INTRODUCTION OF HUMAN IMMUNODEFICIENCY VIRUS 2 INFECTION INTO SOUTH KOREA

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Summary. – Although human immunodeficiency viruses 1 and 2 (HIV-1 and HIV-2) share mode of transmission, their epidemiologic characteristics differ and international spread of HIV-2 has been limited. To investigate the extent of HIV-2 infection in South Korea and to clarify the characteristic of HIV-2 isolates, we describe epidemiological, serological and genetic analyses of five HIV-2 isolates from South Korea. Five of 964 HIV antibody-positive serum specimens showed positive reactivity by HIV1/2 enzyme immunoassay (EIA), HIV-2 Western blot analysis, HIV-2 particle agglutination (PA) test and line immunoassay (LIA) but negative or indeterminate one by HIV-1 PA test and HIV-1 Western blot analysis. To confirm HIV-2 infection by genetic analysis, reverse transcription-polymerase chain reaction (RT-PCR) was performed on five HIV-2 seropositive samples. PCR products from *gag* (197 bp) and *env* gene regions (137 bp) were obtained with three of the five samples with HIV-2 specific *gag* primers and with all the five samples with *env* primers. To obtain larger sequences for a more comprehensive phylogenetic analysis, we performed PCR for a 1191 bp *env* region of HIV-2 but only two such products were obtained. For the phylogenetic analysis, three 197 bp *gag* and two 1191 bp *env* PCR products were cloned and sequenced. Based on the *gag* and *env* sequences alignments, three isolates (KR4063, KR7051 and KR8091) were clustered phylogenetically within HIV-2 subtype A. In conclusion, HIV-2 virus is present in South Korea and was detected in five subjects. Furthermore, the prevalence of HIV-2 infection should be monitored continuously in South Korea to assess the spread of this virus and to assist in the diagnosis of HIV infection.

Key words: HIV-2; PCR; nucleotide sequencing; serological diagnosis; South Korea

Introduction

Since HIV-2 had been isolated in western Africa (Clavel *et al.*, 1986), it has spread virtually worldwide. However, HIV-2 infection is confined to western Africa and most cases of HIV-2 infection outside Africa occurred in France and Portugal (De Cock *et al.*, 1993). Compared to HIV-1, sexual and perinatal transmission of HIV-2 is much less efficient than that of HIV-1 as a result of a low viral burden during the relatively long asymptomatic period (Kanki *et al.*, 1994; Marlink *et al.*, 1994; Simon *et al.*, 1993). Therefore, the number of HIV-2-infected individuals has remained relatively small, bound to limited geographical regions compared to HIV-1 infection (De Cock *et al.*, 1993; Schim *et al.*, 1999).

Molecular epidemiology studies have led to classification of HIV-2 into six specific subtypes from A to F (Chen *et al.*,

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Abbreviations: DTT = dithiothreitol; EIA = enzyme immunoassay; gp = glycoprotein; HIV-1 = human immunodeficiency virus 1; HIV-2 = human immunodeficiency virus 2; LIA = line immunoassay; PA = particle agglutination; PBMC = peripheral blood mononuclear cell; PCR = polymerase chain reaction; RT-PCR = reverse transcription–PCR

Table 1. Primer pairs selected for nested PCR of HIV-2 proviral DNA and cDNA

Region	Position (nt)	Sequence (5′-3′)	PCR product size (bp)	Reaction conditions	Reference
Outer pair				1 min, 94°C	
Gag A	539-561	GTGGGAGATGGGCGCGAGAAACT		1 min, 94 °C; 45 secs, 55 °C; 45 secs, 70 °C (35 cycles)	Grankvist *et al.* (1992)
Gag B	1062-1085	GGATTTCAGGCACTCTCAGAAGGC		3 mins, 70 °C	
Inner pair				1 min, 94 °C	
Gag C	637-660	TAAAACATATTGTGTGGGCAGCGA	197	1 min, 94 °C; 45 secs, 55 °C; 45 secs, 70 °C (25 cycles)	Grankvist *et al.* (1992)
Gag D	810-833	CACGCAGAAGAGAAAGTGAAAGAT		3 mins, 70 °C	
Outer pair				1 min, 94 °C	
Env A	7782-7805	GGGATAGTGCAGCAACAGCAACAG		1 min, 94 °C; 45 secs, 55 °C; 45 secs, 70 °C (35 cycles)	Grankvist *et al.* (1992)
Env B	8259-8281	AAGGGCTATAGGCCTGTTTTCTC		3 mins, 70 °C	
Inner pair				1 min, 94 °C	
Env C	7837-7861	TGTTGCGACTGACCGTCTGGGGAAC	137	1 min, 94 °C; 45 secs, 55 °C; 45 secs, 70 °C (25 cycles)	Grankvist *et al.* (1992)
Env D	7950-7973	GTCTGCCACACTACTGTACCATGG		3 mins, 70 °C	
Outer pair				1 min, 94 °C	
Env E	6300-6324	ATACAGTGCTTRCCAGACAATGATG		1 min, 94 °C; 1 min, 55 °C; 1 min, 70 °C (3 cycles)	Damond *et al.* (1998)
Env B	8259-8281	AAGGGCTATAGGCCTGTTTTCTC		15 secs, 94 °C; 45 secs, 55 °C; 45secs, 70 °C (32 cycles), 5 mins, 70 °C	
Inner pair				1 min, 94 °C	
Env F	6783-6804	TCATGTGAYAARCAYTATTGGG	1191	1 min, 94 °C; 1 min, 55 °C; 1 min, 70 °C (3 cycles)	Damond *et al.* (1998)
Env D	7950-7973	GTCTGCCACACTACTGTACCATGG		15 secs, 94 °C; 45 secs, 55 °C; 45 secs, 70 °C (25 cycles), 5 mins, 70 °C	

1997). The majority of isolates were classified as A subtype, with most of the remaining isolates belonging to B subtype. The virological characteristic and pathogenicity of A subtype have been described (Gao *et al.*, 1994). But for the other subtypes, studies of relative distribution and natural history are scarce. For vaccine development, biological and genetic characterization of HIV-2 isolates must be conducted as well as that of HIV-1 isolates. In addition, prevailing strains by geographical region should be monitored by molecular epidemiology.

According to periodical reports of the Ministry of Health and Welfare of South Korea, 964 South Koreans with HIV have been confirmed as of June 1999. A HIV-2 infection among these subjects was possible since 27% (260/964) of them have been infected abroad by sexual contact or blood transfusion. However, the HIV-2 infection in South Korea has not been monitored until now. With the detection of HIV-2 infection, we can confirm the spreading of HIV-2 in South Korea and establish a HIV testing strategy in which HIV-2 is included. To determine the scale of HIV-2 infection in South Korea and to clarify the characteristics of HIV-2 isolates, we performed serological and genetic analyses on five HIV-2 isolates from South Koreans. This is the first report of HIV-2 infection detected by serological and genetical analyses in South Korea.

Materials and Methods

Subjects. The National Institute of Health of South Korea has performed confirmation of HIV infection on serum specimens showing repeatedly a positive reaction by EIA from over 300 testing sites such as public health centers, blood banks and quarantine stations in South Korea since 1985. Every year about two thousand specimens were tested. According to the test results from 1985 to June 1999, 964 subjects have been confirmed as HIV-seropositive. Among them, five showed positive reactivity by HIV-2 EIA, HIV-2 PA test, and HIV-2 Western blot analysis, and indeterminate reactivity by HIV-1 Western blot analysis.

Serological assays. All referred sera were subjected to HIV-1/HIV-2 EIA (Vironostika® HIV Uni-form II plus 0; Organon Teknika, Wellcozyme® HIV1+2; Murex) and PA (Serodia® – HIV, Fujirebio Inc.) test using commercial kits according to the manufacturer's instructions. If sera positive by HIV1/2 EIA were positive also with HIV-1 PA, they were retested with a HIV-1 Western blot kit (Cambridge Biotech Corp.). The sera positive by HIV-2 PA were retested with a HIV-2 Western blot kit (New Lav Blot II; Sanofi Diagnostic Pasteur). The five HIV-2-positive sera were also tested with (1) a HIV-1/2 Western blot kit (Gene Lab) based on a synthetic peptide from HIV-2 glycoprotein (gp 36) as well as on whole HIV-1 lysates, and (2) by a LIA (PEPTI-LAV 1, 2; Sanofi Diagnostic Pasteur) based on synthetic peptides from a HIV-1 transmembrane protein (gp41) and a HIV-2 transmembrane protein (gp36).

Table 2. Epidemiological history of subjects infected with HIV-2

Subject	Year of HIV-2 detection	Age[a]/ Sex	Occupation	Most likely place of infection	Probable mode of transmission	Disease status
KR8092	'98	19/M	Unknown	Korea	Homosexual contact	Asymptomatic
KR7051	'97	54/M	Sailor	Las Palmas de Gran Canaria	Heterosexual contact with foreign prostitute	Oral candidiasis, herpes zoster rash
KR4075	'94	39/F	Housewife	Korea	Heterosexual contact	Asymptomatic
KR4063	'94	46/M	Sailor	Senegal	Heterosexual contact with foreign prostitute	Asymptomatic
KR1010	'91	28/M	Sailor	Las Palmas de Gran Canaria	Heterosexual contact with foreign prostitute	Asymptomatic

[a]Years.

Table 3. Serological profiles of the sera from HIV-2-infected subjects

Subject	HIV1/2		HIV-1		HIV-2	
	EIA[a]	LIA(HIV1/HIV2)	PA test	Western blot analysis	PA test (titer)	Western blot analysis
KR8091	>6.41	ND/++	–	(gp160),(p31),p24	++ (32,768)	gp140,105,36,p68,56,26,16
KR7051	>7.53	ND/++	–	(gp160)	++ (524,288)	gp140,105,(p68,56),26,16
KR4075	>5.88	± / ++	–	(gp160),p31,24	++ (131,072)	gp140,105,36,p68,56,26,16
KR4063	>9.19	± / ++	±	gp160,(p66,51),p31,55,24	++ (262,144)	gp140,105,36,p68,56,26,16
KR1010	1.41[b]	± / ++	–	gp160,p66,51,31,p55,24	++ (16,384)	gp140,105,36,p68,56,26,16

[a]Absorbancy of sample/cut off absorbancy.
[b]Indirect HIV-1 EIA.

RNA extraction from plasma and RT-PCR. Total RNA was extracted from 250 µl aliquots of plasma with Isogen-LS, an acidic guanidinum-thiocyananate-based reagent (Molecular Research Center Inc.) according to a modification of the method of Nakamura *et al.* (1993). RNA was reverse transcribed in a 20 µl reaction mixture containing 5 U of reverse transcriptase, 1.3 U of RNase inhibitor (Takara Shuzo), 0.2 mmol/l dNTPs, 10 x PCR buffer, 0.01 mol/l dithiothreitol (DTT), and 1 µmol/l antisense primer at 42°C for 60 mins and at 99°C for 5 mins. The obtained cDNA (10 µl) was amplified in a 40 µl reaction mixture containing 10 x PCR buffer, 0.2 mmol/l dNTPs, 1.5 µmol/l sense primer, 0.5 µmol/l antisense primer, and 1.5 U of Taq polymerase (Boehringer Mannheim) according to the nested PCR conditions. The sense and antisense primers are described in Table 1.

DNA extraction from peripheral blood mononuclear cells (PBMCs) and PCR. DNA was extracted from uncultured patients PBMCs with a nucleic acid extraction kit (IsoQuick, ORCA Research Inc). PCR was carried out in a total volume of 50 µl containing 1 µg of genomic DNA, 5 µl of 10 x PCR buffer, 0.2 mmol/l dNTPs, 0.3 mmol/l $MgCl_2$, 1.5 µmol/l primers, and 2.5 U of Taq polymerase (Boehringer Mannheim) using a DNA Thermal Cycler (Perkin Elmer Cetus 2400). The amplification cycles are described in Table 1. The subsequent nested PCR (25 cycles) was carried out under the same conditions as the first PCR with 2 µl of the first PCR product in a volume of 50 µl. DNA isolated from PBMCs of healthy blood donors was used as a control. Nested PCR products were analyzed on a 3.5% or 1% GTG agarose gel after ethidium bromide staining.

DNA sequencing and phylogenetic analysis. Nested PCR products were cloned into pCR® 2.1 Topo-vectors (Invitrogen) by T/A overhang. Recombinant clones containing HIV-2 fragments were purified with 20% polyethylene glycol 8000 and 2.5 mol/l NaCl and sequenced using fluorescent-labeled terminators (ABI Prism 377 DNA sequencer, Applied Biosystems). One or two clones per PCR product were sequenced for the 197 bp *gag* fragment and at least two clones were sequenced for the 1191 bp *env* fragment. Phylogenetic relationships of three South Korean isolates to previously published HIV-2 strains were estimated from comparisons of their nucleotide sequences. Sequences were aligned using Clustal W. MegAline (Dnastar Inc.). Phylogenetic relationships were computed from the distances by the neighbor-joining method.

Results

Epidemiological and clinical characteristics of HIV-2 infected subjects

Epidemiological and clinical characteristics of the five HIV-2 infected subjects are shown in Table 2. Three males, sailors, were most likely infected with the virus by heterosexual contact in regions of western Africa such as Senegal and Las Palmas de Gran Canaria. The fourth male (KR8092) very likely contracted HIV-2 from KR7051 through homosexual contact. The only female was a wife of KR4063. Four subjects were healthy at the time of the diagnosis of HIV infection, while one had symptoms such as oral candidiasis and herpes zoster rash.

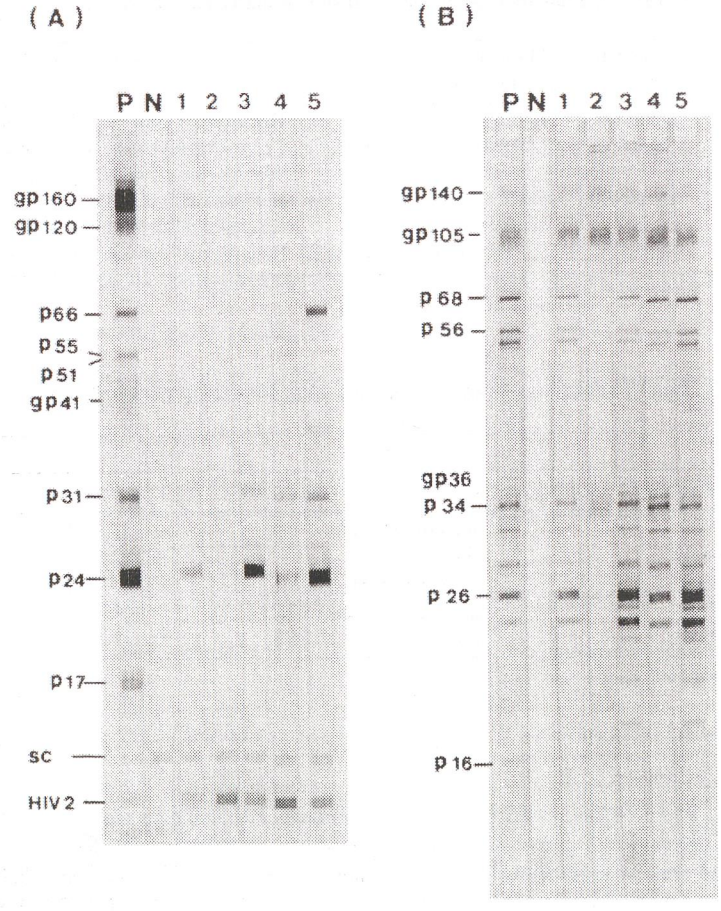

Fig. 1
Western blot profiles of sera from five individuals infected with HIV-2
Serum samples from subjects KR8091 (lane 1), KR7051 (lane 2), KR4075 (lane 3), KR4063 (lane 4), and KR1010 (lane 5) were analyzed for seroreactivity with HIV-1 antigen (A) and HIV-2 antigen (B). P and N, positive and negative controls, respectively.

Analysis of serological profiles in HIV-2 infected subjects

Table 3 shows serological profiles for the five HIV-2-infected subjects which were diagnosed as positive by HIV-1/2 EIA, HIV-2 PA test and HIV-2 Western blot analysis. All the five sera were positive with titers over 1:16,384 in the HIV-2 PA test, while they were negative or indeterminate in the HIV-1 PA test and indeterminate in the HIV-1 Western blot analysis. Fig. 1 shows results of the Western blot analysis for HIV-1 and HIV-2 antigens. Three sera (KR4075, KR4063, and KR1010) showed a strong reactivity with synthetic HIV-2 gp36 in LIA but a weak or no reactivity with synthetic HIV-1 gp41. One serum (KR1010) displayed a weak signal in indirect HIV-1/2 EIA and was repeatedly negative in competitive HIV-1 EIA.

Detection of HIV-2 infection by PCR

The nested PCR was used to characterize viral sequences in PBMC DNA from four subjects. As no PBMCs were obtained in the case of KR8091, we performed the nested PCR with cDNA obtained by RT-PCR. PBMC DNA and cDNA were amplified with the *gag* and *env* primer pairs known to detect HIV-2 sequences. However, the diagnostic HIV-2 *gag* primer pair failed to amplify viral sequences in samples from two subjects (KR4075 and KR1010). Also, these samples did not yield amplification signal with the *env* primer pair despite repeated attempts and various conditions for PCR. Therefore, in these two cases, we tried to amplify the *gag* and *env* region by RT-PCR from fresh plasma samples. However, PCR signals were obtained for the *env* region only. Interestingly, these two samples originated from the individuals with more than 500 CD4+

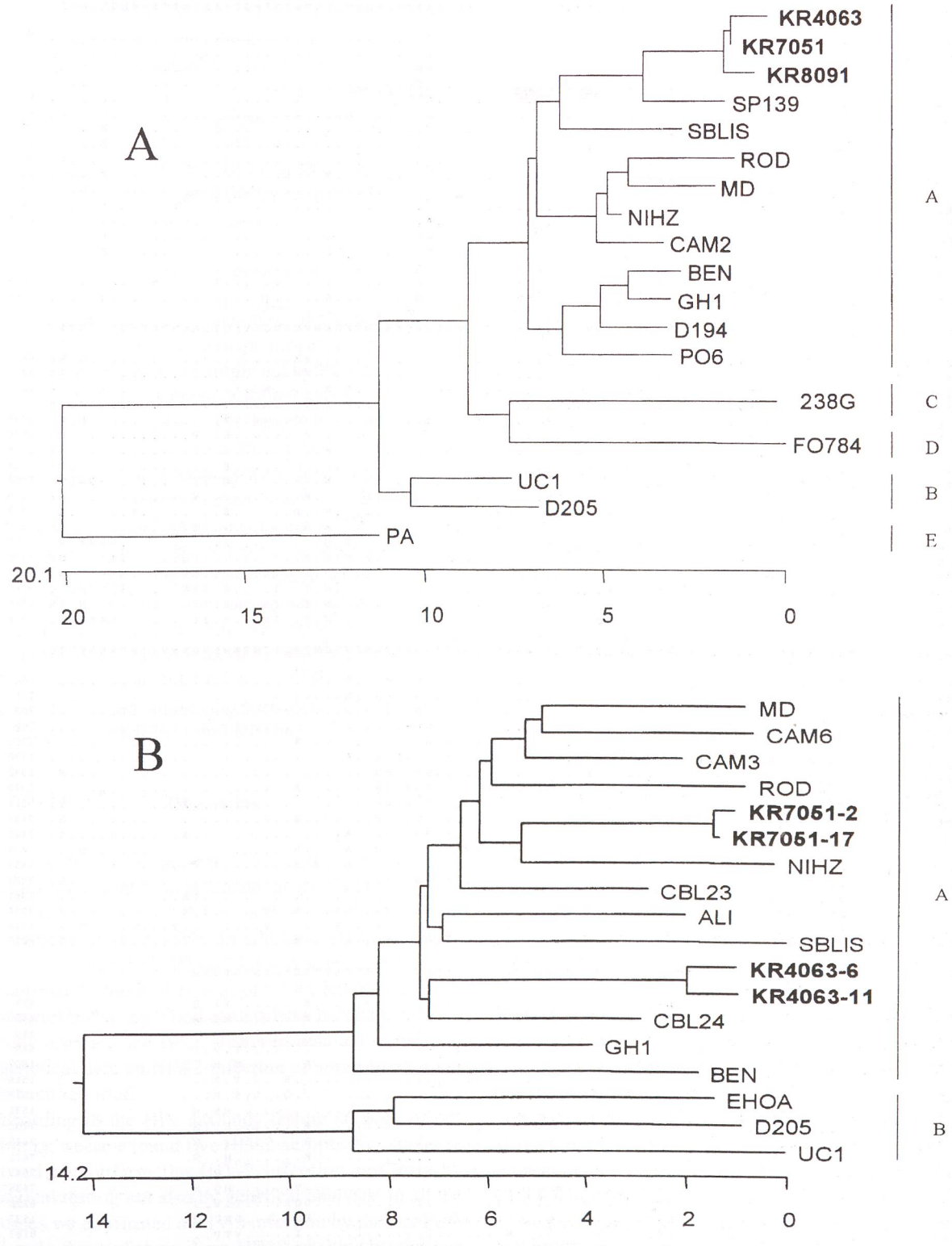

Fig. 2

Phylogenetic analysis based on sequences of parts of gag (A) and env (B) genes of HIV-2 isolates from South Korea (bold shaped) and those of reference HIV-2 strains obtained by blast search

In the *gag* tree, five HIV-2 subtypes (A,B,C,D, E) and in the *env* tree, two HIV-2 subtypes were available. The length of horizontal lines connecting one sequence to another is proportional to the divergence between the sequences.

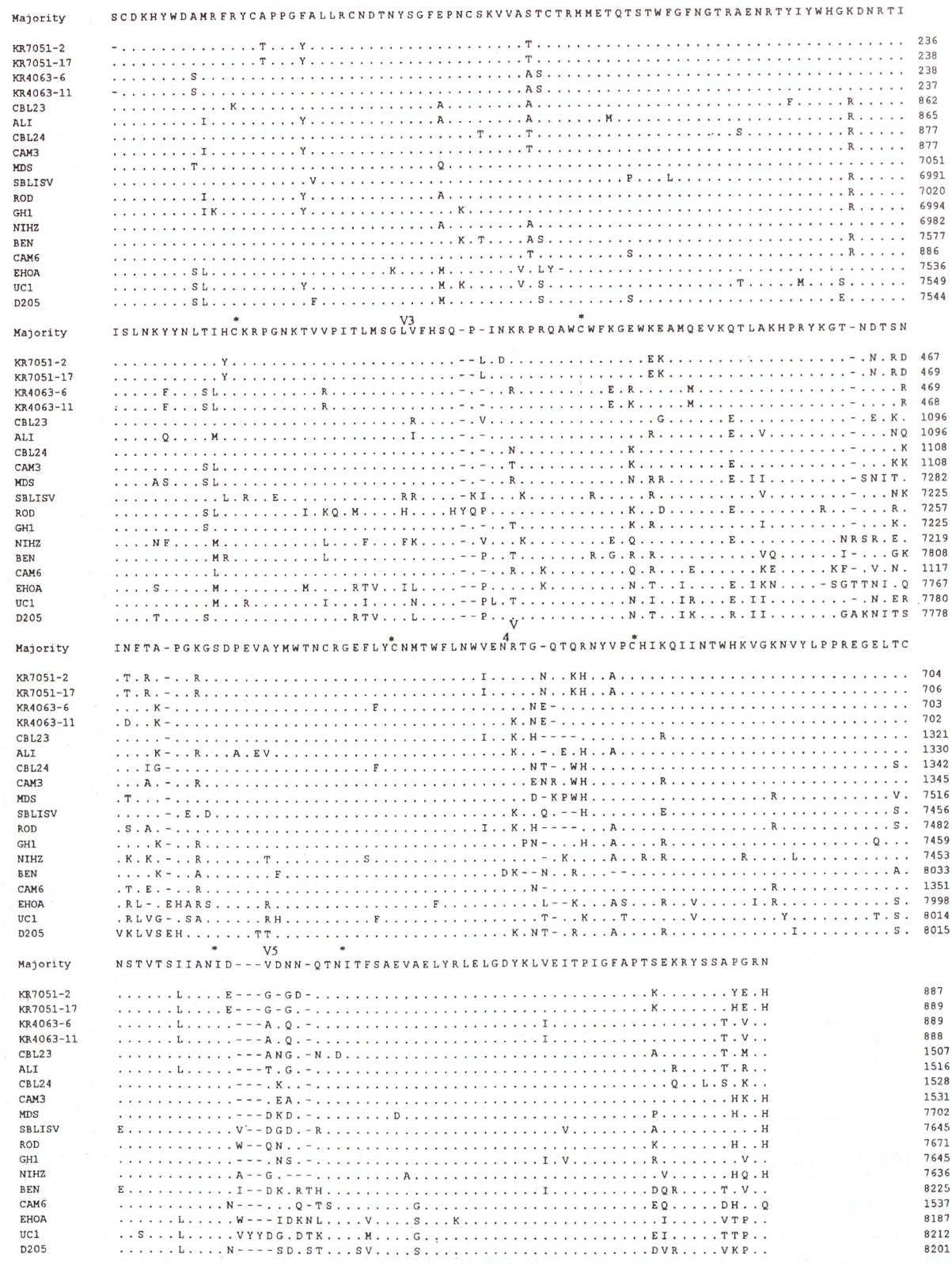

Fig. 3

Amino acid sequences of the HIV-2 C2-C5 regions obtained from two subjects infected with HIV-2 of A subtype

The dots represent amino acids identical to the consensus and the gaps introduced to the alignment are indicated by dashes.

cells/mm³. Finally, PCR signals were obtained with three of the five samples with the HIV-2-specific *gag* primer pair and with all the five samples with the *env* primer pair in PCR or RT-PCR. Table 4 shows the detection of HIV-2 infection by nested PCR in the five subjects. To investigate possible dual infection in those subjects with confirmed HIV-2 infection, we tried to amplify the 1191 bp *env* HIV-1 region (V1-V5 of gp120) from four PBMCs samples but we could not detect any amplification signal.

HIV-2 sequencing and phylogenetic analysis

To investigate the HIV-2 genotype of the isolates from South Korea and to perform a more comprehensive phylogenetical analysis, we amplified by PCR the 1191 bp *env* region. Only two isolates (KR4063 and KR7051) gave a positive result. These two 1191 bp *env* fragments and the three 197 bp *gag* fragments mentioned before were cloned and sequenced. Most sequences were derived from one or two clones. The sequences of each isolate were aligned with those of the corresponding region of various HIV-2 subtypes in the database and phylogenetic relationships were analyzed. As shown in Fig. 2, the isolates KR4063, KR7051, and KR8091 clustered within the known subtype A based on *gag* and *env* alignments. The nucleotide variation of the examined HIV-2 isolates was of 12.8% to 13.1% for the 1191 bp *env* region and of 1.0% to 2.1% for the 197 bp *gag* region. Fig. 3 shows amino acid sequences of HIV-2 gp105 C2-C5 regions of KR7051 and KR4063.

Table 4. Detection of HIV-2 infection by nested PCR

Subjects	HIV-1	HIV-2			CD4 + T cell count[c] (cells/mm³)
	env (1.2 kbp)	*gag* (197 bp)	*env* (137 bp)	*env* (1191 bp)	
KR8091	ND	+++[b]	+++[b]	ND	ND
KR7051	–	+++[a]	+++[a]	+++	121
KR4075	–	–	+++[b]	–	643
KR4063	–	+++[a]	+++[a]	+++	423
KR1010	–	–	++[b]	–	885

[a]Signal intensity of PCR product.
[b]Signal intensity of RT-PCR product.
[c]CD4+ T cell count at that time of PCR.
ND = not done.

Discussion

It is essential for the establishment of HIV testing strategies for a country to elucidate the extent of HIV-1 and HIV-2 infection. In South Korea, a HIV-1 antibody test has been performed since 1985. In addition, HIV-2 antibody testing by a combined HIV-1/2 kit was started in 1990 by the National Institute of Health of South Korea and many laboratories including blood centers have performed HIV-2 antibody testing since 1992. However, detailed serological and genetical data on HIV-2 infection in South Korea have never been reported.

According to the HIV antibody testing strategy of our laboratory, we have found five HIV-2-seropositive subjects. We tried to confirm the HIV-2 infection not only by serological testing but also by genetical analysis. In all the five cases we confirmed a HIV-2 infection by the *env* PCR while only three of them were HIV-2-positive by the *gag* PCR. Earlier studies have shown that it was difficult to isolate HIV-2 from asymptomatic subjects because the proviral load could be very low (Berry *et al.*, 1994; Gao *et al.*, 1994; van der Ende *et al.*, 1996). A nested PCR can be repeatedly negative in some of HIV-2 patients (Damond *et al.*, 1998). Simon (1993) has reported that positive rate of cell and plasma virus content was related to the number of CD4+ cells because HIV-2 viral concentration correlated inversely with the number of CD4+ cells. Further study should be undertaken to elucidate whether the absence of signal in the *gag* region observed by us with the two isolates was related to CD4+ lymphocyte numbers or resulted from genetic variation of HIV-2. The two isolates, KR1010 and KR4075 that failed to produce *gag* PCR products were obtained form healthy patients at asymptomatic clinical stage as one had the CD4+T lymphocyte count of 885/mm³ and the other had that of 643/mm³ based on immunological tests at the time of blood sample collection. Therefore, low viral loads at the asymptomatic stage may lead to failure of PCR.

On the other hand, Ishikawa *et al.* (1998) regarded low HIV-2-positive rates as genetic variation of HIV-2. They confirmed 58 specimens from 68 persons as HIV-2-positive with HIV-2 primers of various nucleotide sequences and enhanced sensitivity by doubling the number of PBMCs for HIV-2 detection from individuals with HIV1/2 dual seroreactivity. However, additional factors should be considered to explain the lower rate of HIV-2 detection compared to that of HIV-1 despite similar genetic variation in *env* and *gag* regions of these viruses (Chen *et al.*, 1997; Gao *et al.*, 1994). In the case of HIV-1 *env* gene, the sensitivity of HIV-1 PCR was up to 100% in our laboratory even though the DNA samples were obtained from PBMCs of asymptomatic patients (data not shown). These data suggest that the low HIV-2 positivity by PCR is due to low viral load rather than high genetic variation.

Numbers of subjects evidenced as infected with HIV-2 in South Korea are one in 1991, two in 1994, one in 1997, and one in 1998. Two of these total five subjects were surveyed as wives or sexual partners of an HIV-2 infected person. Introduction of HIV-2 into South Korea started presumably in early 1990s. Presently, the number of HIV-2-

infected subjects is extremely small compared with 959 HIV-1-infected ones. Leonard *et al.* (1993) have suggested that the combination of serological and PCR tests is required for demonstration of the presence of HIV-2 DNA in dually seroreactive patients. Detection of more HIV-2 infections could be possible by investigating specimens showing positive reaction in both HIV-1 and HIV-2 PA test as well as more than two *env* proteins in HIV-2 Western blot analysis. In addition, the identification of subjects with HIV-2 demonstrates the necessity of including a HIV-2 test in HIV screening in South Korea especially in blood centers and quarantine stations.

Three South Korean HIV-2 isolates were classified as subtype A. Presumably these results are related to the epidemiological history of South Korea with HIV-2 of predominantly A subtype.

In conclusion, HIV-2 has been transmitted into South Korea before 1991 and the newly HIV-2-infected persons have appeared continuously. Therefore, the diagnosis of HIV infection should include the possibility of HIV-1 as well as HIV-2 based on the present transmission situation. Furthermore, our data obtained by genetic analysis of HIV-2 isolates suggest that further study is necessary for the improvement of HIV-2 diagnosis by PCR and development of HIV vaccines.

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