の紫外線を60分間照射した場合に、ウィルスが100%不活性化することを示した。また人工乳中のHIVは酸化チタン+紫外線照射によりp24で2%に減少することを示した。

しかし今回の実験によりマクロファージ系を主とする母乳中に含まれる細胞は、他の細胞と異なり長時間生存可能なことが示された。そこでこれらの細胞を除去した後に酸化チタン+紫外線照射することより、母乳による母子感染を防止できるのではないかと考えた。

現在これらの結果より、孔径8μmの取り外し可能なフィルターを装備し、管壁に酸化チタン処理を行い、充分な時間太陽光線の照射が可能な、特殊搾乳哺乳瓶を試作した。この哺乳瓶の有用性の証明のためには、細胞感染実験を経て、フィールドワークが実施される必要があるが、今後検討を行なう予定である。わが国他の多くの国では、HIV感染母体への抗ウィルス薬の投与、帝王切開による分娩、人工乳による保育などにより母子感染を減少させることが可能となっている。しかし最初に述べたように、多くの開発途上国では抗ウィルス薬の投与、帝王切開による分娩、人工乳による保育などはコスト面からみて不可能である。この哺乳瓶の有用性を証明するには、開発途上国における水の問題、燃料の問題など国の経済状態に関する状況、文化や識字率などの問題を充分に検討した後のフィールドワークが必要である。一方、臨床的に有用性が証明されれば、この哺乳瓶の低コスト化はそれほど困難な問題ではないと考えている。

E　結論

HIVの経母乳感染を防止することを目的とした基礎的実験を行った。その結果をもとに、母乳中の細胞を孔径8μmのフィルターにより除去し、太陽光線を約60分照射することが可能な特殊搾乳哺乳瓶を設計し試作した。

研究協力者　山口晃史（国立成育医療センター）

F　研究発表（論文発表）

1. 和田誠司，左合治彦，松本隆万，川口里恵，杉本公平，尾見裕子，林　聡，小澤伸晃，藤井絵理子，塚原優己，久保隆彦，北川道弘，田中忠夫，名取道也：妊娠中期胎児超音波スクリーニング検査による胎児異常検出率．日本周産期・新生児医学会雑誌　2004；40(1)：24-27　4月

2. Natori M: Ultrasound safety: overview and what we do need in daily clinics for a safe use of diagnostic ultrasound. Proceedings of 7th Congress of the Asian Federation of Ultrasound in Medicin and Biology, International Congress Series No. 1274, 2004:125-128

3. 名取道也：胎児病の診断と治療．1レクチャーシリーズ　1）産婦人科医療における最近のトピック　日本産科婦人科学会雑誌　2004；56(9)：N480-N484　9月

4. 左合治彦，湊川靖之，林　聡，新家　秀，北川道弘，名取道也：胎児超音波スクリーニング検査の実際．産婦人科治療　2004；

89 (5) : 523-530 11月
5. Nakagawa K, Iwasaki W, Sato M, Natori M, Saito H et al: Successful pregnancy, achieved by ovulation induction using a human menopausal gonadotropin low-dose step-up protocol in an infertile patient with Kallmann's syndrome. J Obstet Gynaecol Res 2005;31(2):140-143
6. Yamaguchi K, Yamamoto A, Hisano M, Natori M: Herpes Simplex Virus 2-Associated Hemophagocytic Lympho-histiocytosis in a Pregnant Patient. Obstet Gynecol 2005;Suppl: 1241-1244
7. Hayashi S, Sago H, Kashima K, Kitano Y, Kuroda T, Honna T, Nosaka S, Nakamura T, Ito Y, Kitagawa M, Natori M: Prenatal diagnosis of fetal hydrometrocolpos secondary to a cloacal anomaly by magnetic resonance imaging. Ultrasound Obstet Gynecol 2005 ; 26 : 577-579
8. Hayashi S, Sago H, Hayashi R, Nakagawa S, Kitagawa M, Miyasaka K, Chiba T, Natori M: Manifestation of Mirror Syndrome after Fetoscopic Laser Photocoagulation in Severe Twin-Twin Transfusion Syndrome. Fetal Diagnosis Therapy 2006 ; 21 : 51-54

研究成果の刊行に関する一覧表

1. 高桑好一, 田村正毅, 田中憲一. 産婦人科診療所における妊婦 HIV 抗体検査実施に関するアンケート調査結果 -平成 13 年度厚生労働省エイズ対策研究事業による- 日本産科婦人科学会新潟地方部会会誌. 89 : 31-34, 2003.

2. Suzuki M, Kurabayashi T, Yamamoto Y, Fujita K and Tanaka K: Effect of antioxidant treatment in oligozoospermic and asthenozoospermic men. J. Reprod. Med. 48: 707-712, 2003.

3. Kato S, Saito R, Hiraishi Y, Kitamura N, Matsumoto T, Hanabusa H, Kamakura M, Ikeda Y and Negishi M: Differential prevalence of HIV type 1 subtype B and CRF01_AE among different sexual transmission groups in Tokyo, Japan, as revealed by subtype-specific PCR. AIDS Res. Hum. Retroviruses. 19; 1057-1063, 2003.

4. Tanaka Y, Kato S, Tanaka M, Kuji N and Yoshimura Y : Structure and expression of the human oocyte-specific histone H1 gene elucidated by direct RT-nested PCR of a single oocyte. Biochem. Biophys. Res. Commun. 304, 351-357, 2003.

5. Takakuwa K, Kashima K, Suzuki M, Fujita K, Tamura M, Kaneko S, Kato S, Hanabusa H, Tanaka K: Studies on the IVF-ET for discordant couples where the man is HIV positive and the women is negative using sperm washing technique and highly sensitive PCR method. The Proceedings of the IX Congress of the International Society of Reproductive Immunoloty, 107-111, Medimond, S.r.l., Bologna, Italy, 2004

6. 高桑好一, 田中憲一 : 検査値をどう読むか。 -産婦人科診療へのアプローチ- II 感染症 4 HIV 産科と婦人科, 71 : 56-59, 2004

7. 木内英, 花房秀次.: 必携今日の生殖医療, 精液中 HIV の完全除去法 産婦人科治療 309-314, 2004 増刊

8. Miyake A, Enose Y, Ohkura S, Suzuki H, Kuwata T, Shimada T, Kato S, Narayan O, Hayami M: The quantity and diversity of infectious viruses in various tissues of SHIV-induced monkeys at the early and AIDS stages. Arch Virol 149, 943-955, 2004.

9. Phan TG, Nishimura S, Okame M, Nguyen TA, Khamrin P, Okitsu S, Maneekarn N, Ushijima H: Virus diversity and an outbreak of group C rotavirus among infants and children with diarrhea in Maizuru city, Japan

during 2002-2003. J Med Virol 74(1):173-179, 2004
10. Hansman GS, Doan LTP, Kguyen TA, Okitsu S, Katayama K, Ogawa S, Natori K, Takeda N, Kato Y, Nishio O, Noda M, Ushijima H: Detection of norovirus and sapovirus infection among children with gastroenteritis in Ho Chi Minh City, Vietnam. Arch Virol 149(9):1673-1688, 2004
11. Phan TG, Okame M, Nguyen TA, Maneekarn N, Nishio O, Okitsu S, Ushijima H: Human astrovirus, norovirus (GI, GII), and sapovirus infections in Pakistani children with diarrhea. J Med Virol 73(2):256-261, 2004
12. 高桑好一, 田中憲一. 女性診療のための感染症のすべて.STD 陽性妊婦と新生児. 産婦人科治療, 90 : 732-737, 2005.
13. 高桑好一, 加嶋克則, 鈴木美奈, 藤田和之, 田村正毅, 田中憲一 : 産婦人科診療所における HIV 陽性妊婦の診療について -全国アンケート調査結果の解析. 日本産科婦人科学会新潟地方部会会誌, 93 : 18-21, 2005.
14. Kinai E, Hanabusa H: Renal tubular toxicity associated with tenofovir assessed using urine-beta 2 microglobulin, percentage of tubular reabsorption of phosphate and alkaline phosphatase levels. AIDS. 19:2031-3, 2005.
15. Munkanta M, Terunuma H, Takahashi M, Hanabusa H, Miura T, Ikeda S, Sakai M, Fujii T, Takahashi Y, Oka S, Matsuda J, Ishikawa M, Taki M, Takashima Y, Mimaya J, Ito M, Kimura A, Yasunami M: HLA-B polymorphism in Japanese HIV-1-infected long-term surviving hemophiliacs. Viral Immunol. 18:500-5, 2005.
16. Yagyu F, Okitsu S, Tanamoto K, Ushijima H. Determination of HIV-1 subtypes (A-D, F, G, CRF01_AE) by PCR in the transmembrane region (gp41) with novel primers. J Med Virol. 2005 May;76(1):16-23.
17. Phan TG, Nguyen TA, Shimizu H, Yagyu F, Okitsu S, Muller WE, Ushijima H. Related Identification of enteroviral infection among infants and children admitted to hospital with acute gastroentritis in Ho Chi Minh City, Vietnam. J Med Virol. 2005 Oct;77(2):257-64.
18. Yoshinaga M, Phan TG, Nguyen TA, Yan H, Yagyu F, Okitsu S, Muller WE, Ushijima H. Changing distribution of group A rotavirus G-types and genetic analysis of G9 circulating in Japan. Arch Virol. 151;183-192, 2006.
19. Akihara S, Phan TG, Nguyen TA, Hansman G, Okitsu S, Ushijima H.

Existence of multiple outbreaks of viral gastroenteritis among infants in a day care center in Japan. Arch Virol. 2005 Oct;150(10):2061-75.
20. Phan TG, Nguyen TA, Kuroiwa T, Kaneshi K, Ueda Y, Nakaya S, Nishimura S, Nishimura T, Yamamoto A, Okitsu S, Ushijima H. Viral diarrhea in Japanese children: results from a one-year epidemiologic study. Clin Lab. 2005;51(3-4):183-91.
21. Phan TG, Nguyen TA, Nishimura S, Nishimura T, Yamamoto A, Okitsu S, Ushijima H. Etiologic agents of acute gastroenteritis among Japanese infants and children: virus diversity and genetic analysis of sapovirus. Arch Virol. 2005 Jul;150(7):1415-24.
22. Yamaguchi K, Yamamoto A, Hisano M, Natori M, Murashima A Herpes simplex virus 2-associated hemophagocytic lymphohistiocytosis in a pregnant patient. Obstet Gynecol 105:1241-4, 2005.
23. Takakuwa K, Mitsui T, Iwashita M, Kobayashi I, Suzuki A, Oda T, Torii Y, Matsumoto M, Yahata G, Tanaka K: Studies on the prevalence of human papillomavirus in pregnant women in Japan. J Perinat Med 34: 77-79, 2006.
24. Shingo Kato, Hideji Hanabusa, Satoru Kaneko, Koichi Takakuwa, Mina Suzuki, Naoaki Kuji, Masao Jinno, Rie Tanaka, Kenichi Kojima, Mitsutoshi Iwashita, Yasunori Yoshimura, Kenichi Tanaka. Complete removal of HIV-1 RNA and proviral DNA from semen by the swim-up method: Assisted reproduction technique using spermatozoa free from HIV-1. AIDS 2006 in press

産婦人科診療所における妊婦HIV抗体検査実施に関するアンケート調査結果

―平成13年度厚生労働省エイズ対策研究事業による―

新潟大学医学部産科婦人科学教室
高桑　好一・田村　正毅・田中　憲一

Ⅰ．緒　言

ヒト免疫不全ウイルス（HIV）の本邦における感染率は，諸外国に比べ高いものではないが，確実に増加している。このことは妊娠婦人においても同様であり，妊娠初期検査として，HIV抗体検査を実施することは重要である。出生児への垂直感染の予防を考慮した場合，さらにその重要性は増すが，産婦人科診療所におけるHIV抗体検査の実施状況は明らかではない。そこで，われわれは平成13年度厚生労働省エイズ対策研究事業の一環として，全国の産科診療所を対象として，アンケート調査を実施したので，その結果を報告する。

Ⅱ．研究方法

日本産科婦人科学会，日本母性保護産婦人科医会（現日本産婦人科医会）会員名簿2001年版[1]を参考とし，産婦人科診療所と判断された全施設5938施設に対し，アンケート用紙を送付した。

アンケート内容の概要は①これまでのHIV合併妊娠婦人の診療の有無。②分娩取扱いの有無，分娩件数。③妊娠婦人に対する検査としてのHIV抗体検査施行の有無，などであるが，今回の検討では，妊娠婦人に対する妊娠初期検査としてのHIV抗体検査の実施状況について解析した。

Ⅲ．結　果

(1)　アンケート回収結果

アンケート送付5938施設のうち2676施設から回答を得た。回答施設2676施設のうち，分娩を取り扱っている施設は1504施設（56.2％），これらの施設での分娩総数は約44,3000件であり，分娩を取り扱っていない施設は1062施設（28.9％）であった。また，110施設（4.1％）については不明であった。

(2)　妊娠婦人に対する初期検査としてのHIV抗体検査実施率

分娩を取り扱っている産婦人科診療所で，妊婦の100％にHIV抗体検査を実施している施設の割合いは全国（この質問項目について回答のあった施設数1474施設）では，53.0％であった。

地域別では，北海道33施設中30.3％，東北115施設中46.1％，関東397施設中79.8％，中部238施設中63.0％，近畿253施設中53.0％，中国105施設中39.0％，四国52施設中21.2％，九州256施設中22.7％であった。

各県別のHIV抗体100％実施施設の割合を地域別に表1に示した。また，実施率順を表2に示した。最も高率である県は茨城県であり，94.6％，以下千葉県87.5％，富山83.3県％の順であり，最も低い県は佐賀県の7.1％であり，ついで沖縄県の9.1％，香川県の10.0％であった。

一方，妊婦健診の際にHIV抗体検査をまったく施行していない施設の割合いは，全国（1474施設）では18.6％であった。地方別では，北海道27.3％，東北21.7％，関東2.3％，中部11.3％，近畿17.0％，中国27.6％，四国44.2％，九州38.3％であった。各県別のHIV抗体検査未実施施設の割合を地域別に表3に示した。また割合順を表4に示した。

なお，新潟県におけるHIV抗体100％実施施設の割合は69.0％であり，未実施施設割合は20.7％であった。

各施設における分娩件数別のHIV抗体検査未実施の割合（0％実施）および100％実施の割合を表5に示した。分娩件数800～999件の施設で42.5％と低かったが，分娩件数が多い施設ほどHIV抗体実施率が高い傾向が認められた。

Ⅳ．考　察

HIVの本邦における感染率は，諸外国に比べ高いものではないが，確実に増加している。平成14年11月の厚生労働省のホームページにおけるデータによれば，全国におけるHIV感染者の累積症例数は4982人であり，エイズ発症者数は2488人となっている。また，平成22年（2010年）には，HIV感染者は50000人に達すると推計されている。

従来，HIV感染妊娠婦人の実態および妊娠婦人に対するHIV抗体検査の実施率などの把握のため，産婦人科を有する病院施設に対するアンケート調査が実施されたきた[2]。一方，我が国における分娩の半数近くは

表1　妊娠婦人に対するHIV抗体検査100％実施施設の割合(1)

都道府県	施設数	100％施設	割合
北海道	33	10	30.3％
青森	12	2	16.7％
岩手	17	4	23.5％
秋田	12	8	66.7％
宮城	32	19	59.4％
山形	15	5	33.3％
福島	27	15	55.6％
茨城	37	35	94.6％
栃木	28	23	82.1％
群馬	27	22	81.5％
埼玉	55	42	76.4％
千葉	64	56	87.5％
東京	113	82	72.6％
神奈川	73	57	78.1％
山梨	12	8	66.7％
新潟	29	20	69.0％
富山	12	10	83.3％
石川	16	7	43.8％
福井	9	4	44.4％
長野	20	11	55.0％
静岡	42	28	66.7％
岐阜	25	18	72.0％
愛知	73	44	60.3％
三重	29	17	58.6％
滋賀	14	10	71.4％
京都	33	27	81.8％
奈良	14	10	71.4％
大阪	79	40	50.6％
和歌山	15	4	26.7％
兵庫	69	26	37.7％
岡山	24	8	33.3％
広島	37	20	54.1％
鳥取	8	1	12.5％
島根	9	2	22.2％
山口	27	10	37.0％
香川	10	1	10.0％
愛媛	18	4	22.2％
徳島	12	4	33.3％
高知	12	2	16.7％
福岡	85	17	20.0％
佐賀	14	1	7.1％
長崎	37	10	27.0％
大分	27	4	14.8％
熊本	36	8	22.2％
宮崎	24	6	25.0％
鹿児島	21	11	52.4％
沖縄	11	1	9.1％
不明	25	6	24.0％
総計	1473	780	53.0％

表2　妊娠婦人に対するHIV抗体検査100％実施施設の割合(2)

都道府県	施設数	100％施設	割合
茨城	37	35	94.6％
千葉	64	56	87.5％
富山	12	10	83.3％
栃木	28	23	82.1％
京都	33	27	81.8％
群馬	27	22	81.5％
神奈川	73	57	78.1％
埼玉	55	42	76.4％
東京	113	82	72.6％
岐阜	25	18	72.0％
滋賀	14	10	71.4％
奈良	14	10	71.4％
新潟	29	20	69.0％
秋田	12	8	66.7％
山梨	12	8	66.7％
静岡	42	28	66.7％
愛知	73	44	60.3％
宮城	32	19	59.4％
三重	29	17	58.6％
福島	27	15	55.6％
長野	20	11	55.0％
広島	37	20	54.1％
平均			53.0％
鹿児島	21	11	52.4％
大阪	79	40	50.6％
福井	9	4	44.4％
石川	16	7	43.8％
兵庫	69	26	37.7％
山口	27	10	37.0％
山形	15	5	33.3％
岡山	24	8	33.3％
徳島	12	4	33.3％
北海道	33	10	30.3％
長崎	37	10	27.0％
和歌山	15	4	26.7％
宮崎	24	6	25.0％
岩手	17	4	23.5％
島根	9	2	22.2％
愛媛	18	4	22.2％
熊本	36	8	22.2％
福岡	85	17	20.0％
青森	12	2	16.7％
高知	12	2	16.7％
大分	27	4	14.8％
鳥取	8	1	12.5％
香川	10	1	10.0％
沖縄	11	1	9.1％
佐賀	14	1	7.1％

表3　妊娠婦人に対するHIV抗体検査実施0％施設の割合(1)

都道府県	施設数	0％施設	割合
北海道	33	9	27.3％
青森	12	5	41.7％
岩手	17	9	52.9％
秋田	12	0	0.0％
宮城	32	5	15.6％
山形	15	3	20.0％
福島	27	3	11.1％
茨城	37	0	0.0％
栃木	28	0	0.0％
群馬	27	0	0.0％
埼玉	55	0	0.0％
千葉	64	1	1.6％
東京	113	7	6.2％
神奈川	73	1	1.4％
山梨	12	0	0.0％
新潟	29	6	20.7％
富山	12	1	8.3％
石川	16	3	18.8％
福井	9	2	22.2％
長野	20	0	0.0％
静岡	42	3	7.1％
岐阜	25	3	12.0％
愛知	73	9	12.3％
三重	29	2	6.9％
滋賀	14	3	21.4％
京都	33	2	6.1％
奈良	14	2	14.3％
大阪	79	8	10.1％
和歌山	15	4	26.7％
兵庫	69	22	31.9％
岡山	24	8	33.3％
広島	37	4	10.8％
鳥取	8	5	62.5％
島根	9	1	11.1％
山口	27	11	40.7％
香川	10	6	60.0％
愛媛	18	5	27.8％
徳島	12	5	41.7％
高知	12	7	58.3％
福岡	85	46	54.1％
佐賀	14	9	64.3％
長崎	37	3	8.1％
大分	27	14	51.9％
熊本	36	12	33.3％
宮崎	24	9	37.5％
鹿児島	21	1	4.8％
沖縄	11	4	36.4％
不明	25	11	44.0％
総計	1473	274	18.6％

表4　妊娠婦人に対するHIV抗体検査実施0％施設の割合(2)

都道府県	施設数	0％施設	割合
秋田	12	0	0.0％
茨城	37	0	0.0％
栃木	28	0	0.0％
群馬	27	0	0.0％
埼玉	55	0	0.0％
山梨	12	0	0.0％
長野	20	0	0.0％
神奈川	73	1	1.4％
千葉	64	1	1.6％
鹿児島	21	1	4.8％
京都	33	2	6.1％
東京	113	7	6.2％
三重	29	2	6.9％
静岡	42	3	7.1％
長崎	37	3	8.1％
富山	12	1	8.3％
大阪	79	8	10.1％
広島	37	4	10.8％
福島	27	3	11.1％
島根	9	1	11.1％
岐阜	25	3	12.0％
愛知	73	9	12.3％
奈良	14	2	14.3％
宮城	32	5	15.6％
平均			18.6％
石川	16	3	18.8％
山形	15	3	20.0％
新潟	29	6	20.7％
滋賀	14	3	21.4％
福井	9	2	22.2％
和歌山	15	4	26.7％
北海道	33	9	27.3％
愛媛	18	5	27.8％
兵庫	69	22	31.9％
岡山	24	8	33.3％
熊本	36	12	33.3％
沖縄	11	4	36.4％
宮崎	24	9	37.5％
山口	27	11	40.7％
青森	12	5	41.7％
徳島	12	5	41.7％
大分	27	14	51.9％
岩手	17	9	52.9％
福岡	85	46	54.1％
高知	12	7	58.3％
香川	10	6	60.0％
鳥取	8	5	62.5％
佐賀	14	9	64.3％

表5　分娩件数別HIV抗体検査実施率

	施設数	実施0％施設数	（率）	実施100％施設数	（率）
～200件	595	149	25.0％	260	43.7％
200～399	494	68	13.8％	290	58.7％
400～599	221	37	16.7％	133	60.2％
600～799	91	10	11.0％	57	62.6％
800～999	40	8	20.0％	17	42.5％
1000件～	33	2	6.1％	23	69.7％
総計	1474	274	18.6％	780	52.9％

産婦人科の有床診療所で行われており，HIV感染妊娠婦人の実態および妊娠婦人に対するHIV抗体検査の実施率などをより正確に把握するためには，これらの施設に対するアンケート調査は重要である。

このような観点から，産婦人科診療所を対象としたアンケート調査を実施した。ただし，病院施設と異なり，アンケートを送付すべき産婦人科診療所の実態の把握は容易ではない。今回は「日本産科婦人科学会，日本産婦人科医会会員名簿」より産婦人科診療所と判断された全施設を選択し，アンケート用紙を送付した。回答は2676施設（45.1％）から寄せられ，分娩を取り扱っている施設が1504施設であった。これらの施設で取り扱われた分娩総数は約44,3000件であり，我が国における年間分娩件数（約120万件）を考慮すると，分娩を取り扱うほとんどの産婦人科診療所から回答が寄せられたものと判断している。

調査の重要な項目として，妊婦健診の際にHIV抗体の検査を実施しているか否かについて質問を行ったが，分娩を取り扱っている産婦人科診療所で，妊婦の100％にHIV抗体検査を実施して施設の割合いは全国で53.0％であった。一方，HIV抗体検査をまったく施行していない施設の割合いは18.6％であった。地方別では関東，中部地方などで妊婦に対するHIV抗体の実施率が高く，九州，中国，四国地方などでは低いことが判明した。

妊娠初期検査でHIV陽性が判明した場合，妊娠中に抗HIV剤の服用を行い，帝王切開術を行うことにより，母体から新生児への垂直感染を阻止しうることが明らかになっており[2]，検査を行うことは有用である。ただし，患者（妊娠婦人）の自己決定権の観点から，インフォームドコンセントは重要であり，同意を得てからHIV抗体の検査を行うことが重要であると判断される。

V．結　語

本邦におけるHIV感染妊娠婦人はそれ程多いものではないが，HIV感染症例を管理する機会は確実に増えるものと考えられ，その際には，妊娠，分娩期間をとおして，医学的管理のみならず精神的なケアも含め，きめ細かい管理が必要とされる[3]。一方，最近では，HIV感染男性，非感染女性に対する体外受精-胚移植（妻への二次感染をほぼ0％としての妊娠補助）を実施している[4]。このように産婦人科医がHIV感染に取り組む機会は，今後増加するものと考えられ，妊娠婦人に対するHIV抗体検査の実施にあたっても，十分な知識をもって対応することが必要であるものと，判断される。

謝　辞

このたびの研究にご協力いただきました，日本産科婦人科学会，日本産婦人科医会の会員の諸先生方に深謝いたす次第です。

本研究は厚生労働科学研究費補助金（エイズ対策研究事業）により実施されたものです。

参考文献

1）日本産科婦人科学会，日本母体保護産婦人科医会会員名簿：2001年1月出版

2）平成13年度厚生労働科学研究費補助金（エイズ対策研究事業）「妊産婦のSTDおよびHIV陽性率と妊婦STD及びHIVの出生児に与える影響に関する研究」研究報告書，主任研究者：田中憲一

3）高桑好一，鈴木美奈，田中憲一他：HIV感染妊婦の管理経験　日産婦新潟地方会誌　86：10-12，2002

4）高桑好一，鈴木美奈，藤田和之，花房秀次，田中憲一：不妊症と感染症の現在－　不妊治療とウイルス感染症：化学療法の領域　17：1122-1126，2001

Reprinted from *The Journal of Reproductive Medicine®*
Vol.48 No.9, September 2003

Effects of Antioxidant Treatment in Oligozoospermic and Asthenozoospermic Men

Mina Suzuki, M.D., Takumi Kurabayashi, M.D., Yasuaki Yamamoto, M.D., Kazuyuki Fujita, M.D., and Kenichi Tanaka, M.D.

OBJECTIVE: To evaluate the effect of sairei-to, an herbal medicine, as an antioxidant in oligozoospermia and asthenozoospermia (nonnormozoospermia).

STUDY DESIGN: Forty-seven nonnormozoospermic and 16 normozoospermic men were the subjects of this prospective clinical study. After sairei-to (9.0 g/d) was administered daily to the 2 groups for 3 months, sperm parameters, serum hormones and superoxide dismutase (SOD) activity in the serum and the seminal plasma was analyzed. The testicular artery was also assessed.

RESULTS: After therapy, serum hormones and SOD activity did not change significantly in either group. Although men with normozoospermia did not undergo a significant change in sperm conditions or testicular artery flow, total sperm concentration (17.1 ± 20.0 versus $28.7 \pm 35.5 \times 10^6$/mL, $P = .02$) and sperm motility ($30.1\% \pm 21.6$ versus $45.8\% \pm 24.4$, $P < .0001$) were significantly increased, and the pulsatility index of the testicular artery (2.03 ± 0.84 versus 1.64 ± 0.48, $P = .04$) was significantly decreased in nonnormozoospermia.

CONCLUSION: Treatment with the herbal antioxidant sairei-to improves sperm condition and testicular artery flow in nonnormozoospermia. (J Reprod Med 2003; 48:707–712)

Sairei-to, an antioxidant supplement, has a beneficial effect on nonnormozoospermic men....

Keywords: infertility, male; antioxidants; sairei-to; oligozoospermia; asthenozoospermia.

Male infertility has complex pathophysiologic causes. In particular, unexplained disturbances of spermatogenesis are common clinical challenges. Recent studies have shown that sperm function is influenced largely by the presence of oxygen radicals. Spermatozoa have been shown to release hydrogen peroxide and superoxide radicals,[1] and peroxidation of spermatozoal lipids leads to loss of sperm motility.[2] Human sperm are particularly sensitive to free radicals because of their high content of polyunsaturated fatty acids and lack of DNA repair mechanisms.[3,4] In addition, superoxide radicals have been implicated in a capacitation reaction.[5,6] Different enzymes and compounds present in seminal fluid—glutathione peroxidase/reductase, superoxide dismutase (SOD), pyruvate, taurine, hypotaurine, urate, and

From the Department of Obstetrics and Gynecology, Niigata University School of Medicine, Niigata, Japan.

Address reprint requests to: Mina Suzuki, M.D., Department of Obstetrics and Gynecology, Niigata University School of Medicine, Niigata, Japan.

Financial Disclosure: The authors have no connection to any companies or products mentioned in this article.

vitamin C and E—have antioxidative activity.[7] The seminal plasma of infertile men, particularly those whose semen has poor sperm motility, has lower antioxidant levels than that of fertile men.[8] Therefore, antioxidant treatment with vitamin C and alpha-tocopherol (vitamin E) has been studied. Some reports have indicated that vitamin C and E may improve the fertilization rate, possibly owing to a reduction in lipid peroxidation.[9-11] In contrast, other reports have indicated that mixed vitamin E and C supplementation does not affect sperm function.[12] The effectiveness of antioxidant treatment for male subfertility thus remains unresolved.[13]

Measurements of SOD levels in the seminal plasma and blood flow of the testicular artery may be useful methods of assessing sperm condition.

In light of their clinical effects, the Chinese herbal medicines hochu-ekki-to, guizhi-fuling-wan and hachimi-jio-gan are considered promising drugs for improving sperm quality,[14-16] although their mechanisms of action have not been detailed. Sairei-to contains similar antioxidant elements—that is, saiko, takusha, hange, taisou, ninjin, ougon, taisou and keihi. In the field of internal medicine, sairei-to is used as an antioxidant in nephrosis and is reported to have effects on glomerular nephritis[17,18] because it eliminates superoxides and controls hyperoxidation. Therefore, we considered that sairei-to might be useful as an alternative to vitamin C and alpha-tocopherol in the treatment of male infertility.

In assessing treatments in infertile men, it is important to evaluate systematically each factor involved in successful fertilization. Ultrasonography has been the method of choice for detecting varicocele and tumors of the scrotum.[19,20] Another objective of our study was to find sensitive indicators of serum and ultrasonographic findings that would reflect the effects of antioxidant treatment.

Materials and Methods

We studied 47 nonnormozoospermia and 16 normozoospermic men who visited the outpatient clinic of the Department of Obstetrics Gynecology, Niigata University Hospital, between January 1999 and January 2001. Nonnormozoospermic men were diagnosed as having oligozoospermia, with $<20\times10^6$ sperm/mL of semen, and/or asthenozoospermia, defined as sperm motility <50% according to WHO criteria.[21] The patients received 9.0 g/d of sairei-to for 12 weeks. Semen and blood samples for laboratory testing were collected, and the blood flow in the paratesticular artery was measured at baseline and at the end of week 12. Informed consent was obtained from all patients.

Sperm Parameters

After a 3–7-day period of abstinence from emission, ejaculates were collected from the nonazoospermic patients for evaluation of infertility. Each specimen was allowed to liquefy at room temperature before routine analysis (volume, sperm count, motility, morphology) was done according to WHO criteria.[22] After analysis, the specimen were centrifuged (3,000 g, 20 minutes) to separate the spermatozoa from the seminal plasma.

Hormonal Analysis

Before and after medication for 3 months, levels of luteinizing hormone (LH), follicle stimulating hormone (FSH), testosterone (T) and prolactin (PRL) in the serum were measured by radioimmunoassay.

SOD Analysis

SOD, as an antioxidant substance, was quantitatively analyzed in the serum and seminal plasma. SOD activity was measured by the inhibition of nitroblue tetrazolium (NBT) reduction by superoxide anions generated by the combination of xanthine (XO) plus xanthine oxidase (XOD).[23] The reaction mixture was buffered with phosphate-buffered saline (PBS) (0.1 M, pH 8.0) and contained XO (0.40 mmol/L), NBT (0.24 mmol/L), and 0.1 mL serum or seminal plasma (A) or 0.1 mL distilled water (B). The reaction of A or B was started by adding XOD (0.049 U/mL). For blank samples, 0.1 M PBS was added to A (C) and B (D). The 4 samples were incubated at 37°C for 20 minutes; then the reaction was stopped by adding 2.0 mL sodium dodecylsulfate (69 mmol/L), and extinction was read at 560 nm. We defined Ea as the extinction of A with added XOD, Eb as the extinction of B with added XOD, Ec as the extinction of C with added 0.1 M PBS and Ed as the extinction of D with added 0.1 M PBS. SOD activity was defined as $\{(Eb-Ed)-(Ea-Ec)\}\times100/Eb-Ed$.

Table I *Semen Parameters Before and After Sairei-to Treatment in Oligozoospermic and/or Asthenozoospermic Men*

	Total (n = 47)			Oligo (n = 32)			Astheno (n = 41)		
Parameter	Before	After	*P*	Before	After	*P*	Before	After	*P*
Sperm concentration ($\times 10^6$/mL)	17.1 ± 20.0	28.7 ± 35.5	.015	6.2 ± 6.1	16.8 ± 23.9	.008	17.8 ± 21.3	31.2 ± 37.2	.013
Motile sperm concentration ($\times 10^6$/mL)	5.5 ± 7.5	15.1 ± 21.6	.004	2.7 ± 3.8	9.5 ± 17.1	.023	5.1 ± 7.8	16.1 ± 22.8	.003
Motile sperm rate (%)	30.1 ± 21.6	45.8 ± 24.4	< .0001	31.8 ± 24.3	43.9 ± 25.9	.001	23.9 ± 14.5	43.5 ± 22.8	< .0001
Progressive motile sperm concentration ($\times 10^6$/mL)	2.1 ± 2.9	6.6 ± 9.8	.002	1.1 ± 1.8	3.6 ± 6.6	.02	2.0 ± 2.9	7.0 ± 10.4	.002

Values are mean ± SD.
Total = sperm concentration < 20×10^6/mL or percentage of motile sperm < 50, Oligo = sperm concentration < 20×10^6/mL, Astheno = percentage of motile sperm < 50.

Testicular Artery Flow

Doppler examinations were carried out on supine patients by the same examiner (Y.Y.) with a Sonovista color II scanner (Mochida Co. Ltd., Tokyo, Japan) attached to a 7.5-MHz linear-type probe. The flow velocity waveforms of the paratestis were recorded over 4 or 5 cardiac cycles. Resistant index (RI) was calculated from the following equation:

RI = (peak systolic velocity-end diastolic velocity)/end diastolic velocity.

The pulsatility index (PI) was calculated from the following equation:

PI = (peak systolic velocity-end diastolic velocity)/time averaged mean peak velocity.

For statistical analysis, RI and PI were used as averages of the right and left sides.

Statistical Analysis

Statistical analysis was performed with Stat View 4.5 software (Abacus Concepts, Berkeley, California). The mean values for each group were expressed with their SD. The Wilcoxon matched pairs test was used to compare pairs of values. Correlations between sperm parameters and other parameters were evaluated by linear regression analysis and the F-test. A value of $P < .05$ was defined as statistically significant.

Results

Sperm Condition

Of the 47 participants, 32 were classified as having oligozoospermia and 41 as having asthenozoospermia. Concentrations of total, motile and progressively motile sperm, as well as the percentage of motile sperm, were significantly increased after 3 months of therapy (Table I).

Hormonal Analysis

There were no remarkable changes in the serum levels of LH, FSH, PRL and T at the end of antioxidant therapy in the infertile group (Table II).

SOD Analysis

The concentration of SOD in seminal plasma (26.3 ± 5.1%) was much higher than that in serum (12.1 ± 1.7%). SOD activity in serum and seminal plasma did not change significantly after antioxidant therapy (26.3 ± 5.1% versus 24.0 ± 5.4%, $P = .37$; 12.1 ± 1.7% versus 11.7 ± 2.2%; $P = .059$).

In normozoospermic men, average SOD activity in seminal plasma was 23.4%. Therefore, infertile men were divided into 2 groups according to whether their seminal plasma SOD activity was ≥ 23.4% or < 23.4% before antioxidant treatment. The former group (high SOD) had significantly de-

Table II *Serum Hormonal Analysis of Sairei-to Treatment in Infertile Men*

Variable	Before	After	*P* value
LH (mIU/mL)	3.1 ± 1.7	3.4 ± 1.7	.24
FSH (mIU/mL)	9.4 ± 8.8	9.7 ± 8.0	.81
PRL (ng/mL)	9.1 ± 3.7	10.2 ± 5.7	.19
Testosterone (ng/mL)	4.4 ± 1.1	4.8 ± 1.1	.07

N = 47.
Values are mean ± SD.

Table III Characteristics of Patients

Characteristic	Normo (n = 16)	Nonnormo (n = 47)	P value
Age (yr)	35.3 ± 4.9	35.3 ± 5.6	.97
Height (cm)	174.8 ± 6.8	171.3 ± 4.8	.036
Weight (kg)	71.7 ± 11.0	68.7 ± 9.0	.295
Sperm concentration ($\times 10^6$/mL)	70.4 ± 43.4	17.1 ± 20.0	< .0001
Motile sperm concentration ($\times 10^6$/mL)	45.2 ± 28.5	5.5 ± 7.5	< .0001
Motile sperm rates (%)	64.3 ± 8.6	30.0 ± 21.6	< .0001
Progressive motile sperm concentration ($\times 10^6$/mL)	11.9 ± 8.0	2.1 ± 2.9	< .0001
SOD activity in serum (%)	11.5 ± 1.3	12.1 ± 1.7	.24
SOD activity in seminal plasma (%)	23.4 ± 27.8	26.3 ± 25.7	.108
RI of paratesticular artery	0.73 ± 0.08	0.79 ± 0.06	.01
PI of paratesticular artery	1.51 ± 0.5	2.03 ± 0.8	.029

Values are mean ± SD.
Normo = normozoospermia, Nonnormo = oligozoospermia and/or asthenozoospermia.

creased SOD activity in serum (12.2 ± 1.7% versus 11.5 ± 1.8%, $P = .01$) and seminal plasma (29.0 ± 4.0% versus 26.5 ± 4.3%, $P = .015$) after antioxidant treatment (Table III). The latter SOD group (low SOD) had no significant change (serum, 11.9 ± 1.3% versus 11.3 ± 0.7%; $P = .093$; seminal plasma, 21.0 ± 1.7% versus 20.8 ± 4.4%; $P = .83$).

Assessment of Blood Flow in the Testicular Artery

The paratesticular artery in nonnormozoospermic men had higher resistance than in normozoospermic men (Table IV). Although RI and PI of the paratesticular artery in normozoospermic men did not change with therapy (RI, 0.73 ± 0.08 versus 0.70 ± 0.10, $P = .053$, PI, 1.51 ± 0.48 versus 1.39 ± 0.46, $P = .069$), RI and PI in the paratesticular artery in infertile men decreased significantly after antioxidant treatment (RI, 0.79 ± 0.06 versus 0.75 ± 0.06, $P = .038$; PI, 2.03 ± 0.84 versus 1.64 ± 0.48, $P = .036$) (Figures 1 and 2).

Discussion

There are two types of antioxidant: preventive and chain breaking. SOD, catalase and glutamine peroxidase are of the former type, and vitamin C and E of the latter. Sairei-to contains many antioxidant elements, the presence of which has been confirmed by neutrocyte chemiluminescence, but the precise nature of these elements is unknown. In our study, the sperm parameters of oligozoospermic or asthenozoospermic men were significantly improved by supplementation with sairei-to. The aim of therapy for infertile couples is pregnancy, and improving sperm condition should lead to higher pregnancy rates.

It is difficult to identify the mechanism of sairei-to's action. Supplementation of preparation media with antioxidants does not improve sperm motility.[24] Therefore, sairei-to may not affect spermatozoa directly. It is possible that sairei-to stimulates Leydig cells and thus promotes the production of testosterone, which leads to stimulation of Sertoli cells and spermatogenesis. In our trial, testosterone levels increased after sairei-to treatment, although the changes were not significant ($P = .07$). The epididymis contains extremely large amounts of mRNA-encoding SOD, and seminal plasma from the caudal epididymis has very high SOD activity in rats.[25] Therefore, the epididymis in men may also be an important source of SOD. If this is so, then

Table IV Changes in SOD Activity

	Total			High SOD group (n = 15)			Low SOD group (n = 9)		
SOD activity	Before	After	P value	Before	After	P value	Before	After	P value
In serum (%)	12.1 ± 1.7	11.7 ± 2.2	.37	12.2 ± 1.7	11.5 ± 1.8	.01	11.9 ± 1.3	11.3 ± 0.7	.093
In seminal plasma (%)	26.3 ± 5.1	24.0 ± 5.4	.059	29.0 ± 4.0	26.5 ± 4.3	.015	21.0 ± 1.7	20.8 ± 4.4	.83

Total = oligozoospermic and asthenozoospermic men.
High SOD group: SOD activity in seminal plasma ≥ 23.4%.
Low SOD group: SOD activity in seminal plasma < 23.4%.

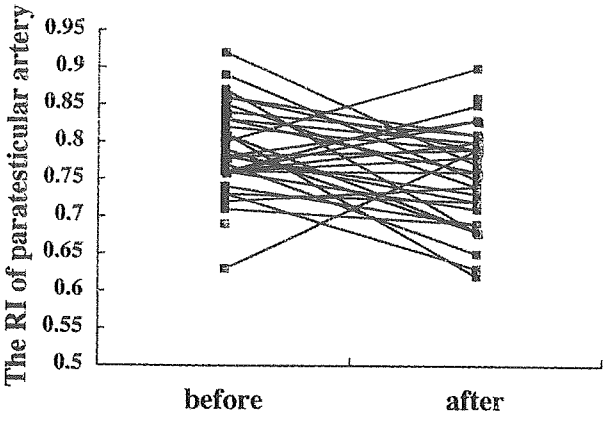

Figure 1 RI of the paratesticular artery in infertile men before and after sairei-to treatment.

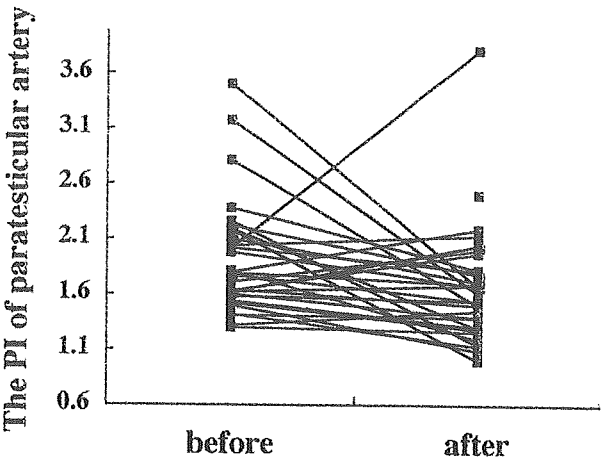

Figure 2 PI of paratesticular artery in infertile men before and after sairei-to treatment.

sairei-to might affect the epididymis and thus secondarily influence sperm condition.

There are some reports on the relationship between SOD and spermatozoa. The ratio of SOD to xanthine oxidase may be a diagnostic factor, useful in the determination of male infertility.[26] High SOD activity in seminal plasma would suppress that reaction in the male genitourinary system,[27] and SOD may play a physiologic role in maintaining a balance between superoxide and hydrogen peroxide; therefore, high levels of SOD might be associated with impaired sperm function.[28] It has also been reported that a high level of SOD-like activity may reflect a defect in the maturation of spermatozoa[29] and that high SOD activity is 1 defect in oligozoospermia.[30] In our study, sairei-to supplementation suppressed SOD levels in the high-SOD group and may act to normalize SOD levels and thus secondarily control spermatogenesis. In contrast, the effect of sairei-to might be similar to that of NO in relaxing the seminal arteries.

Recently, remarkable progress has been made in the use of ultrasonography as a diagnostic tool, and color Doppler sonography of the testis may be useful in the differential diagnosis of azoospermia.[31] We investigated the change in the RI and PI of the testicular artery with sairei-to. No change was observed in normozoospermic men, but in oligozoospermic and asthenozoospermic men there were changes in the RI and PI of the paratesticular artery. Like NO, SOD might affect the seminal arteries by relaxing them.

Sairei-to, an antioxidant supplement, has a beneficial effect on nonnormozoospermic men, and measurements of SOD levels in the seminal plasma and blood flow of the testicular artery may be useful methods of assessing sperm condition. Further studies are needed.

References

1. Alvarez JG, Touchstone JC, Blasco L, et al: Spontaneous lipid peroxidation and production of hydrogen peroxidase and superoxide in human spermatozoa: Superoxide dismutase is a major enzyme protectant against oxygen toxicity. J Androl 1987;8:338–348
2. Rao B, Soufir JC, David G: Lipid peroxidation in human spermatozoa as related to midpiece abnormalities and motility. Gamete Res 1989;4:127–134
3. Aitken RJ, Clarkson JS: Cellular basis of defective sperm function and its association with the genesis of reactive oxygen species by human spermatozoa. J Reprod Fertil 1987;81: 459–469
4. Griveau JF, Le Lannou D: Reactive oxygen species and human spermatozoa physiology and pathology. Int J Androl 1997;20:61–69
5. De Iamirande E, Gagnon C: A positive role for the superoxide anion in triggering hyperactivation and capacitation of human spermatozoa. Int J Androl 1993;16:21–25
6. Grivevau JF, Renard P, le Lannou D: Superoxide anion production by human spermatozoa as a part of the ionophore-induced acrosome reaction process. Int J Androl 1995;18: 67–74
7. Lewis SEM, Sterling ESL, Yung IS, et al: Comparison of individual antioxidants of sperm and seminal plasma in fertile and infertile men. Fertil Steril 1997;67:142–147
8. Lewis SEM, Yung IS, Boyle PM, et al: Total antioxidant ca-

pacity of seminal plasma is different in fertile and infertile men. Fertil Steril 1995;64:868–870

9. Rolf C, Cooper TG, Yeung CH, et al: Antioxidant treatment of patients with asthenozoospermia or moderate oligoasthenozoospermia with high-dose vitamin C and vitamin E: A randomized, placebo-controlled, double-blind study. Hum Reprod 1999;14:1028–1033
10. Geva E, Bartoov B, Zabludovsky N, et al: The effect of antioxidant treatment on human spermatozoa and fertilization rate in an in vitro fertilization program. Fertil Steril 1996;66: 430–434
11. Efrossini K, Jean MR, Hillary JP, et al: A double-blind randomized placebo cross-over controlled trial using the antioxidant vitamin E to treat reactive oxygen species associated male infertility. Fertil Steril 1995;64:825–831
12. Ford WC, Whittington K: Antioxidant treatment for male subfertility: A promise that remains unfulfilled. Hum Reprod 1998;13:1416–1419
13. Ten J, Vendrell FJ, Cano A, et al: Dietary antioxidant supplementation did not affect declining sperm function with age in the mouse but did increase head abnormalities and reduced sperm production. Reprod Nutr Dev 1997;37:481–492
14. Amano T, Hirata A, Namiki M: Effects of Chinese herbal medicine on sperm motility and fluorescence spectra parameters. Arch Androl 1996;37:219–224
15. Ishikawa H, Ohashi M, Hayakawa K, et al: Effects of Guizhi-Fuling-Wan on male infertility with varicocele. Am J Chinese Med 1996;24:327–331
16. Usuki S: Hachimi-jio-gan changes serum hormonal circumstance and improves spermatogenesis in oligozoospermic men. Am J Chinese Med 1986;14:37–45
17. Adachi T, Fukuta M, Ito Y: Effect of superoxide dismutase on glomerular nephritis. Biochem Pharmacol 1986;35:813–817
18. Aoyagi K, Nagase S, Narita M, et al: Role of active oxygen on methylguanidine synthesis in isolated rat hepatocytes. Kidney Int (suppl) 1987;22:229–233
19. Goullet E, Rigot JM, Blois N, et al: Role of systemic scrotal ultrasonography in the management of male infertility. Progr Urol 2000;10:78–82
20. Resim S, Cek M, Fazlioglu A, et al: Echo-color doppler ultrasonography in the diagnosis of varicocele. Int Urol Nephrol 1999;31:371–382
21. World Health Organization: WHO Laboratory Manual for the Examination of Human Semen–Cervical Mucus Interaction. Cambridge, Cambridge University Press, 1987
22. World Health Organization: WHO Laboratory Manual for the Examination of Human Semen and Sperm–Cervical Mucus Interaction. Third edition. Cambridge, Cambridge, University Press, 1992
23. Zini A, Garrels K, Phang D: Antioxidant activity in the sperm of fertile and infertile men. Urology 2000;55:922–926
24. Donnelly ET, McClire N, Lewis SEM: Antioxidant supplementation in vitro does not improve human sperm motility. Fertil Steril 1999;72:484–495
25. Perry ACE, Jones R, Hall L: Isolation and characterization of a rat cDNA clone encoding a secreted superoxide dismutase reveals the epididymis to be a major site of its expression. Biochem J 1993;293:21–25
26. Kurpisz M, Miesel DS, Jedrzejczak P: Seminal plasma can be a predictive factor for male infertility. Hum Reprod 1996;11: 1223–1226
27. Peeker R, Abramsson L, Marklund SL: Superoxide dismutase isoenzymes in human seminal plasma and spermatozoa. Mol Hum Reprod 1997;3:1061–1066
28. Aitken RJ, Buckingham DW, Carreras A, et al: Superoxide dismutase in human sperm suspension: Relationship with cellular composition, oxidative stress, and sperm function. Free Radic Biol Med 1996;21:495–504
29. Gavella M, Lipovac V, Vucic M, et al: Relationship of sperm superoxide dismutase–like activity with other sperm-specific enzymes and experimentally induced lipid peroxidation in infertile men. Andrologia 1996;28:223–229
30. Nivsarkar M, Patel RY, Mokal R: Modulation of sperm membrane conformation by pentoxifylline in oligospermia: A biophysical investigation of sperm membrane in vitro. Biochem Biophys Res Commun 1996;23:791–795
31. Foresta C, Garolla A, Bettella A, et al: Doppler ultrasound of the testis in azoospermic subjects as a parameter of testicular function. Hum Reprod 1998;13:3090–3093

AIDS RESEARCH AND HUMAN RETROVIRUSES
Volume 19, Number 11, 2003, pp. 1057–1063

Sequence Note

Differential Prevalence of HIV Type 1 Subtype B and CRF01_AE among Different Sexual Transmission Groups in Tokyo, Japan, as Revealed by Subtype-Specific PCR

SHINGO KATO,[1] YUKI SAITO,[1] RIE TANAKA,[1] YOSHIYUKI HIRAISHI,[1] NAOTO KITAMURA,[1] TOMOKO MATSUMOTO,[2] HIDEJI HANABUSA,[3] MITSUHIRO KAMAKURA,[4] YASUO IKEDA,[2] and MASAYOSHI NEGISHI[2,5]

ABSTRACT

We determined the subtype of HIV-1 in 89 infected individuals attending three reference hospitals located in the Tokyo metropolitan area of Japan. Subtyping was performed with subtype-specific polymerase chain reaction (PCR) distinguishing subtype A, B, C, and CRF01_AE and/or phylogenetic analysis of HIV-1 *env* C2V3C3 sequences. Subtype-specific PCR provided unequivocal results in 97% of samples. Sixty-five subjects were infected with subtype B, 16 with CRF01_AE, 4 with subtype A, 1 with CRF02_AG, and 3 with subtype C. Among 31 Japanese individuals infected through heterosexual contact, 13 were infected with subtype B and 12 with CRF01_AE. All of the 41 Japanese men infected through homosexual contact harbored subtype B. These results indicate that subtype B is exclusively predominant in a homosexually transmitted group, whereas subtype B and CRF01_AE are evenly predominant in a heterosexually transmitted group.

HIV-1 IS CHARACTERIZED BY HIGH GENETIC VARIABILITY and is classified into three groups (M, O, and N). Group M strains are most globally prevalent and currently comprise 11 subtypes (A1, A2, B, C, D, F1, F2, G, H, J, and K) and 15 circulating recombinant forms (CRFs) [for an overview of HIV and simian immunodeficiency syndrome (SIV) nomenclature, see http://hiv-web.lanl.gov/]. The global distribution of each subtype or CRF shows its own distinct geographic pattern. For example, subtype A and CRF02_AG prevail in central African countries and some north African countries; subtype B is predominant in the Americas, Western Europe, and Australia; subtype C is mainly observed in southern African countries, Ethiopia, and India; CRF01_AE (also referred to as env subtype E) is circulating in Southeast Asia.[1] In Japan, patients with hemophilia infected through contaminated blood coagulation factor products imported from North America in the early 1980s have subtype B viruses as a matter of course.[2] The recent trends of HIV/AIDS in Japan indicate a steady increase in both heterosexual and homosexual Japanese males. The implication is that infection through heterosexual contact is linked to the epidemic in Southeast Asia through commercial sex.[3] Homosexual behaviors in Japan are thought to be strongly linked to those in Western countries. Therefore, the subtype prevalence is expected to be different between the two groups infected through homosexual and heterosexual contact.

The most accurate HIV-1 subtyping is phylogenetic analysis of nucleotide sequences determined in several distinct regions of the viral genome. However, this procedure is expensive and time consuming. Thus, we developed a simple but powerful subtype-specific polymerase chain reaction (PCR) that can distinguish subtypes A, B, C, and CRF01_AE in the *env* C2V3C3 region. By using this new technique together with

[1]Department of Microbiology and Immunology, Keio University School of Medicine, Tokyo 160-8582, Japan.
[2]Department of Infectious Disease, Keio University Hospital, Tokyo 160-8582, Japan.
[3]Department of Hematology, Ogikubo Hospital, Tokyo 167-8515, Japan.
[4]Faculty of Nursing and Medical Care and Clinic for Infectious Diseases, Keio University, Fujisawa, Kanagawa 252-8530, Japan.
[5]Department of Infectious Disease, Tokyo Metropolitan Komagome Hospital, Tokyo 3-18-22, Japan.

conventional phylogenetic analysis, we determined HIV-1 subtypes among subjects attending three reference hospitals in the Tokyo metropolitan area and investigated characteristic subtype prevalence among different transmission risk groups.

Study subjects included a total of 89 nonhemophiliac HIV-1-infected individuals attending Keio University Hospital (n = 26), Tokyo Metropolitan Komagome Hospital (n = 60), and Ogikubo Hospital (n = 3), which are located in the Tokyo metropolitan area. From February 1998 to March 2002, these hospitals provided us with blood samples with informed consent, together with anonymous epidemiological data, including age, sex, nationality, most likely route of infection, and $CD4^+$ cell count. The study group was composed of 77 males and 12 females; 81 Japanese, 2 Thai, 2 Myanmar, 2 Americans, 1 German, and 1 Filipino, all residing in Japan. Modes of infection were heterosexual contact (n = 37), homosexual contact (n = 43), and unclear (n = 9).

The *env* C2V3C3 region was chosen for the target of subtype-specific PCR because HIV-1 sequences diverge most in this region and, especially, subtypes B and CRF01_AE, which predominate in Japan, can be easily distinguished. HIV-1 sequences were obtained from the HIV Sequence Data Base (http://hiv-web.lanl.gov/) to deduce the consensus sequences of each subtype. The primers specific for subtypes A, B, C, and CRF01_AE (designated JA11QA, JA11VB, JA11XC, and JA11YE, respectively) were designed such that its sequence should be identical to the consensus sequence of the corresponding subtype but should have at least three mismatches to those of the other subtypes near the 3′ end.

Subtype-specific PCR was carried out as follows. Blood samples were collected in 1% sodium citrate. Peripheral blood mononuclear cells (PBMCs) were separated by Ficoll-Paque (Pharmacia, Uppsala, Sweden) density centrifugation. Cellular DNA was prepared with the QIAamp Blood Kit (Qiagen, Hilden, Germany). The *env* V3 proviral sequences were amplified from 1 μg of cellular DNA by nested PCR as follows. The reaction mixture of 100 μl for both first and second round PCR was composed of 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 4 mM $MgCl_2$, 0.2 mM each dNTP, primers described below, and 2.5 U AmpliTaq DNA polymerase (Roche, Branchburg, NJ). The first round PCR was performed with primers of 0.5 μM JA9AE (5′-CACAGTACAATGCACACATG-3′, nucleotide positions 6943–6962 in NL4-3[4]), 0.5 μM JA9B (5′-CACAGTACAATGTACACATG-3′, 6943–6962), 0.5 μM JA12A (5′-GCAATAGAAAAATTCTCCTC-3′, 7369–7350), and 0.5 μM JA12B (5′-ACAGTAGAAAAATTCCCCTC-3′, 7369–7350). The thermoprofile was 94°C for 5 min; 30 cycles of 94°C for 15 sec, 56°C for 30 sec, 72°C for 60 sec; 72°C for 5 min. One two-hundred-fiftieth of the first-round PCR product is used in each of the second-round PCR, comprising five reactions described below. Upstream primers in all second-round PCR reactions were a mixture of 0.33 μM JA10UB (5′-CTGTTAAATGGCAGTCTAGC-3′, 6992–7011), 0.33 μM JA10UC (5′-CTGTTAAATGGTAGTCTAGC-3′, 6992–7011), and 0.33 μM JA10UG (5′-CTGTTAAATGGCAGTTTAGC-3′, 6992–7011). Downstream primers are different in each reaction: 1 μM JA11QA (5′-CCCCTCCTGAGGAGTTAGCA-3′, 7316–7297) specific for subtype A, 1 μM JA11VB (5′-CACAATTAAAACTGTGCATTACAA-3′, 7347–7328) for subtype B, 1 μM JA11XC (5′-TTGTTTTATTAGGGAAGTGTTC-3′, 7289–7268) for subtype C, 1 μM JA11YE (5′-AAATTCCCCTCTACAATTAAAATGA-3′, 7360–7336) for CRF01_AE, or a mixture of 0.33 μM JA11LAE (5′-AATTTCTAGATCCCCTCCTG-3′, 7327–7308), 0.33 μM JA11LB (5′-AATTTCTGGGTCCCCTCCTG-3′, 7327–7308), and 0.33 μM JA11LC (5′-AATTTCTAGGTCCCCTCCTG-3′, 7327–7308) for subtype-independent amplification. The thermoprofile was 94°C for 5 min; 28 cycles of 94°C for 15 sec, 60°C for 30 sec, 72°C for 60 sec; 72°C for 5 min. The PCR amplification products were detected by electrophoresis on 2% agarose gel and visualized by ethidium bromide staining. The subtype was judged from two criteria: one subtype-specific reaction pro-

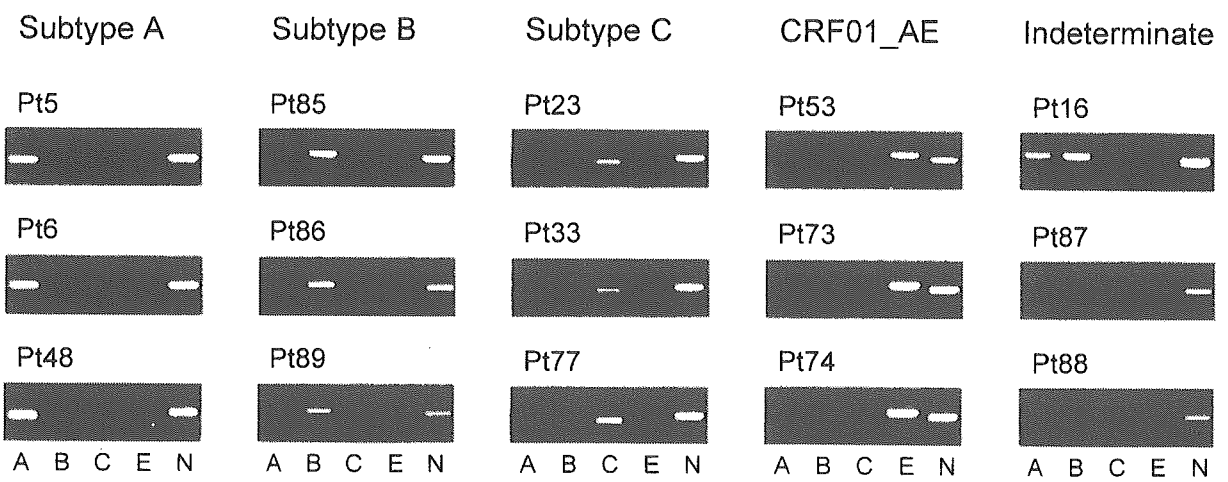

FIG. 1. Typical results of the subtype-specific PCR. The first-round PCR products were subjected to second-round PCR using subtype-specific primers for subtype A (lanes A), subtype B (lanes B), subtype C (lanes C), CRF01_AE (lanes E), and subtype-independent primers (lanes N). Usually, strong bands were detected only in lane N and one of the other lanes, allowing identification of the subtype. Three samples in all those tested were indeterminate because of cross-reaction (Pt16) or insufficient amplification by subtype-specific primers (Pt87 and Pt88).

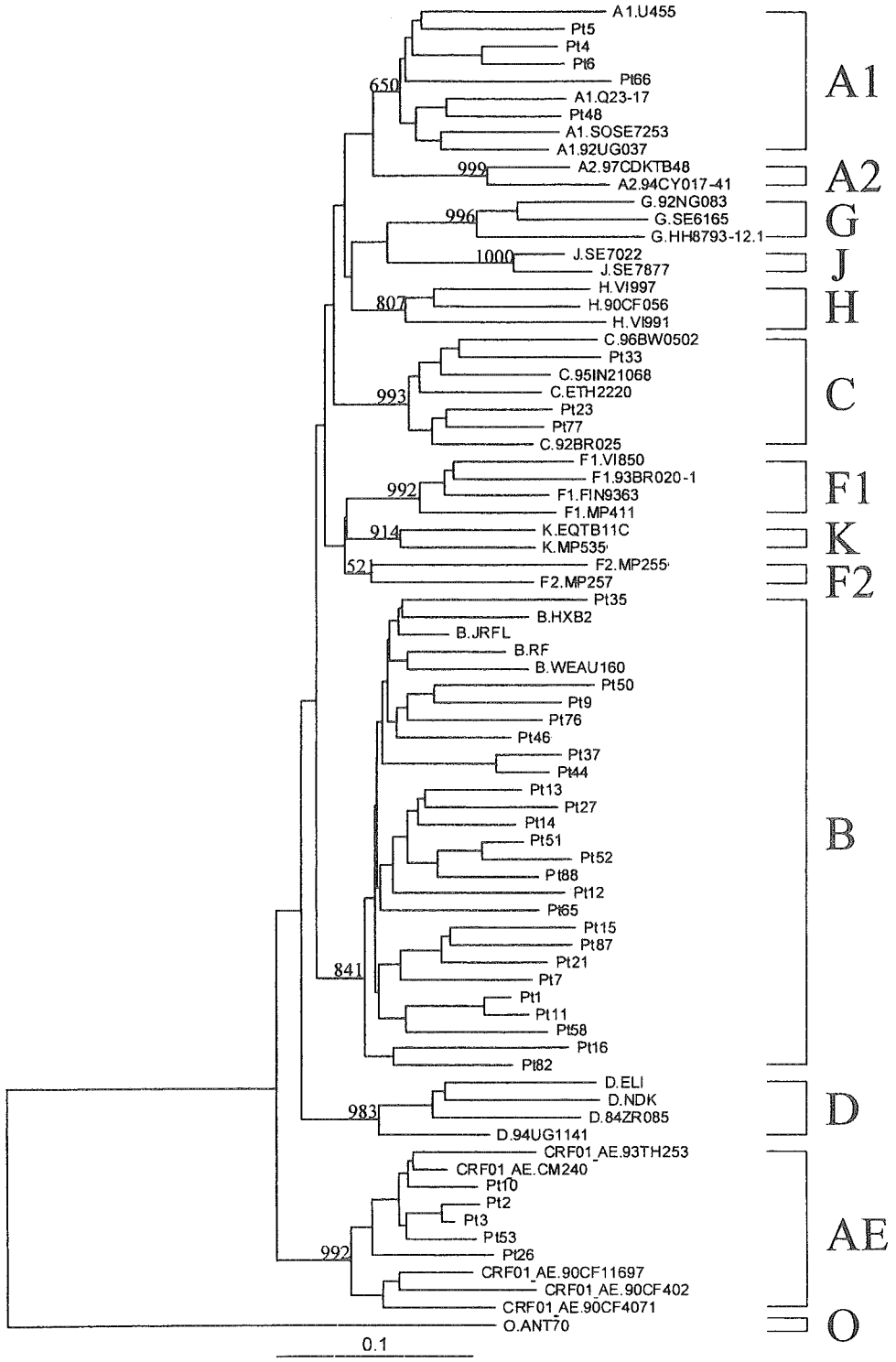

FIG. 2. Phylogenetic analysis of HIV-1 strains from this study and reference HIV-1 strains from group M subtypes A to K (as indicated) and CRF01_AE (indicated by AE), using group O ANT 70 as the outgroup sequence. The phylogenetic tree was constructed by the neighbor-joining method from pairwise distance matrixes calculated by the Kimura two-parameter method, based on aligned 294-nucleotide sequences in the C2V3C3 region. Bootstrap values of 1000 resamplings are indicated on branch nodes of each subtype. The scale bar represents 10% of genetic diversity.

vided a band as strong as the subtype-independent reaction primer did; the other subtype-specific primers generated no or a significantly weaker band (less than 20% in intensity).

The second-round PCR products amplified by using a subtype-independent primer mixture were purified with the QIAquick PCR Purification Kit (QIAGEN) and subjected to automated dideoxy sequencing using the Dye Terminator Cycle Sequencing FS Ready Reaction Kit and DNA Sequencer 370A (Applied Biosystems, Foster City, CA).

DNA sequences of HIV-1 samples and reference sequences of subtypes were aligned using CLUSTAL W version 1.8[5] and corrected manually to ensure that gaps did not alter the reading frame. The following reference sequences obtained from the HIV Sequence Data Base: Q23-17, SOSE7253, U455, and 92UG037 for subsubtype A1; 97CDKTB48 and 94CY017–41 for subsubtype A2; HXB2, RF, JRFL, and WEAU160 for subtype B; 92BR025, 96BW0502, ETH2220, and 95IN21068 for subtype C; ELI, NDK, 84ZR085, and 94UG1141 for subtype D; VI850, 93BR020–1, FIN9363, and MP411 for subsubtype F1; MP255 and MP257 for subsubtype F2; HH8793-12.1, 92NG083, and SE6165 for subtype G; VI991, VI997, and 90CF056 for subtype H; SE7887 and SE7022 for subtype J; EQTB11C and MP535 for subtype K; 90CF11697, 90CF402, 90CF4071, CM240, and 93TH253 for CRF01_AE; and Group O ANT70 as an outgroup sequence. Phylogenetic analysis was performed with the CLUSTAL W version 1.8 software package.[5] Phylogenetic trees were constructed by the neighbor-joining method[6] from pairwise distance matrixes calculated with the Kimura two-parameter model.[7] The robustness of the generated trees was evaluated by bootstrap analysis of 1000 resamplings.

DNA samples extracted from PBMCs of 89 infected individuals were subjected to subtype-specific nested PCR in the *env* C2V3C3 region. This PCR involved a set of the second-round PCR reactions using different subtype-specific downstream primers designed for distinguishing subtypes A, B, C, and CRF01_AE in addition to primers matching with the sequence highly conserved among subtypes. The subtype was judged by whether the DNA band produced by one subtype-specific primer had an intensity similar to that by subtype-independent primers and was significantly stronger than those by the other subtype-specific primers, which were usually undetected (see Fig. 1 for typical results). Subtype-independent primers provided clear bands in all cases. Only three samples were judged indeterminate because of cross-reaction (Pt16) or insufficient amplification by subtype-specific primers (Pt87 and Pt88) (Fig. 1). The other 86 samples (97%) were unequivocally subtypable with subtype distributions of 5 A, 62 B, 3 C, and 16 AE. To confirm and supplement the results by subtype-specific PCR, amplification products from 38 samples (5 A, 21 B, 3 C, 5 AE, and 3 dubious results) by subtype-independent primers were sequenced. The phylogenetic tree (Fig. 2) shows a clear separation of different HIV-1 subtypes, supported by high bootstrap values (the lowest was 521 for subsubtype F2). All the subtype results of subtype-specific PCR were in agreement with phylogenetic analysis. All the subtype A sample sequences clustered with subsubtype A1 reference sequences. The three dubious samples (Pt16, Pt87, and Pt88) were found to belong to subtype B. There was no phylogenetic clue for their ambiguity in subtype-specific PCR.

Subtyping and epidemiological data are summarized in Table 1. Overall, subtype B is present in 65 (73%) of the 89 samples studied. Sixteen subjects (18%) are infected with CRF01_AE, 4 (4%) with subtype A, 3 (3%) with subtype C, and 1 with CRF02_AG. There is a remarkable association between subtype distribution (B vs. AE) and transmission risk groups. All

TABLE 1. HIV-1 SUBTYPE DISTRIBUTION AMONG RESIDENTS IN THE TOKYO METROPOLITAN AREA OF JAPAN, FEBRUARY 1998–MARCH 2002

Characteristic	*Total samples*	*Subtype A*	*Subtype B*	*Subtype C*	*CRF01_AE*
All samples	89	5	65	3	16
Mean age (years)	41	32	42	38	40
Mean $CD4^+$ counts ($\times 10^6$/liter)	418	207	434	585	336
Citizenship					
Japanese	81	5	61	2	13
Thai	2	0	0	0	2
Myanmar	2	0	1	0	1
American	2	0	1	1	0
German	1	0	1	0	0
Filipino	1	0	1	0	0
Sex					
Male	77 (74)[a]	2 (2)	61 (59)	2 (2)	12 (11)
Female	12 (7)	3 (3)	4 (2)	1 (0)	4 (2)
Transmission risk					
Heterosexual	37 (31)	4 (4)	15 (13)	3 (2)	15 (12)
Male	25 (24)	1 (1)	11 (11)	2 (2)	11 (10)
Female	12 (7)	3 (3)	4 (2)	1 (0)	4 (2)
Homosexual[b]	43 (41)	0	43 (41)	0	0
Unclear[b]	9 (9)	1 (1)	7 (7)	0	1 (1)

[a]Numbers in parentheses are the subject number among Japanese.
[b]All subjects in these categories were male.

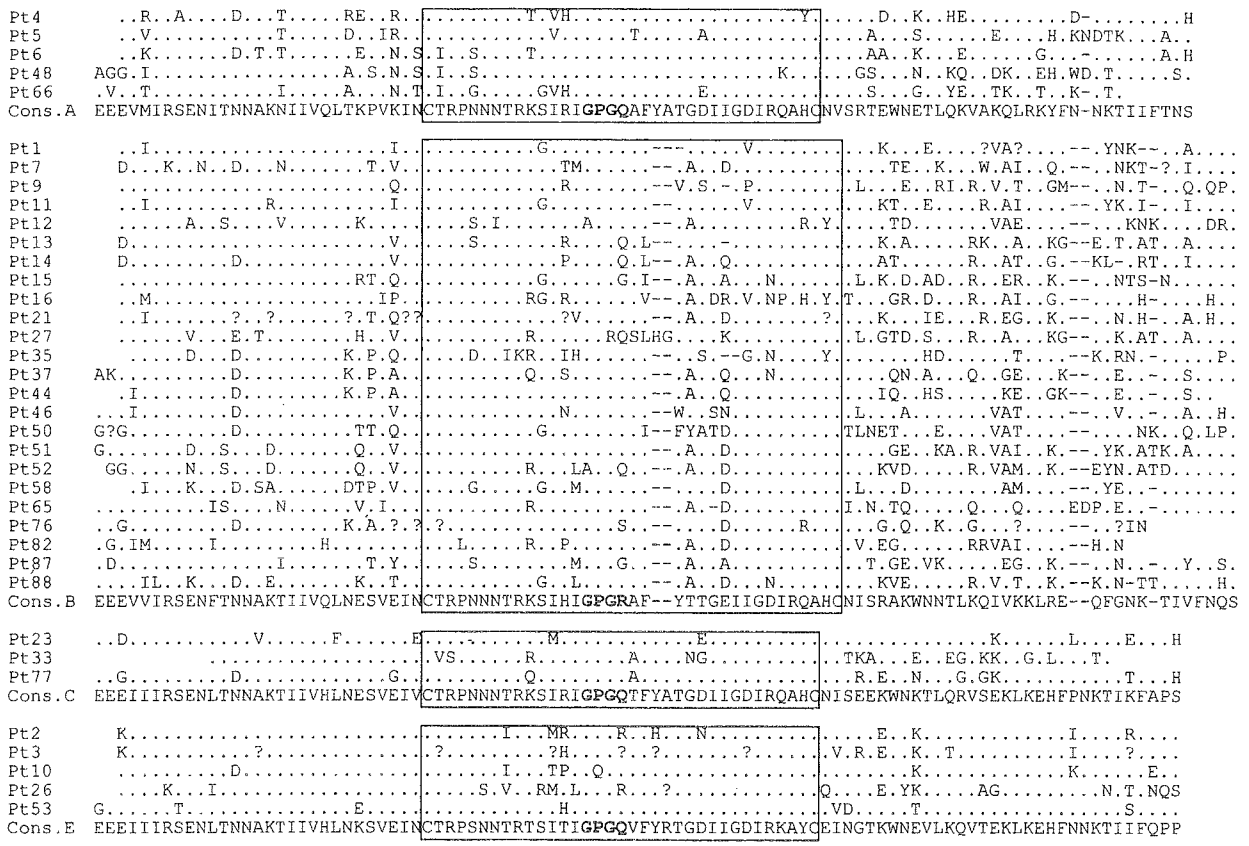

FIG. 3. Alignments of deduced amino acid sequences of the C2V3C3 region of the sequenced HIV-1 strains. Sequences are grouped by subtype and aligned with each corresponding consensus sequence (Cons.). Dots indicate amino acids identical to the consensus, dashes are gaps introduced to obtain the correct alignment, question marks indicate indeterminate amino acid positions due to considerable overlapping of different nucleotide peaks in sequencing, and asterisks indicate the introduction of translation stop codons. The V3 region surrounded by box and crown motif is indicated in boldface. The first and last amino acids shown correspond to positions 267–364 in the subtype B HXB2 Env sequence.

of the 43 subjects with homosexual transmission risk carried subtype B. On the other hand, subtype B and CRF01_AE were equally frequent (42% and 39%, respectively) among individuals with heterosexual transmission risk. Phylogenetic analysis does not show any clustering of subtype B samples from different transmission risk groups. As for a heterosexual transmission group, there is no significant correlation between subtype prevalence and sex (χ^2 test, $p = 0.29$).

Figure 3 shows the alignment of multiple amino acid sequences deduced from the C2V3C3 nucleotide sequences used for phylogenetic analysis, grouped according to each subtype consensus. Most of the strains encode V3 loops composed of 35 amino acids bounded by a conserved cysteine–cysteine disulfide bridge. One strain (subtype B Pt27) has 37 residues, one strain (subtype B Pt9) has 36 residues, and two strains (subtype B Pt35 and Pt65) have 34 residues in the V3 loop. The crown motifs of the V3 loop are predominantly GPGR for subtype B (68%) and GPGQ for all other subtypes (85%). Inter- and intrasubtype diversity is noticed in the cytotoxic T lymphocyte epitope (amino acid positions 311–320)[8] and the principal antibody-neutralizing determinant (positions 309–317)[9] in the V3 loop. Interestingly, the diversity is also observed in the 5′ portion of the C3 region.

The V3 genotype allows prediction of the viral phenotype. HIV-1 strains are phenotypically divided into two types: T cell tropic/syncytium-inducing (SI) viruses usually using CXCR4 as the coreceptor and macrophage tropic/non-syncytium-inducing (NSI) viruses primarily using CCR5 as the coreceptor. The emergence of SI strains in the course of infection coincides with an accelerated loss of $CD4^+$ T lymphocytes and a rapid disease progression to AIDS, whereas NSI strains persist through the early phase of infection.[10] The majority of NSI strains possess the amino acid sequence S/GXXXGPGXXXXXXXE/D (X representing any amino acid) around the V3 crown motif independently of the subtype.[11] SI strains are associated with positively charged residues at positions 306 (the eleventh amino acid from the 5′ end of V3) and 322 (the twenty-fifty amino acid from the 3′ end of V3)[11] and an overall V3 net charge above +6.[12] In our study, irrelevant to the subtype, most of the V3 loops sequenced have uncharged or negatively charged amino acids at the above positions and have an amino acid motif predictive of CCR5 usage. The presence of basic residues at position 322 was observed for two of the sequences (subtype B Pt16 and Pt27). Both of these sequences have an overall V3 loop charge of +8. They have already developed AIDS at