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Renal tubular toxicity associated with tenofovir assessed using urine-beta 2 microglobulin, percentage of tubular reabsorption of phosphate and alkaline phosphatase levels

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Despite its wide use, the renal tubular toxicity of tenofovir has not been fully evaluated. Twelve weeks after initiating a tenofovir-containing HAART regimen, a high urine-beta 2 microglobulin level was observed in 12 out of 17 patients, the percentage of tubular reabsorption of phosphate decreased from 96.0 to 91.1% and alkaline phosphatase increased from 294 to 365 U/l, whereas serum creatinine and phosphorus remained largely unchanged. Patients with the above findings should be monitored carefully for renal tubular toxicity.

Tenofovir disoproxil fumarate (TDF), a nucleotide analogue of adenosine 5′-monophosphate, is a recommended regimen in the Department of Health and Human Services guidelines because its efficacy, safety and especially lower mitochondrial toxicity have been demonstrated in randomized studies [1–4]. Two other nucleotide analogues, cidofovir and adefovir, have been found to cause nephrotoxicity [5–7]. Although TDF-related Fanconi syndrome, acute renal failure and diabetes insipidus have been reported [8–12], most studies concluded that TDF has little nephrotoxicity because no increased serum creatinine and decreased serum phosphorus were observed [1–4]. Fanconi syndrome is complicated and results in the urinary loss of amino acids, glucose, uric acid, phosphorus, bicarbonate and low molecular weight proteins as a result of proximal tubular dysfunction. Laboratory findings show high urine-beta 2 microglobulin (BMG), glucosuria, hyperphosphaturia [low percentage of tubular reabsorption of phosphate (%TRP)], hypophosphoremia, hyperuricuria, hypouricemia and metabolic acidosis. Considering these laboratory characteristics, it is necessary to use a more sensitive marker than serum creatinine or creatinine clearance to detect TDF-related tubular dysfunction.

Seventeen HIV-1-infected patients followed at Ogikubo Hospital were treated with TDF-containing regimens. Serum creatinine, serum phosphorus, serum uric acid, serum alkaline phosphatase (ALP), urine-BMG and %TRP were monitored every 3 months. As a control group, 12 patients treated with zidovudine or stavudine-containing regimens excluding TDF were monitored using the same markers. Statistical analysis compared the results using the Wilcoxon matched-pairs test or Mann–Whitney U test.

The median (range) baseline characteristics for patients in the TDF and control groups were: age 36 (28–51) and 34 (28–71) years; HIV-1 RNA in serum less than 50 (< 50–100 000) and less than 50 (< 50–58 000) copies/ml; CD4 cell count 430 (48–1243) and 376 (210–790) cells/μl; serum creatinine level 0.70 (0.59–1.36) and 0.66 (0.56–1.20) mg/dl. There was no significant difference between the two groups with regard to age, HIV-1 RNA, CD4 cell count and serum creatinine level.

A data summary is shown in Fig. 1. High urine-BMG (>200 μg/l) was observed in 71% (12/17) of patients at 12 weeks in the TDF group. The mean urine-BMG increased from 411 μg/l at baseline to 2214 μg/l at 12 weeks ($P = 0.10$) and 2559 μg/l at 24 weeks ($P = 0.36$). Urine-BMG was 2020 μg/l at baseline in one antiretroviral-naive patient, whose CD4 cell count was 48 cells/μl. The mean urine-BMG for the TDF group was significantly higher at 12 weeks than the control group (174 μg/l at 12 weeks, $P = 0.003$). The mean %TRP had a rapid decrease from 96.0% at baseline to 91.1% at 12 weeks and 90.8% at 24 weeks ($P = 0.03$ and 0.03). Low %TRP (< 90%) was observed in 19% (3/16) of patients at 12 weeks and 29% (4/14) of patients at 24 weeks, whereas the control group was 0% (0/12) of patients.

In the TDF group, the mean serum creatinine level increased from 0.70 mg/dl at baseline to 0.73 mg/dl at 12 weeks ($P = 0.16$) and 0.74 mg/dl at 24 weeks ($P = 0.04$). The incidence of an abnormal serum creatinine level (> 1.0 mg/dl) was 6% (1/17) and this single case had no increase from baseline to 24 weeks. Serum phosphorus showed no significant decline from 3.4 mg/dl at baseline to 3.3 mg/dl at 12weeks and 3.4 mg/dl at 24 weeks ($P = 0.44$ and 0.40). Serum uric acid decreased from 5.9 mg/dl at baseline to 5.8 mg/dl at 12 weeks and 5.5 mg/dl at 24 weeks ($P = 0.37$ and 0.05). ALP significantly increased from 294 U/l at baseline to 365 U/l at 12 weeks and 401 U/l at 24 weeks ($P = 0.0002$ and 0.0001).

In the TDF group, two cases of tubular toxicity were found. The first case presented as Fanconi syndrome with hypophosphatemic osteomalacia 23 months after starting TDF. His laboratory data showed high urine-BMG (55 100 μg/l), low %TRP (14.9%), hypophosphatemia (1.3 mg/dl), hypouricemia (0.8 mg/dl) and elevated ALP (3194 U/l). Serum creatinine showed mild elevation

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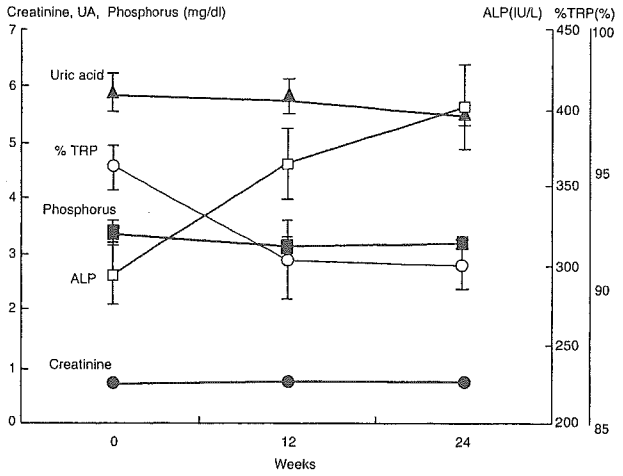

Fig. 1. Summary of data on patients. In 17 patients treated with tenofovir disoproxil fumarate-containing regimens; serum creatinine, serum phosphorus, serum uric acid (UA), serum alkaline phosphatase (ALP), urine-beta 2 microglobulin and the percentage of tubular reabsorption of phosphate (%TRP) were measured every 3 months. Data are shown as mean ± SE.

(0.84 mg/dl at baseline and 1.06 mg/dl at 23 months). In this case, tubular dysfunction was reversible and recovered in 3–4 months after discontinuing TDF, although osteomalacia with elevated ALP still persisted. The second case showed TDF-related tubular dysfunction with mild diabetic nephropathy and normal creatinine clearance. His urine-BMG increased to 55 100 μg/l (16 weeks), and %TRP decreased to 42% (12 weeks). After discontinuing TDF, urine-BMG and %TRP improved to 370 μg/l and 85%, respectively, in 4–8 weeks.

Our study showed a high incidence of elevated urine-BMG in the TDF group. Although urine-BMG is also increased in other renal diseases and HIV progression [13], urine-BMG increased after initiating TDF-containing regimens. Urine-BMG is a sensitive marker for tubular dysfunction and its increased excretion precedes the impairment of phosphate reabsorption [14,15]. Serum creatinine showed no abnormally high values, and neither serum phosphorus nor serum uric acid showed significant changes during the study.

In the TDF group, %TRP declined rapidly and ALP elevated significantly from baseline, but serum phosphorus only decreased slightly. Although TDF causes both severe hypophosphatemia and ALP increase in macaques [16], randomized studies and our result did not show hypophosphatemia. The reason for the discrepancy is not clear. Although the aetiology of hypophosphatemia is multifactorial [17], normocalcemia is maintained by 1,25-dihydroxyvitamin D-induced calcium mobilization from bone [18]. The same homeostatic mechanism may work to maintain serum phosphorus in TDF-related tubular toxicity. Our study observed a case of TDF-related Fanconi syndrome with hypophosphatemic osteomalacia. He had no renal disease and the HIV-1-RNA level in serum was suppressed (< 50 copies/ml). This case suggests that Fanconi syndrome cannot be detected by monitoring serum creatinine, can produce severe osteomalacia, and is reversible upon discontinuing TDF. Our study also observed TDF-induced tubular dysfunction with mild diabetic nephropathy with normal serum creatinine and creatinine clearance.

When TDF-containing HAART is initiated in patients with chronic kidney disease, careful monitoring is necessary. Serum creatinine and serum phosphorus are not able to detect tubular toxicity. TDF-related nephrotoxicity might be underestimated with a less sensitive marker for serum creatinine.

In conclusion, patients using TDF should be regularly monitored for urine-BMG, %TRP and ALP levels. Although mild urine-BMG elevation does not directly imply the clinical severity of renal disease, persistent %TRP decrease and ALP elevation suggest renal tubular toxicity and may indicate that TDF should be discontinued.

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HIV testing among female sex workers in Andhra Pradesh, India

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Of 6648 female sex workers (FSW) in 13 districts of Andhra Pradesh state in India, only 7.9% reported having undergone HIV testing, and three-quarters of the rest were unwilling to undergo HIV testing in the future. The risk of HIV infection as a result of the non-use of condoms was higher among FSW who reported not having been tested and were also unwilling to get tested, and they also held significantly more negative beliefs about HIV/AIDS.

Voluntary counselling and testing (VCTC) for HIV is currently being scaled up in India [1,2]. The uptake of VCTC can reduce the rates of sexually transmitted infections and high-risk sexual practices [3,4]; however, stigma and discrimination are among the major barriers to the uptake of VCTC [5]. HIV transmission in India is largely linked to sex work [1,2]. HIV prevalence among female sex workers (FSW) in the Indian state of Andhra Pradesh was estimated to be 16% (range 8–41%) in 2004 [6]. Knowledge about the rate of and willingness for HIV testing among FSW is important for planning comprehensive HIV prevention. We report these data from a large sample of FSW in Andhra Pradesh.

This study was carried out as part of the baseline for an impact assessment study of the Frontiers Prevention Project, which aims to reduce the spread of HIV through the provision of HIV interventions among high-risk population groups. The necessary ethical approval for this study was obtained.

Forty geographical sites (72 cities/towns/villages) in 13 districts of Andhra Pradesh state were identified where access to FSW was considered feasible through non-governmental organizations (NGO) having links with them. FSW were contacted and recruited at bus and rail stations, cinema theatres, parks, brothels, their homes, small informal hotels, and some other places. A total of 7251 FSW 16 years of age and older were contacted for participation between July 2003 and April 2004. Confidential interviews were conducted after obtaining their written informed consent. Relevant to this report, FSW were asked if they had undergone HIV testing. The place and cost of the HIV test, whether they had received the test results, and the reasons for undergoing HIV testing were documented for those who reported having been tested. The willingness to undergo testing in the future was documented for those who had heard of HIV/AIDS but had not been tested for HIV. Those who had heard of HIV/AIDS also responded to two statements reflecting negative beliefs about individuals living with HIV/AIDS. The FSW who had not heard of HIV/AIDS were considered as not having undergone HIV testing for this analysis. Self-reported condom non-use for penetrative sex with the last three clients was documented as an indication of HIV risk behaviour. The 95% confidence intervals (CI) of the estimates of the assessed variables were adjusted for the design effect of cluster sampling [7]. Multivariate analysis was performed to assess the associations for having undergone HIV testing and an unwillingness to undergo HIV testing using SPSS and Stata software. The 95% confidence intervals of the estimates were adjusted for the design effect of cluster sampling [7].

Of the FSW contacted, 6648 (91.7%) participated, with a median age of 27 years (range 16–54 years), of whom 523 (7.9%, 95% CI 5.0–10.8%, design effect 19.5) reported having undergone HIV testing. Among the 4843 FSW who had heard of HIV/AIDS but had not undergone HIV testing, 3548 (73.2%, 95% CI 69.9–76.7%, design effect 7.5) were unwilling to undergo testing in the future.

The major predictors of having undergone HIV testing included being a non-street-based FSW, participation in a support group, sex work duration greater than 5 years, and higher income; and those for the unwillingness to undergo HIV testing included FSW age 16–17 years, no participation in a support group, and lower income (Table 1).

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HLA-B Polymorphism in Japanese HIV-1–Infected Long-Term Surviving Hemophiliacs

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ABSTRACT

Approximately 30% of patients with hemophilia in Japan were infected with human immunodeficiency virus (HIV) in early 1980s through contaminated blood products. In 1995, a cohort of HIV-infected, asymptomatic patients with hemophilia was set up for follow-up study. Although the patients met the criteria for long-term non-progressor (LTNP) at the entry to the cohort, some of them later developed lymphopenia during five more years of observation. We collected blood samples from 80 long-term survivors; 42 of them did not require antiviral therapy, but the rest were under treatment. Analysis of HLA-B genotype revealed that carriers of known HIV-resistant alleles such as HLA-B*5701, B*5801, and alleles of B27 antigenic group were not increased in frequency, but that HLA-B*1507 was increased in the cohort (6.25% vs. 1.03%, OR = 6.40, $p = 0.039$). We also observed the decrease in carriers of HLA-B*5401 (3.75% vs. 14.95%, OR = 0.22, $p = 0.016$). HLA-B*5401 is a relatively common allele in East Asian populations and belongs to the same B22 antigenic group as B55 and B56 which were reported to associate with rapid progression. Our data indicated that HLA class I is one of the host factors involved in the retardation of HIV disease progression as also reported in the previous studies; however, the alleles associated with this resistance were not the same because of divergent host genetic background.

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INTRODUCTION

Approximately 0.1% of the adult Japanese population is infected with the human immunodeficiency virus (HIV) (1). The number of reported HIV cases, particularly through heterosexual contact, is steadily rising with a doubling of annual cases from the numbers seen in the 1990s to those reported recently (2), but the major source of infection was imported blood products administrated to patients with hemophilia at the beginning of the HIV endemic in Japan (3). The first reported cases of AIDS in Japan were noted in patients with hemophilia in 1985. Thereafter a national HIV/AIDS surveillance study was conducted. By 1998, it was reported that 1404 patients with hemophilia were infected with HIV and that 502 among them had died, after developing AIDS (2). Approximately 30% of hemophiliac patients were found to be HIV carriers within the seven years from 1985 when heated plasma concentrates were generally used. In 1995, we set up a cohort of HIV-infected, asymptomatic patients with hemophilia for follow-up study. Upon the entry to the study, it had already been more than 10 years since exposure to the virus for all patients.

In the natural course of HIV infection, initial viremia accompanied by general manifestations such as fever takes place in the acute phase. Then, most of patients recover more or less from peak viremia to certain levels of viral load or "set points," presumably at least in part through the elimination of viral infected cells by cytotoxic T lymphocyte (CTL) response of the host immune system (4). After years of an asymptomatic period, a considerable number of the patients develop AIDS characterized by the deficit and dysfunction of CD4-positive T lymphocytes along with the re-elevation of viral load, if they do not take any anti-viral measures, such as highly active anti-retroviral treatment (HAART). In the latter viremic phase, the viral genome will have accumulated several mutations, some of which are advantageous to escape from host immune surveillance (4). A small part of infected patients maintain low viral loads, high $CD4^+$ cell count and remain asymptomatic for seven years or more. These are known as long term non-progressors (LTNP) (5). The presence of slow progressors and LTNP has attracted research interests in general because they may hold the underlying biological mechanisms against HIV disease progression.

CTL responses are HLA class I restricted and most immune evasion mutations tend to take place in the coding sequence of the CTL epitope. The CTL epitopes vary with polymorphisms in the peptide binding groove of HLA class I molecule. Therefore HLA class I polymorphism is one candidate factor which elucidates the individual difference in anti-viral response and clinical features. Indeed, there have been many studies which describe that some HLA-alleles are associated with resistance to HIV, and that other alleles were associated with susceptibility (5). In the present study, we examined HLA-B polymorphism in the cohort of Japanese patients with hemophilia who had been infected with HIV for 10 years or longer without progression to AIDS.

MATERIALS AND METHODS

Patient samples. All the protocol of the present study was approved by Ethics Reviewing Board of Yamanashi University and the Medical Research Institute of Tokyo Medical and Dental University as well as all hospitals in which the samples were taken. Upon the set-up of the cohort of HIV-infected Japanese patients with hemophilia in 1995, all patients were infected for longer than ten years but asymptomatic without any antiviral measures. Blood samples were collected from 80 well-characterized patients who were selected from the cohort after obtaining written informed consent. At the time of sample collection, 42 of them were still asymptomatic and maintained their $CD4^+$ T cells at certain level (no less than $200/mm^3$), but the rest were under anti-retroviral treatment (HAART) because of development of CD4 reduction less than $200/mm^3$. Quantification of viral RNA in the plasma was carried out by the Roche Amplicor versions 1.5 assay (Roche Diagnostics, NJ) on the sample collection and will be reported elsewhere in detail. The DNA was prepared from the blood samples by the use of Wizard genomic DNA purification kit (Promega, WI).

HLA-B typing. HLA-B genotype was determined by using Dynal RELI SSO HLA-B typing kit (Dynal Biotech, Oslo, Norway) and Pattern Matching Program (Dynal Biotech) and/or sequence-based typing (SBT) method essentially according to the 13th International HLA Workshop and Congress (IHWC) technical manual published on the IHWC web site ⟨www.ihwc.org⟩. Detailed method of SBT is available on request. In brief, the sequences around exons 2 and 3 were amplified in separate PCR. Amplified DNA was treated with ExoSAP-IT (Amersham Biosciences, NJ) according to the manufacturer's recommendation, and then the sequence was determined by using BigDye Terminator v1.1 cycle sequencing kit (Applied Biosystems, CA) and ABI 3100 Genetic Analyzer (Applied Biosystems).

Statistical analysis. Frequency of carriers of each allele between patient group and controls were compared. The controls were from random sampling of healthy volunteer ($n = 194$). Odds ratio (OR) was calculated by Woolf's formula (7), for which Haldane's modification was applied upon necessity (8). Statistical significance of the difference in frequencies was evaluated by χ^2 test with Yates' correction (9).

TABLE 1. CARRIERS OF HLA-B ALLELES IN JAPANESE LONG-TERM SURVIVING, HIV-INFECTED HEMOPHILIACS

Allele	*HIV-infected hemophiliacs (n = 80) (%)*	*Controls (n = 194) (%)*	*Odds ratio*[a]	p
B*0702	12.5	11.9	1.06	ns
B*1301	2.5	0.5	4.95	ns
B*1302	1.3	0.0	(7.34)	ns
B*1501	22.5	15.0	1.65	ns
B*1502	0.0	1.6	(0.34)	ns
B*1507	6.3	1.0	6.40	0.039
B*1511	2.5	2.6	0.97	ns
B*1518	2.5	5.2	0.47	ns
B*2704	1.3	0.0	(7.34)	ns
B*2705	0.0	0.5	(0.80)	ns
B*3501	7.5	10.3	0.71	ns
B*3701	1.3	0.0	(7.34)	ns
B*3802	0.0	1.0	(0.48)	ns
B*3901	3.8	4.6	0.80	ns
B*3902	0.0	0.5	(0.80)	ns
B*3904	0.0	0.5	(0.80)	ns
B*4001	12.5	10.3	1.24	ns
B*4002	21.3	11.3	2.11	0.052 (ns)
B*4003	2.5	0.5	4.95	ns
B*4006	6.3	10.8	0.55	ns
B*4402	3.8	0.5	7.52	ns
B*4403	11.3	13.4	0.82	ns
B*4501	0.0	0.5	(0.80)	ns
B*4601	7.5	11.3	0.63	ns
B*4801	2.5	7.7	0.31	ns
B*5101	21.3	11.3	2.11	0.052 (ns)
B*5102	0.0	0.5	(0.80)	ns
B*5201	17.5	27.3	0.56	ns
B*5401	3.8	15.0	0.22	0.016
B*5502	6.3	6.2	1.01	ns
B*5504	0.0	0.5	(0.80)	ns
B*5601	1.3	2.6	0.48	ns
B*5603	1.3	1.0	1.22	ns
B*5801	2.5	1.0	2.46	ns
B*5901	3.8	5.7	0.69	ns
B*6701	6.3	2.1	3.17	ns
Bw4/Bw6				
Bw4	58.8	52.1	1.31	ns
Bw4-80Thr	21.3	15.5	1.48	ns
Bw4-80Ile	45.0	41.8	1.14	ns
Bw6	90.0	87.6	1.27	ns
Supertype				
B7	50.0	54.1	0.84	ns
B27	10.0	19.0	0.47	ns
B44	55.0	42.7	1.63	ns
B58	2.5	1.0	2.46	ns
B62	55.0	50.0	1.22	ns

[a]Odds ratio was calculated by Woolf's formula. Value in parenthesis was from Haldane's modification when Woolf's formula was inappropriate. The p value was calculated by χ^2 test with Yates' correction.

ns, not significant.

TABLE 2. HLA-B SUPERTYPES AND OUTCOME OF LONG-TERM SURVIVING, HIV-INFECTED HEMOPHILIACS

Supertype	*Non-progressors (n = 42) (%)*	*Patients under HAART (n = 38) (%)*	*Odds ratio*[a]	p
B7	42.9	57.9	0.55	ns
B27	2.4	18.4	0.11	0.044
B44	54.8	55.3	0.98	ns
B58	4.8	0.0	(4.75)	ns
B62	57.1	42.1	1.83	ns

[a]See footnote of Table 1.

RESULTS

Analysis of HLA-B alleles. HLA-B genotype of 80 hemophiliac patients was determined and frequency of carriers of respective alleles was calculated (Table 1). HLA-B*5701, B58 alleles, and B27 alleles that were reported to be associated with resistance to HIV infection in the North American, European and African populations were not found or significantly changed in frequency in our cohort (6,10). Instead, the frequency of HLA-B*1507 carrier was significantly increased in the patients in comparison to the controls (6.25% vs. 1.03%, OR = 6.40, $p = 0.039$). Carriers of HLA-B*4002 and those of HLA-B*5101 were also marginally increased. On the contrary, carriers of HLA-B*5401 were significantly decreased in patients (3.75% vs. 14.95%, OR = 0.22, $p = 0.016$). Bw4 and Bw6 are mutually exclusive public epitopes on most of HLA-B alleles and are shared by a small group of HLA-A alleles. The Ile80 subtype of Bw4 was reported to associate to HIV-resistance presumably via interaction with its receptor on natural killer cells, KIR-3DS1 (11). However, the frequencies of Bw-public antigens or subtypes of Bw4 were not different between patients and controls (Table 1).

Most HLA class I alleles fall in to several groups called as supertype in terms of their preference in binding to antigen peptide (12). Therefore, the alleles classified to the same HLA supertype are assumed to be roughly equivalent in their function. Among five known HLA-B supertypes, B58 supertype was reported to confer the host resistance to HIV (13). The frequencies of HLA-B supertypes were not different between patients and controls (Table 1).

Can HLA-B predict the long-term prognosis? Although the patients had been so-called non-progressors or slow progressors for more than ten years at the entry of the cohort, some of them developed immune dysfunction and required introduction of HAART to prevent further progression. When we compared the HLA-B alleles and supertypes between patients who had not introduced HAART ($n = 42$) and those who were under HAART ($n = 38$), B27 supertype carrier was more in patients under treatment (Table 2). The alleles of B27 supertype found in the patients were HLA-B*1518,

TABLE 3. HIV LOAD AND HLA-B SUPERTYPE CARRIERS[a]

Outcome	*HIV load (copies RNA/mL)*	*Supertype carriers (%)*				
		B7	*B27*	*B44*	*B58*	*B62*
Non-progressors ($n = 40$)[b]						
$<10^2$	9	55.6	0.0	77.8	0.0	55.6
10^2–10^3	11	36.4	9.1	54.5	0.0	54.5
10^3–10^4	12	50.0	0.0	50.0	8.3	50.0
$10^4<$	8	25.0	0.0	50.0	12.5	75.0
Patients under HAART ($n = 38$)						
$<10^2$	23	69.6	8.7	60.9	0.0	34.8
10^2–10^3	3	66.7	33.3	0.0	0.0	66.7
$10^3<$	12	33.3	33.3	58.3	0.0	41.7

[a]Each outcome group of patients was stratified according to the viral load.
[b]Data was not available for two non-progressors.

B*2704, B*3901and B*4801, the presence of which may unfavorable for the host to control virus for extended period (12). When the patients were further classified according to the viral load in the peripheral blood, no HLA supertypes affected of the levels of HIV load after seven to eight years of observation period in both outcome groups (Table 3). Therefore, HLA-B could not predict the long-term prognosis of non-progressors in the present study.

DISCUSSION

The rate of progression from HIV infection to AIDS is controlled by several host and viral factors. Polymorphisms in chemokine and chemokine receptor genes have been repeatedly observed to associate with host resistance, which may affect viral propagation in the host organism through the changes in infectivity (14,15). HLA class I polymorphism is another key factor for host resistance against HIV. Several alleles such as HLA-B*5701, B58 alleles and B27 alleles were demonstrated to be associated with resistance and others such as HLA-B*-0702, B35, B53, and B22 alleles were associated with rapid progression (6,10,16–19). In addition to association of specific alleles, groups of functionally equivalent allele are also associated with the progression of disease and the viral load, which force the viral genome to evolve so as to fit to the prevalent host HLA polymorphism (13). An increase of B27 super type in patients under HAART in comparison to LTNP in the present study appeared to be discrepant with the previous findings in which B27 supertype was associated to resistance. This might be because of difference in observation period after infection of the studies or difference in genetic background of the patient ethnic group.

HLA-B*1507 was associated to long term survival upon HIV infection in our cohort. Although HLA-B*1507 encodes B62 serological antigen, data concerning binding peptide motif for this allele has not been available yet. Therefore, no supertype was designated for HLA-B*1507 (12). Bioinformatics has been applied to infer the supertype for the alleles without peptide binding data, but the results require to be confirmed by experiments (20). Analysis of escape mutations in HLA-B*1507 carriers would be helpful for understanding of the mechanism of resistance against HIV. On the contrary, we observed decreased frequency of HLA-B*5401 in the long-term survivors. LTNP group alone reached to significant level (not listed in the table). HLA-B*5401 were not evaluated in the previous studies with non-Asian ethnic groups, because the frequency of this allele is very low in the population. Because HLA-B*5401 belongs to B22 antigenic group together with B55 and B56 alleles, which were reported associate with rapid progression in MACS and other cohorts, this allele might be unfavorable for the host (18). This would be an important public issue because HLA-B*5401 is the second most prevalent allele in the Japanese, and also highly prevalent in China (21).

We collected samples from the cohort of HIV infected hemophiliac patients under close follow-up by multiple hospitals in Japan for over fifteen years. The number of the samples may not sufficient to reveal the presence of genetic factors with weak to moderate effect. It is not practical to increase the sample size to confirm our results without compromising sampling criteria. As approximately one third of hemophiliac patients who were exposed to contaminated blood products were actually infected with HIV in the early 1980s, there should be a group of hemophiliac patients who were exposed but sero-negative (ESN). Indeed, there were many hepatitis virus type C (HCV) carriers among hemophiliac patients presumably infected through blood products. A study with such ESN patients may provide us some other aspects of information on host–HIV interaction.

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Determination of HIV-1 Subtypes (A–D, F, G, CRF01_AE) by PCR in the Transmembrane Region (gp41) With Novel Primers

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HIV-1 has a huge genetic diversity. So far, nine subtypes have been isolated, namely, subtypes A, B, C, D, F, G, H, J, and K. Epidemiological study provides information which may help in the development of HIV-1 prevention programs or health policies. In the future, subtyping may also be critical for vaccine development, and an effective anti-viral drug will need to be effective for different subtypes of HIV virus. The analysis of the nucleotide sequence of the v3 region is considered the most reliable method for determining the HIV-1 subtype. However, the procedures for determining the v3 sequences are complicated and time consuming, requiring expensive reagents, equipment, and well-trained personnel. The polymerase chain reaction (PCR) method using subtype-specific primers for HIV-1 subtyping is easier and faster. The objective of this study was to develop subtype-specific primers for subtyping PCR. The specific primers were designed for subtypes A, B, C, D, F, G, and CRF01_AE, and these primers could be applied to assay for various HIV-1 subtypes in the clinical samples. The specific primers were designed for each subtypes in the gp41 region. The result of PCR was compared with the subtypes which was determined by the v3 sequence. The results of subtyping by PCR using the newly designed primers could detect 29 of 33 patients tested, and all matched those obtained by nucleotide sequencing of the env v3 region except for three subjects, which were differentiated as CRF02_AG. The newly designed primers functioned accurately and conclusively. In comparison with PCR as a method for the determination of subtypes, sequence analysis requires better-trained personnel, more expensive reagents, and more equipment and time. ***J. Med. Virol. 76:16–23, 2005.*** © 2005 Wiley-Liss, Inc.

KEY WORDS: HIV-1; subtyping; PCR

INTRODUCTION

HIV-1 has numerous genetic variations and can be divided by group, subtype, and subsubtype. The majority of HIV-1 strains belong to the M (Major) group, which causes pandemic HIV-1 infection. The O (Outlier) group and the N (Non-M/Non-O) group are seldomly found. Both the O and N groups have high genetic diversity from the M group [Charneau et al., 1994; Gurtler et al., 1994; Vanden Haesevelde et al., 1994; Loussert-Ajaka et al., 1995; Simon et al., 1998].

The M group has thus far been subdivided into nine isolated subtypes: A, B, C, D, F, G, H, J, and K [Carr et al., 1998; Robertson et al., 2000]. Although some isolates from Cyprus and Greece (94CY032, PVMY, and PVCH) were recognized previously as subtype I, they were shown to be a recombinant strain upon reanalysis. Subtype K was recognized initially as subtype F3, and later designated subtype K. Subtypes A and F are divided, respectively, into subsubtypes A1 and A2, and F1 and F2 [Triques et al., 1999, 2000]. Subtypes B and D are similar [Robertson et al., 2000; Triques et al., 2000], and although they should be reclassified as a single subtype, for the sake of historical consistency, they remain classified as different subtypes.

HIV-1 has been divided into not only many subtypes but also many circulating recombinant forms (CRFs), and so far 15 CRFs have been isolated [Carr et al., 1998]. For example, in the CRF01_AE strain, only the env region belongs to subtype E; the rest of the regions

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belong to subtype A, and the original full-length of subtype E is no longer found [Carr et al., 1996; Gao et al., 1996].

Worldwide, the incidence of HIV-1 subtypes is as follows: subtype C (47.0%), subtype A (27.2%), subtype B (12.3%), subtype D (5.3%), and CRF01_AE (3.2%) [Osmanov et al., 2002]. Certain subtypes prevail in distinct geographical areas, and different subtypes may predominate for each of the different means of infection [Essex, 1999]. In Thailand, subtype B is predominant among those infected through intravenous drug use, and subtype E among those infected through sexual intercourse [Ou et al., 1992, 1993; Weniger and Brown, 1996; Lole et al., 1999].

The gp120 of the HIV-1 envelope gene comprises five variable domains interspersed with conserved regions. The third variable (v3) region plays an important role in biological properties such as cell tropism, cytopathic effect, and pathogenicity [Robert-Guroff et al., 1994; Palker et al., 1988; Takahashi et al., 1988]. Therefore, analysis of the nucleotide sequence of the v3 region is considered as the most reliable method for determining the HIV-1 subtype. However, the procedures for determining the v3 sequences are complicated and time consuming, requiring expensive reagents, equipment, and well-trained personnel. For determining HIV-1 subtypes of a large number of samples, the heteroduplex mobility assay (HMA) and the peptide enzyme-linked immunosorbent assays (PELISAs) are usually used [Cheingsong-Popov et al., 1994; Wasi et al., 1995; Gaywee et al., 1996; Novitsky et al., 1996; Delwart et al., 1998]. Both of these methods often show cross-reaction or are not reliable. Recently, new methods were attempted to detect subtypes and recombinants [Hoelscher et al., 2002; Plantier et al., 2002]. However, these new methods are more difficult and complicated than PCR. Furthermore, the cost of the single PCR method in particular is approximately 10 times less than that of the sequence method, and thus consideration should be given to its preferential use in developing countries in the future [Yagyu et al., 2002].

The objective of this study was to develop subtype-specific primers for subtyping PCR, since the PCR method is easy, fast, cheap, and accurate. Therefore, specific primers were designed for subtypes A, B, C, D, F, G, and CRF01_AE, and these primers could be applied to examine various HIV-1 subtypes in clinical samples. This method could be applied with several advantages in developing countries, which face a large number of people living with HIV/AIDS.

MATERIALS AND METHODS

Clinical Specimens

Blood samples were collected from HIV-1 carriers with informed consent (Table I). All the subjects were positive originally for anti-HIV-1 antibody. Six samples were from Brazil and the patients had a history of treatment. Six samples from Japan, 2 samples from Kenya, 2 samples from Thailand, 1 sample from Tanzania, and 15 samples from Africa, for all of which a history of treatment was not available. The collection site of one sample was not specified nor the history of medication was known. Blood sample was obtained without any anti-coagulant and incubated at 56°C for 1 hr and stored at −20°C.

DNA Extraction

Stored samples were thawed at room temperature, centrifuged at 12,000*g*, and the supernatants were discarded. The cell pellets were suspended in 500 μl of DNA extraction buffer (150 mM NaCl, 10 mM Tris-HCl [pH 8.0], 10 mM EDTA), 5 μl of 10% SDS and 5 μl of Proteinase K (25 mg/ml) were added, and incubated at 56°C for 1 hr. Following phenol and phenol/chloroform extractions, DNA was ethanol-precipitated at −85°C for 1 hr. After centrifugation at 12,000*g* for 15 min, DNA pellets were rinsed with ice-cold 70% ethanol and dried. The dried pellet in each tube was dissolved in 15 μl of distilled H_2O and stored at −20°C until use.

PCR of Cellular Beta-Actin

To examine the integrity of the DNA samples, cellular *beta-actin* gene was amplified as described previously with b-F and b-R primers [Yagyu et al., 2002] (Table II). The PCR products were subjected to 1% agarose gel electrophoresis at 100 V for 30 min and stained with ethidium bromide.

Differentiation of Subtype B and E by PCR With Subtype-Specific Primers

Nested PCR with two sets of primers were carried out for differentiating subtypes B and E as described previously [Yagyu et al., 2002]. The primers were BECO5 and BECO3 for the first round and BE-ANCH, B-SPEC, and E-SPEC for the second round in PCR reaction (Table II). The PCR products were subjected to 1% agarose gel electrophoresis at 100 V for 30 min and stained with ethidium bromide.

Determination of Subtypes by PCR With Novel Designed Primers

The primers were BECO5 and BECO3 for the first round and BE-ANCH, B-SPEC, C-SPEC, E-SPEC, and F-SPEC for the second round in PCR reaction (Table II, Fig. 1). The primers were 5′D and 3′D, or 5′A, 3′A, 5′E, 3′E, 5′G, and 3′G for the alternative second round PCR (Table II).

A reaction mixture was made by adding 5.0 μl of 10 × PCR buffer with 22.5 mM $MgCl_2$ (Roche Diagnostics, Indianapolis, IN), 0.3 μl of enzyme mix (Roche Diagnostics), 1.5 μl each of dATP, dCTP, dGTP, and dTTP (25 mM each), 1.5 μl each primer (33 pM each), 1.0 μl of template DNA solution and adding distilled H_2O, up to 50 μl. The cycle condition was 93°C for 1 min, 50°C for 1.5 min, and 72°C for 2 min, for 30 cycles.

The second round PCR products were subjected to 2% agarose gel electrophoresis at 100 V for 1 hr and stained with ethidium bromide.

TABLE I. Samples and Subtyping Results

Sample	Sex	Age	Sample taken in	HV+ from when	Symptom	The way of infection	Drug therapy	Sampling date	v3 sequence	PCR differentiate subtype B and E[b]	PCR with newly designed primer[c]	PCR with newly designed primer[d]	PCR with newly designed primer[e]
BRON01	M	34	Brazil	Sep/95	AC	Sexual	+	Nov/99	B	B	B	—[g]	B
BRON02	M	24	Brazil	Aug/95	AC	Sexual	+	Nov/99	B	B	B	—[g]	B
BRON03	M	54	Brazil	Nov/96	AC	Sexual	+	Nov/99	B	B	B	—[g]	B
BRON04	F	31	Brazil	Aug/98	AC	Sexual	+	Nov/99	F	—[f]	F	—[g]	—[g]
BRON05	M	33	Brazil	Mar/97	AIDS	Sexual	+	Nov/99	B	B	B	—[g]	B
BRON06	M	36	Brazil	Mar/93	AIDS	Sexual	+	Nov/99	B	B	B	—[g]	B
YoD	—[a]	—[a]	Japan	—[a]	—[a]	—[a]	—[a]	—[a]	D	B	B	—[g]	D
OS01	F	—[a]	Tanzania	—[a]	—[a]	Sexual	—[a]	—[a]	D	B	B	—[g]	D
OS02	F	—[a]	Japan	—[a]	—[a]	Sexual	—[a]	—[a]	D	B	B	—[g]	D
OS03	F	—[a]	Thailand	—[a]	—[a]	Sexual	—[a]	—[a]	CRF01 AE	CRF01 AE	CRF01 AE	CRF01 AE	—[g]
OS04	F	—[a]	Thailand	—[a]	—[a]	Blood transfusion	—[a]	—[a]	CRF01 AE	CRF01 AE	CRF01 AE	CRF01 AE	—[g]
OS05	M	—[a]	Japan	—[a]	—[a]	Sexual	—[a]	—[a]	CRF01 AE	CRF01 AE	CRF01 AE	CRF01 AE	—[g]
OS06	—[a]	—[a]	—[a]	—[a]	—[a]	Sexual	—[a]	—[a]	CRF01 AE	CRF01 AE	CRF01 AE	CRF01 AE	—[g]
OS07	F	—[a]	Japan	—[a]	—[a]	Sexual	—[a]	—[a]	CRF01 AE	CRF01 AE	CRF01 AE	CRF01 AE	—[g]
OS08	F	—[a]	Kenya	—[a]	—[a]	Sexual	—[a]	—[a]	CRF02 AG	CRF01 AE	CRF01 AE	A	—[g]
OS09	M	—[a]	Japan	—[a]	—[a]	Sexual	—[a]	—[a]	CRF01 AE	CRF01 AE	CRF01 AE	CRF01 AE	—[g]
OS10	F	—[a]	Kenya	—[a]	—[a]	Blood transfusion	—[a]	—[a]	G	CRF01 AE	CRF01 AE	G	—[g]
NG01	F	41	Africa	—[a]	—[a]	Sexual	—[a]	Sep/2002	A	CRF01 AE	CRF01 AE	A	—[g]
NG02	F	38	Africa	—[a]	—[a]	Sexual	—[a]	Sep/2002	A	—[f]	—[f]	—[f]	—[g]
NG03	F	21	Africa	—[a]	—[a]	Sexual	—[a]	Sep/2002	D	B	B	—[g]	D
NG04	M	14	Africa	—[a]	—[a]	Sexual	—[a]	Sep/2002	C	CRF01 AE	C	—[g]	—[g]
NG05	F	36	Africa	—[a]	—[a]	Sexual	—[a]	Sep/2002	A	CRF01 AE	CRF01 AE	A	—[g]
NG06	F	26	Africa	—[a]	—[a]	Sexual	—[a]	Sep/2002	CRF02 AG	CRF01 AE	CRF01 AE	A	—[g]
NG07	F	20	Africa	—[a]	—[a]	Sexual	—[a]	Sep/2002	C	CRF01 AE	C	—[g]	—[g]
NG08	F	61	Africa	—[a]	—[a]	Sexual	—[a]	Sep/2002	CRF02 AG	—[f]	—[f]	—[g]	—[g]
NG09	F	34	Africa	—[a]	—[a]	Sexual	—[a]	Sep/2002	A	CRF01 AE	CRF01 AE	A	—[g]
NG10	F	35	Africa	—[a]	—[a]	Sexual	—[a]	Sep/2002	C	CRF01 AE	C	—[g]	—[g]
NG11	M	33	Africa	—[a]	—[a]	Sexual	—[a]	Sep/2002	D	B	B	—[g]	D
NG12	M	40	Africa	—[a]	—[a]	Sexual	—[a]	Sep/2002	D	—[f]	—[f]	—[g]	—[f]
JP02	M	31	Japan	1994	AC	Sexual	+	Jul/1999	B	—[f]	—[f]	—[g]	—[f]
KA14	—[a]	—[a]	Africa	—[a]	—[a]	—[a]	—[a]	—[a]	G	CRF01 AE	CRF01 AE	G	—[g]
KA18	—[a]	—[a]	Africa	—[a]	—[a]	—[a]	—[a]	—[a]	F	—[f]	—[f]	—[g]	—[g]
KA43	—[a]	—[a]	Africa	—[a]	—[a]	—[a]	—[a]	—[a]	G	CRF01 AE	CRF01 AE	G	—[g]

[a] No data.
[b] Using primer BECO5 and BECO3 for the first round PCR and BE-ANCH, B-SPEC, and E-SPEC for the second PCR.
[c] Using primer BECO5 and BECO3 for the first round PCR and BE-ANCH, B-SPEC, E-SPEC, and newly designed subtype-specific primers C-SPEC and F-SPEC for the second round PCR.
[d] Using primer BECO5 and BECO3 for the first round PCR and BE-ANCH, B-SPEC, E-SPEC, and newly designed subtype-specific primers 5′A, 3′A, 5′E, 3′E, 5′G, and 3′G for the second round PCR.
[e] Using primer BECO5 and BECO3 for the first round PCR and BE-ANCH, B-SPEC, E-SPEC, and newly designed subtype-specific primers 5′D and 3′D for the second round PCR.
[f] No product generated.
[g] Not done.

TABLE II. Sequence of Primers and Positions

Name	Sequence (5′–3′)	HXB2 no.[a]
J5′-2KSI	ATAAGCTTGCAGTGTAGCAGAAGAAGA	7003–7029
5′C2V3	TGTACACATGGAATTAGGCCAG	6963–6984
3′V3	ATGAATTCATTACAGTAGAAAAATTCCC	7363–7391
3′C2V3	ATTTCTGGGTCCCCTCCTGAGG	7313–7334
BECO5	GGCATCAAACAGCTCCAGGCAAG	7938–7960
BECO3	AGCAAAGCCCTTTCTAAGCCCTGTCT	8766–8791
BE-ANCH	TCCTGGCTGTGGAAAGATACCTA	7963–7985
B-SPEC	GTCCCCTCGGGGCTGGGAGG	8384–8403
E-SPEC	GTCTCAGTCCCTTGAGACTGCTG	8585[b]
F-SPEC	AACAGCTCTACCAGCTCTTTGCAAA	8720–8744
C-SPEC	AGACCCCAATACTGCACAAGACTT	8615–8638
5′E	CAGGAAAGGAATGAAAAGGATTTGTTA	8181–8207
3′E	ATAACCCTATCTGTCCACCCC	8693–8713
5′A	GANAACATGACCTGGCTGC	8094–8112
3′A	TCTATAACCCTATCTGTCCAGCCA	8693–8716
5′G	ACAATTACACATACCACATATACAGCC	8131–8757
3′G	TCTATAACCCTATCTGTCCAGTT	8694–8716
5′D	ACCACTAATGTGCCCTGGAACT	8037–8058
3′D	AGGAGGGTCTGAAATGACAGA	8356–8386
b-F	AGAGATGGCCACGGCTGCTT	
b-R	ATTTGCGGTGGACGATGGAG	

[a] HXB2 no. indicates primer position corresponding to nucleotide number of HXB2.
[b] HXB2 does not have sequence corresponding to primer, because of gap.

PCR of the v3 Region of the *gp120* Gene

The v3 region of HIV-1 provirus was amplified by nested PCR using primers 5′C2V3 and 3′V3 for the first round PCR reaction, and J5′-2KSI and 3′C2V3 for the second round reaction as described previously [Yagyu et al., 2002] (Table II). The PCR products were then subjected to 1% agarose gel electrophoresis at 100 V for 30 min, recovered from the gel, and used as a template for nucleotide sequencing.

Nucleotide Sequencing and Analysis

The sequencing reaction was carried out with a dideoxynucleotide cycle sequencing kit (Perkin Elmer, Wellesley, MA) using J5′-2KSI and 3′C2V3 as sense

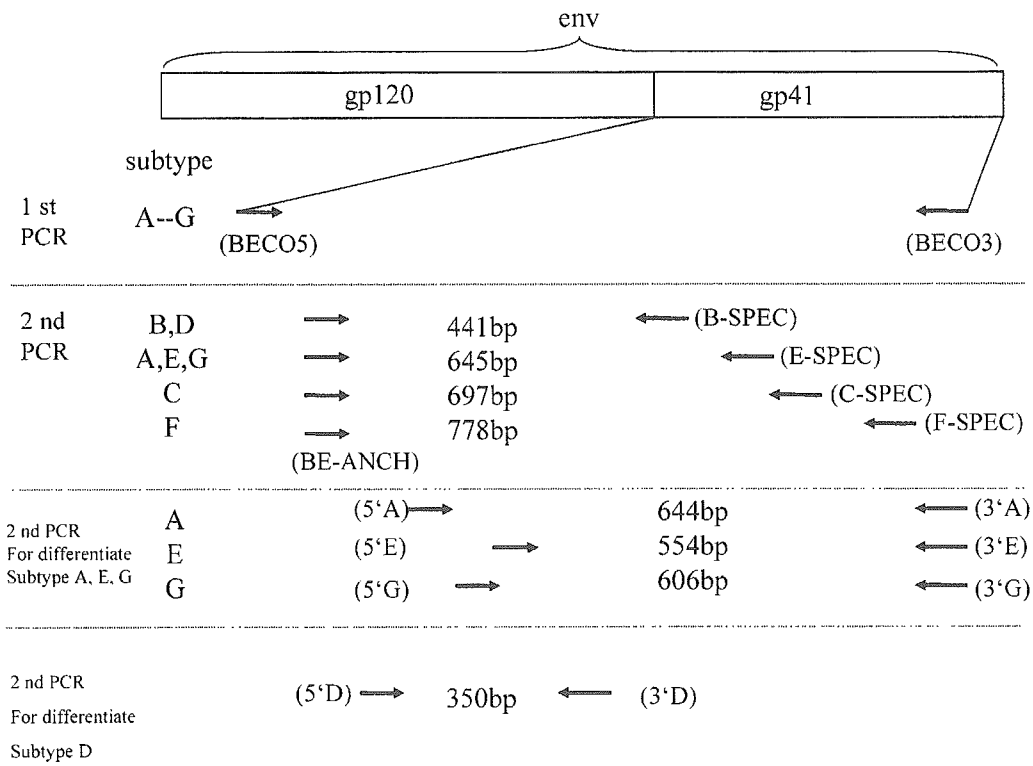

Fig. 1. Primer location and length of polymerase chain reaction (PCR) products.

and anti-sense primers for v3 region, respectively (Table II). The reaction products were analyzed by an automated DNA sequencer (ABI). The sequences of samples were aligned with references strains of subtypes A, B, C, D, F, G, CRF01_AE, and CRF02_AG, and SIV for analysis of v3 region by Clustal X [Thompson et al., 1997]. The subtypes were determined by phylogenetic trees, which were constructed using the neighbor-joining (NJ) method [Saitou and Nei, 1987].

RESULTS

Differentiation of Subtypes B and E by PCR With Subtype-Specific Primers

PCR to differentiate subtypes B and CRF01_AE was performed using samples from 33 subjects. A 441-bp PCR product, which was amplified between BE-ANCH and B-SPEC, was generated from the samples of 10 subjects, and a 645-bp PCR product, which was amplified between BE-ANCH and E-SPEC, was generated from the samples of 17 subjects. However, six subjects were negative, no PCR product was generated (Table I). All samples were positive for cellular beta-actin. This experiment was carried out to determine whether or not the primers B-SPEC and E-SPEC would react with the different subtypes. This information was considered useful for designing of new primers.

Determination of HIV-1 Subtype by Nucleotide Sequence Analysis of the v3 Region

In order to differentiate correct subtypes of the subjects, sequence analysis of the v3 region was performed, since this is considered the most reliable method. PCR of the v3 region of all samples generated successfully products of approximately 350 bp, which were then directly sequenced. The phylogenetic tree showed that the number of subjects of subtypes A, B, C, D, F, G, CRF01_AE, and CRF02_AG were 4, 6, 3, 6, 2, 3, 6, and 3, respectively (Tables I and III).

Designing of Env-Specific Primers for Differentiating Subtypes A, B, C, D, F, G, and CRF01_AE

For the first round PCR, the subtype-common primers, BECO5 and BECO3, were designed to differentiate subtypes B and CRF01_AE. Between BECO5 and BECO3, the subtype-unique conserved sequences of subtypes C and F were found, and used to design subtype C- and F-specific primers, respectively. However, the unique sequences specific for subtypes A, D, G, and CRF01_AE that could be used as anti-sense primers were not found. The results showed that the samples which were positive by PCR using BE-ANCH and subtype E-specific primers could be either subtype A, E, or G according to the sequence alignment, and this observation was in good agreement with a previous study [Yagyu et al., 2002]. Therefore, when the sample was positive for subtype A, E, or G by using the subtype E-specific primer, it was necessary to perform the second round PCR by using the first PCR product as the template with the alternative sets of subtype-specific primers, 5′A, 3′A, 5′E, 3′E, 5′G, and 3′G (Table II, Fig. 2). Similarly, the sample which was positive for subtype B or D by using the subtype B-specific primer should be differentiated between subtypes B and D in the second round PCR using the first round PCR product as the template with the alternative set of primers 5′D and 3′D (Table II, Fig. 2).

Determination of Subtypes With Subtype-Specific Primers

The novel subtype-specific primers designed in this study were verified for specificity by testing with clinical specimens of the known subtypes. The primers used for the first round PCR were BECO5 and BECO3, and then the second round PCR was performed using the first round PCR product as the template, BE-ANCH as the 5′ primer and B-SPEC, C-SPEC, E-SPEC, and F-SPEC as the 3′ primers (Table II, Figs. 1, 2, and 3A). The primers C-SPEC and F-SPEC generated products of 675 and 778 bp, and the samples were identified as subtype C and F, respectively. A PCR product of 440 bp was also generated, possibly due to the annealing of the B-SPEC primer to the proviral DNA of subtypes B and D in the sample. Then, using the first PCR product as template, the second PCR was performed with 5′D and 3′D primers for detecting subtype D, and a 350-bp product was generated (Figs. 1, 2, and 3B). Similarly, E-SPEC annealed to subtypes A, E, and G, and generated a 650-bp product. Then, using the first PCR product as template, the second PCR was performed with a mixture of the primers 5′A, 3′A, 5′E, 3′E, 5′G, and 3′G, and

TABLE III. Subtypes Determined by v3 Sequence and PCR Using Novel Primers

Subtype by v3 sequence	No. of samples with subtype by PCR using novel primers								
	A	B	C	D	F	G	CRF01_AE	ND	Total (n = 33)
A	3							1	4
B		5						1	6
C			3						3
D				5				1	6
F					2				2
G						3			3
CRF01_AE							6		6
CRF02_AG	2							1	3

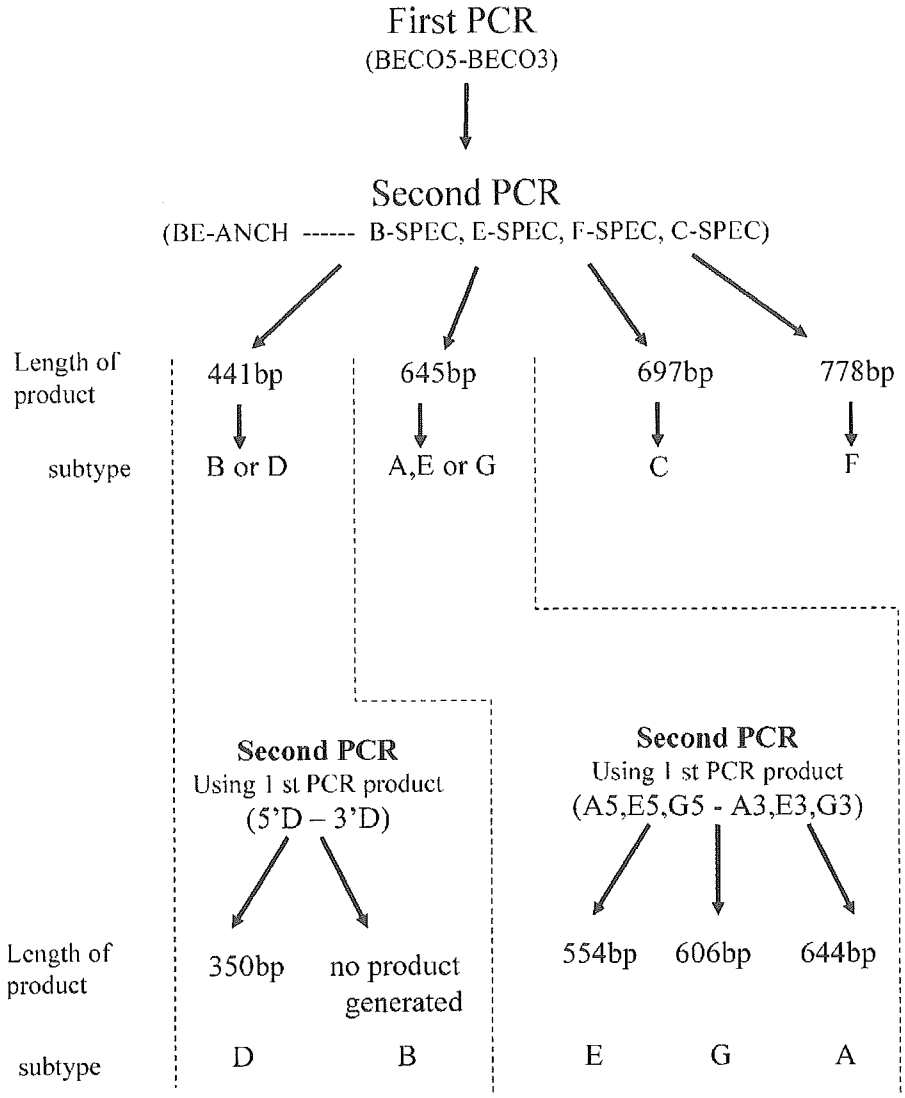

Fig. 2. Procedure of PCR for differentiation of subtypes with newly designed primers.

products of 644, 554, and 606 bp were generated for subtypes A, E, and G, respectively (Figs. 1, 2, and 3C). A total of 33 subjects were tested by this procedure using the newly designed primers. When BE-ANCH was used as a 5′ end primer in combination with B-SPEC, E-SPEC, C-SPEC, and F-SPEC as 3′ end primers, and the first round product was used as the template, PCR products of 441, 645, 697, and 778 bp were generated from 10, 14, 3, and 2 out of the 33 subjects, respectively (Tables I and III). In addition, when the alternative second round PCR for differentiation of subtypes A, G, and CRF01_AE was performed using the first round PCR product as a template and a mixture of the 5′A and 3′A, 5′E and 3′E, 5′B and 3′G primer pairs, 644 bp (subtype A), 554 bp (CRF01_AE), and 606 bp (subtype G) products were generated from 5, 6, and 2, out of the 13 subjects, respectively. Furthermore, when 5′D and 3′D were used in the alternative second round PCR to differentiate subtype D from subtype B, a 350 bp (subtype D) product was generated from 6 out of 12 subjects. The other 6 of the 12 subjects were therefore identified as subtype B.

DISCUSSION

The PCR for differentiation of subtype B and CRF01_AE was carried out as described previously [Yagyu et al., 2002]. The primers B-SPEC and E-SPEC have cross-reaction with other subtypes, except for subtype B and CRF01_AE. The B-SPEC primer anneals subtypes B and D, and the E-SPEC primer anneals subtypes A, C, G, and CRF01_AE. The result of PCR of cellular beta-actin was positive for all samples, suggesting that the DNA in samples was intact by the time of testing and the extraction procedure was successful. The samples that did not generate any products were considered not to have been amplified by the B-SPEC and E-SPEC primers, possibly because there was an insufficient copy number of the HIV-1 provirus or because it was subtype F [Yagyu et al., 2002].

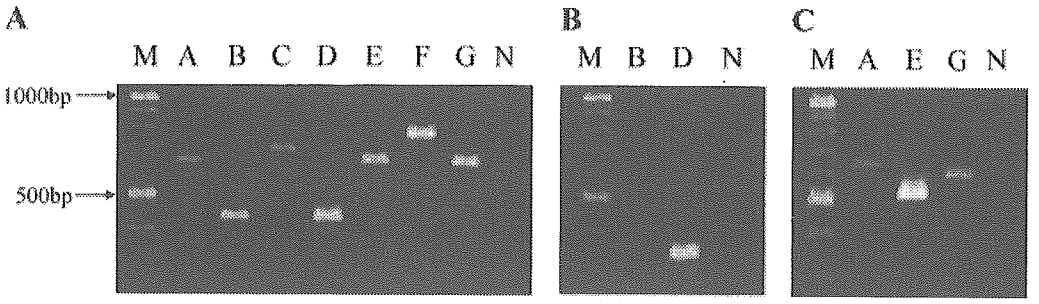

Fig. 3. Determination of subtypes with subtype-specific primers. **A**: PCR with primers (BECO5 and BECO3 for the first round PCR and, BE-ANCH, B-SPEC, E-SPEC, and newly designed subtype-specific primers C-SPEC and F-SPEC for the second round PCR) of subtype A (**lane A**), B (**lane B**), C (**lane C**), D (**lane D**), CRF01_AE (**lane E**), F (**lane F**), and G (lane F). PCR products were subjected to agarose gel electphoresis with 100 bp ladder markers (**lane M**) and negative control (**lane N**). **B**: PCR with primers (BECO5 and BECO3 for the first round PCR and, 5′D and 3′D for the second round PCR) of subtype B (**lane B**) and D (**lane D**). PCR products were subjected to agarose gel electrophoresis with 100 bp ladder markers (**lane M**) and negative control (**lane N**). **C**: PCR with primers (BECO5 and BECO3 for the first round PCR and, 5′A, 3′A, 5′E, 3′E, 5′G, and 3′G for the second round PCR) of subtype A (**lane A**), CRF01_AE (**lane E**), and G (**lane G**). PCR products were subjected to agarose gel electphoresis with 100 bp ladder markers (**lane M**) and negative control (**lane N**).

Among the patients (BRON04, NG02, NG08, NG12, JP02, and KA18) who were negative for PCR for differentiation of subtypes B and E, BRON04 and KA18 were subtype F as expected. And, the subjects, NG02, NG08, NG12, and JP02, were subtypes A, CRF02_AG, D, and B, respectively.

The newly designed subtype-specific primers could be used as anti-sense primers against BE-ANCH, and could generate PCR products of different lengths. Specific primers for subtypes C and F which act as the anti-sense primers against BE-ANCH were also designed. However, it was rather difficult to design a subtype-specific for A, B, D, G, or CRF01_AE, because subtypes A, E, and G and subtypes B and D, respectively, were similar to each other. After the second PCR with primers BE-ANCH, B-SPEC, C-SPEC, E-SPEC, and F-SPEC, the sample which was positive for the subtype B-specific primer was further differentiated between subtypes B and D by using the subtype D-specific primers 5′D and 3′D. By this strategy, the primers were able to recognize only subtype D, since it was impossible to design a subtype B-specific primer for the second PCR. Since the subtypes B and D are very closely related, an attempt was made to differentiate the subsubtypes by PCR. The results showed that PCR was able to separate them incompletely in this experiment. However, because subtypes B and D are predominant in different countries, the differentiated subtypes B and D could provide important information on the course of an HIV strain newly imported to a particular country.

Three samples (OS08, NG06, NG08) of CRF02_AG determined by nucleotide sequence were differentiated as subtype A by PCR with the novel primers. The envelope gene of CRF02_AG consists of subtype A and G. Therefore, it may be impossible to design specific primers for CRF02_AG, when only the genome of gp41 is used for subtype determination.

The subtypes of all subjects determined by PCR using newly designed primers were in complete agreement with those determined by nucleotide sequence analysis of the v3 region except for CRF02_AG. Nevertheless, there were four patients, who did not generate any positive signals. This indicates that the relatively lower sensitivity of PCR with novel primers might not be due to the low copy number of proviral DNA; rather, it might be due to mismatched of the primer, since point mutations readily occur in the HIV-1 genome.

The newly designed primers functioned accurately and conclusively. In comparison with PCR as a method for the determination of subtypes, sequence analysis requires better-trained personnel, more expensive reagents, and more equipment and time. The PCR method is useful for developing countries in which the burden of HIV/AIDS has increased dramatically. However, although the PCR method has the above advantages, it also has a shortcoming in that it differentiates subtypes only in the gp41 region. In order to evaluate this method accurately, a larger number of samples should be tested.

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Identification of Enteroviral Infection Among Infants and Children Admitted to Hospital With Acute Gastroentritis in Ho Chi Minh City, Vietnam

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A total of 276 fecal specimens collected from infants and children admitted to hospital with acute gastroenteritis in Ho Chi Minh City, Vietnam from October 2002 to September 2003, were tested for the presence of enteroviruses by RT-PCR and virus isolation. Enteroviruses were detected in 27 patients by RT-PCR corresponding to 9.8%. However, only four enterovirus strains could be isolated by cell culture with two different cell lines CaCo2 and Vero, showing specific cytopathic effect (CPE). The results clearly indicate that RT-PCR is a sensitive, specific assay to investigate the true burden of acute gastroenteritis due to enteroviruses in clinical fecal specimens. In the present study, enteroviruses were identified throughout the year except in May and the highest number was in December. Enteroviruses were subjected to molecular analysis by sequencing. It was found that enterovirus strains detected were classified further into two distinct genetic clusters (I, II) and demonstrated the great genetic diversity among them. Based on genetic analysis, 5′ noncoding region (5′ NCR) sequences suggested the predominant presence of Vietnamese enteroviruses with the greatest similarities to coxsakieviruses (51.9%) and echoviruses (29.6%). Interestingly, two of the sequenced specimens of enteroviruses were similar to a new strain called enterovirus 74. This report is the first detection of enteroviral infection in feces from infants and children admitted to hospital with acute gastroenteritis in Vietnam. ***J. Med. Virol. 77:257–264, 2005.*** © 2005 Wiley-Liss, Inc.

KEY WORDS: RT- PCR; enteroviruses; Vietnam

INTRODUCTION

Viral gastroenteritis is a common disease with a high morbidity reported worldwide especially in infants and the elderly [Murray and Lopez, 1997; Kosek et al., 2003]. Acute gastroenteritis has been reported as a major cause of morbidity and mortality of infants and young children both in developed and developing countries and ranks among the principal causes of all deaths [Parashar et al., 2003; Thapar and Sanderson, 2004]. Enteroviruses are recognized as the major etiologic agents of gastroenteritis in children and young animals [Melnick, 1996; Mark and Raymond, 2001]; they are associated with sporadic cases and outbreaks of gastroenteritis. The transmission routes of these viruses are multifold and can be foodborne, spread from person-to-person or by other still unknown modes of transmission [Melnick, 1996; Mark and Raymond, 2001; Numanovic et al., 2004].

Enteroviruses are non-enveloped enteric RNA viruses belonging to the family *Picornaviridae* that infect not only humans but also a wide range of mammals including cattle and pigs. Enteroviruses infect billions of people worldwide and cause clinical diseases such as poliomyelitis, myocarditis, encephalitis, aseptic meningitis, hand-foot, and mouth diseases (HFMD), and other acute and chronic illnesses [Melnick, 1996; Mark and Raymond, 2001]. Infected humans and animals excrete feces, which contain infectious enteroviruses [Wait and Sobsey, 2001]. Several reports on the identification of enteroviruses in seawater, sand, sewage, and oysters have been published [Nestor et al., 1984; Le Guyader

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et al., 1994; Amvros'eva et al., 2004; Dubois et al., 2004; Numanovic et al., 2004; Wetz et al., 2004]. These viruses were also used as an indicator of fecal contamination in drinking and bathing water [Havemeister, 1993; Ley et al., 2002; Katayama et al., 2004].

Enteroviruses form a group of viruses with numerous serotypes; they are subgrouped into poliovirus, coxsackieviruses A and B, echovirus and enteroviruses 68 to 71 on basis of their pathogenetic effects in humans and experimental animals. Classification of enteroviruses based on the genetic clusters (species A to D, and polioviruses) in the coding region and sequences of the VP1 genes correlates well with the current serotype (polio- coxsackie- and echovirus types) [Oberste et al., 1999; Thoelen et al., 2004]. However, enteroviruses are classified into two genetic clusters I and II on the basis of their 5′ noncoding region (5′ NCR) which makes exact epidemiological analysis and correlation of the findings with the serotypes difficult [Caro et al., 2001; Thoelen et al., 2004]. Specifically, it is noticed that coxsackieviruses A1, 11, 13, 15, 17–22, and 24 are clustered along with polioviruses (1–3) and enterovirus 70, whereas the rest of the coxsackieviruses A are clustered along with coxsackieviruses B, echovirus, and the other enteroviruses (68, 69, and 71) [Melnick, 1996; Mark and Raymond, 2001; Siafakas et al., 2002]. Recently, new enterovirus serotypes (73, 74, 75, 76, 77, and 78) have been shown to infect humans [Oberste et al., 2001; Norder et al., 2003; Bailly et al., 2004; Oberste et al., 2004].

The objectives of this study were to describe the prevalence of enteroviruses in clinical fecal specimens collected from infants and children with acute gastroenteritis in Ho Chi Minh City, Vietnam; and to determine the age-related distribution and seasonal pattern of enteroviral infections. Additionally, the genetic diversity of enterovirus strains is described.

MATERIALS AND METHODS

Fecal Specimens

Two hundred and seventy-six fecal specimens were collected from infants and children admitted to hospital with acute gastroenteritis in Ho Chi Minh City, Vietnam during the period of October 2002 to September 2003. The work done in Ho Chi Minh City included the report of clinical manifestations and bacterial tests (campylobacter, clostridium, *escherichia coli*, and salmonella). A research group in Japan determined all fecal specimens were to be negative for rotavirus, adenovirus, sapovirus, norovirus, and astrovirus. The fecal specimens were diluted with distilled water as an optional diluent to 10% suspensions, and clarified by centrifugation at 10,000*g* for 10 min; the supernatants were collected and stored at −30°C until use for the detection of enteroviruses.

Extraction of Viral Genomes

The viral genome was extracted from 140 μl of a 10% fecal suspension using a spin column technique according to the instructions in the QIAamp® Viral RNA Mini Kit Handbook, Germany.

Positive Control

A positive control of poliovirus 2 (Sabin) with 10^9 PFU/ml used in this study was provided by Dr. Hideaki Shimizu from Kawasaki City Institute of Public Health, Kawasaki, Japan.

Reverse Transcription (RT)

For RT, 7.5 μl of extracted RNA was added with a reaction mixture consisting of 2.05 μl of 5x First Strand Buffer (Invitrogen, Carlsbad, CA), 0.75 μl of 10 mM dNTPs (Roche, Mannheim, Germany), 0.75 μl of 10 mM DTT (Invitrogen), 0.75 μl (200 U/μl) of SuperScript Reverse Transcriptase II (Invitrogen), 0.375 μl (1 μg/μl) of random primer (hexa-deoxyribonucleotide mixture) (Takara, Shiga, Japan), 0.5 μl (33 U/μl) of RNase Inhibitor (Toyobo, Osaka, Japan), and 2.325 μl MilliQ water. The total of reaction mixture is 15 μl. The RT step was carried out at 42°C for 1 hr, followed by 99°C for 5 min and then held at 4°C.

Polymerase Chain Reaction (PCR)

Enteroviruses were detected by RT-PCR of extracted viral RNA with specific primers published previously [Zoll et al., 1992]. Briefly, a pair of specific primers previously published F1 (sense; 5′-CAAGCACTTCTG-TTTCCCCGG-3′) and R1 (antisense; 5′-ATTGTCACCA-TAAGCAGCCA-3′) for amplifying the 5′-NCR of enteroviruses was widely used to amplify viral genomes and specifically generated amplicon size of 440 bp. Exactly, 2.5 μl of cDNA was mixed with a reaction mixture containing 2.5 μl of 10x TaqDNA polymerase buffer (Promega, Madison, WI), 2.0 μl of dNTPs (2.5 mM/μl), 0.4 μl of each specific primer (33 μM), and 0.125 μl (2.5 mM/μl) of Tag DNA polymerase (Promega). MilliQ water was added to make up a total volume of 25 μl. The PCR was carried out at 94°C for 3 min followed by 35 cycles of 94°C 30 sec, 55°C 30 sec, 72°C 60 sec, and a final extension at 72°C for 7 min, and then held at 4°C.

Electrophoresis

The PCR products (DNA) were electrophoresed in a 1.5% agarose gel, followed by staining with ethidium bromide (EtBr) for 20 min then visualized under ultraviolet (UV) light, and the results were recorded by photography.

Cell Culture

Fecal specimens (including all RT-PCR positive samples) were tested further for enteroviruses by a second method; virus isolation in cell culture [Guney et al., 2003]. Briefly, clinical specimens were inoculated into 24-well plates covered with monolayers of two different cell lines, CaCo2 and Vero. These cells were

incubated for at least 2 weeks at 34°C in an atmosphere containing 5% of CO_2 in the air. Subsequently, the cells were examined daily for cytopathic effects (CPEs) characteristic for enteroviruses. Cultures exhibiting no CPE by the end of observation period were passaged blindly for another two weeks before reporting as negative.

Nucleotide Sequencing and Phylogenetic Analysis

The nucleotide sequences of PCR products (DNA) positive for enteroviruses were determined with the Big-Dye terminator cycle sequencing kit and an ABI Prism 310 Genetic Analyzer (Applied Biosystems Inc., Foster City, CA). Sequence analysis was undertaken using E-CLUSTAL W (Version 1.6). Enterovirus strains and their accession numbers used in this study are as follows: CoxA13/Flores (AF465511), CoxA24/Joseph (AF068881), CoxA11/Belgium-1 (AF499636), CoxA17/G-12 (AF499639), CoxA22/Chulman (AF499643), CoxA4/ P-2319/Kanagawa/03 (AB162739), CoxA2/P-2242/Kanagawa/03 (AB162722), Echo13/DelCarmen (AF405311), CoxB3/Nancy (AJ420884), Echo17/CHHE-29 (AY302543), Echo19/Djum/91 (AF44780), Echo6/Kh7/92 (AF447481), CoxB1/P-2346/Kanagawa/03 (AB162737), CoxB2/10178 (AY373056), Echo11/Pz/87 (AF447476), Echo11/HUN-1337 (AJ577593), Echo11/Kar/87 (AY167104), Poliovirus1/Sabin (V02281), Poliovirus2/Sabin (X00595), Poliovirus3/Sabin (K00043), and Porcine/Entero8/Sek1562/9 (AY392556).

RESULTS

Detection of Enteroviruses

Two seventy-six fecal specimens from infants and children admitted to hospital with acute gastroenteritis in Ho Chi Minh City, Vietnam during October 2002 and September 2003 were collected and then tested for the presence of enteroviruses by RT-PCR and by virus isolation. The results shown in Table I show that enteroviruses were detected in 27 of 276 (9.8%) specimens as determined by RT-PCR. However, only 4 of these samples were positive for enteroviruses showing CPE in cell culture. Figure 1 shows an example of enteroviruses found in Vietnamese clinical fecal specimens by RT-PCR.

Seasonal Variation of Enteroviral Infection

Enteroviruses were detected in samples during each month except for May with the highest incidence in December (Fig. 2). The ages of patients with enteroviral infection ranged from 4 months to 4 years; the highest rate was in the age groups of 6–11 and 12–23 months (37%, 10 of 27) and the lowest infection rate was found in children 24–35 months of age (3.7%, 1 of 27). The number of male infected by enteroviruses with acute gastroenteritis accounted for 48.1% (13 of 27).

Dehydration Related to Enteroviral Infection

The findings in Table I show that the majority (92.6%; 25 of 27) of infants and children infected with enterovirus associated diarrhea did not suffer from dehydration known as grade A; only two cases (patients 178 and 994) displayed mild dehydration (B grade).

Nucleotide Sequences and Phylogenetic Analyses of Enteroviruses

The PCR products of the 5′ NCR were sequenced in order to characterize further the genetic relationship among the 27 isolates of enteroviruses detected in infants and children with acute gastroenteritis in Ho Chi Minh City, Vietnam. The partial nucleotide sequences were compared to each other as well as to those of reference enterovirus strains available in the DDBJ DNA/GenBank database by BLAST. A total of 27 nucleotide sequences of enterovirus isolates were analyzed by phylogenetics and grouped using the 5′ NCR classification scheme of Siafakas et al. [2002].

All sequences of Vietnamese enteroviruses were classified into two distinct genetic clusters I and II, accounting for 40.7% (11 of 27) and 59.3% (16 of 27), respectively (Fig. 3). The highest homology on the nucleotide level between enterovirus isolates in this study with other reference strains registered previously in the DDBJ DNA/Genbank database was in the range of 88%–99%. The highest homology of Vietnamese enteroviruses at the nucleotide level was 100% and the lowest only 58%. The homologies among them in cluster I about 74%–100% and in cluster II 82%–100% were also noted. Genetic analysis of the isolates' 5′ NCR sequences suggested the predominant presence of Vietnamese enteroviruses with a closest match to coxsakieviruses (including types A and B) (51.9%; 14 of 27) and echoviruses (29.6%; 8 of 27). Of interest, two of the sequenced specimens positive for enteroviruses, EV/143/VN/02 and EV/268/VN/03, turned out to have the highest homologies at the nucleotide level with a new strain called enterovirus 74 isolated from stool in 1975 in California, USA (known as the Entero74/USA/CA75-10213). Furthermore, it was found that the nucleotide identity between EV/143/VN/02 and Hou7-1181/90 was 95%, and between EV/143/VN/02 and Hou7-1181/90 was 97%. The 94% similarity between these two Vietnamese enterovirus isolates was also noted.

DISCUSSION

Acute gastroenteritis is considered as a very common disease causing illness worldwide [Murray and Lopez, 1997; Kosek et al., 2003]. Enteroviruses cause infantile viral gastroenteritis and are associated with sporadic cases as well as with outbreaks [Melnick, 1996; Mark and Raymond, 2001; Parashar et al., 2003]. Even though numerous reports of enteroviral infection in many countries have been published, no specific epidemiological reports about this disease and the prevalence of enteroviruses in healthy children in Vietnam are