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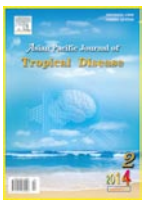

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Phylogenetic analysis of HIV-1 *pol* gene: first subgenomic evidence of CRF29-BF among Iranian HIV-1 patients

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PEER REVIEW

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ABSTRACT

Objective: To identify the dominant subtype among the HIV-1 strains circulation in Iran.
Methods: In this cross sectional study 100 HIV positive patients participated. HIV-1 RNA was extracted from plasma. RT nested-PCR was performed and the final products were sequenced and phylogenetically analyzed; reference sequences were downloaded from Los Alamos, aligned with Iranian pol sequences in the study and analyzed by neighbor-joining method.
Results: The results of the phylogenetic analysis showed that HIV-1 subtype CRF-35AD was the dominant subtype among HIV-1 infected patients in Iran; this analysis also suggested a new circulating recombinant form that had not previously been identified in Iran: CRF-29BF.
Conclusions: The impact of HIV diversity on pathogenesis, transmission and clinical management have been discussed in different studies; therefore, analyses of HIV genetic diversity is required to design effective antiretroviral strategies for different HIV subtypes.

1. Introduction

Investigating subtypes of HIV-1 circulation in a country is important due to its effects on pathogenesis, immune response and escape, vaccine development, transmission, disease progression, drug resistance and treatment response[1,2].

HIV-1 has been classified into groups, subtypes, sub-subtypes and recombinant forms and the unique circulation forms, with high and increasing prevalence in pandemic. Four distinct genetic groups (M, N, O and P) with group M responsible for the global HIV/AIDS pandemic; Nine group M subtypes (A-D, F-H, J, and K)[3], along with 61 circulating recombinant forms (CRFs) have been identified[4].

The rate of HIV-1 in Iran has been increasing in the last few years. According to the Centers for Disease Control, a total of 26 556 people living with HIV had been identified in Iran in June 2013[5,6]. The HIV transmission routes in all of the cases which have been registered since 1986 in order of magnitude are sharing injection equipment among intravenous drug users (IDUs) (68.4%), sexual intercourse (12.3%), blood transfusion (0.9%), and mother-to-child transmission (1.2%). The route of transmission for the remaining 17.2% is unknown[5,7]. A recent study showed that all hemophiliacs were infected with HIV-1 subtype B and all IDUs were infected with HIV-1 subtype A[8].

Previous molecular epidemiological analysis of HIV-1 in Iran reported two distinct results; It was firstly suggested that the predominant strain circulation among IDUs was subtype A[9-12] but recent studies of *pol*, *gag* and *env* gene segments reported that the predominant strain was CRF-3AD, a subtype A/D recombinant strain in Iranian population[3,8,13,14]. Therefore, we analyzed the subgenomic sequence of HIV-1 in 100 patients to confirm

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the predominant HIV-1 strain circulating in Iran which may significantly influence the diagnostic and therapeutic strategies[15].

2. Materials and methods

In this cross sectional study, HIV infected participants were collected from Infectious Disease Department of Imam Khomeini Hospital, Tehran, Iran. Ethical approval was obtained from Iranian Research Center for HIV/AIDS, and the consent was taken from all participants prior to blood collection.

One hundred participants were enrolled in this study. Blood samples were collected in sterile ethylene diamine tetraacetic acid-containing tubes, and plasma was separated and stored at -80 °C for ongoing process.

2.1. HIV RNA extraction and cDNA synthesis

Total RNA was extracted from 140 μL of each plasma sample using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany), following RNA viral denaturation at 70 °C for 10 min, cDNA synthesis was performed at 42 °C for 60 mins, using 10 IU of M-MuLV reverse transciptase (Fermentas), 1 mmol/L of antisense outer primers, 1.0 mmol/L deoxy-nucleotide tri-phosphates and 10 IU RNAse inhibitor (Fermentas).

2.2. Nested PCR amplification

First-round PCR was performed using outer primers: RTForward 15′ GTA GGA CCT ACA CCT GTC AA 3′, RTReverse 15′ TGT TAG TGC TTT GGT TCC CCT 3′ (2096-3056). Briefly, the amplification profile consisted of denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 50 seconds, annealing at 55 °C for 40 seconds, extension at 72 °C for 55 seconds, and a final extension phase at 72 °C for 3 min. An aliquot (about 25 μL) of the primary PCR products was used for 35 cycles of nested PCR as follows; initial denaturation at 95 °C for 5 min, 35 cycles of denaturation at 94 °C for 50 seconds, annealing at 58 °C for 40 seconds and polymerisation at 72 °C for 50 seconds, with a final elongation at 72 °C for 3 min. Primers used in nested PCR were as follows, RTForward 2 5′ ATG GCC CAA AGG TTA AAC AAT GG 3′, RTReverse2 5′ TTC TGT ATA TCA TTG ACA GTC CAG 3′(2189-2929). The final length of the product was 740 bp. Then the nested PCR products were checked by 2% agarose gel electrophoresis.

2.3. Purification and DNA sequencing

The PCR products were purified by Gel Purification kit (Bioneer, Global Genomics Partner), according to manufacturer's instructions and sequenced on both strands (bi-directionally) by the dideoxy chain termination method (ABI PRISM 3730 DNA analyzer automated DNA Sequencer, Applied Biosystem, Foster city, CA, USA).

2.4. Phylogenetic analysis

The *pol* region has been identified as a reliable region for HIV-1 subtyping[16,17]. To determine the HIV subtype in Iran, *pol* gene sequences were aligned with reference sequences available at the Los Alamos HIV database (www.hiv.lanl.gov) using CLUSTAL W software; Phylogenetic trees were reconstructed using a Neighbor-Joining method with Molecular Evolutionary Genetics Analysis (MEGA) software, version 5 and also Recombination Detection Program (RDP4) and DataMonkey (http://www.datamonkey.org/dataupload_scueal.php) tools. The Kimura 2-parameter model was used with a transition/transversion ratio of 1.5 and statistical support of the tree structures was obtained by 1 000 bootstrap replicates[18,19]. Bootstrap values of >70% were considered significant. To improve the accuracy of recombinant forms, RDP4 sofware was used too.

2.5. GenBank accession numbers

The *pol* nucleotide sequences reported in this study have been deposited in GenBank under accession numbers KF029508-KF029592.

3. Result

Among one hundred patients participated in the study, 86 gene sequences were obtained; The inability to amplify 16 samples can be due to low viral load in those 16 samples. The majority (81.0%) of participants were male, with a mean age of 37 years. In most (66.3%) a history of injection drug use was reported. A percentage of 9.7% of patients had hemophiliacs, 16.3% had sexual contacts and the route of transmission was unknown in 7.7% of participants.

Phylogenetic analysis (Figure 1) showed that subtype CRF-35AD (80.23%) was by far the most common subtype , followed by subtypes: B (15.11%), CRF-29BF (3.48%) and C (1.16%) in studied samples. CRF-29BF recombinant strain was reported for the first time in the study in Iran. Sequences identified in the present study are labeled with the prefix "IR.KB".

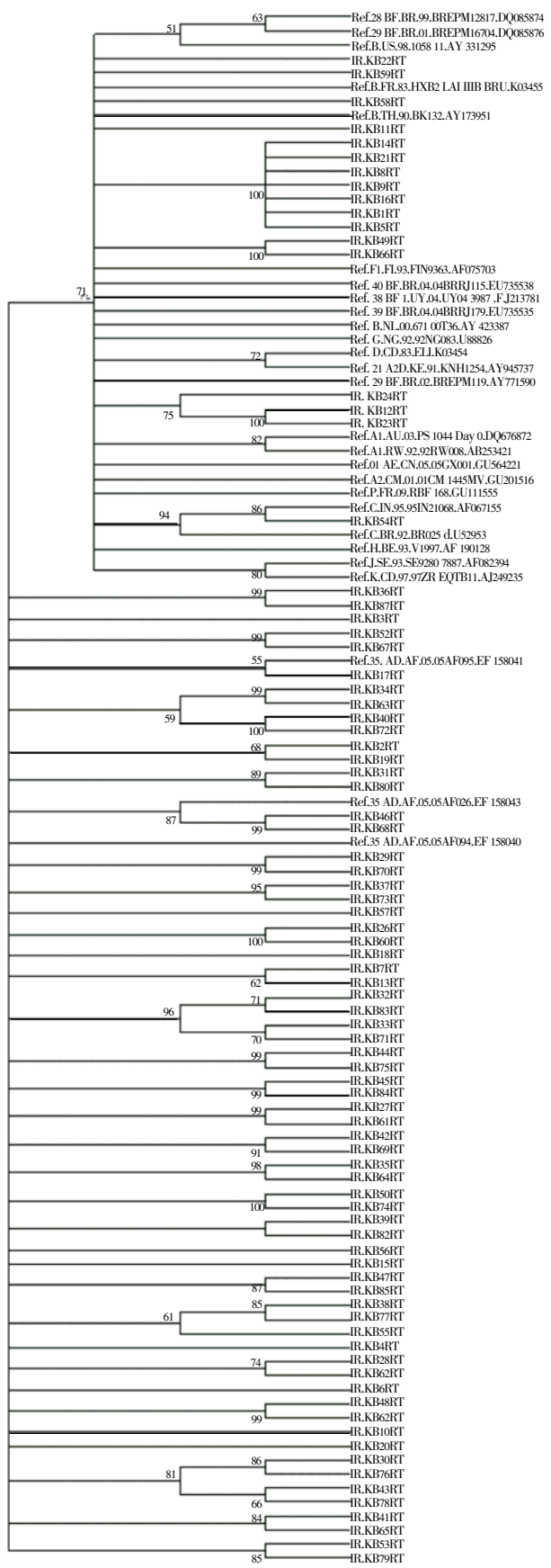

Figure 1. Un-rooted phylogenetic tree constructed using kimura two parameter matrixes and neighbor-joining method, based on *pol* sequence (Recommended subtype reference sequences and Iranian *pol* gene sequences in the study).
Iranian sequences determined in this study were indicated by the IR.KB prefix.

4. Discussion

Consistent with some recent reports, HIV-1 subtype CRF-35AD was found to be the dominant circulating viral subtype in Iran in this study[16,17], but the result differed from previous studies which reported that the subtype A is the most prevalent in HIV infected patients in Iran[9-12,14].The *pol* region of these viruses appeared to be similar to that of recombinant viruses from Afghanistan, This connection among Iran and Afghanistan sequences shown in this study can be explained by drug trafficking and/or immigration[3,20].

Additionally, our data suggested a growing diversity of HIV subtypes in Iran. The phylogenetic analyses of the *pol* gene suggested new circulating recombinant form which was not previously identified in Iran: CRF-29BF. Further sequencing of the *env* and *gag* genes will be needed to verify this finding. The results of some studies which were undertaken before were different with the latest findings including this study, probably because CRF-29BF and CRF-35AD reference sequences were not available in the HIV database website in previous years.

Knowing the distribution of HIV variants in Iran, along with corresponding epidemiologic factors, will help to assess the implications of any differences in transmissibility. the apparent segregation of HIV-1 subtypes by type of risk behavior rather than as a result of virologic factors (cell tropism, coreceptor specificity) could derive from genetic, demographic, economic, and social factors that separate the different risk groups for HIV-1 infection. Moreover, the overwhelming predominance of the C subtypes in areas where unprotected heterosexual intercourse is the main transmission route could result from a founder effect with a fast-colonization outcome[21]. The public health implications of such findings varies from prevention to treatment strategies. As the main transmission route of HIV in Iran remains IDUs, strengthened harm reduction program is needed to prevent it's transmission to the population. This molecular epidemiological information will also be extremely relevant for guiding the development and implementation of diagnostic as well as preventive and therapeutic approaches in Iran.

Conflict of interest statement

We declare that we have no conflict of interest.

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