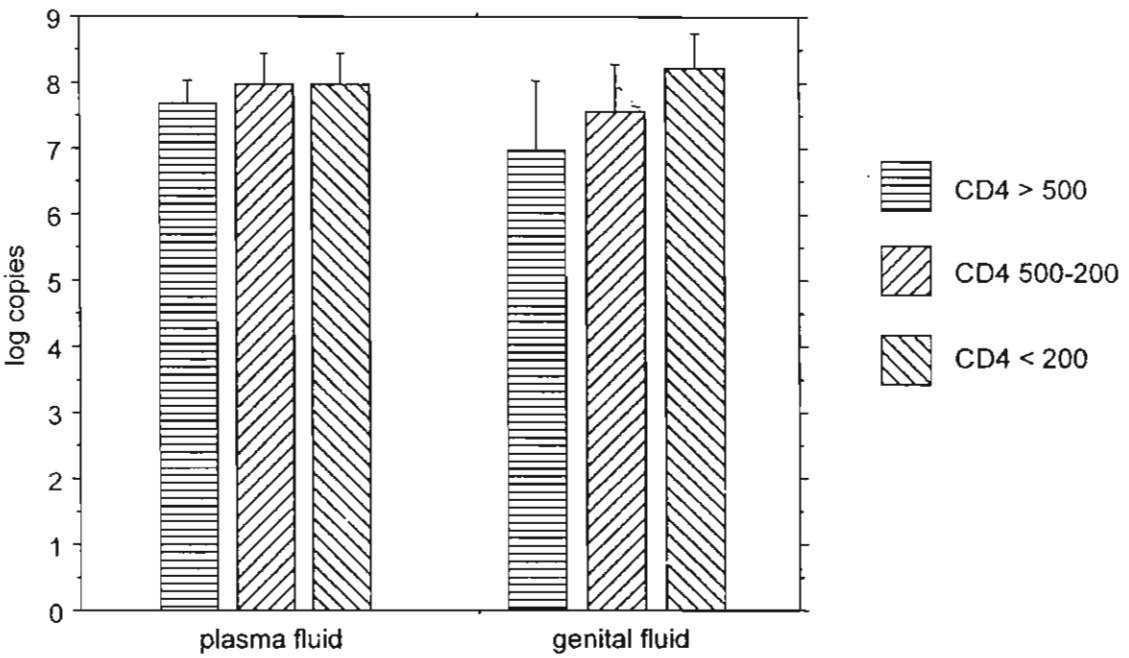


Figure 8a HIV-1 viral RNA copy number quantified by dot blot hybridization in relation with blood CD4 lymphocyte count.

The relationship of blood CD4+ lymphocyte count and HIV-1 viral RNA copy number in plasma and genital fluid samples from infected couples were studied. Blood CD4 lymphocyte count was classified in to 3 groups. In both plasma and genital fluid samples, the lowest mean RNA copy number of 7.7 log and 7 log, respectively was found in CD4 >500 cells/mm^3group.

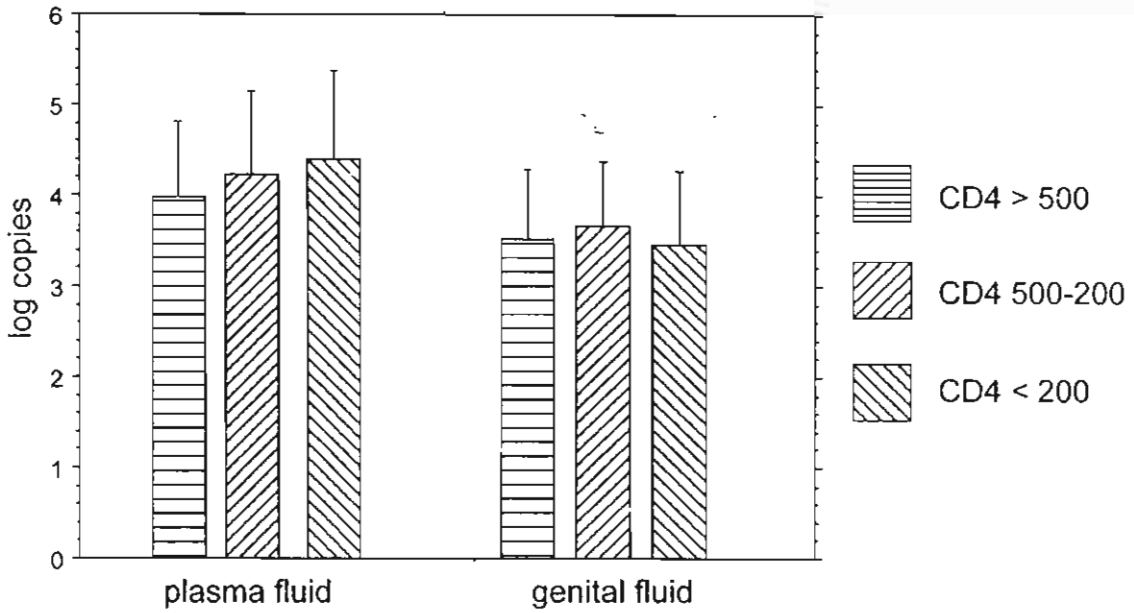
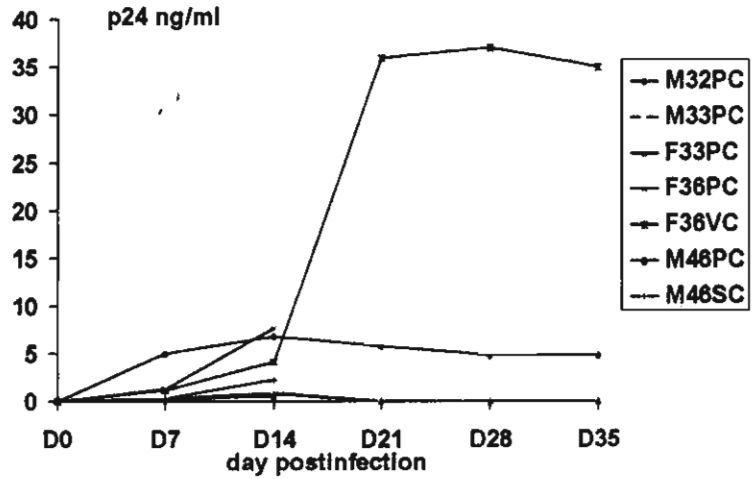


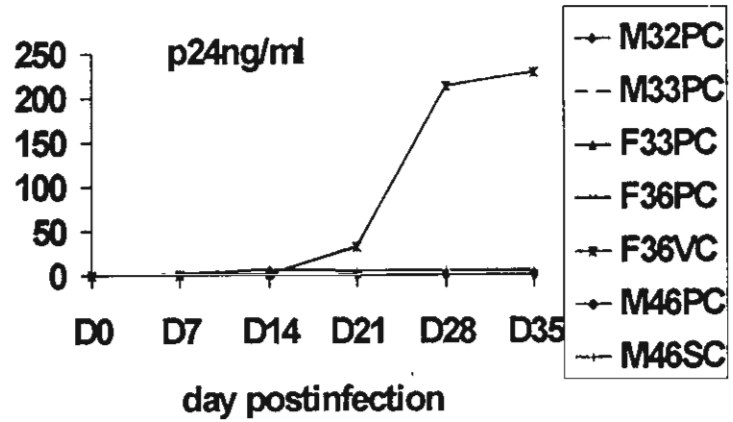
Figure 8b HIV-1 viral RNA copy number quantified by ROCHE AMPLICOR HIV-1 MONITOR Test in relation with blood CD4 lymphocyte count.

The relationship of blood CD4+ lymphocyte count and HIV-1 viral RNA copy number in plasma and genital fluid samples from infected couples were studied. The lowest mean viral RNA copy number of 4.0 log RNA in plasma sample was found in CD4 >500 cells/mm^3 group while the lowest viral RNA copy number of 3.5 log was found in CD4 < 200 cells/mm^3.

Fig. 3 Infectivity assay of HIV-1 isolates in primary maccrophage

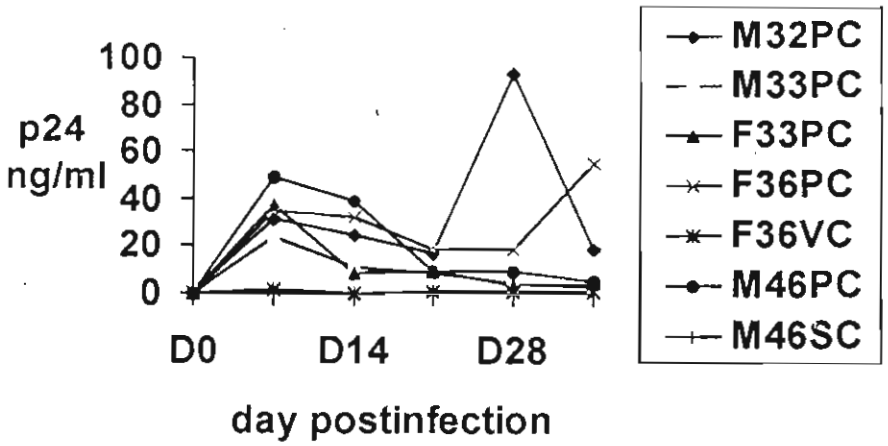


CAV



CFV

Fig.10Infectivity assay of HIV isolates inMT2 cell line



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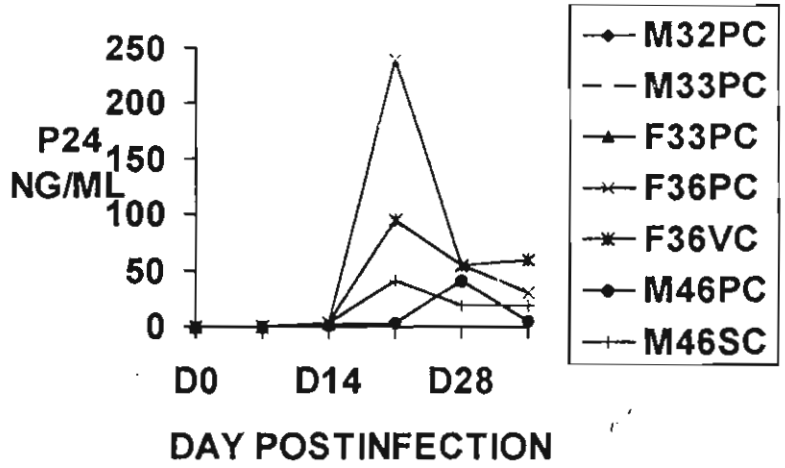
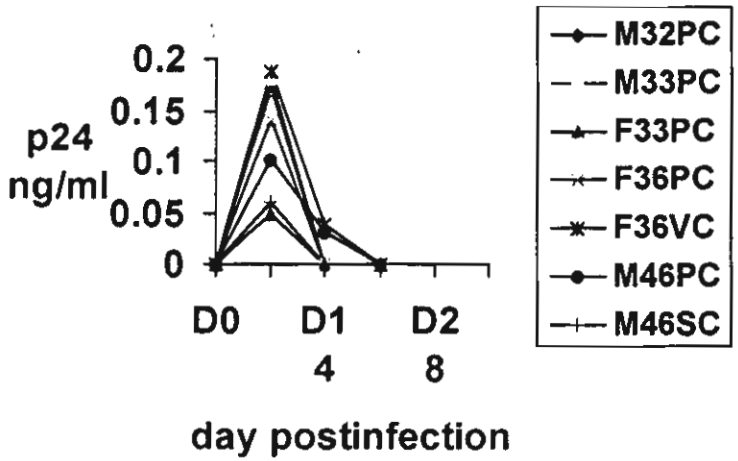


CFV

Fig.11 INFECTIVITY ASSAY OF HIV ISOLATES IN SW 837 RECTAL EPITHELIAL CELL LINE



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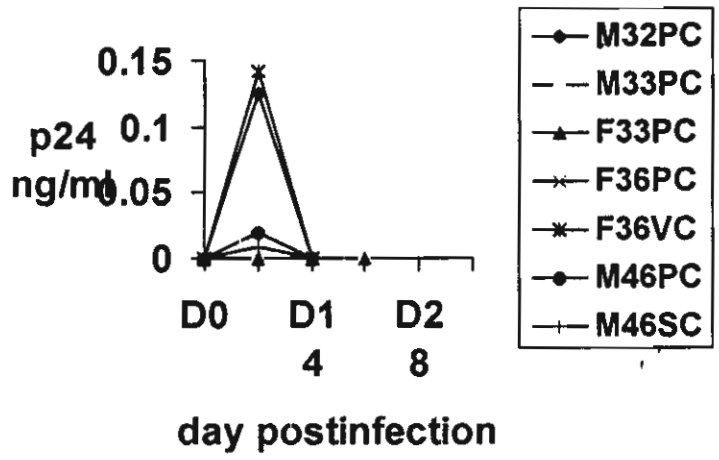
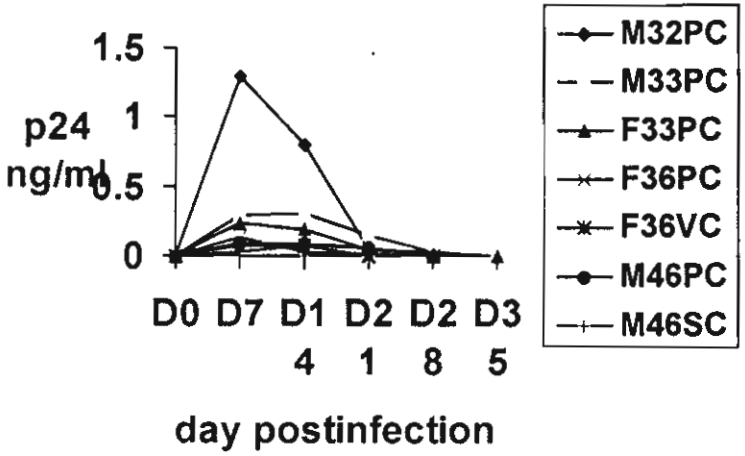
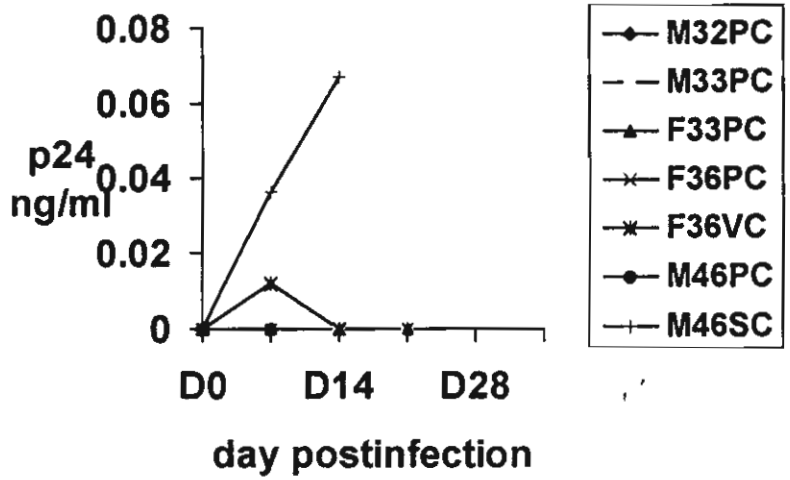


CFV

Fig. 12INFECTIVITY ASSAY OF HIV ISOLATES IN HT29 COLONIC EPITHELIAL CELL LINE

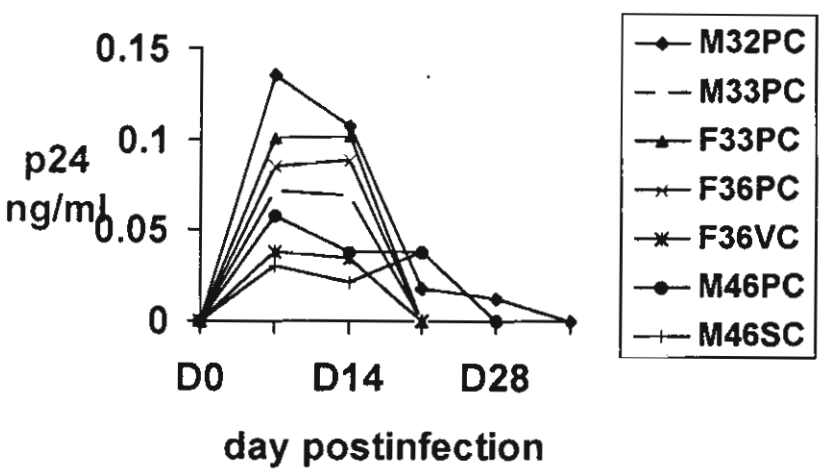


CAV

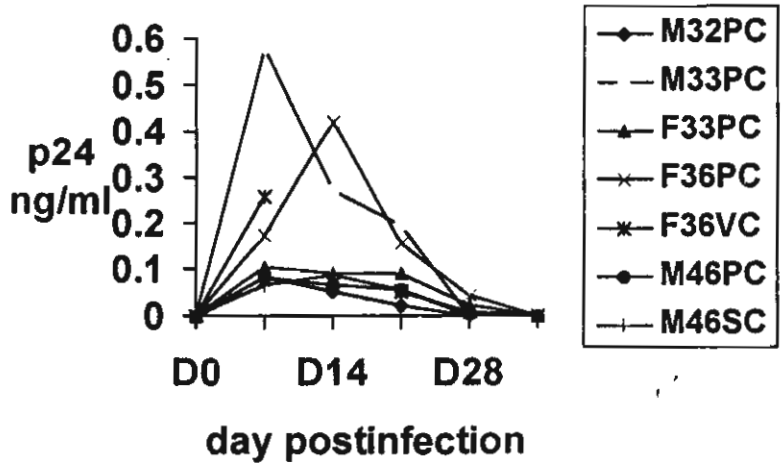


CFV

Fig.13 INFECTIVITY ASSAY OF CAV HIV ISOLATES IN CERVICAL EPITHELIAL CELL LINE



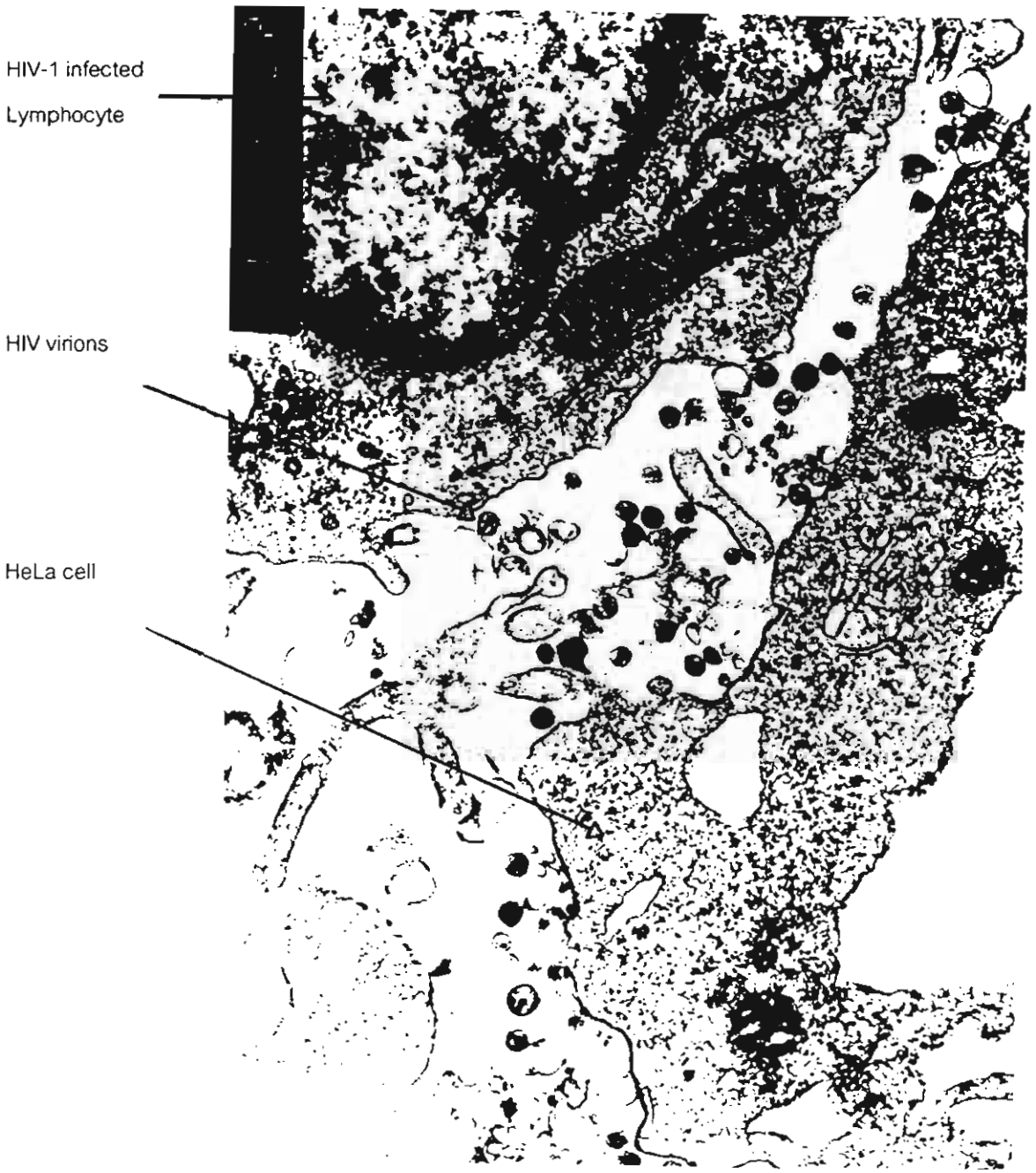
ME180 CELL



HELA CELL

รูปที่ 14 ภาพ Ultrastructure **ที่ได้จากการถ่ายภาพด้วยกล้องจุลทรรศน์อิเล็กตรอน ของ** Hela epithelaial cell line **ที่ติดเชื้อเอ็ชไอวี** F36VC a) **ภาพขยาย** 39,600 **เท่า ลูกศรบน แสดงเซลล์ลิมโฟไซท์ และลูกศรล่างแสดงเซลล์** HeLa **จะเห็นอนุภาคไวรัสอยู่ตรงกลาง** b) **ภาพขยาย** 70,000 **เท่า แสดงภายใน** HeLa cell **ที่ลูกศรชี้ คืออนุภาคไวรัสเชื้อเอ็ชไอวี**

14 a)



14 b)

รูปที่ 15 แสดงการเรียงตัวของ amino acid ยาวประมาณ 500 amino acid ของ whole envelope protein ของเชื้อเอ็ชไอวี ที่ได้จากการทำ nucleotide sequencing ของ env gene และนำมา translate consensus ของ HIV-1 subtype E envelope protein ถูกแสดงอยู่ด้านบนสุด . = amino acid ที่เหมือนกับ consensus , - = amino acid deletion ที่ตำแหน่งนั้น เทียบกับ concensus

```
                          10         20         30         40         50
HIV1EK          1 MRVKETQMNW PNLWKWGTLI LGLVIICSAS DNLWVTVYYG VPVWRDADTT   50
1V08VC          1 ---------- ---------- ---------- -------... ....T.....   50
54QV20VC        1 ---------- ---------- ---------- -------... .........L   50
IAM31SC         1 ---------- ---------- ---------- -------... ....K.....   50
M46SC           1 ---------- ---------- ---------- -------... ....K.....   50
F36VC           1 ---------- ---------- ---------- -------... ....K..E..   50
M46PC           1 ---------- ---------- ---------- -------... ..........   50
F36PC           1 ---------- ---------- ---------- -------... .......K..   50
                          60         70         80         90        100
HIV1EK         51 L-FCASDAKA HETEVHNVWA THACVPTDPN PQEIYLENVT ENFNMWNNNM   100
1V08VC         51 .F-....V.. .......... .Y....P... ....H..... ......K...   100
54QV20VC       51 PYR...H... .D........ .Y........ ..A..... ......K...   100
IAM31SC        51 .F-..... .......... .......... ......G... ......K...   100
M46SC          51 .F-..... .......... .........D .......... ......K...   100
F36VC          51 SF-..... .......... .......... .......... ......K...   100
M46PC          51 .-..... .......... .......... .......... ......K...   100
F36PC          51 .-..... .......... .......... .......... ......K...   100
                         110        120        130        140        150
HIV1EK        101 VEQMQEDVIS LWDQSLKPCV KLTPLCVTLI CTNAKLTNAN -LTNVNNITN   150
1V08VC        101 .......... .......... .........R .P.....K.. AFST.E.L.-   150
54QV20VC      101 .........SQ ...H...... .S.......R ....NW..NA NS.K...-.-   150
IAM31SC       101 .......... .......... .........N ....T..EDK NK..DS..I-   150
M46SC         101 .........Q ...H...... .........H .....W.H.. -R.K.I...I   150
F36VC         101 A.......NQ ...H...... .........N .....W---- -W11..YT..   150
M46PC         101 .........Q .......... .........H .......H.. -P........   150
F36PC         101 .........Q .......... .........N ...K...L-- ---.......   150
                         160        170        180        190        200
HIV1EK        151 VSNIIGNITD EVRNCSFNMT TELRDKKQKV HALFYKLDIV QIGDKN-SSE   200
1V08VC        151 ----V..... .........I. ..I...R... .......... ..E.NKN...   200
54QV20VC      151 TNILL.-L.. .......... ..II..Q... .........I P..NR.D...   200
IAM31SC       151 GNIT...M.- .......... ..I.....R. Y......... ...N.SN...   200
M46SC         151 TPVFM..L.. .......... ..II..Q... .........I ....R.D...   200
F36VC         151 GP.R..... K.......... ...I...... .......... ....RY-...   200
M46PC         151 .P.FM..L.. .......... ..II..Q... .........I ......D...   200
F36PC         151 GP..V..... .......... ..II...... ......V... ....RY-...   200
                         210        220        230        240        250
HIV1EK        201 YRLINCNTSV IKQACPKISF DPIPIHYCTP AGYAIFKCND KNFNGTGPCK   250
1V08VC        201 .......... .......... .......... .....L.... ..........   250
54QV20VC      201 .M........ .......... .......... .....L.... ..........   250
IAM31SC       201 .M........ .....QQ... .......... .....L.... ....R.....   250
M46SC         201 .M........ .......... .......... .......... ..........   250
F36VC         201 .......... .......V.. .......... .....L.... ..........   250
M46PC         201 .M........ .......... .......... .......... ..........   250
F36PC         201 .......... .......V.. .......... .....I.... ..........   250
                         260        270        280        290        300
HIV1EK        251 NVSSVQCTHG IKPVVSTQLL LNGSLAEEEI IIRSENLTDN AKTIIVHLNK   300
1V08VC        251 .......... .......... .......... ........N. .......F..   300
54QV20VC      251 .......... .......... ...T...... V.......N. ..........   300
IAM31SC       251 .......... .NS....... .R.T...... V......... ..........   300
M46SC         251 .......P.. .......... .......... ........N. ..........   300
F36VC         251 .......P.. .......H.. .......... ........N. ..........   300
M46PC         251 .......P.. .......... .......... ........N. ..........   300
F36PC         251 .......... .......... .......... L.....PT.. ..S.......   300
                         310        320        330        340        350
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HIV1EK      301 SVGINCTRPS NNTRTSITIG PGQVFYRTGD IIGDIRKAYC EINGTKWNRV  350
1V08VC      301 ..E.....  ..........  ..........  ...N..Q...  K.......ET  350
54QV20VC    301 ..E......Y ....IRM.M.  ..K......E  .V........  ........KA  350
IAM31SC     301 ..E...I...  .....RM...  ..........  .T........  ........KA  350
M46SC       301 ......S..Y ...IG....  ..K......E  .V........  ........KL  350
F36VC       301 ..........  A.I.Y.....  ..R.......  ......Q...  ........K.  350
M46PC       301 ..........  ..........  ..K.......  .......P..  ........KA  350
F36PC       301 ..........  A.I.VV....  ..R......N  L....D..H.  ........K.  350
                        360         370         380         390         400

HIV1EK      351 LKQVTEKLKE HFNNKTIIFQ PPSGGDLEIT MHHFNCRGEF FYCNTTRLFN  400
1V08VC      351 ....A...R.  ..I.....L.  ..........  ......K...  ......K.L.  400
54QV20VC    351 ..........  ....R..L..  ..........  ......K...  ......K.L.  400
IAM31SC     351 .....G....  ....T..T..  .........Q  .......E..  ......K.L.  400
M46SC       351 ..........  ..........  ..........  ......K...  ......K...  400
F36VC       351 .TP....F..  ..P....V..  ..........  ..........  ......Q...  400
M46PC       351 ..........  ..........  ..........  ......K...  ......K...  400
F36PC       351 ..........  ..P....D..  ..........  ........K.  ......Q...  400
                        410         420         430         440         450

HIV1EK      401 NTGIGNETMN GCNGTITLPC KIKQIINMWQ GAGQAMYAPP ISGKINCVSN  450
1V08VC      401 ..C.....VG  ...N..I...  ..........  ..........  .K.W.R....  450
54QV20VC    401 ..C.E...VG  ...N..I...  ..........  ..........  .K.W.R....  450
IAM31SC     401 ..C.K...TE  ...D..I...  ..........  ..........  .R.S.....  450
M46SC       401 ..C.....VG  ...N..I...  ..........  ..........  .K.W.R....  450
F36VC       401 ..C...K.KE  ...S...I..  R.......S.  KV........  .K..FS....  450
M46PC       401 ........VG  ...N..I...  ..........  ..........  .K.W.R....  450
F36PC       401 ......K.KE  ..........  ..I.......  EV........  .K..FS....  450
                        460         470         480         490         500

HIV1EK      451 ITGILLTRDG GANTTTNETF RPGGGNIKDN WRSELYKYKV VQIEPLGIAP  500
1V08VC      451 ..........  .---------  ----------  ----------  ----------  500
54QV20VC    451 .....Y....  .---------  ----------  ----------  ----------  500
IAM31SC     451 .....Y....  .---------  ----------  ----------  ----------  500
M46SC       451 ..........  .---------  ----------  ----------  ----------  500
F36VC       451 ........N.  .---------  ----------  ----------  ----------  500
M46PC       451 .S........  .N.A.-....  ..........  ..........  ..........  500
F36PC       451 .......K..  .-.I......  K.A.......  LK........  ......E...  500
                        510         520         530         540         550

HIV1EK      501 TRAKRRVVER EKRAVGIGAM IFGFLGAAG.  ..........  ..........  550
1V08VC      501 ----------  ----------  ---------.  ..........  ..........  550
54QV20VC    501 ----------  ----------  ---------.  ..........  ..........  550
IAM31SC     501 ----------  ----------  ---------.  ..........  ..........  550
M46SC       501 ----------  ----------  ---------.  ..........  ..........  550
F36VC       501 ----------  ----------  ---------.  ..........  ..........  550
M46PC       501 .-........  .........L  ..........  ..........  ..........  550
F36PC       501 ....KK..K.  K.K..E.K.L  ..........  ..........  ..........  550
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Shedding of HIV-1 Subtype E in Semen and Cervico-Vaginal Fluid

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CHANTAPONG WASI, M.D.*

Abstract

The uneven expansion of HIV-1 subtypes in each transmitted group raises the possibility that some viruses have less/more potential by qualitative/quantitative for heterosexual transmission compared to others. In Thailand, HIV-1 subtype E is mainly spread *via* heterosexual route and accounts for about 95 per cent of the infected cases. To determine whether high sexual infectivity of HIV-1 subtype E is due to the presence of a virus in genital fluid, we conducted a study to characterize shedding of HIV-1 in seminal and cervico-vaginal fluids of 30 HIV-1 subtype E infected Thai couples by PCR and virus isolation methods. All subjects had no HIV-associated diseases and other sexually transmitted diseases. HIV-1 subtype E DNA was detected in 22/30 (77.33%) of cervico-vaginal and also 22/30 (77.33%) of seminal fluid samples. The isolation rate of HIV-1 from semen and cervico-vaginal secretion was 36.67 per cent and 16.67 per cent, respectively. Number of HIV-1 subtype E DNA copies in the blood is reversely correlated with the number of blood CD4+ T cells, while that in genital fluid was not related to CD4+ T cell count. An increase in shedding of HIV- DNA subtype E in female genital tract compared to other HIV subtypes reported by other investigators might be one reason to explain the rapid spread of subtype E by heterosexual transmission in Thailand.

There are three patterns of human immunodeficiency virus type 1 (HIV-1) transmission; pattern I, homosexual and injecting drug user groups by exchanging contaminated blood; pattern II, heterosexual transmission by sexual contact; pattern III, mother-to-child route[1,2]. Heterosexual transmission has accounted for about 75 per cent of HIV-1 infected cases worldwide[3]. More than

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90 per cent of HIV-1 infection in developing countries like Thailand is transmitted *via* pattern II, while, pattern I transmission has been far more common than pattern II in western countries. Hence, there are epidemiological differences in sexual HIV-1 transmission observed in developing countries compared with the developed ones.

HIV-1 subtypes or clades have been classified according to HIV env and gag gene sequences obtained from about 669 strains worldwide and designated as A-H and O[4]. Subtype E accounts for only 10 per cent of HIV-1 strains isolated worldwide but more than 90 per cent of them are present in Thailand. Approximately 95 per cent of heterosexually acquired cases in Thailand are E subtype, although the B subtype used to be a more common genotype among injecting drug users[5]. In Thailand, the segregration of HIV-1 subtype B and E by mode of transmission was demonstrated.

Some preliminary data suggested that among HIV-1, some clades may differ in their transmissibility and virulence as well. There is also evidence from mother-infant pairs suggesting selective transmission of certain maternal HIV-1 variants, and also suggestive evidence for differential transmissibility of two different subtypes through sexual contact[6,7]. In Thailand, the risk of infection to a male per sexual encounter with an infected female is 10-fold higher than that occurring in the U.S.A. (3% & 0.3%)[8]. The uneven expansion of HIV-1 subtypes in each transmitted group raises the possibility that some viruses have less/more potential by qualitative/quantitative for heterosexual transmission compared to others. Preliminary *in vitro* findings suggested that HIV-1 subtype E from heterosexual Thais can grow more efficiently in Langerhans cells than subtype B from homosexual Americans[9]. HIV-1 subtypes may have differing sexual infectivity related to the level of viremia, cell tropism, presence of virus in genital fluid and related sexually transmitted diseases. The prevalence of HIV-1 subtype B in seminal and cervico-vaginal fluids, reported by other investigators, was 70-80 per cent and 30-50 per cent, repectively[10-14].

To compare the detection of HIV-1 subtype E in genital fluid with those reported on subtype B[10-14], we made a cross-sectional study of 30 asymptomatic HIV-1 subtype E infected couples (husband-wife pairs) who attended Siriraj Hospital, Bangkok, Thailand. The prevalence of HIV-1 subtype E in genital fluids detected by PCR and cocultivation methods and the correlation between amount of proviral DNA in blood and number of CD4+ T cell were determined in this study.

MATERIAL AND METHOD

Subjects

Thirty HIV-1 seropositive women, in the age range of 18-37 years, attending Siriraj Hospital, Bangkok underwent an interview with written consent, physical examination including pelvic examination, STD screening, and CD4 lymphocyte count. None of the subjects in this study had HIV-associated diseases. The exclusion criteria were STD, genital ulcer, or bleeding in the vaginal canal. Cervico-vaginal (C-V) secretions were collected by a swab at the endocervix and the vaginal walls and the swab was placed into 5 ml of RPMI 1640 medium in a sterile tube. Ten millilitres of clotted and EDTA blood were collected for HIV serological assay and virus isolation by culture and polymerase chain reaction (PCR).

Husbands of seropositive women, in the age range of 21-40 years, attending the clinic were also enrolled with HIV seropositivity. All husbands underwent an interview with written consent, physical examination, and collection of donated EDTA blood and semen specimens achieved by masturbation. None of the husbands had a history of recent exposure, HIV-related diseases or clinical examinations suggesting active infection with other sexually transmitted pathogens.

Specimen processing

The unclotted (EDTA) blood was divided for determining the lymphocyte subset count performed at Department of Immunology, Siriraj Hospital (FACScan, Becton-Dickenson, U.S.A.) and separating plasma and peripheral blood mononuclear cells (PBMCs) by Ficoll-Hypaque gradient (Lymphoprep, Becton-Dickenson), which were used for cocultivation and frozen at -70°C for further analysis by PCR. Plasma was used to investigate for anti-HIV antibody by ELISA methods (Vironostika HIV UniformII, Organon and Genelavia Mixt, Sanofi) and Western blot technique (HIV blot 2.2, Diagnostic Biotech).

Semen was processed within 2 hours[15]. Phosphate buffered saline (PBS) was used to dilute semen in 1:1 dilution. The diluted specimens were centrifuged at 2,940 x g for 2 minutes. The supernatant (seminal plasma) was filtered (0.45 mm) and the pellet (seminal cells) was suspended in PBS for HIV-1 coculture and frozen at -70°C for further analysis by PCR.

C-V secretions in 5 ml of culture medium were centrifuged at 2,940 x g for 2 minutes, the supernatant and cell pellet were divided and tested separately. Samples were examined under the microscope upon arrival in the laboratory, and again after centrifugation, to confirm the absence of red blood cells. The resuspended cell-pellet was used for HIV-1 coculture and frozen at -70°C for PCR analysis.

HIV-1 cultures[16,17]

HIV was isolated from PBMCs, cells from semen and C-V secretion of each infected case by cocultivation with 3-to-4-day-old phytohemagglutinin stimulated donor cells at a concentration of 1 x 10^7 cells in 10 ml RPMI 1640 medium supplemented with 15 per cent fetal calf serum and 10 units/ml recombinant human interleukin-2. Cellular and fluid/plasma fractions were cocultivated separately. Cultures were maintained for 6 weeks before considered negative and p24 antigen assay (Coulter, U.S.A.) was used to detect HIV in the culture.

Detection of HIV proviral DNA by PCR

Cell lysate

The PBMCs, seminal cells, and C-V cells were lysed in PCR lysis buffer (10 mM Tris-HCl *p*H 8.3, 25 mM $MgCl_2$, 50 mM KCl, 0.45% NP-40, 0.45% Tween 20 and 10 mg/ml proteinase K) at the concentration of 10^6 cells per 100 µl of lysis buffer for 1 hour at 56°C, thereafter, proteinase K was inactivated for 10 min at 95°C. The lysates were stored at -70°C until used for PCR. 25 µl of lysate, equivalent to 2 µg of genomic DNA or 2.5 x 10^5 cells, was used in each amplification[18].

The 8E5 T cell line stably infected with HIV-1, each cell containing one copy of integrated HIV proviral DNA defective in the pol gene, was used as positive control at 25 copies per reaction. To check reagents for contamination of HIV-1 amplicons, a reagent and negative control samples, which were provided by CDC, U.S.A., were included in every amplified reaction.

Primers

Oligonucleotide primers specific for gag gene; SK380/390 and SK38/39[19], and env gene; ED3/4 and ED5/12[20] were used for nested PCR. The sequences of these primers are shown as follows:

Primer	Gene	Sequence (5'-3')	Location
SK380	gag	GAGAACCAAGGGGAAGTGACATAGGAG	684-712
SK390	gag	TAGAACCGGTCTACATAGTCTCTAAAGGG	903-894
SK38	gag	ATAATCCACCTATCCCAGTAGGAGAAAT	1551-1578
SK39	gag	TTTGGTCCTTGTCTTATGTCCAGAATGC	1665-1638
ED3	env	TTAGGCATCTCCTATGGCAGGAAGAAGCGG	5537-556
ED14	env	TCTTGCCTGGAGCTGCTTGATGCCCCAGAG	7538-7509
ED5	env	ATGGGATCAAAGCCTAAAGCCATGTG	6134-6159
ED12	env	AGTGCTTCCTGCTGCTCCCAAGAACCCAAG	7388-7359

Amplification

The PCR assay was performed, as previously described,[21] briefly, 25 µl of cell lysates were amplified for 30 cycles in 50 µl volume containing 2.5 mM for gag gene amplification or 1.25 mM for env gene amplification of $MgCl_2$. The amplification cycle of primary and secondary PCR for gag gene was 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min and for env gene was 94°C for 15 sec, 55°C for 45 sec, 72°C for 1 min and final extension at 72°C for 5 min. The amplified product from the second PCR of gag gene (118 bases long) and env gene (1,200 bases long) was electrophoresed through a 1 per cent low melting point agarose gel (Sigma, U.S.A.) and visualized by ethidium bromide staining under UV light transilluminator.

Quantitation of HIV-1 proviral DNA[22]

The quantitative polymerase chain reaction (qPCR) was performed as described above. For each PCR assay, two-fold dilutions equal to 1 to 2560 copies of the plasmid control, HIVZ6 (Perkin Elmer) were amplified in duplicate and used as standards for copy number quantitation. Amplified HIV-1 DNA products (lysate of 10,000 cells) and positive controls were dot blotted, denatured, and hybridized with a fluorescein-labelled specific oligonucleotide probe (ECL, Amersham). The hybridized blot was exposed to X-ray film. Detection signal was accomplished using an Image analysis (Biomed Instruments Inc., AAB) to measure the density of samples in autoradiogram compared with those of positive control dilutions.

HIV-1 subtype identification

Heteroduplex mobility assay (HMA)[20]

5 µl of nested PCR product as using primers ED3/14 and ED5/12 was mixed with either 5 µl of water (for homoduplex) or 5 µl of single PCR product as using ED5/12 of reference plasmid specified for HIV-1 subtype B or E in a 500 µl eppendorf PCR tube containing 1.1 µl of 10X heteroduplex annealing buffer (1M NaCl, 100 mM Tris-HCl *p*H 7.8, and 20 mM EDTA). Heteroduplex formation was done by denaturing the mixture at 94°C for 2 min in DNA Thermal Cycler (Perkin Elmer 4800), then cooling rapidly in wet ice. Heteroduplex reaction was mixed with 3 µl of 5x Ficoll/loading dye and load onto a 5 per cent non-denaturing polyacrylamide gel. The electrophoresis was performed at 200 Volts for 6 hours, then the gel was stained in ethidium bromide solution for 15 min and photographed under UV light. Electrophoresis pattern of each unknown sample was compared to that of the standard of subtype B and E. The subtype of each PCR product sample was unambiguously assigned by this method as the amplified products would form fast migrating heteroduplexes with standard DNA of the corresponding subtype in 5 per cent polyacrylamide gels, and slow migrating heteroduplexes with the other subtype.

Peptide ELISA (PELISA)

The PELISA used in this study has been described previously[23], with 14 amino acids long specific for Thai A (env subtype E: TSITIGPGQVFYRT) and Thai B (env subtype B: KSIHLGPGQAWYTT). 100 µl of peptide solution at concentration of 5mg/ml in 20 mM carbonate buffer *p*H 9.6 were immobilized on each well of the microtiter plate by incubation for 16-18 hours at 4°C. The next day, antigen was aspirated and plates were blocked with 200 µl/well of PBS con-

Table 1. Detection of HIV-1 subtype E[a] in PBMCs, semen, and C-V secretion of 30 HIV-1 seropositive husband-wife pairs.

Positive result	PBMCs-husband (%)	PBMCs-wife (%)	Semen (%)	C-V secretion (%)
PCR[b]	30/30 (100.00)	29/30 (96.67)	22/30 (73.33)	22/30 (73.33)
Culture[c]	18/30 (60.00)	11/30 (36.67)	8/30 (26.67)[d]	5/30 (16.67)[d]

[a] HIV-1 subtype E characterized by HMA and PELISA
[b] PCR result of gag and env genes amplification
[c] coculture with PHA activated donor PBMCs
[d] coculture from cells pellet of seminal/C-V fluid

taining 5 per cent dry skimmed milk powder. In the test assay, serum samples at a dilution of 1:400 in blocking buffer were added to the antigen-coated plates and incubated for 1 hour at 37°C. After six washes with washing buffer (PBS containing 0.05% Tween 20), anti-human IgG peroxidase conjugate (Sigma, U.S.A.) diluted in blocking buffer was applied to a well for 1 hour at 37°C. The color was developed with orthophenylenediamine dihydrochloride substrate after a further six washes. Absorbance at 492 nm against 620 nm was measured. A cutoff of 0.3 was used throughout the study, with dual-reactions further classified as monoreactive.

Table 2. HIV-1 proviral DNA in semen and cervico-vaginal secretion of husband-wife pair from corresponding couple.

Group	PCR result in seminal/C-V fluid	Pair (%)
1.	+/+	15/30 (50.00)
2.	+/-	6/30 (20.00)
3.	-/+	6/30 (20.00)
4.	-/-	3/30 (10.00)

RESULT

Prevalence of HIV-1 subtype E in genital fluid

Thirty HIV-seropositive asymptomatic couples were enrolled at Siriraj Hospital, Bangkok and studied on a single occasion and classified into three groups according to blood CD4+ T cell count; 1) 13 cases with CD4+ T cell count more than 500, 2) 39 cases with CD4+ T cell count between 200-

Table 3. Quantitation of HIV-1 DNA in PBMC, semen and cervico-vaginal secretion.

Couple	Husband			Wife		
	CD4 level	copy number of HIV in		CD4 level	copy number of HIV in	
		PBMC	Seminal cells		PBMC	C-V cells
1	274	200	12	136	8	4
2	422	400	80	430	100	NA
3	504	40	2	125	80	12
4	604	80	NA	534	40	NA
5	438	16	8	339	8	NA
6	245	4	4	162	200	2
7	378	16	NA	256	16	4
8	228	2	40	155	100	2
9	382	12	2	159	2	2
10	402	12	2	469	2	2
11	108	100	2	389	4	2
12	267	40	2	725	2	2
13	301	60	2	506	20	2
14	203	80	2	264	60	2
15	525	12	2	359	8	2
16	135	400	2	437	2	NA
17	489	4	NA	229	20	8
18	448	8	2	246	100	2
19	235	80	20	447	12	NA
20	388	8	2	307	NA	NA
21	558	4	2	415	80	2
22	633	4	NA	468	2	2
23	565	4	NA	298	100	20
24	551	200	4	373	2	2
25	251	80	4	600	2	2
26	599	8	NA	638	4	NA
27	471	100	2	244	20	NA
28	253	200	2	497	100	4
29	229	80	NA	439	40	16
30	54	200	NA	363	60	4

NA = not amplificable

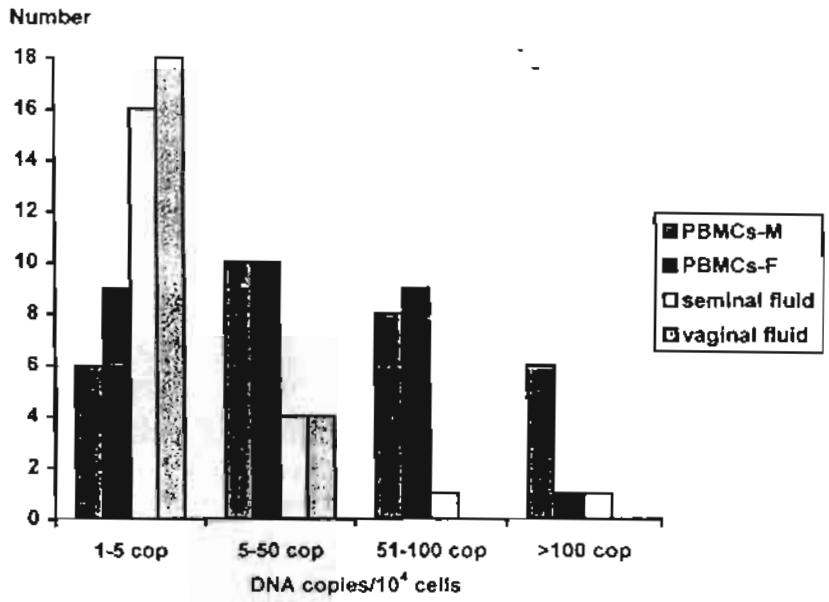

Fig. 1. Quantitation of HIV-1 proviral DNA subtype E in 10,000 cells from blood, seminal and C-V fluids.

500, and 3) 8 cases with CD4+ T cell count less than 200. Cervico-vaginal secretion and semen specimens were obtained from all couples. HIV-1 proviral DNA was detected in 59 (98.3%) of 60 PBMCs, 22 (73.33%) of 30 C-V samples, and also 22 (73.33%) of 30 semen samples by PCR amplification at gag and env genes (Table 1). All of these sixty cases were infected with HIV-1 subtype E, which was identified by heteroduplex mobility assay (HMA) and PELISA. While, HIV-1 isolated by coculture method was only 5 in 30 (16.67%) from cells of C-V secretion samples and 8 in 30 (26.67%) from seminal cells. We could not isolate any HIV-1 from fluid part of semen and C-V secretion by culture method. The rate of HIV-1 culture positive from PBMCs of husbands and wives were 18/30 (60.0%) and 11/30 (36.67%), respectively. Blood CD4+ T cell count of culturable HIV-1 specimens from PBMCs all was less than 500. HIV-1 proviral DNA presence in both seminal and C-V fluids of corresponding couple was found in 15 (50.0%) from 30 couples (Table 2). Only three couples (10.0%) had no HIV-1 proviral DNA in neither semen nor C-V secretion. All of these couples had blood CD4+ T cell count more than 500.

Quantitation of HIV-1 proviral DNA in blood and genital fluid (Table 3, Fig. 1-3)

Of 44 HIV-1 DNA positive cases in genital fluid, 35 (79.5%) of them contained less than 5 copies of proviral DNA per 10,000 cells, and only 9 cases, which had blood CD4+ T cell count in group 2 and 3, contained more than 5 copies of proviral DNA per10,000 cells (Table 3 and Fig. 1). HIV-1 proviral DNA copies in genital fluid were not correlated with blood CD4+ T cell count (Fig. 3). While the amount of HIV-1 proviral DNA in the blood varies from less than 5 to more than 100 copies/10,000 cells and reversely correlated with blood CD4+ T cell count (Table 3 and Fig. 2). In group 1 with CD4+ T cell count more than 500, 7/13 (53.8%) cases had HIV-1 proviral DNA less than 5 copies/10,000 cells, while, only 7/39 (17.9%) and 1/8 (12.5%) cases in group 2 and 3 with CD4+ T cell count between 200-500

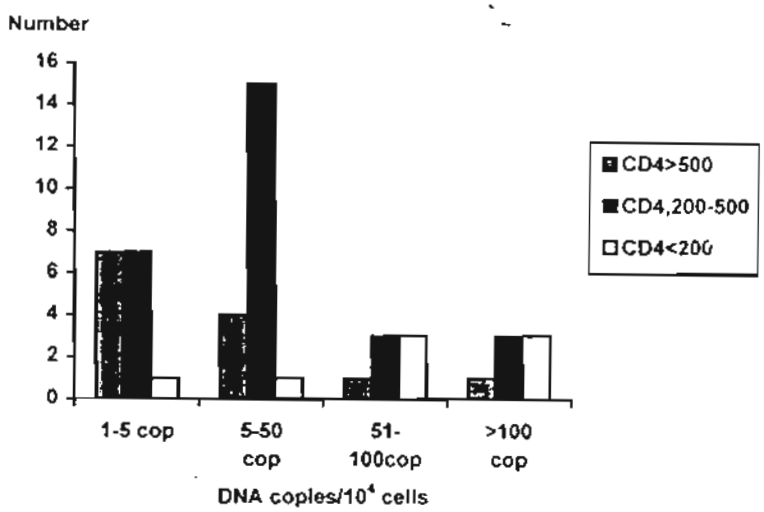

Fig. 2. Quantitation of HIV-1 proviral DNA in 10,000 cells of PBMCs in relation to blood CD4+ T cell count.

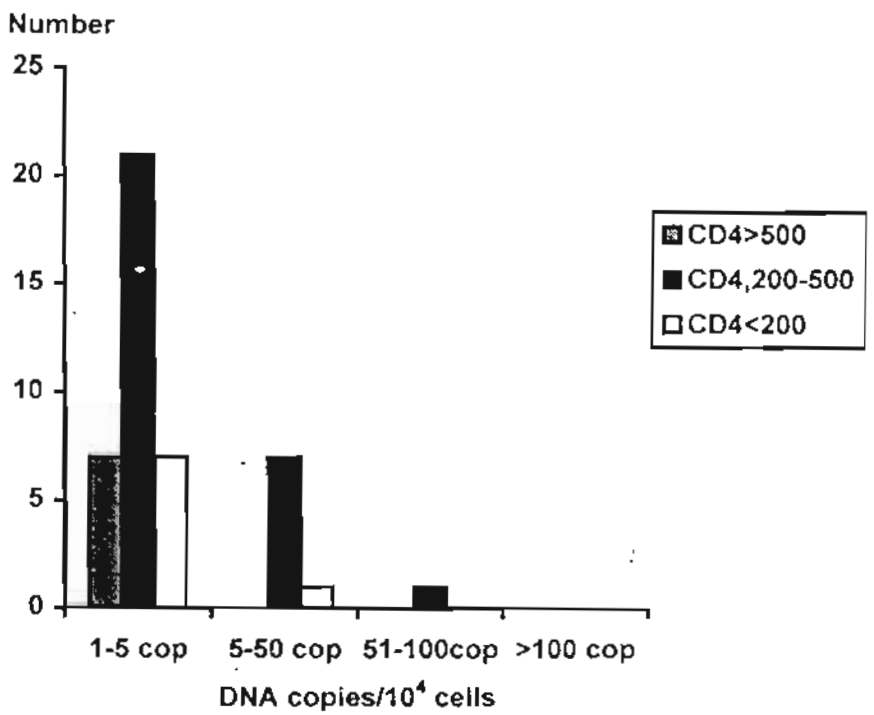

Fig. 3. Quantitation of HIV-1 proviral DNA/10,000 cells in seminal and cervico-vaginal fluids in relation to blood CD4+ T cell count.

and less than 200, respectively, had HIV-1 DNA less then 5 copies/10,000 cells.

DISCUSSION

HIV-1 subtype E is predominantly spread in Thailand mainly in heterosexual transmission groups, while in developed countries, HIV-1 subtype B is more predominant. The reasons for rapid transmission of HIV *via* sexual route in Thailand might be because of more mucosal or Langerhans cell tropism of subtype E than subtype B or higher amount of HIV subtype E secreted in seminal/vaginal fluids. We found that the prevalence of subtype E HIV-1 DNA detected by PCR in seminal and C-V fluids were similar as 73.33 per cent, on contrary, other investigators reported that subtype B was found only 30-50 per cent in C-V fluid[10-12]. The high secretion of subtype E HIV-1 DNA in C-V fluid of Thai women did not correlate with other STDs because of exclusion criteria at the enrollment. The shedding of HIV-1 subtype B DNA in semen was about 70-80 per cent[13-15] which is similar to what we found in our study for subtype E. The culture rate of HIV-1 subtype E from C-V fluid was much lower than that of subtype B, but, from seminal fluid there was no difference. Cocultivation with phytohemagglutinin activated PBMCs might not be suitable for isolation of HIV-1 subtype E from C-V fluid.

HIV subtype E copy level in blood among individuals ranged from 2 to 400 copies per 10^4 PBMC. This is similar to those observed ranges in subtype B[24-26]. There was an increase in proviral copies with a decrease in CD4+ T cell count which was also observed by others in subtype B[24,27,28]. Contrary, HIV-1 copy level in genital fluid did not correlate well with blood CD4+ level, 35/44 (79.5%) of cases with less than 5 copies of HIV-1 proviral DNA in genital fluid.

The increase in the presence of HIV-1 DNA of subtype E in C-V fluid in this study was more than that report of subtype B; may render Thai women more infectious to sexual partners. Larger scale studies should be undertaken to obtain better understanding of sexual transmission of HIV-1 subtype E and to help formulate HIV prevention policies.

ACKNOWLEDGEMENT

The authors would like to thank Dr. Tim Mastro and Ms. Nancy Young from The HIV/AIDS Collaboration for providing synthetic peptide for performing PELISA, Drs. Jim Mullins and Eric Delwart for supplying the kit and teaching HMA, and nursing staff at STD clinic, Department of Obstetrics-Gynecology, Siriraj Hospital.

This work is supported by Thailand Research Fund Grant No. RSA/1/2538.

(Received for publication on January 13, 1997)

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Journal of Acquired Immune Deficiency Syndromes and Human Retrovirology
18:323–331 © 1998 Lippincott-Raven Publishers, Philadelphia

V3 Sequence Diversity of HIV-1 Subtype E in Infected Mothers and Their Infants

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Summary: To elucidate genetic characteristics of HIV-1 subtype E involved in vertical transmission, V3 regions of HIV-1 subtype E isolated from 17 infected mothers (M1–M17) and their infants (I1–I17) at 1 month after birth were sequenced after cloned into pCRII vectors. At least three clones of each sample were collected. All mothers were asymptomatic and had been infected through a heterosexual route. Nine infants (I9–I17) showed mild symptomatic and immunosuppression within the first year of life. The interpatient nucleotide distance of mothers and infants in this group (0.065 ± 0.008) were of greater diversity than those of a nonimmunosuppression group (0.039 ± 0.006) by a significant amount (Fischer's exact test, $p = .003$). The substitution with asparagine (N) at threonine (T) at position 13 and aspartic acid (D) at position 29 of the V3 sequence were significantly associated with nonimmunosuppression in the first year of life (*F*-test, $p = 0.003$). Either a single or multiple viral variants could transmit from mothers to their infants. **Key Words:** HIV-1 subtype E—Vertical transmission—V3 sequences.

At least 1.5 million children are infected with HIV-1 worldwide (1). Most children with HIV infection have derived it from the mother either in utero, at the time of birth, or postpartum by breast-feeding, with transmission rates varying from 14% to 39% (2) in global rates and 24.2% and 28% in Thai cohorts (3,4).

Although several studies have shown that high maternal viral load was associated with transmission, this can also occur at low viral load (5–7). Many studies in vertical transmission of HIV-1 subtype B suggested that maternal antibodies (8–11), $CD4^+$ T-cell count (6,12), and virus phenotype (13–18) were factors associated with mother-child transmission. Certain subtypes may be predominantly associated with specific modes of transmission: for example, subtype B with homosexual contact and intravenous drug use (essentially in blood) and subtype E, predominantly found in Thailand, with heterosexual transmission through a mucosal route (19–21).

Vertical transmission of HIV-1 is complex. It is possible that HIV-1 subtype may relate with transmissibility. HIV-1 subtype E in Thailand was spread heterosexually. In this situation, the genetic variants of HIV-1 in vertical transmission must be studied. V3 appears to be the major determinant of several biologic properties of HIV. Mutations in this region could potentially affect mother-to-child transmission, in that the V3 loop is an important determinant for viral neutralization and cellular tropism. Therefore, the V3 region of HIV-1 subtype E involved in mother-to-infant transmission was studied.

To further elucidate the molecular characteristic of

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Manuscript received September 26, 1997; accepted February 25, 1998.

HIV-1 subtype E involved in vertical transmission, we have observed the genetic diversity of V3 sequences from proviral DNA in 17 HIV-1-infected mother-infant pairs.

MATERIALS AND METHODS

Patients

Ethylenediamine tetraacetic acid (EDTA)-treated blood specimens were collected from 17 HIV-1-seropositive mother-child pairs, designated M1 through M17 and I1 through I17, respectively, who received medical care in the pediatric clinic at Siriraj Hospital, Bangkok, Thailand in 1994 and 1995. All mothers were asymptomatic and had been infected with HIV heterosexually. Blood samples were taken from the mothers at the initial visit, then from 1 day to 30 days after delivery and from the infants, at 1 month and every 4 to 6 months for 2 years.

DNA Preparation

Viral DNA for polymerase chain reaction (PCR) amplification was isolated from peripheral blood mononuclear cells (PBMCs) of the patients. PBMCs were isolated by Ficoll-Hypaque density gradient centrifugation and plasma was kept at −80°C for viral load assay. PBMCs were lysed in PCR-lysis buffer (10 mM Tris-HCl pH 8.3, 2.5 mM $MgCl_2$, 50 mM KCl, 0.45% NP-40, 0.45% Tween 20, and 100 μg/ml proteinase K) at a concentration of 10^7 cells per milliliter of lysis buffer for 1 hour at 56°C followed by 10 minutes at 95°C in a water bath to deactivate the enzyme.

Polymerase Chain Reaction Amplification

HIV-1 proviral DNA lysate was amplified by nested PCR as described in the procedure of Delwart et al. (22). The outer primers were ED3 (5'-TTAGGCATCTCCTATGGCAGGAAGAAGCGG at position 5956-5985 of the HXB2CG genome, Genbank accession number K03455) and ED14 (5'-TCTTGCCTGGCGCTGTTTGATGCCCCAGAC, position 7960-7931). The inner primers were ED5 (5'-ATGGGATCAAAGCCTAAAGCCATGTG, position 6556-6581), and ED12 (5-AGTGCTTCCTGCTGCTCCCAAGAACCCAAG, position 7822-7792). Nested PCR was carried out with a total volume of 50 μl, containing 10 μl of the cell lysate, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.25 mM $MgCl_2$, 0.2 mM of each dNTP, 10 pmol of each primer and 2.5 units of Taq DNA polymerase (Gibco-BRL, Grand Island, NY, U.S.A.). The amplifications were carried out in a Perkin-Elmer Thermocycler (Perkin-Elmer, Norwalk, CT, U.S.A.) for 30 cycles with step of 94°C for 15 seconds, 55°C for 45 seconds, 72°C for 1 minute, and final extension at 72°C for 5 minutes and 2 μl of the first reaction product were used as template in a second round PCR with the inner primers at the same condition.

Cloning and Sequencing

The second round PCR reactions were detected for the DNA amplification product (~1200 bp) by horizontal 0.8% agarose gel electrophoresis in Tris-borate EDTA (TBE) buffer at 100 V for 30 minutes. The PCR products were purified from gel slices with Geneclean (Bio 101 Inc., La Jolla, CA, U.S.A.) and cloned into pCR II vector using the TA Cloning system (Invitrogen, San Diego, CA, U.S.A.) according to the procedure in the manufacturer's instructions. Three clones with the inserted *env*-amplified DNA isolated from mothers and infants, at 1 month after birth, were selected and sequenced by dideoxy sequencing with Sequenase Version 2.2 (United States Biochemical Corp., Cleveland, OH, U.S.A.). The sequencing primer used for V3 was 5'-CTGTTAAATGGCAGTCTAGCT (sequence derived from HIV-1 LAI, Genbank accession no K02013; primer CB 207) (23).

Sequence Analysis

The 105 nucleotide sequences of the V3 region of the HIV-1 *env* gene from 17 mother-infant pairs were translated to the corresponding amino acids and aligned by using DNASIS version 2.1 (Hitachi Software Engineering, San Bruno, CA, U.S.A.). Pairwise distances define as the proportion of nucleotide differences between two aligned nucleotide sequences were performed to study the extent of genetic variation within sequence set and between sets by using software ESEE and MEGA (kindly provided by Dr. Marcia Kalish, Centers for Disease Control and Prevention, Atlanta, GA, U.S.A.) (24). The value of means was present as means ± standard error of means.

Plasma Viral RNA Quantification

Plasma was thawed and used for HIV-1 RNA quantification by Amplicor HIV Monitor test (Roche Molecular Systems, Somerville, NJ, U.S.A.). The quantification assay was done according to the manufacturer's instruction. Briefly, RNA was extracted from 0.2 ml plasma and known amount of quantification standard (QS). Reverse transcription (RT) and PCR were carried out in a single reaction by using rTth DNA polymerase and biotinylated primers specified for *pol* genes (SK431 and SK462). Fivefold serial dilutions of the amplified product were made. The biotinylated HIV-1 and QS amplicons were detected with an avidin–horseradish peroxidase conjugate and a chromogenic substrate mixture. Absorbance was measured at 450 nm. The HIV-1 RNA copy number was calculated from the known input copy number of the QS RNA.

RESULTS

Clinical Status of Patients

The clinical status, age, and lymphocyte levels and plasma viral RNA at initial visit (first month after delivery) of the mothers and infants and gender of infants were summarized in Table 1. All mothers were asymptomatic and had never received any antiretroviral drug. They were neither intravenous drug users nor partners of intravenous drug users. Infants (I1–I8) showed as mildly symptomatic with HIV-related diseases and evidence of immunosuppression by having $CD4^+$ T-lymphocyte depletion to lower than one fourth of blood $CD4^+$ cell count at 1 month old within 1 year of age (25). The others (I9–I17) comprised an infant group that remained asymptomatic and manifested no immunosuppression in the first year of life.

TABLE 1. *Demographic data, CD4⁺ T lymphocyte levels, and plasma viral RNA of HIV-1–infected mothers and their infants at their initial visits*[a]

Mothers					Infants				
		CD4 lymphocytes					CD4 lymphocyte		
No.	Age (y)	cells/mm³	%	Plasma RNA (copies/ml)	No.	Sex	cells/mm³	%	Plasma RNA (copies/ml)
M1	23	379	11.91	78,406	I1	F	2,437	20.93	1,381,852
M2	29	411	17.3	66,936	I2	M	2,137	31.31	570
M3	26	586	23.24	16,692	I3	M	1,946	24.56	3,536,269
M4	22	276	9.33	ND	I4	F	2,114	27.01	ND
M5	28	400	24.79	44,892	I5	F	3,650	38.76	381,529
M6	31	1,609	27.04	453,665	I6	F	2,231	24.38	15,434,887
M7	23	685	25.96	ND	I7	F	2,416	33.97	ND
M8	24	541	18.46	ND	I8	F	2,256	26.53	ND
M9	26	875	30.63	ND	I9	F	2,975	38.2	ND
M10	27	657	20.44	ND	I10	M	2,543	42.92	ND
M11	24	791	17.76	ND	I11	F	4,347	35.99	ND
M12	26	516	21.3	ND	I12	F	1,748	26.76	ND
M13	26	480	15.99	18,949	I13	M	2,117	25.11	12,823,848
M14	22	413	17.34	54,582	I14	F	4,565	49.64	205,739
M15	27	849	35.37	40,567	I15	F	2,393	32.56	1,237,191
M16	28	428	18.52	61,251	I16	M	1,633	26.04	3,193,585
M17	22	ND	ND	ND	I17	M	3,075	45.15	ND

[a] Mothers at first month after delivery, infants at age 1 month.
ND, not done.

Plasma HIV-1 RNA Quantitation

A study of plasma HIV-1 RNA quantification was determined in nine mother-infant pairs as shown in Table 1. No correlation was found between the level of HIV-1 RNA in mothers and their corresponding infants. Thus, HIV-infected infants appeared to have a higher viral load early in life. The level of HIV-1 RNA in plasma and blood CD4 at first month of the infants' lives was not associated with immune status.

V3 Region Sequences

The multiple alignments of the amino acid sequences of V3 of HIV-1 *env* from PBMC DNA of the 17 mother-infant pairs were shown in Figure 1. The degree of variability of distances of the V3 nucleotide sequences is shown in Figure 2. The net charge was calculated from the number of positively charged amino acids (R and K) minus the number of negatively charged amino acids (D and E).

Comparison of V3 Nucleotide Sequences of Mothers and Infants

V3 nucleotide sequences of HIV-1 isolated from each mother (M1–M17) were heterogeneous with mean intrasample variations from 0% to 7.87% with total mean value of 3.54% ± 0.6% as shown in Figure 2. The mean distance for intrasample of each mother's isolate of M1 to M8 (0.034 ± 0.010) was no different from those of M9 to M17 (0.037 ± 0.008). The interpatient nucleotide sequences distance among all mothers showed a large variation, ranging from 1.21% to 12.30% (mean, 0.070 ± 0.003). The mean distances among M1 to M8 (0.071 ± 0.005) and among M9 to M17 (0.070 ± 0.004) were almost exactly the same.

The variability within each infant (I1–I17) ranged from 0% to 7.62% (mean, 0.023 ± 0.006). The mean distance of each I1 to I8 (0.028 ± 0.010) and the mean distance of each I9 to I17 (0.018 ± 0.006) were not significantly different.

The V3 nucleotide sequence distance of 17 mother-infant pairs showed a large variation, with a range of differences from 0 to 23.81% (data not shown; *p*-distance mean value, .051 ± .006). Further analysis of pairwise sequences showed that the nucleotide distance of M1/I1 to M8/I8 (mean, 0.065 ± 0.008) were more heterogeneous than those of M9/I9 to M17/I17 (mean, 0.039 ± 0.006; Mann-Whitney U test, $p = .0269$). The pairwise distance of mother-infant intersample pairs was significantly higher than intrasample distance of each of mothers and infants (Wilcoxon matched-pair signed-rank test, $p = .0352$ and $p = .0016$, respectively). To compare the nucleotide distances between the mothers' samples and the infants, we have plotted each value of mother and infant pairs on a bar chart (Fig. 2). Most pairs showed less distance in the infants' samples than their mothers',

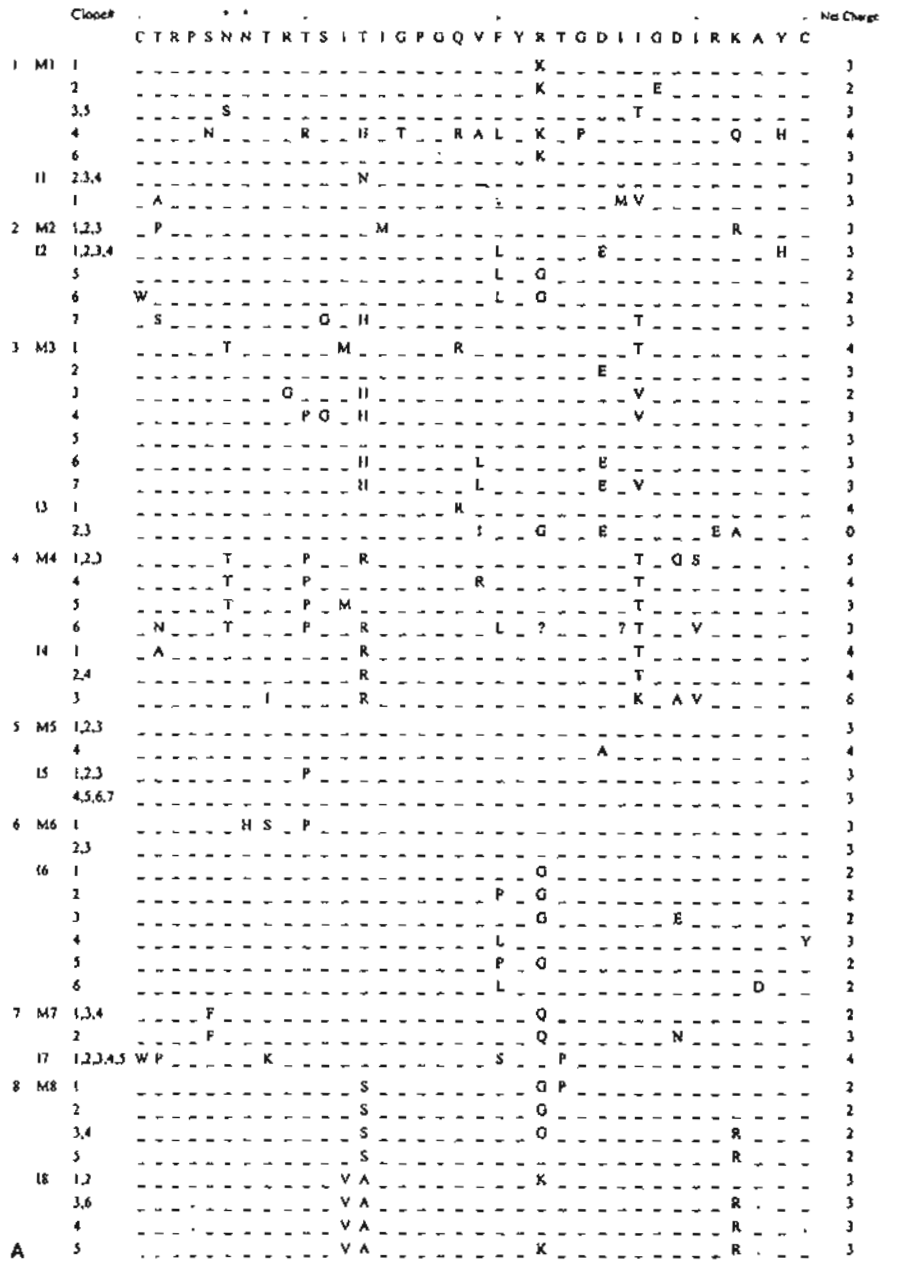

FIG. 1. Amino acid sequence multiple alignments of the V3 region of the envelope gene of HIV-1 subtype E from 17 infected mother-infant pairs at 1 month after birth. The top row shows consensus sequence of subtype E. Amino acids that match the consensus at the top of the alignment are indicated by a minus sign. The double asterisks above the alignment indicate the *N*-link glycosylation sites. The question marks indicate positions that could not be resolved. Amino acid positions above the alignment are numbered according to their positions in the V3 loop. Net charges were calculated by the number of positively charged amino acids (R and K) minus the number of negatively charged amino acids (D and E).

whereas pairs 4, 5, and 16 were similar. Seven of eight pairs from the M1/I1 to M8/I8 group have nucleotide distances >6%, whereas eight of nine from M9/I9 to M17/I17 group showed the distances <6%. These data demonstrated that in mother-infant pairs of M1/I1 to M8/I8, the infants' sequences were far from the mothers' sequences. Therefore, a significant correlation was found between a mother-infant pair's nucleotide distance >6% and rapid disease progression in the infected infants (Fisher's exact test, $p = .003$).

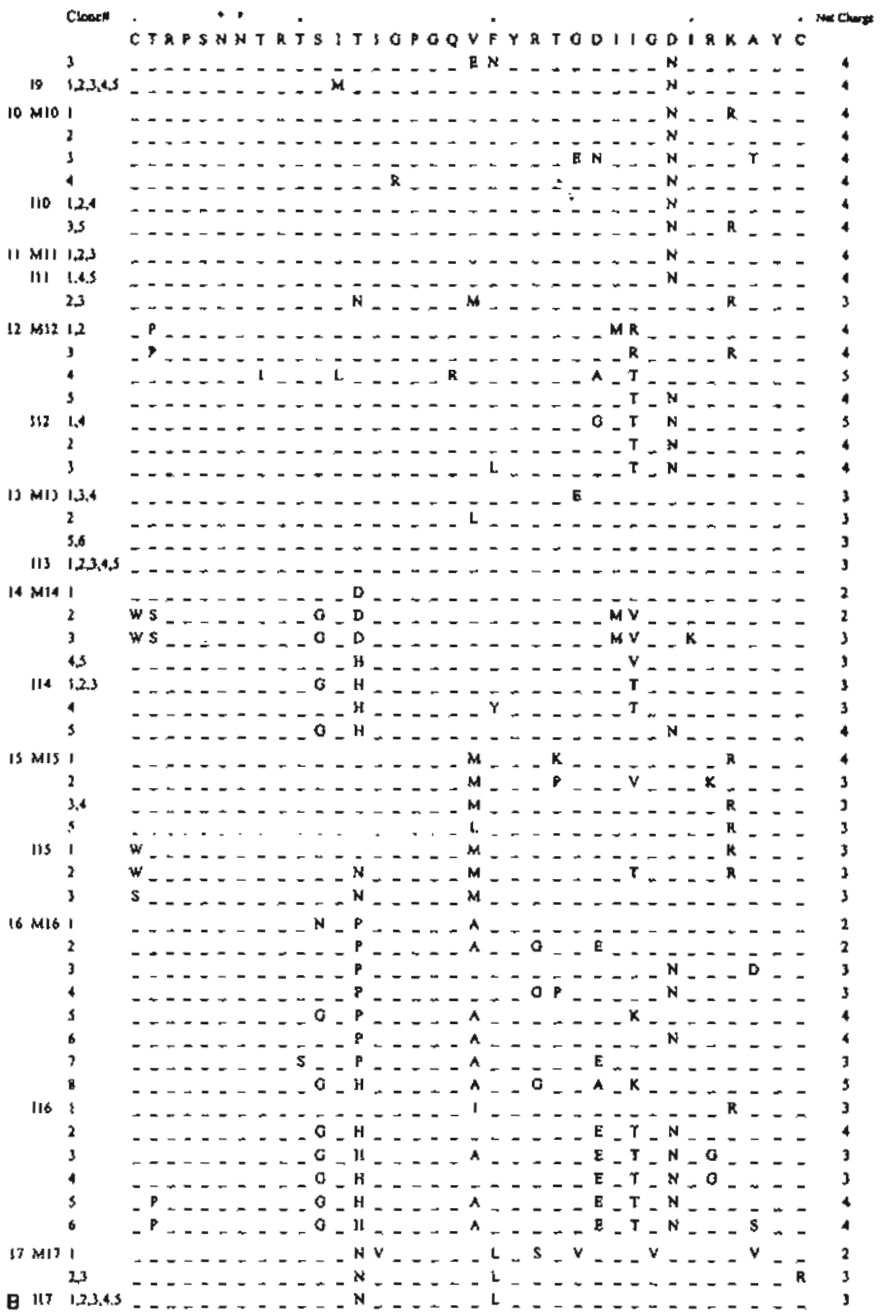

FIG. 1. Continued.

Amino Acid Sequence Variation

The amino acid sequence alignments of the 17 mother-infant pairs are presented in Figure 1. From all 158 V3 sequences in this study, arginine (R position 3), proline (P position 4 and 16), glycine (G position 17), and tyrosine (Y position 21) were conserved in all sequences. Amino acids at positions 3 to 7 and 9 (RPSNNXR) of the V3 sequence were conserved in all infant sequences. The GPGQ motif was nighly conserved in both mothers' and infants' sequences. Although GPGR was found in only one clone of M3, it was also found in her infant (I3). Clone number 4 of M1 had V3 sequence close to consensus of HIV-1 subtype B but it was not found in her

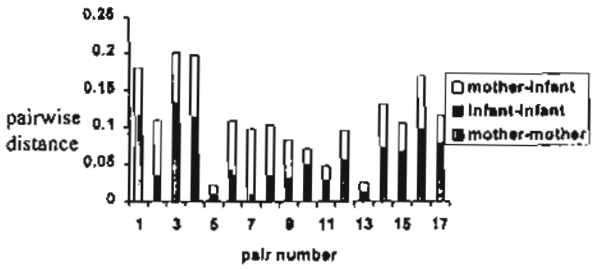

FIG. 2. Mean pair-wise distances of the V3 nucleotide sequence within the mother sets, within the infant sets, and between mother-infant sets.

infant. Only five infants (I5 and I10–I13) had V3 amino acid sequences completely identified with the corresponding mothers' sequences, whereas V3 sequences of I1, I2, I6, and I7 showed amino acid substitutions different than the mothers' sequences. Most HIV-1 V3 sequences in infants shared some amino acid similarity with their mothers. No evidence was found of specific variants transmitted from mothers to infants.

The substitutions of acidic amino acid at position 29 of the V3 loop from aspartic acid (D) to asparagine (N) was found in 6 of 9 of I9 to I17 V3 sequences, which was not found in I1 to I8. Substitution with N was found at threonine (T) position 13 of V3 sequences of I1, I11, I15, and I17. Thus, I9 to I17 group had N substitution at position 13 and/or 29 of the V3 sequence. The N at position 29 and/or 13 was significantly associated with a lack of immunosuppression status group in the first year of life (Fisher's exact test, $p = .003$; Φ coefficient value = 0.764). This N substitution at positions 13 and 29 in these infants was clearly derived from those of the mothers (M9, M10, M11, M12, M15, M16, M17). The loss of D negative charge at the position 29 gave more positive charge to the V3 amino acid sequences in this group (Mann-Whitney U test, $p = .0032$).

Pattern of *N*-Linked Glycosylation of Mother and Infant Sequences

The *N*-linked glycosylation sites at position 6 and 7 close to the first cysteine of the V3 sequence were completely conserved in all sequences of infants (Fig. 1). These positions in some clones of M1, M3, M4, and M6 were substituted with S or T or H; however, the transmitted clones to the corresponding infants had N at these positions.

DISCUSSION

Many of the previous studies of V3 sequences of mother and child in perinatal transmission had been focused on HIV-1 subtype B. HIV-1 subtype E was demonstrated to be more sexually transmissible than subtype B and is the most serious public health problem in Thailand and other Southeast Asian countries (26). The V3 sequences pattern had been implicated as important in HIV-1 perinatal infection, in that genetic variation within V3 has been found to influence host antibody response as well as affinities for epitopes within V3 and was related to the transmitted variants (10,27). This region was also an important determinant for replicative capacity and cellular tropism (14–18). Therefore, we have analyzed the proviral DNA V3 region sequences of the envelope gene from 17 HIV-1 subtype E–infected mother-infant pairs.

The previous published reports of HIV-1 subtype B (28–31) showed that the V3 sequences of mothers had a high degree of genetic diversity compared with their corresponding infants. Although most of our results (Fig. 2) also confirmed the earlier published reports, pairs 4, 5, 8, and 16 had similar genetic diversity as reported by Briant et al. (32). The viral variation of newly infected infants, especially the V3 loop, were different, which may be necessary to escape the immune response or cell tropism in the individual. Most earlier published reports (28–29, 32) indicated the selection of the minor genotypes or variants from the heterogeneous virus population that mothers transmitted to their infants. However, Scarlatti et al. (30) showed that the transmitted virus could exist as either major or minor variants. Although we cannot infer from the data whether major or minor variants in the virus population of the mothers were transmitted, we can observe from the V3 amino acid sequences that both a single variant or several variants of HIV-1 were able to transmit and replicate in the infants as shown in the previous reports (31,32). Most infants showed single cluster homogeneous viral sequences, which were closely related with their mothers' sequences (Fig. 1). This particular feature was obvious in infants I4, I5, I9, I10, I12, I13, I14, I15, and I17. These events may be the selection of a single transmitted variant and its subsequent variation or may be the selection after infection by more than one variant. It is also possible that these variants may be the minor variants from the mother or may reflect the duration of transmission and sampling. However, 13 samples showed two different clones that are closely related to two different clones from the mother and this evidence also confirmed the transmission with more than one variant to the infant (31–33).

The pattern of transmitted and nontransmitted viral sequences could not be identified from the comparison of the amino acid sequence of the mother with sequences of their respective infants. The GPGQ motif at the tip of V3

loop was predominant in HIV-1 subtype E in Thailand (23,34–35). The tip of the V3 loop, GPGQ, showed itself as highly conserved in sequences of mothers and their infants, which might reflect the important function for viral entry into cells (36).

The glycosylation of envelope might determine the pathogenesis in individuals, which finding was suggested to effect the infectivity and cellular host range by the carbohydrate binding protein on the cell surface or a macrophage endocytosis receptor (37). *N*-glycosylation site at positions 6 and 7 close to the C of the V3 loop were perfectly conserved in all infants. Although the substitution of N in some clones of M1, M3, M4, and M6 were found, the transmitted variances also have N at these positions.

In our study, I9 through 117 remained asymptomatic, whereas 11 through I8 showed immunosuppression and were diagnosed with pediatric AIDS within the first year of life. The nucleotide sequences of asymptomatic infants were significantly closer to maternal sequences than those of the AIDS cases. Our data suggested that one or two genotypes from the mother was selected and transmitted to the infant and then became diverse by the time.

From the studies of HIV-1 subtype B, the substitution with N or P at position 13 of the V3 domain lead to lowering the ability of neutralizing antibodies to bind the virus (38). The mutation to positively charged amino acid arginine (R) at position 11 and 25 of the V3 loop were shown to be associated with the SI phenotype of HIV-1 subtype A, B, C, D, and E (39). In addition, the mutation of aspartate to asparagine (D to N) at position 29 of the V3 loop was modulated by the SI phenotype (R at position 11 and 25) or enhanced by virus infectivity in T cells, which were major factors for disease progression in HIV infection (16). Thus, all viral variants in this study were predicted to be non-SI phenotype by V3 amino acid sequence prediction. The observed V3 sequences of HIV-2, which provide the long incubation period to the development of AIDS, were used to determine that HIV-2 contains N at both sites of the tip of V3 loop, at position 6, and at position 27. The other primate immunodeficiency viruses that give a long-term disease progression also contain N at both sites of the tip of V3 loop: simian immunodeficiency virus from macaque (SIVmac), simian immunodeficiency virus from sooty mangabeys (SIVsmn), and simian immunodeficiency virus from African green monkey (SIVagm) at position 6 and 27; simian immunodeficiency virus from mandrills (SIVmnd) at position 6 and 29; simian immunodeficiency virus from chimpanzee (SIVcpz) at position 6, 7, 22 and 25; simian immunodeficiency virus from Sykes' monkeys (SIVsyk) at position 6 and 11 (40–46). From our result, the comparison of the V3 amino acid sequences of asymptomatic and symptomatic infants showed the significant correlation of N at position 13 (I) and/or 29 (D) with the slow-progressor infant group. Thus, N substitution at position 13 (I) and/or 29 (D) may be an indicator for slow-progressor HIV-infected infants.

The transmission could occur early or late during pregnancy (33). Wolinsky et al. (28) and Ahmad et al. (29) suggested that a minor genotype of maternal virus was transmitted to the infants, whereas Scarlatti et al. (30) showed that the transmitted virus represented either a minor or a major population which was present in the mother. The V3 infant sequences were less diverse than those of their mothers in many published reports (28–30). Three infants from the study of Briant et al. (32) showed more heterogeneous than their mothers. The *N*-linked glycosylation site proximal to the first cysteine of V3 loop absent in the infant sets described by Wolinsky et al. (28), but other investigators reported that this glycosylation site remained conserved between mother and infant sequences (29–30,32–33). The pattern of *N*-linked glycosylation site in the HIV-1 envelope glycoprotein is generally well conserved and can play an important role in protein folding, viral infectivity, and immunogenicity by limiting epitope recognition by T lymphocytes, masking potential epitopes or actually forming an epitope (47–50).

Acknowledgments: This work was supported by Siriraj-China Medical Fund, Thailand's Ministry of University Affairs Grant, and Thailand Research Fund Grant No. RSA/1/2538. We would like to thank Dr. Marcia Kalish for her advice in nucleotide sequence analysis.

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