

Molecular Biology Confirmation and Analysis of Suspected Transfusion-Associated HIV Cases

Abstract

“Blood, a gift for life, thank you.” – is a slogan used to encourage people to donate their blood enthusiastically to help those in need. However, in recent years, some individuals were found to be using blood donation as an opportunistic way to establish if they were HIV infected. If the donors’ blood is in the window period for HIV detection, it would be tragic for recipients to receive this blood, which might lead to transfusion infection. This year (2004), three suspected blood transfusion-associated HIV cases were registered in a research project to investigate the possibility of such transmission. Blood samples were collected from both donors and recipients of each case. Samples were examined using molecular biology techniques in our virology laboratory, the Division of Laboratory Research and Development, CDC, Taiwan. First, HIV nucleic acids were extracted from blood samples. Then, Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Nest Polymerase Chain Reaction (Nest PCR) techniques were applied with specific primers targeting the envelope gene (*env*) and the polymerase gene (*pol*) in the HIV genome. Subsequently, amplification

products were sequenced. Relations between transfusing donors and recipients were determined by sequence similarity and homology.

Introduction

HIV (human immunodeficiency virus), the so-called AIDS virus, is the primary cause of the acquired immunodeficiency syndrome (AIDS). Since the initial description of the human immunodeficiency virus in 1983, the number of HIV-1 infected individuals has increased dramatically.[1,2] According to WHO (World Health Organization) estimates, approximately 74.9 million people were infected with HIV by the end of 2003, and about 30 million people died of AIDS.

HIV belongs to the Lentivirus subgroup of the Retroviridae virus family. Using electron-microscopic estimation, the size of the HIV particle is around 110 nm. Essentially, there are two main parts: the inner core, and the viral membrane. The viral membrane encloses the particle, and has glycoproteins embedded in it. [3] The inner core contains the viral RNA, enzymes such as reverse transcriptase, integrase, viral protease, and regulatory proteins for virus replication. The full HIV genome is encoded on one long strand of RNA, 9.2 Kb in length, and carries nine genes. By comparing difference in the genomic sequence, there are two types of HIV viruses defined, HIV-1 and HIV-2. The two viruses were observed in Eastern Africa and West Africa, respectively. The serologic patterns of the two viruses are distinct from each other. HIV-2 is more like certain types of simian immunodeficiency virus (SIV), while HIV-1 is more like the immunodeficiency virus discovered in chimpanzees. HIV-1 can be classified into three groups according to sequence diversion: M group, O group, N group. [4] "M" is the main group of viruses associated with the HIV-1 global pandemic, and it contains 11 subtypes (subtype

A to K) [17] according to their difference in env gene sequence. The diversity among these subtypes is over 20%. [5,6] O is the "outlier" group, and group N is sometimes referred to as the "new" group. Subtypes are not yet defined in group O or N.

HIV is transmitted primarily through the exchange of blood and body fluids. Recommendations from WHO and CDC (Center for Disease Control and Prevention, USA) were applied in our laboratory confirmation for HIV infection. A series of screening tests such as ELISA, PA, etc., followed by confirmation tests such as Western Blot were performed. Definition of an HIV confirmed case is two positive results in screening tests and followed by a positive result in a confirmation test. These methods are based on detecting the presence of antibodies to HIV in human blood. Since HIV antibodies generally do not reach detectable levels early on in the infection, there will be a so-called window period when HIV cannot be diagnosed. This article indicates that recipients were infected with HIV by receiving donors' blood, which was still in the window period for detection.

Materials and Methods

Samples Collection and Cases Background

Case 1:

In May 2004, our lab received a blood sample from a male (Mr. Wang), in his thirties, who suffered from chronic illness. In this case, the patient's past history included surgery and blood transfusion. Recently, his infection with HIV was confirmed. By tracing the blood transfusion history, a 20-year-old donor (Mr. Lin) was discovered, who was found to have HIV serum antibodies three months after donating his blood. Therefore, we suspected that this was a case of transfusion

infection.

Case 2:

A 50-year-old female (Mrs. Yang) with a simple lifestyle, sought medical treatment because of her suspicious symptoms. A test for HIV infection was ordered, and the lab data showed a positive HIV viral antibody test. Later, a specimen of her husband's blood was also confirmed seropositive. The doctor suspected that this patient was infected through transfusion. An investigation of her transfusion records revealed that Mrs. Yang once had received blood components from a donor (Mr.Yu) who was later identified to be HIV seropositive. Blood samples from Mr. And Mrs. Yang and the donor were collected and transported to our lab for further analysis.

Case 3:

A military officer (Mr. A) in his thirties tested positive for HIV serum antibody in a hospital laboratory in September, 2004. His sexual partner was also confirmed to have HIV infection in August. According to his blood donation record, the last blood donation was in May 2004. His blood components were given to three recipients, two of whom died; one survived, but became HIV infected. The latter patient was a female (Mrs. M) in her sixties, who was diagnosed seropositive in September. This appeared to indicate a transfusion infection, and the advanced laboratory confirmation analysis would be performed in October.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) [3]

1. Reverse Transcription

The reaction solution was composed of 10 µl of viral RNA, 75mM KCl, 50 mM Tris-HCl, 3 mM MgCl₂, 10 mM DTT, 0.5 mM dNTP mixture, 0.5 of

Rnasin (38U/ μ l), and 50 pmole of antisense primers (including primer 35R, primer 1-R-RT21, and primer R-RR-probe). These primer sequences are available in (Table 1) and (Table 2). Conditions for the cDNA transcription are as follows: the primer/RNA template mix is thermally denatured at 70°C for 10 minutes, and 100 units of MMLV-reverse transcriptase (Promega, Cat #M1701) was added to the reaction tube. Incubation at 37 °C for 90 minutes allowed cDNA synthesis.

2. PCR

(a) First round PCR

cDNA obtained from reverse transcription was used as template. Specific primers were applied, which targeted env gene (primers 44F/35R) and pol gene (primers 1-F-RT18/1-RRT21, primers F-MAW26/R-RR-probe) (Table 1) (Table 2). Template was added to the reaction tube containing 50 mM KCl, 10 mM Tris-HCl, 1.5 mM MgCl₂, 0.1% Triton-X 100, 1mM dNTP mixture, specific primers (50 pmole of each) and 5 units of Taq polymerase (Invitrogen). Reaction was carried out under the following conditions: initial denaturing at 94°C for three minutes, then, denaturing at 94°C for one minute, annealing at 48°C for one minute, extension at 72°C for two minutes, for 35 cycles, and finally an additional fifteen minutes at 72°C.

(b) Nest-PCR

Nest- PCR was performed with 5 μ l of previous PCR product. Reaction mixtures consisted of 50 mM KCl, 10 mM Tris-HCl, 1.5 mM MgCl₂, 0.1% Triton-X 100, 1 mM dNTP mixture, 5 units of Taq polymerase (Invitrogen) and primers 33F/48R, primers 2-F-RT19/2-R-RT20 for 50 pmole of each (Table 1) (Table 2). The thermo setting was: 94°C for 3 minutes in advance, 35 cycles of 94°C for 1 minute, 52°C for 1 minute, 72°C for 2 minutes and a plus 15 minutes at

72°C.

Sequencing

Purified nested PCR products ($OD_{260/280} > 1.8$) from selected PCR amplicons were used for automated sequencing with ABI Prism^(TM) BigDye^(TM) Terminator Cycle Sequencing Ready Reaction Kit 108 (Applied Biosystems). To each sample, an adequate amount of nucleic acid, 3 µl of premix (including Tris-HCl buffer, MgCl₂ in pH 9.0, dNTP mixture, labeled A-dye terminator, C-dye terminator, G-dye terminator, T-dye terminator, AmpliTag DNA polymerase FS with thermally stable pyrophosphatase) were added, as well as 3.2 to 5.0 pmole of primers (primers Nest-PR-F/Nest-PR-R, primers 33F/48R, primers 2-R-RT20/2-F-RT19) used for the PCR (Table 1) (Table 2). The final volume of 10 µl was achieved by adding water, and the solution was overlaid with paraffin oil. The conditions for the sequencing reactions were 25 cycles of 94°C for 30 sec, 55°C for 15 sec, and 60°C for 4 min, stopped at 4°C. Sequencing was then run in a DNA sequencer.

Percentage of Sequence Similarity (Similarity %)

Sequences were aligned by using multiple-and-pairwise sequence alignment software, MegAlign, DNASTAR, Inc.. Jotun Hein Method and Clustal Method were applied. Similarity analysis was determined by the genetic distance within 345 bp fragment of C2V3 region in env gene, 348 bp fragment of RR region in pol gene, and 967 bp fragment of RT region in pol gene, respectively.

Phylogenetic Tree Analysis [6,7]

Sequences of all three cases (donors and recipients) along with reference sequences from subtype HIV strains: TW20, TW71, TW115, TW8637, W112,

TW8623, HIVMN, HIVSF2, TW78, THAI-B, TW8602, TW8610, TW31, HIVJY1, IVNDK, W61, TW8616, THAI-E, TW8604, TW8629, TW98, TW8621, IVD747, HIVNOF, BV217, I525A, HIVKENY, HIVZ321, HIVB7944, VI557, HIVMP51, HIVAN70C were analyzed. Phylogenetic trees were constructed by the neighbor-joining method with Molecular Evolutionary Genetics Analysis (MEGA) version 2.1, and bootstrap analysis 1000 times.

Results

Samples from the donor (Mr. Lin) and recipient (Mr. Wang) in case 1 were both used to extract viral RNA, and molecular biology techniques were applied with specific primers targeting envelope gene (*env*) and polymerase gene (*pol*) in the viral genome. Sequences were obtained and compared with Stanford HIV database in subtype sequence homology. Data showed that the isolated genome had high homology to subtype B and Australia SM17e virus strain (data not shown). Therefore, sequences of C2V3 region, RR region, and RT region were subsequently aligned by use of MegAlign software. The percentages of similarity were 99.6% in the C2V3 region, 99.4% in the PR region and 99.6% in the RT region (Fig. 1) (Fig. 2). Phylogenetic analysis of *pol* gene sequences showed that these two strains were clustered with B subtype HIV reference strains. The bootstrap value was 100, which indicated a close relationship between donor and recipient strains. Taken together, HIV genome of donor and recipient were highly homologous, over 99%, and phylogenetic analysis suggested the same lineage of virus strains.

In case 2, genomic sequences of the donor (Mr. Yu), the recipient (Mrs. Yang) and Mrs. Yang's husband were obtained by use of molecular biology techniques as mentioned above. Sequences of envelope gene (*env*) and polymerase gene (*pol*)

were compared with Stanford HIV database in subtype sequence homology. Data showed that sequences of the infected married couple were highly homologous to subtype A/E and 01TH.OUR7881 (Thailand); on the contrary, the sequence of the donor was homologous to subtype B and 271767WKO (USA) virus strains (data not shown). By comparison, similarity in pol gene sequences of Mr. and Mrs. Yang was 98.6%, while comparing with the donor sequence it was merely 88.7% and 89.4%, respectively. The phylogenetic tree in (Fig.4) showed that isolated strains of Mr. And Mrs. Yang were clustered together with A/E subtype reference strains, and the bootstrap value was 93 (Fig. 5). Meanwhile, the donor strain was clustered with virus subtype B. As a result, a positive association linked HIV infected husband and wife, and apparently not the donor.

The donor (later a patient) in case 3 was a military officer (Mr. A) who was confirmed HIV infected. In addition, his sexual partner was also found to be seropositive in August. According to donation records, his blood components were given to three recipients, two of whom died; and one 60 year old female (Mrs. M) survived, but was diagnosed HIV seropositive in September. Samples were collected from Mr. A and Mrs. M, and advanced laboratory conformation analysis was performed. Specific primers were designed to target envelope gene (env) and polymerase gene (pol). Sequences were obtained and compared with NCBI and Stanford HIV database in subtype sequence homology. Isolated viral genome had a high homology to subtype B and p3c080-10 (USA) virus strain (data not shown). When comparing env gene and pol gene sequences, there was a similarity of 99.4% in the C2V3 region and 96.9% in the PR region (Fig. 6). A cluster of virus strains isolated from Mr. A and Mrs. M was observed in subtype B lineage, and the bootstrap value was 100.

Discussion

This year (2004), we applied molecular biology techniques on three suspected transfusion-associated cases. Sequences similarity and phylogenetic analysis of viral nucleic acids indicated that the case 1 recipient and case 3 recipient were possibly infected through transfusion. The sum total of HIV cases infected through transfusion since 1988 is 14. However, in case 2, a low degree in sequence similarity, and difference in subtype classification indicated a loose relationship between donor and recipient couple.

The immune system protects the body from organisms causing invasive disease. Natural immune responses including inflammation and phagocytosis, act immediately or within hours of a pathogen's appearance in the body. Once the body's intrinsic immune cells (Antigen Presenting Cell, APC) recognize foreign materials (virus or bacteria), they stimulate a specialized group of white blood cells that causes CD4⁺ helper T cells to become activated. With cytokine secretion, helper T cells can stimulate B cells to produce antibodies that bind specific antigens and immobilize them, preventing them from causing infection. This is called "acquired immunity". [8-10] The infectious window period defines the time period when an individual is both infected and infectious prior to sero-conversion, which could be six to twelve weeks for HIV infection. [11] In Taiwan, the diagnostic method for HIV in blood components detects the presence of serum antibody. Using the current method, the window period between HIV infection and its detection by laboratory methods poses a challenge for diagnosis. In this study, it is possible that recipients in case 1 and case 3 were infected through receiving donated blood, which was in the window period for detection.

In addition, individuals who use blood donation as a way to establish if they are HIV infected, pose an unpredictable threat to the public. To ensure the health

of the population, guidelines are set when donating blood. Donors may not have certain designated diseases; persons in the high risk groups for HBV, HCV, and HIV infections are not allowed to donate blood. In addition, we also set up a telephone line for donors who are aware that they may possibly be infected or suspect they may be HIV infected. With their voluntary feedback, transfusion infection cases can be further reduced.

Serum samples were collected for analysis; however, the particles of RNA virus such as HIV were not stable in the environmental conditions of the collection samples. Instability of viral RNA quality had some impact on nucleic acid extraction and RT-PCR experiments. In our cases, when amplifying C2V3, PR, and RR regions with three pairs of specific primers, not all fragments were amplified in every specimen. This is possibly due to unstable viral RNA or differences in their nucleic sequences. Hence, proviral DNA extracted from PBMCs (Peripheral Blood Mononuclear Cells) was recommended for HIV analysis, because DNA is more stable than RNA, and DNA replication without retro-transcription produces less sequence diversity, which may reduce errors in comparison sequences from donor strains and recipient strains. Moreover, applications of two-step PCR and Nest-PCR techniques can greatly enhance PCR sensitivity in detecting clinical specimens.

Phylogenetic analysis is a powerful tool in many areas such as molecular biology, epidemiology, and even in criminology. Using software computation and statistical analysis with different parameters, evolution distances can be calculated and determined among nucleic acid or amino acid sequences. In case 1 and case 3, phylogenetic analysis of env gene and pol gene sequences showed donor-recipient strains with a 98% similarity, and were clustered within same subtype lineage. The bootstrap value was close to 100, which indicated an evolutionary correlation.

However, in case 2, different subtype lineages were observed between Mrs. And Mr. Yang (subtype A/E) and the donor (Mr. Yu) (subtype B). Difference of pol gene sequences among different HIV subtypes is 10 to 15%. Thus, we may theorize a loose association between the donor and the recipient couple. Though whether Mr. and Mrs. Yang were definitely infected through receiving (donated) blood could not be ruled out.

In order to prevent HIV transfusion infections, people should have a clear concept of blood donation through continuous education, and should not use donation as a way to test for HIV infection. In addition, improved methods to detect HIV in the window period need to be established. Today, many countries apply molecular methods called NAT (Nucleic Acid Amplification Testing) [12] to detect directly HIV nucleic acids, and to perform qualitative and quantitative experiments. By utilizing RT-PCR, Nest-PCR techniques initially, in addition to the Real-time PCR technique currently used, which detects viral nucleic acids with fluorescence dyes (SYBR Green I) and fluorescence-labeled probe (TaqMan probe or Hybridization probe), or even using the Multiplex RT-PCT technique [13,14], which increases its sensitivity and specificity by adding several primer pairs in a single reaction tube, the methods of testing efficiently and accurately are increased. For instance, the window period for HIV detection can thereby be shortened from 21 days to 11 days; for HBV detection, from 59 days to 34 days; for HCV detection, from 82 days to 23 days. Also, false negative results of serum antibody tests can be reduced. [15] To ensure a safe blood supply, early in 1999, the AABB (American Association of Blood Banks) evaluated the feasibility of the NAT process. [12] Since July 1999, NAT was used to screen for HBV, HCV, and HIV in donated blood at a Red Cross Blood transfusion center in Japan. [15] Later, a transfusion center in France tested donated blood with NAT in July 2004. [16]

Evaluation reports showed that it is very positive improvement to apply NAT as a clinical screening procedure. Test reports would be available within one day, and may be very helpful in reducing transfusion infections. To our concern, the growth of the HIV infected population and a trend toward the infection of younger people was observed in Taiwan. Statistical data showed a total of 6,635 people infected with HIV up until September 2004. Thus, to lower the HIV incidence and prevalence, the best way to reduce the risk of contracting the HIV virus is to practice safer sex. Further, the use of NAT on blood and blood components will reduce the possibility of virus transmission through transfusion.

A method of direct detection of viral nucleic acids antigen is being developed to be used routinely in donated blood testing. Currently, the Department of Health, Executive Yuan, Taiwan, R.O.C. and Chinese Blood Donation Association are revising diagnostic procedures to ensure better safety. Nonetheless, individuals should not donate blood if they do not practice safe sex or if they have sexually transmitted diseases, or could be in the HIV window period. Meanwhile, the Department of Health has already established several screening stations at ten hospitals, including the National Taiwan University Hospital, National Cheng Kung University Hospital, Taipei Veterans General Hospital, Kaohsiung Medical University Hospital, Tri-Service General Hospital, Kaohsiung Veterans General Hospital, Chang Gung Memorial Hospital, Health Bureau Taoyuan General Hospital, Taichung Veterans General Hospital, and Chi-Mei Hospital. Quick results, services free of charge and anonymous HIV testing are provided at these stations for people who suspect themselves to be infected with HIV. The main purpose of these initiatives is to reduce HIV infection through transfusion, and keep the population free from the risk of HIV.

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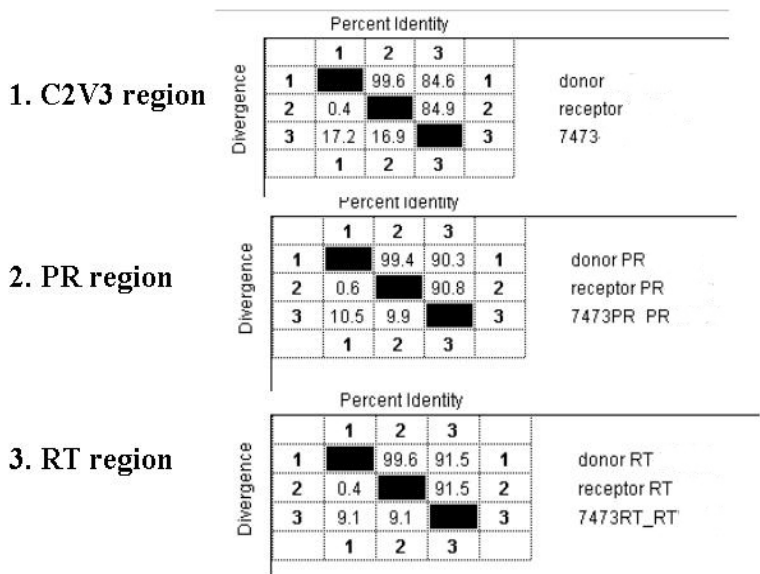
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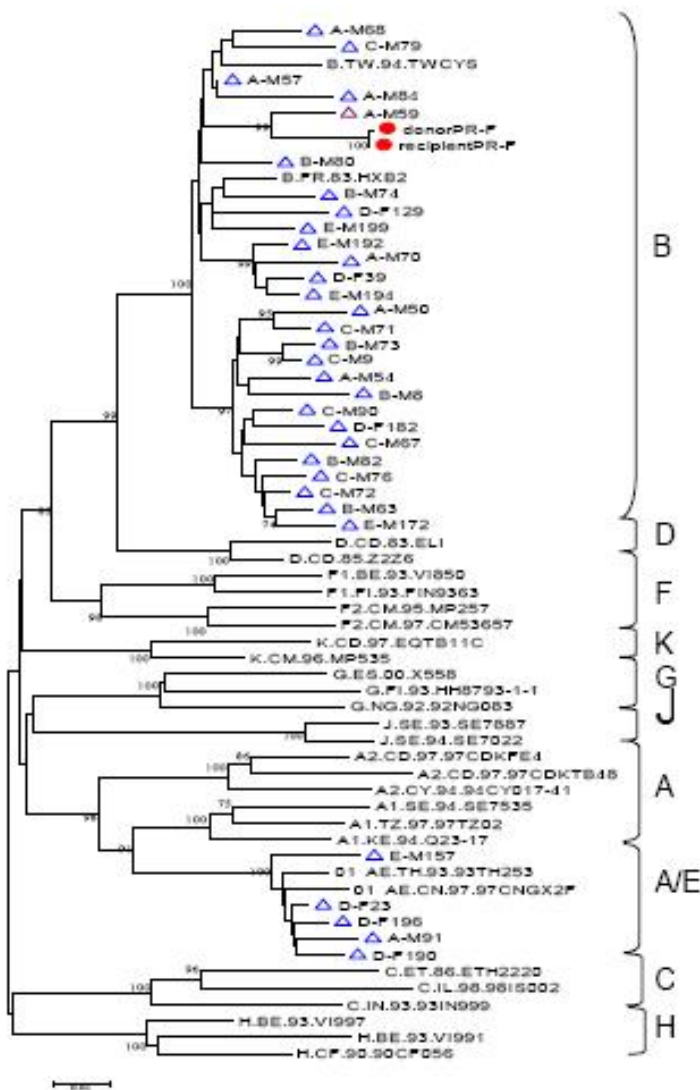


Fig. 1. Alignment of C2V3 region (env gene) sequences derived from infected donor and recipient in case 1.



(7473 is the HIV-1 positive control strain.)

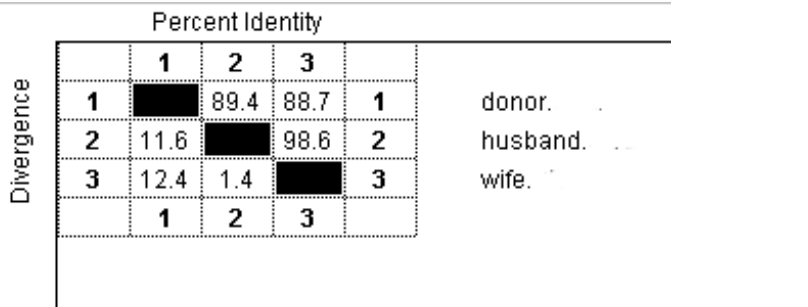
Fig. 2. Sequence similarity and genomic diversity analysis of three different regions in env gene and pol gene derived from infected donor and recipient in case 1.



(△represents the HIV reference strains in Taiwan; the sequences data were obtained from Taipei Municipal Venereal Disease Control Center)

Fig. 3. Phylogenetic trees of *pol* gene sequences derived from infected donor and recipient in case 1.

(1).



(2).

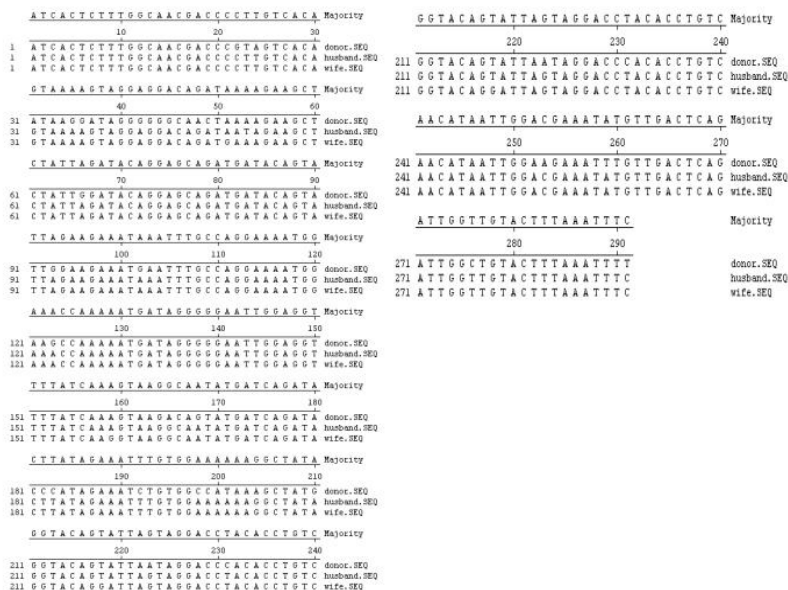
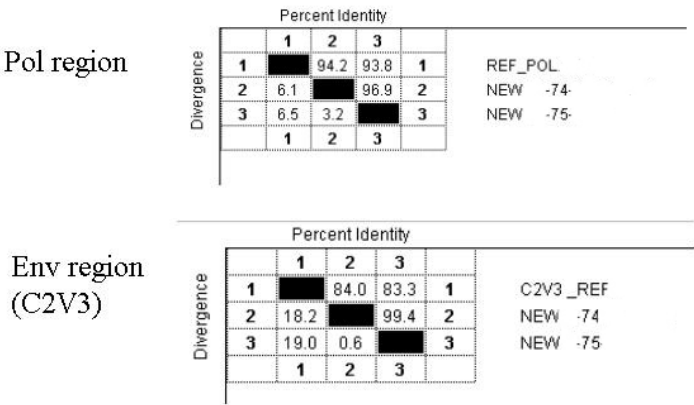


Fig. 4. (1) Sequence similarity and genomic diversity analysis of *pol* gene derived from infected donor, recipient, and her husband (Mrs. And Mr. Yang) in case 2. (2) Alignment of *pol* gene sequences

Fig. 5. Phylogenetic trees of *pol* gene sequences derived from donor, recipient, and her husband (Mrs. And Mr. Yang) in case 2.



*New -74 represents the donor, and New-P75 represents the recipients; REF_POL and C2V3_REF are HIV-1 positive control sequences.

Fig. 6. Sequence similarity and genomic diversity analysis of C2V3 region (*env* gene) and *pol* gene derived from infected donor and recipient in case 3.

Fig. 7. Phylogenetic trees of C2V3 region (*env* gene) sequences derived from infected donor and recipient in case 3.

Table 1. PCR and Nested PCR primer pairs used to amplify HIV protease gene and reverse transcriptase genes

Primer	Sequence ^a	Gene	Polarity
1-F-RT18	GGAAACCAAAATGATAGGGGGAA	Pol	Sense
1-R-RT21	TTGGAGG	Pol	Anti-sens
2-F-RT19	CTGTATTTCTGCTATTAAGTCTTTTG	Pol	e Sense
2-R-RT20	ATGGG	Pol	Anti-sens
F-MAW26	GGACATAAAGCTATAGGTACAG	Ga	e Sense
R-PR-prob	CTGCCAGTTCVAGCTCTGCTTC	g	Anti-sens
e	TTGGAAATGTGGAAAGGAAGGAC	Pol	e Sense
Nest-F-P	GGCAAATACTGGAGTATTGTATGG	Ga	Anti-sens
R	CAACTCCCCCTCAGAAGCAGGAGC	g	e
Nest-R-P	CGATAGACAACATCCATTCTGGCT	Pol	
R	TTAATTTTACTGGTACAGT		

Table 2. PCR and Nested PCR primer pairs used to amplify HIV C2V3 region in env gene

Primer	sequence	Polarity
44F	5'-ACAGTRCARTGYACACATGG-3'	Sense
35R	5'-CACTTCTCCAATTGTCCITCA-3'	Anti-sens
33F	5'-CTGTTIAATGGCAGICTAGC-3'	Sense
48R	5'-RATGGGAGGRGYATACAT-3'	Anti-sens