

Genetic Characterization of HIV-1 Strains Among the Injecting Drug Users in Nagaland, India

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Abstract: Global HIV-1 surveillance has led to the detection of its new recombinant forms. This study was carried out for the first time to elucidate the genetic characterization and evolutionary relationship of HIV-1 strains among injecting drug users of Nagaland, northeastern India. A total of 156 injecting drug users participated in this study voluntarily. Among them 18 were seropositive for HIV-1 (11.5%).

The Heteroduplex Mobility Assay (HMA) of HIV-1 based on p24-p7 region of *gag* gene and C2-V3 region of *env* gene revealed 11 samples to be subtype C (*gag/env*), 1 sample as subtype B (*gag/env*) and 6 samples to be recombinants between subtype C and B. Also, the sequencing and phylogenetic analysis of *gag* (p24-p7) and *env* (C2-V3) genes from eighteen samples of Nagaland IDUs with different global HIV-1 strains showed the presence of Indian, African, Thai and their recombinant forms. However, more recombinant strains based on different genomic regions of HIV-1 were detected using Multiregional Hybridization Assay (MHA) where 8 out of 18 samples were found to be recombinants between subtype C and B. Thus, multiregional hybridization assay along with heteroduplex mobility assay can serve as an efficient tool in the characterization of recombination pattern among the newly emerging HIV-1 recombinants.

Keywords: Injecting drug users, nagaland, multiregional hybridization assay, phylogenetic analysis, heteroduplex mobility assay.

INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) is a double single stranded RNA virus. It belongs to the Family: *Retroviridae*, Genus: *lentivirus* and Species: *Human immunodeficiency virus 1*. India has been witnessing the rapid spread of HIV-1 infection throughout the north-eastern border region since a very long time [1, 2]. Nagaland, one of the northeastern states of India, bordering Myanmar and Manipur and close to the “golden triangle”, is one of the major opium and heroin producing areas of south-east Asia. It is the sixth highly prevalent state in the country with a prevalence rate of 1.27 %. Sexual route of transmission has taken predominance in this area with 78.73%, followed by transmission with infected needles and syringes contributing to 12.21 %, as reported by Nagaland states AIDS control society. The high rate of HIV-1 transmission among injecting drug users of Manipur, a neighboring state of Nagaland, through sharing of contaminated needles and syringes was reported earlier [1, 2]. Previous studies from Manipur detected the presence of Thai-B as the second major subtype after subtype-C, circulating among IDUs [3-5]. Recent studies have revealed the occurrence of different multigenomic variants of HIV-1 through multiregional hybridization assay in Manipur which might pose serious

challenge to effective candidate vaccine design against newly evolving HIV-1 viruses in the northeastern states of India [6]. But the nucleotide sequencing of the whole 9 kb HIV-1 genome is a tedious as well as expensive procedure. However, a breakthrough has been achieved by the combined implementation of heteroduplex mobility assay along with multiregional hybridization assay. This parallel combination has been successful to reveal a detailed picture of emerging recombination patterns in different genomic regions of HIV-1. In order to bring forth the present scenario of HIV-1 strains in north eastern states of India, we have selected Nagaland where the characterization of HIV-1 strains has not been conducted till date. Thus the present study focuses towards the trend of increasing HIV-1 recombinant strains in the northeastern states of India.

MATERIALS AND METHODOLOGY

Study Design

It was a community based cross-sectional study. Male IDUs of ages 31- 40 years were contacted through secondary informant method followed by snowballing. All IDUs were explained about the purpose of this study and requested to participate voluntarily. Experienced social workers interviewed them to study their demography, risk behavior and risk perception using a field tested questionnaire. Interview was followed by collection of about 5 ml blood in vacutainer containing EDTA. HIV was tested by enzyme linked immunosorbent assay (Immunogenetics, Belgium) followed by tridot assay (Standard Diagnostics, Bioline, Korea). Peripheral blood mononuclear cells were separated

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from the whole blood by Ficoll-Hypaque gradient centrifugation [7]. DNA was extracted by using the QIAamp DNA Blood Mini Kit 250 (QIAGEN, Germany, Hilden) according to the manufacturer's protocol.

Heteroduplex Mobility Assay

The HIV-1 strains were subjected to *gag* and *env* Heteroduplex Mobility Assay as described earlier [5, 8]. Briefly, the amplicons (460bp, 500bp) for *gag* (p24-p7) and *env* (C2-V3) were amplified through nested PCR using standard primers (NIH AIDS Research and Reference Reagent Program, NIH, USA). For *gag* HMA, 4.5µl of the amplicon of the unknown sample was mixed with 4.5µl of the reference amplicon in presence of 1µl of 10x annealing buffer (1M NaCl, 100mM Tris-HCl [pH 7.8], 20mM EDTA). For *env* HMA, 5µl of the amplicon was mixed with 5µl of the reference amplicon in the presence of 1.1µl of 10x annealing buffer. It was then denatured at 94°C for 2 minutes followed by renaturation by snap freezing in ice to form Heteroduplex molecules. The mixture were then loaded on a 5% Polyacrylamide Gel (for *gag*-HMA, 5% Polyacrylamide / 20% Urea; 5% polyacrylamide for *env*) in 1X TBE buffer and electrophoresed at 250 V for 2 hours 30 minutes. Based on the relative mobility of the heteroduplex molecules towards homoduplexes, subtypes were assigned. Heteroduplex molecules formed between the unknown sample and the most closely related subtype exhibited the fastest mobility.

Multiregional Hybridization Assay

The MHA_{bc} v.2 study was a Taqman assay based on real time PCR method performed using probes designed from eight different genomic regions (p17, pro, rt, int, tat, gp120, gp41 and gpnef) of HIV-1 as described earlier [6, 9]. Briefly, the first round PCR was conducted at ABI9600 Thermal cycler (Applied Biosystems) with outer primers designed for specific genomic regions, 10x PCR buffer, 25mM MgCl₂, 10mM dNTPs and 1U AmpliTaq Gold DNA polymerase (Applied Biosystems) in a final volume of 50µl. The second round PCR was carried out in 96 well ABI PRISM 7900HT sequence detection system (Applied Biosystems) using inner primers and probes designed for specific genomic regions in a final volume of 25µl. The probes were labeled with either 6-carboxy fluorescein (FAM) or 6-carboxytetramethylrhodamine (TET) at the 5' end and Black Hole Quencher (BHQ) at the 3' end. The fluorescence intensity was measured by SDS v.2 software (Applied Biosystem, USA). The PCR product generated was checked by conducting dissociation curve analysis through SybrGreen PCR Master Mix (Applied Biosystems) which distinguishes PCR product from primer dimmers of lower thermal stability.

DNA Sequencing, Phylogenetic and Simplot Analysis

Amplicons of the *gag* & *env* gene segments were purified by a QIA quick PCR purification kit (QIAGEN, Germany, and Hilden) and were subjected to cycle sequencing reactions using fluorescent dye-labeled dideoxy nucleotides in an ABI PRISM 3100 automated sequencer following the manufacturer's protocol. The sequences were edited manually using BIOEDIT sequence alignment editor program (version 5.0.6; Department of Microbiology, North

Carolina State University) [<http://www.mbio.ncsu.edu/Bioedit/BioDoc.pdf>]. The edited sequences were Blast searched and further aligned with the reference sequences from different geographic regions available in the HIV database (<http://www.hiv.lanl.gov/content/index>) for phylogenetic analysis using the Molecular evolutionary genetics analysis software version 4 (MEGA 4) [10].

Gene Bank Accession Numbers

The Gen- Bank accession numbers for the nucleotide sequences of *gag* (p24-p7) and *env* (C2-V3) reported in this paper are EU541498, EU526648- EU526656, EU526658- EU526666, EU526668- EU526669, HM-130667, HQ897949- HQ897962.

RESULTS

HMA Analysis

Out of 18 HIV seropositive samples, the *gag* heteroduplex mobility assay of all the 18 samples of Nagaland injecting drug users showed 17 samples as subtype C while one as subtype B (nag120) (Table 1). On the other hand, *env* heteroduplex mobility assay showed 11 samples as subtype C and rest of the 7 samples e.g. nag1, nag12, nag23, nag 57, nag120, nag135 and nag153 as subtype B. (Fig. 1) shows the subtype specific heteroduplex mobility for *env* (C2-V3) and *gag* (p24-p7) genes of "nag 1".

MHA Analysis

Multiregional hybridization assay was carried out for all the 18 samples (Table 1). The analysis showed that nag 25, nag 33, nag 49, nag 65, nag 81, nag 86, nag 111, nag 113 and nag 157 belonged to subtype C. However, subtype B probe reacted with nag120. Multigenomic recombination was detected for the samples nag 1, nag 12, nag 23, nag 57, nag 88, nag 135, nag 152 and nag 153. The samples nag 23, nag 88 and nag152 showed both dual probe reactivity and multigenomic recombination pattern.

Phylogenetic Analysis

The B/C recombination pattern with respect to *gag* (p24-p7) and *env* (C2-V3) of the recombinant samples was confirmed by phylogenetic analysis. Phylogenetic analysis of the *gag* (p24-p7) gene of Nagaland injecting drug users with the reference subtype C and subtype B sequences available in the database (<http://www.hiv.lanl.gov/content/index>) clearly showed that the samples nag 1, nag 23, nag 25, nag 33, nag 49, nag 57, nag 65, nag 81, nag 86, nag 88, nag 111, nag 135 clustered with subtype C HIV-1 strains from Africa and nag 12, nag 113, nag 152, nag 153, nag 157 clustered with Indian subtype C. For nag 120, *gag* (p24-p7) gene clustered with subtype B strains from China (Fig. 2). On the other hand, phylogenetic analysis of the *env* C2-V3 gene with other global HIV-1 strains showed that nag 25, nag 33, nag 65, nag 86, nag 88, nag 111, nag 113 formed cluster with subtype C reference sequences from Africa and nag 49, nag 81, nag 152, nag 157 formed cluster with Indian subtype C (Fig. 3). However nag 1, nag 57, nag 135, nag 153 formed a unique cluster close to Thai B sequences and nag 12, nag 23 and nag 120 clustered with subtype B sequences from China and Myanmar. The results obtained from the phylogenetic analysis were further validated through Simplot analysis

Table 1. Genotyping Results of HIV-1 Positive IDU Samples Based on MHAbce v.2 and HMA. 18 Samples were Subjected to MHA Using Probes Specific for Subtype C, Subtype B and Subtype AE. “NR” Denotes No Probe Reactivity; B/C Denotes Dual Probe Reactivity

Sample. No.		MHAbce v.2								HMA	
		<i>p17</i>	<i>pro</i>	<i>rt</i>	<i>int</i>	<i>tat</i>	<i>gp120</i>	<i>gp41</i>	<i>nef</i>	p24-p7	C2-V3
1.	nag1	C	NR	C	B	C	B	B	C	C	B
2.	nag12	C	C	C	C	NR	B	B	B	C	B
3.	nag23	C	C	B/C	B	C	B	NR	C	C	B
4.	nag25	C	C	C	C	C	C	C	C	C	C
5.	nag33	C	C	C	C	C	C	C	C	C	C
6.	nag49	C	C	C	C	C	C	NR	C	C	C
7.	nag57	C	C	C	C	C	B	NR	B	C	B
8.	nag65	C	C	C	C	NR	C	C	C	C	C
9.	nag81	C	C	C	C	C	C	C	NR	C	C
10.	nag86	C	C	C	C	C	C	C	C	C	C
11.	nag88	C	C	B	B/C	C	C	C	C	C	C
12.	nag111	C	C	C	C	C	C	C	C	C	C
13.	nag113	C	C	C	C	C	C	C	NR	C	C
14.	nag120	B	B	B	B	B	B	B	B	B	B
15.	nag135	C	C	C	C	C	B	B	NR	C	B
16.	nag152	C	NR	C	B	B/C	C	C	NR	C	C
17.	nag153	C	NR	B	C	C	B	B	NR	C	B
18.	nag157	C	NR	C	C	C	C	C	C	C	C

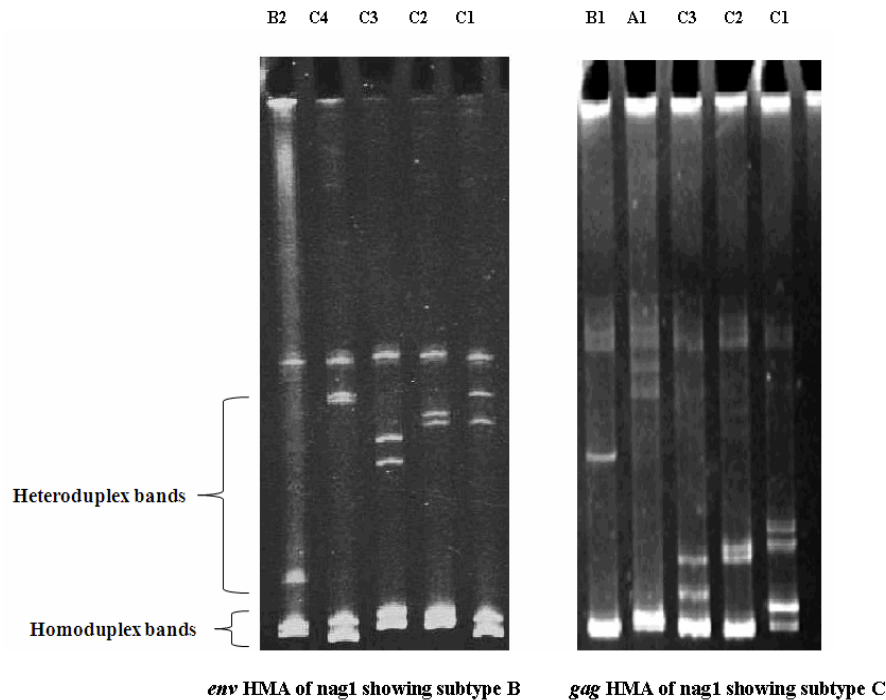


Fig. (1). Heteroduplex mobility assay of “nag 1” for *env* (C2-V3) and *gag* (p24-p7) genes. Heteroduplex and homoduplex bands are indicated in the figure. The lanes are marked according to subtype specific references. The heteroduplex with subtype B reference amplicons (B2) exhibits the fastest mobility as compared to subtype C reference amplicons (C1, C2, C3, C4) in case of *env* gene. For *gag* gene, the heteroduplex with subtype C reference amplicons (C1, C2, and C3) exhibits the fastest mobility as compared to subtype B (B1) and subtype A (A1) reference amplicons.

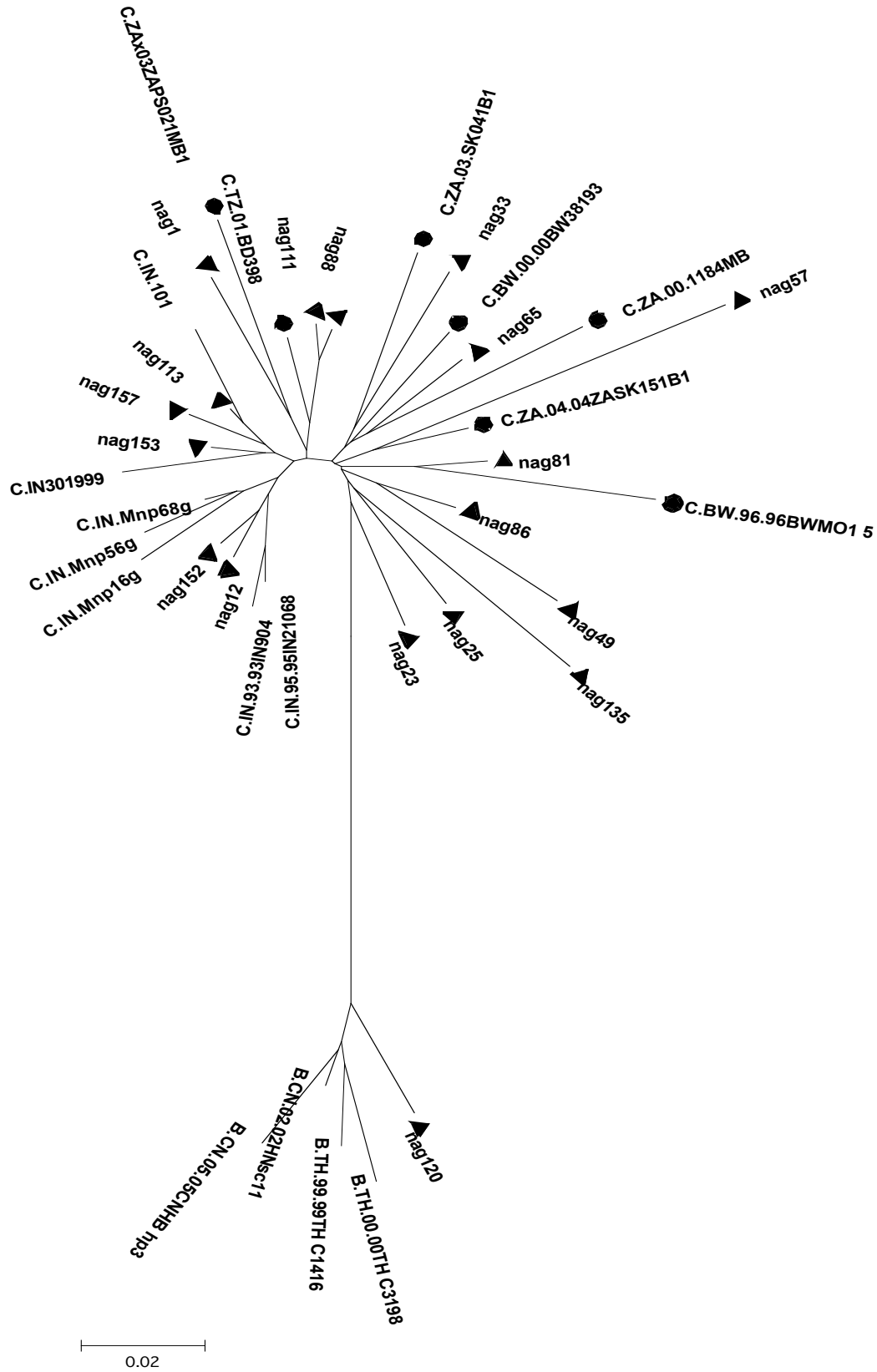


Fig. (2). Phylogenetic analysis of *gag* gene (p24-p7) of the HIV-1 strains isolated from the Nagaland IDU samples. The Nagaland IDU strains are denoted as “nag”▼ and African strains as “●”. The accession number for *gag* (p24-p7) sequences are nag 1 (HQ897949), nag 12 (EU 526655), nag 23 (EU 526652), nag 25 (HQ897951), nag 33 (HQ897952), nag 49 (EU 526653), nag 57 (EU 526648), nag 65 (EU 526649), nag 81 (HQ897953), nag 113 (HQ897950), nag 86 (EU 526650), nag 88 (EU 526651), nag 111 (EU 526654), nag 120 (EU 526656), nag 135 (EU541498), nag 152 (HQ897954), nag 153 (EU 526658), nag 157 (HQ897955).



Fig. (3). Phylogenetic analysis of *env* gene (C2-V3 region) of the HIV-1 strains isolated from the Nagaland IDU samples. The Nagaland IDU strains are denoted as “nag” ▼ and African strains as “●”. The accession number for *env* (C2-V3) sequences are nag 1 (HQ897961), nag 12 (EU 526665), nag 23 (HM 130667), nag 25 (HQ897956), nag 33 (HQ897957), nag 49 (EU 526663), nag 57 (EU 526659), nag 65 (EU 526660), nag 81 (HQ897958), nag 88 (EU 526661), nag 86 (EU 526662), nag 111 (EU 526664), nag 113 (HQ897962), nag 120 (EU 526666), nag 135 (EU 526669), nag 152 (HQ897959), nag 153 (EU 526668), nag 157 (HQ897960).

(data not shown). While focusing on individual HIV-1 isolates, the information revealed regarding the genotyping pattern of *gag* (p24-p7) and *env* (C2-V3), can be divided into seven categories as shown (Table 2). First, *gag* B/ *env* B showed by nag 120; second, *gag* C (African)/ *env* C (African) showed by nag 65, nag 86, nag 88, nag 111, nag 33, nag 25; third, *gag* C (African) / *env* C (Indian) showed by nag 49, nag 81; fourth *gag* C (Indian) / *env* C (Indian) showed by nag 152, nag 157; fifth *gag* C (Indian) / *env* C (African) showed by nag 113; sixth *gag* C (African)/ *env*

(Thai B) showed by nag 1, nag 23, nag 57, nag 135 and the seventh one *gag* C (Indian) / *env* (Thai B) showed by nag 12 and nag 153.

DISCUSSION

Due to its geographical proximity with the golden triangle, Manipur and the adjoining states of Nagaland have made the north-eastern region vulnerable to HIV infection [1, 2]. Rampant injecting practices have contributed to making these areas as the main abode for HIV. Sharing of

Table 2. Different Genotypic Categories of HIV-1 Strains Among IDUs of Nagaland

Sample ID	Genotype categories	<i>gag</i>	<i>env</i>
nag 33, nag 25, nag 65, nag 86, nag 88, nag 111	<i>gag</i> (African)/ <i>env</i> (African)	C	C
nag 113	<i>gag</i> (Indian)/ <i>env</i> (African)	C	C
nag 152, nag 157	<i>gag</i> (Indian)/ <i>env</i> (Indian)	C	C
nag 49, nag 81	<i>gag</i> (African)/ <i>env</i> (Indian)	C	C
nag 1, nag 23, nag 57, nag135	<i>gag</i> (African)/ <i>env</i> (Thai)	C	B
nag 12, nag 153	<i>gag</i> (Indian)/ <i>env</i> (Thai)	C	B
nag 120	<i>gag</i> (Thai)/ <i>env</i> (Thai)	B	B

injecting equipments frequently occurs among injecting drug users and drug traders as a part of their drug-purchasing behavior, thereby, leading to a possibility of introduction of different HIV-1 subtypes into circulation. Similar observations have been found in studies from China where HIV epidemic due to different subtypes has been spreading along heroin trafficking routes [11]. Genetic diversity of HIV-1 might also be related to cross-border migration and sharing practices of IDUs of different ethnicity (host factor). The IDUs who had sexual relationship with other IDUs were especially at the brink of acquiring HIV infection. These findings suggest that an IDU having these risk behaviors has multiple chances of getting exposed to different circulating subtypes of HIV-1 thereby facilitating the development of novel recombinant strains. Various other studies have documented the rapid spread of recombinant strains of HIV among IDUs due to unsafe injecting practices [12-14].

The sequencing and phylogenetic analyses of *gag* and *env* genes in this study clearly indicate that the HIV-1 strains from Africa are in circulation along with Indian strains. The introduction of African HIV-1 strains might have occurred in the past and had become stable in the northeastern IDU population. Though it is well known that the B/ C recombinants were evolved from recombination between subtype C and subtype B viruses due to dual infection, however, the recombination pattern in different genes of HIV-1 detected by multiregional hybridization assay, raises the possibility of recombination within recombinant strains and the generation of mosaic recombinant forms of HIV-1. The occurrence of Indian C (*gag*) /Thai-B (*env*), African C (*gag*) / Thai-B (*env*), Indian C (*gag*) / African C (*env*), African C (*gag*) / Indian C (*env*) recombinant forms of HIV-1 among the injecting drug users of Nagaland brings about the possibility of incorporation of recombinant HIV-1 strains in Nagaland IDUs. This gives an alarming signal of increasing population with inter and intragenomic recombinant forms of HIV-1 which could lead to serious epidemic among injecting drug users of the north-eastern region. Multiregional hybridization assay along with heteroduplex mobility assay enabled us to scan the whole HIV-1 genome within a short period of time. In Multiregional Hybridization Assay, dual probe reactivity and failure of probe hybridization in a few cases could have been the outcome of genetic diversity of HIV-1 within the clade [9]. The full length HIV-1 gene

amplification of the samples with B/C recombination is in process to track the possibility of newly circulating recombinant forms among Nagaland injecting drug users. Moreover, it remains to be investigated whether recombination may confer selective advantage over parental viruses.

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