29 % (± 3.77 %, n=14), respectively and the mean *env* gene intrasubtype B′ divergence in blood and semen was 9.2 (1.5-16.8 %, n=26) % and

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16.25 % (± 4.7 %, n=5), respectively. The compartmentalization of HIV-1 V3 and *gag* nucleotide divergence between the blood and semen were shown mean (± SD) 3.34 % (± 3.38 %), (0-6.67 %, n=5), 0.88 % (±0.54 %), (0-2.24 %, n=14).

The gag protein sequence subtype E of pair PBMC and seminal cells showed from 95.3 % to 100 % homology, and subtype B′ showed from 97.5% to 100% homology. The most V3 crown motif predominate in subtype E was GPGQ, and next GPGR, but not differ in subtype B. The HIV-1 V3 protein divergence between the blood and semen was shown mean 8.02 % (± 5.08 %), (2.9-14.3 %, n=5) and 6.30 % (± 6.16 %), (0-17.1 %, n=5), respectively. There was no significant divergence between pair semen and blood (Mann-Whitney Test, $p>0.05$, n=5). There was no significant divergence between same directed sequence and cloning (Mann-Whitney Test, $p>0.05$, n=4), (directed sequence 2.9-11.4 %, mean 7.18 %, ±3.68 %), (cloning 3.8-14.3 %, mean 6.78 %, ±6.09 %), however, the analysis V3 crown motif between semen and blood should be used more population.

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APPENDIX

Stock solution

1. 10% Ammonium persulfate

   Ammonium persulfate 1 gm

   Distilled water to 10 ml

2. KCl

   KCl 7.46 gm

   Distilled water to 100 ml

3. Tris HCl 1M pH 8.0

   Tris hydroxymethyl aminomethane 12.1 gm

   Distilled water 80 ml

   Adjust pH to 8.0 with concentrated HCl then mix and add distilled water to 100 ml.

   Steriled by autoclaving at 121°c with pressure 15 lb/square inch for 15 minutes.

4. $MgCl_2$ 1M

   $MgCl_2.6H_2O$ 20.3 gm

   Distilled water to 100 ml

5. 10X TBE buffer

   Tris base 85.3 gm

   Distilled water to 500 ml

Reagent for PCR technique

1. PCR lysis buffer

   1M KCl 5 ml

   1M Tris HCl (pH 8.3) 1ml

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Reagent for PCR technique (cont.)

1M $MgCl_2$	0.25 ml
NP-40	0.45 ml
Tween-20	0.45 ml
Distilled water	100 ml

Sterilize by autoclaving at 121°c with pressure 15 lb/square inch for 15 minutes at store at 4°c

2. Proteinase K (10 mg/ml)

Proteinase K	10 mg
10 mM Tris HCl (pH 7.5)	1 ml

To prepare working solution, 10 µl of proteinase K solution was added to 1 ml of lysis buffer just before used.

3. Deoxynucleotide triphosphate (dNTPs) mixture.

Each dATP, dGTP, dCTP, dTTP was supplied in each vial 100 mM concentration.

dATP	25 µl
dGTP	25 µl
dCTP	25 µl
dTTP	25 µl

Distilled water 900 µl

4. Loading dye (6X)

Bromphenol blue	100 mg
Sucrose	20 gm
0.5X TBE buffer	50 ml

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Reagent for PCR technique (cont.)

5. 0.5X TBE buffer

10X TBE buffer 50 ml

Distilled water to 950 ml

6. Ethidium bromide solution (10 mg/ml)

Ethidium bromide 1 gm

Distilled water 100 ml

Store in the dark at room temperature

7. Agarose gel for electrophoresis

2 % Agarose gel

Agarose gel 0.6 gm

TBE buffer (0.5X) 30 ml

1 % Agarose gel

Agarose gel 0.3 gm

TBE buffer (0.5X) 30 ml

8. Oligonucleotide primers for amplification and sequencing

Prepare each primer at 100 pmole/µl in sterile deionized water, store at –20 °c

Reagent for Semen collection

1. Viral transport Medium (VTM Medium)

Penicillin (10^5 Unit/ml) 1 ml

Streptomycin (10^5 µg/ml) 1 ml

Nystatin (10^4 Unit/ml) 2 ml

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Reagent for Semen collection (cont.)

RPMI 1640 (sterile by filtrated) to bring to 100 ml. Stored at 4°c for 6 months. The final concentrations of the components is 200 units/ml of nystatin, 1000 units/ml of penicillin, and 1000 µl/ml of streptomycin.

2. Culture medium

Penicillin (10^5 Unit/ml)	100 µl
Streptomycin (10^5 µg/ml)	100 µl
Fetal bovine serum (heat inactivated)	20 µl
Glutamine (1M)	200 µl

RPMI 1640 (sterile by filtrated) to bring to 100 ml. Stored at 4°c. The final concentration of the components is 100 Unit/ml penicillin, 100 µg/ml streptomycin and 2 mM glutamine

2. Freezing Medium

20 % DMSO	20 ml
Fetal bovine serum (heat inactivated)	80 ml

Storage at 4 ° c temperature

Reagent for cPCR technique

1. 30.8 % (w/v) Acrylamide-bisacrylamide

Acrylamide (Biorad)	30.0 gm
Bisacrylamide (Biorad)	0.8 gm
Distilled water	100 ml

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Reagent for cPCR technique (cont.)

Sterilize by 0.2 mm filter before use, kept in dark at 4 ° c for 1 month

2. 10 % (w/v) Ammonium persulfate

Ammonium persulfate	1.0 gm

Dissolved in 10 ml of distilled water, aliquot into 1 ml and kept in –20 ° c

3. 10% polyacrylamide gel (for 6 ml)

30.8 % (w/v) polyacrylamine	2 ml
TEMED (N,N,N',N'-Tetramethylethylenediamine)	3 µl
10 % (w/v) Ammonium persulfate	30 µl
5X TBE buffer	1.2 µl
Distill water	2.75 ml

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