Identification of Human Immunodeficiency Virus Type-1 Subtypes by Heteroduplex Mobility Assay

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Abstract

Background: Human immunodeficiency virus type-1 (HIV-1) has developed marked genomic sequence differences over the course of an epidemic because of an error prone reverse transcriptase (RT), which rapidly incorporates mutations resulting in genomic diversity, altered cell tropism, immune escape and variable resistance to antiretroviral drugs. The best preventive strategy for HIV control is development of an efficacious prophylactic vaccine using the most appropriate (antigenically related) subtypes. On the basis of phylogenetic analysis, HIV strains can be separated into major group “M” consisting of genetic subtypes A-K, “N”, the new group and “O”, the outlier group.

Methods: Heteroduplex mobility assay (HMA) is a rapid, economical and reliable technique of subtyping HIV-1. It is based on the principle of determining the genomic relatedness and divergence of the unknown sample with the known reference plasmid HIV-1 subtypes by studying the mobility patterns of the resulting heteroduplexes formed on the polyacrylamide gel.

Result: A total of 70 HIV-1 seropositive samples obtained from service personnel, their families and civilians from service hospitals were analyzed and their subtype distribution studied. 66 (94.28%) were HIV-1 subtype C and two (2.85%) subtype B. In two (2.85%) samples, the subtype distribution was homotypic recombinant, one each of subtype C1 & C2 and C2 & C4 respectively.

Conclusion: Service personnel and their families represent a divergent population from different regions of India. An analysis of subtypes in these HIV-1 seropositive individuals will help in understanding the geographical distribution and evolution of the virus. Determination of HIV-1 subtypes has significant implications for development of candidate vaccine for India.

MJAFI 2007; 63 : 249-252

Key Words : HIV-1; Heteroduplex mobility assay; Subtypes; India

Introduction

Human immunodeficiency virus (HIV) is the most significant emerging pathogen which has produced a world wide epidemic [1,2]. It is divided into three genetic groups based on phylogenetic reconstruction using nucleotide sequences [3-6]. Majority of the isolates fall into the “M” or Major group while a small number constitutes the “O” or Outlier group and the “N” or New group. The “M” group again has been subdivided into distinct subtypes “A” to “K”. Subtype “C” is the predominant subtype in India and South Africa. It is further divided into genotypes- C1 (Malawi), C2 (Zambia), C3 (India) and C4 (Brazil). Majority of the affected appear to be infected initially with a single strain of HIV-1, which evolves over a period of time into a swarm of related viruses or quasispecies during the course of infection [7]. By the time a person has been infected for several years, presumably owing to immune selection pressure and lack of fidelity of reverse transcriptase (RT), innumerable mutations within distinct

regions of the envelope gp120, results in the emergence of viral variants and genomic diversity [8].

Study of the genetic diversity of HIV-1 is significant in understanding the molecular epidemiology of HIV-1, biological behaviour of the virus, modes of transmission, geographical distribution of the subtypes and selection of the most prevalent subtype in a defined region for selecting a candidate vaccine. Heteroduplex mobility assay (HMA) [9], is a rapid, reliable and an economical tool for ascertaining genetic diversity and relatedness among HIV-1 subtypes. It can be used to study the molecular epidemiology of HIV-1, selection of candidate vaccine and is suited for developing countries like India.

Material and Methods

Seventy samples from HIV-1 seropositive individuals were collected after taking informed consent and the epidemiological data was recorded (Table 1).

Ten millilitres of blood from the cubital vein was collected in a sterile tube with acid citrate dextrose (ACD) as

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anticoagulant. The peripheral blood mononuclear cells (PBMCs) were separated from the whole blood by layering over Ficol-Hypaque in the ratio of 2:1 (Sigma Chemical Co, USA) and centrifuging at 1600 rpm in swing bucket rotor centrifuge for 30 minutes at room temperature. The plasma, was collected separately in the sterile tube. The buffy coat containing the PBMCs was collected in a 15ml conical tube (Costar, USA) and washed thrice with phosphate buffer saline by spinning at 1000 rpm for 10 minutes. Qiaamp blood kits (Qiagen from Genetix) and Nucleospin kits (from Invitrogen) were used to extract DNA from the PBMCs. Nested polymerase chain reaction (PCR) protocol was used to generate sufficient quantities of 0.7- kb amplicon for HMA (Fig.1). For analysis of the HIV-1 *env* gene, well-conserved primer sequences were chosen (HMA HIV-1 *env* subtyping kit, protocol version 3) (Table 2).

Heteroduplexes were formed by mixing 5ml of the sample DNA from the third round PCR with 5ml of the third round PCR product from the analogous fragment of *env* derived from a reference strains (subtypes A-E) along with one millilitre of HMA annealing buffer (100mM NaCl, 10mM Tris pH 7.2, 2mM EDTA). The mixed PCR products were denatured at 94°C for two minutes in a Tec gene thermocycler (Care Biomedicals) and reannealed quickly on wet ice. Samples were mixed with gel loading dye prior to loading on the gel [9]. Electrophoresis was done using 5% non-denaturing acrylamide gel (using a 30% acrylamide, 0.8% bisacrylamide stock) in TBE buffer, running at 250 volts for three hours and

Fig. 1 : ES7 and ES8 amplified HIV-1 PCR products

Table 1

Age/sex distribution of the cases studied

S No.	Age (years)	Total No. (70)	Sex	
			Male	Female
1.	< 10	5	3	2
2.	11-20	3	0	3
3.	21-30	17	11	6
4.	31-40	28	22	6
5.	41-50	12	11	1
6.	51-60	5	4	1

then stained in 0.5x TBE containing 0.7%(w/v) ethidium bromide for one hour prior to visualization. Retarded mobilities of heteroduplexes reflect proportionate nucleotide mismatches between the sample and the reference strain. The sample/reference heteroduplexes, which migrated closest to the corresponding homoduplex, determined the subtype designation (Fig. 2). Homoduplexes, which represent 100% homology, showed fastest mobility in gel.

The heteroduplex mobility ratio was calculated from the mobility distance of the heteroduplexes and homoduplexes from the well bottom [9].

$$\text{Heteroduplex Mobility Ratio} = \frac{\text{Distance from the well bottom to the mid point of the heteroduplex bands}}{\text{Distance from the well bottom to the mid point of the homoduplex bands}}$$

Results

The predominant mode of transmission of HIV-1 in the study was sexual contact. The infection was transmitted by heterosexual route in 60, by blood product in one, by vertical transmission in five and history of transmission was not known /revealed in four (Table 3). The geographical distribution of samples is given in Table 4 and the distribution of HIV-1 subtypes in Table 5. The correlation of subtypes with the modes of transmission reveals that majority acquired HIV-1 infection through heterosexual contact and the predominant HIV-1 subtype was subtype C3, followed by subtype C2, subtype C4, subtype B2 and one case each of homotypic recombinant subtype C2 & C4 and C1& C2 (Fig.3). Five samples of vertical transmissions were of subtype C3. The only case due to transfusion of blood and blood product was of subtype C3 and four cases with unknown/ undisclosed

Table 2

Nested PCR primers used in amplifying HIV-1 *env* sequences

Primers

1st round

ED3 5'- TTAGGCATCTCCTATGGCAGGAAGAAGCGG (5956-5985*)
ED14 5'-TCTTGCCTGGAGCTGTTTGATGCCCCAGAC (7960-7931*)

These amplify a ~2.0Kb fragment spanning from the first exon of rev to the transmembrane protein gp41 coding region of *env*.

2nd round

ED5 5'- ATGGGATCAAAGCCTAAAGCCATGTG (6556-6581*)
ED12 5'- AGTGCTTCCTGCTGCTCCCAAGAACCAAG (7822-7792*)

These amplify an internal fragment of ~1.25Kb spanning the V1-V5 coding region of gp120

3rd round

ES7 5'-CTGTTAAATGGCAGTCTAG (7001-7020*)
ES8 5'-CACTTCTCCAATTGTCCTCA (7667-7647*)

These amplify an internal fragment of ~0.7Kb spanning the V3-V5 coding region of gp120

* Coordinates from the HIV-1 -HXB2 genome (GenBank accession no. K03455)

Fig. 2 : Interpretation of heteroduplex gels

history were of C3 subtype (Tables 3, 5).

Discussion

Extensive variability of HIV-1 has potential impact on disease epidemiology, diagnosis, therapy and prevention of infection. The marked diversity of HIV-1 is because of introduction of genetically diverse simian immunodeficiency viruses (SIVs) into humans and error prone reverse transcriptase which lacks proof reading function during viral replication resulting in 1-3 nucleotide change each time the provirus is synthesized. There is

Table 3
Distribution and correlation of subtypes with modes of transmission

Modes of transmission	Total cases	HIV-1 subtypes				
		A	B	C	D	E
Heterosexual	60	—	2	58	—	—
Mother to child transmission	5	—	—	5	—	—
Transfusion of blood and blood products	1	—	—	1	—	—
Unknown	4	—	—	4	—	—

Table 4
Geographical distribution of HIV-1 subtypes

Geographical location	Total No. cases	HIV-1 subtypes					
		B	C2	C3	C4	C1/C2	C2/C4
Northern India	14	—	1	13	—	—	—
Southern India	13	—	1	11	1	—	—
Central and Eastern India	6	—	1	4	—	1	—
Western India	37	2	3	31	—	—	1

Table 5
Distribution of HIV-1 subtypes as per cases

HIV-1 subtypes	Number of cases
C3	59
C2	6
C4	1
C1/C2	1
C2/C4	1
B2	2

- Lane 1 - Sample + Sample
- Lane 2 - Sample + A
- Lane 3 - Sample + B1
- Lane 4 - Sample + B2
- Lane 5 - Sample + B3
- Lane 6 - Sample + C1
- Lane 7 - Sample + C2
- Lane 8 - Sample + C4
- Lane 9 - Sample + C3
- Lane 10 - Sample + D
- Lane 11 - Sample + E
- Lane 12 - Marker

Result : C2 and C4 (Homotypic recombinant)
Fig. 3 : Heteroduplex mobility assay

rapid turn over of virion in HIV-1 infected individuals (10^9 virions per day), which results in *in-vivo* swarm of closely related viral variants called quasispecies [4]. Reverse transcriptase is highly recombinogenic and radically different genomic combinations are generated in individuals infected by genetically different viruses resulting in circulating recombinant forms (CRFs).

DNA sequencing is the gold standard for studying genomic diversity of HIV. However it is labour and cost intensive. HMA was used for detecting and estimating the degree of genetic similarity or divergence between HIV strains. The distinction between intraclade and interclade subtypes was made on the basis of heteroduplex DNA mobility. The structural distortions of the DNA double helix caused by nucleotide mismatch reduces the electrophoretic mobility of the DNA i.e. the larger the gap, the slower the heteroduplex mobility [11,12]. The sample showing fastest mobility with the appropriate reference strain is the subtype of the unknown strain (Fig 2). Various intraclade and interclade subtypes having similar mobility ratio were considered homotypic and heterotypic recombinants respectively.

The present study shows that HIV-1 subtype C3 is the most prevalent subtype in India. Earlier studies from India have revealed presence of multiple subtypes with predominance of subtype C [13-19]. The presence of CRFs has also been reported from India [20]. The

predominance of subtype C in India may be due to complex highly efficient viral transmission in the population due to specific virological and host factor interaction. Predominant heterosexual transmission of the subtype C strain in the population may also influence the evolution of these strains in India [21].

The highest rate of spread of HIV-1 for Africa appeared during the 1980s at about the same time the epidemic was spreading in the United States and Europe [22]. In the mid 1980s while North America, Europe and Africa were affected by the HIV epidemic, Thailand and the rest of Asia appeared to be spared. Thereafter HIV-1 has spread rapidly in South and Southeast Asia. Extensive molecular characterization of circulating strains in the Thailand has revealed simultaneous existence of epidemics caused independently by subtypes of B and E [22]. The subtype B sequence predominating in the intravenous drug user population in Thailand and the HIV-1 subtype B in the north-eastern region of India show similarity and this may be due to the international drug trafficking in the "golden triangle" located at the convergence of the international borders of Thailand, Myanmar and Laos.

Although subtype C remains the predominant strain, transmission of multiple HIV-1 subtypes and the emergence of the recombinants within the same communities in India may have important implications in understanding the molecular epidemiology of HIV and for the design of effective candidate vaccine well suited for Indian subcontinent.

Conflicts of Interest

None identified

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