Shedding of HIV-1 Subtype E in Semen and Cervico-Vaginal Fluid

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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

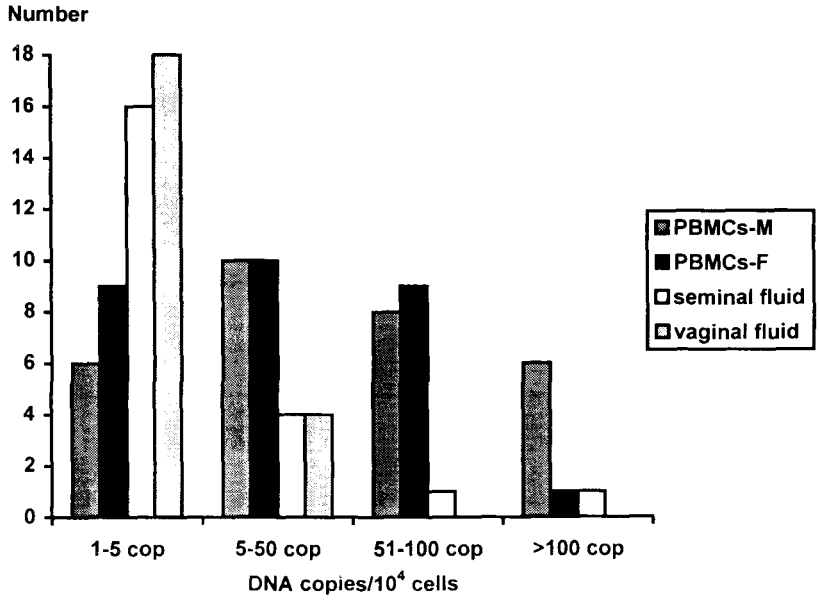

Fig. 1. Quantitation of HIV-1 proviral DNA subtype E in 10,000 cells from blood, seminal and C-V fluids.

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

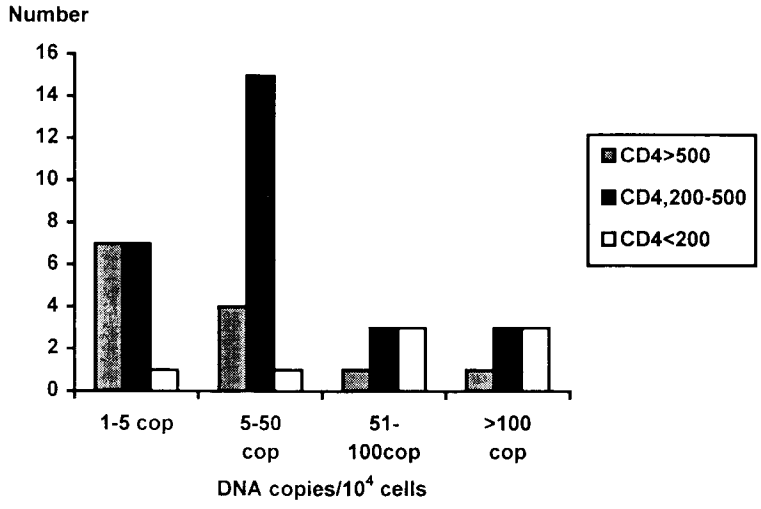

Fig. 2. Quantitation of HIV-1 proviral DNA in 10,000 cells of PBMCs in relation to blood CD4+ T cell count.

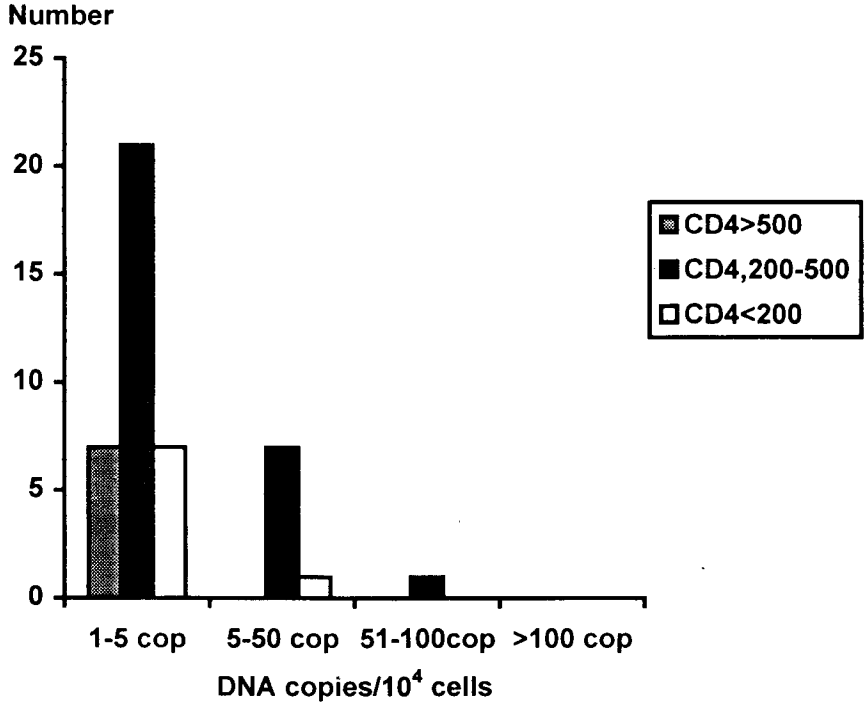

Fig. 3. Quantitation of HIV-1 proviral DNA/10,000 cells in seminal and cervico-vaginal fluids in relation to blood CD4+ T cell count.

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.

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การตรวจหาเชื้อ เอ็ช ไอ วี 1 สับทัยป์ อี ในน้ำอสุจิ และสารคัดหลั่งของช่องคลอดและปากมดลูก

รวงผึ้ง สุทเธนทร์, พ.บ., ปร.ด.., พงษ์ศักดิ์ ชัยศิลป์วัฒนา, พ.บ.**, อนุวัตร รุ่งพิสุทธิพงษ์, พ.บ.**, ปิยนท วิรัชศิลป์, วท.บ.*, ขวัญจิตร สำราญทรัพย์, วท.บ.*, ภัทรวรรณ ไชยกุล, พย.บ.***, พิไลพันธ์ พุธวัฒนะ, วท.บ, ป.ร.ด.*, จันทพงษ์ วะสี, พ.บ.**

เชื้อ เอ็ช ไอ วี 1 ที่ระบาดในไทยด้วยวิธีการติดต่อทางเพศสัมพันธ์ มากกว่า 95% เป็นสับทัยป์ E ดังนั้นเชื้อเอ็ช ไอ วี 1 สับทัยป์ E น่าจะมีความสามารถในการแพร่กระจายทางเพศสัมพันธ์ ได้ดีกว่าบางชนิด ซึ่งอาจมีสาเหตุจากที่เชื้อชนิดนั้นสามารถผ่านเยื่อบุของระบบสืบพันธ์ ได้ดีกว่า เพราะมีความจำเพาะต่อเซลล์บริเวณนั้นมากกว่าหรืออาจเพราะว่าเชื้อชนิดนั้นถูกพบได้บ่อยกว่าชนิดอื่นในสารหลั่งของระบบสืบพันธุ์ เช่น น้ำอสุจิ หรือ สารคัดหลั่งในช่องคลอดและปากมดลูก เพื่อศึกษาถึงความเป็นไปได้ของเหตุผลประการหลัง คณะผู้วิจัยจึงได้ขอเก็บ น้ำอสุจิ และ สารคัดหลั่งของช่องคลอดและปากมดลูก จากคู่สามีภรรยาที่ติดเชื้อเอ็ช ไอ วี ที่อยู่ในระยะไม่มีอาการ และไม่มีโรคทางเพศสัมพันธ์อย่างอื่นด้วยจำนวน 30 คู่ ซึ่งทั้งหมดติดเชื้อไวรัสสับทัยป์ E (ตรวจสอบไวรัสด้วยวิธี heteroduplex mobility assay และ PELISA) จากการศึกษาสามารถตรวจพบยีโนมของเชื้อเอ็ช ไอ วี โดยวิธีปฏิกิริยาลูกโซ่โพลิเมอเรส ได้ 73.33% ทั้งในน้ำอสุจิ และ สารคัดหลั่งในช่องคลอดและปากมดลูก เมื่อเปรียบเทียบกับของเชื้อเอ็ช ไอ วี สับทัยป์ B ที่มีรายงานมาก่อน พบว่าเชื้อ เอ็ช ไอ วี สับทัยป์ B และ E ถูกตรวจพบในน้ำอสุจิได้เท่ากัน แต่ในสารคัดหลั่งของช่องคลอดและปากมดลูก สามารถพบสับทัยป์ E (73.33%) ได้มากกว่าสับทัยป์ B (30–50%) แต่อัตราการแยกเชื้อเอ็ช ไอ วี โดยวิธี cocultivation ได้ 36.67% และ 16.67% จากน้ำอสุจิ และ สารคัดหลั่งในช่องคลอดและปากมดลูก ตามลำดับ ปริมาณของยีโนมของเชื้อเอ็ช ไอ วี ในเลือดแปรผกผันกับปริมาณเซลล์ CD4 ขณะที่ปริมาณของยีโนมของเชื้อเอ็ช ไอ วี ใน น้ำอสุจิ และ สารคัดหลั่งในช่องคลอดและปากมดลูก จะไม่ขึ้นอยู่กับปริมาณเซลล์ CD4 ของผู้ติดเชื้อเอ็ช ไอ วี ระยะไม่มีอาการ การที่สามารถพบเชื้อเอ็ช ไอ วี สับทัยป์ E ได้บ่อยในสารหลั่งของช่องคลอดและปากมดลูก อาจเป็นเหตุผลหนึ่งที่ทำให้เชื้อเอ็ช ไอ วี สับทัยป์ E ระบาดได้รวดเร็วทางเพศสัมพันธ์ในประเทศไทย

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