Implementation of a Multiregion Hybridization Assay to Characterize HIV-1 Strains Detected among Injecting Drug Users in Manipur, India

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Key Words

Drug users • Heteroduplex mobility assay • HIV strains • Hybridization • Multiregion hybridization assay

Abstract

We have implemented the latest technology of a multiregion hybridization assay (MHAbce, version 2) for the molecular characterization of HIV-1 among injecting drug users (IDUs) of Manipur, India. This study provides a more detailed analysis on the basis of probes designed from eight different genomic regions of HIV-1, to achieve a clear picture of HIV-1 genomic diversity in Manipur. Out of 30 samples, 15 were found to be of subtype C, 1 of subtype B, 5 with dual-probe reactivity, 8 with multigenomic recombination pattern and 1 sample showed both dual-probe reactivity and multigenomic variations. In contrast, the heteroduplex mobility assay (HMA) with respect to gag and env genes revealed 21 samples to be of subtype C (gag C/env C), 3 samples of subtype B (gag B/env B) and 6 samples of B/C recombinants (gag C/env B). MHAbce illustrates the occurrence of inter- and intragenomic variants and dual infection in an IDU population from India. It also indicates the possibility of the presence of new circulating recombinant forms of HIV-1 strains, which might have been difficult to trace by HMA alone.

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Manipur, one of the states in the northeastern region of India bordering Myanmar, is very close to the 'Golden Triangle' (composed of northern Thailand, eastern Myanmar and western Laos). These regions are the world's leading opium- and heroin-producing area. Reported cases of HIV-infected individuals are quite high in Manipur (>1% HIV positivity among women in antenatal clinics and >5% among patients in sexually transmitted diseases clinics) [1] due to international drug trafficking and sharing of contaminated needles and syringes [2, 3]. As reported earlier, the number of injecting drug users (IDUs) estimated all over the world is 13.2 million (0.3% of the estimated 4 billion adult population), the majority of which (10.3 million) live in developing countries [4].

The presence of dual-subtype infection, intersubtype recombination between subtype C and Thai B, has been reported in Manipur [5, 7] using a heteroduplex mobility assay (HMA) based on gag (p24-p7) and env (C2-V3) regions. In HMA, amplicons of gag and env genomic regions from an unknown subtype were mixed individually with amplicons of different known (reference) subtypes separately followed by rapid denaturation and renaturation to form heteroduplexes. The heteroduplex formed between the unknown sample and its closest subtype exhibits fastest mobility in polyacrylamide gel. As HMA is restricted to two genomic regions only, the real scenario of genetic diversity of HIV-1 cannot be ascertained. A multiregion hybridization assay (MHA) has

Fig. 1. The probes for the MHAbce v.2 assay were designed as shown in black circles within the HIV-1 genome covering all the genes. The p24-p7 region in the gag gene and the C2-V3 region in the env gene used for HMA is shown in gray rectangles.

been developed based on real-time PCR using subtype-specific TaqMan probes within different regions of the HIV-1 genome [8–11]. This technology is a powerful tool in tracking new circulating recombinant HIV-1 strains.

Here we report for the first time the implementation of MHAbce (version 2; v.2) among the IDU population from Manipur (India) to characterize HIV-1 strains with improved scope and clarity.

A total of 62 blood samples were collected from IDUs in Manipur after obtaining their informed consent. HIV-1 testing was done by ELISA (Immunogenetics, Zwijndrecht Belgium) followed by the TRIDOT assay according to the policy of the country's National AIDS Control Program. Thirty samples were found to be HIV-1 seropositive and were used for genotyping studies. HMA was performed with respect to 500 bp of env (C2-V3) and 460 bp of gag (p24-p7) genomic regions (fig. 1) and also confirmed by DNA sequencing. Results of HMA revealed that 21 samples were of subtype C (gag C/env C), 3 samples of subtype B (gag B/env B) and 6 samples of B/C (gag C/env B) recombinants (table 1).

In MHAbce v.2, multiple short fragments were amplified throughout the HIV-1 genome (fig. 1) to assess the hybridization of clade-specific fluorescent probes in real-time PCR. The viral RNA was extracted from plasma using a viral RNA mini kit (Qiagen GmbH, Hilden, Germany). The primers and probes (Applied Biosystems, Foster City, Calif., USA) were used with respect to subtype B, C and AE [12]. The genomic regions within *p17*, protease (*pro*), reverse transcriptase (*rt*), integrase (*int*), first exon of *tat* (*tat*), C3 in *gp120* (*gp120*), and *gp41/nef* were amplified in separate first-round PCR using cDNA synthesized by reverse transcriptase PCR from viral RNA. The second round real-time PCR was performed in a final volume of 25 µl with TaqMan 2× Universal

PCR Master Mix (Applied Biosystems) including an aliquot of each amplicon from first-round PCR products distributed in separate sets of second-round PCRs with subtype-specific probes for (*p17*, *pro*, *rt*, *int*, *tat*, *gp120*, *gp41* and *nef*) gene fragments. The *gp120* probes were labelled with reporter dye 6-carboxytetramethylrhodamine and Black Hole Quencher at 5'- and 3'-ends, respectively, whereas all the other probes were labelled with 6-carboxy fluorescein (FAM) at the 5'-end as reporter dye and with Black Hole Quencher at the 3'-end. During the amplification, emission of fluorescence started due to the breakage of linkage between reporter and quencher. The fluorescence was measured and analyzed using the SDS v2.1 software (Applied Biosystems). The second-round real-time PCR amplification was performed in a 96-well ABI PRISM 7900HT sequence detection system (Applied Biosystems) with the following steps: hold at 95° for 10 min, 40 cycles at 95° for 10 s, 60° for 1 min and for the *gp120* region, 60° at 2 min. The threshold was set based on negative controls where the first-round PCR product was omitted. The results were considered positive when the threshold cycle was <35 and there was an exponential increase in the normalized fluorescence intensity for five consecutive cycles. The identity of amplicons was verified by conducting melting-curve analysis with SybrGreen (95° for 15 s, 30° for 15 s, 95° for 15 s at 10% ramp rate), which distinguished the expected PCR products from primer dimers of lower thermal stability. The melting-curve analysis was a real-time PCR containing 2× TaqMan SybrGreen PCR Master Mix (Applied Biosystems) and similar amounts of inner primers and template as used in second-round real-time PCR except for the probes. The melting temperature (T_m) of the duplexes was the maximum peak region obtained in the $-dRn/Dt$ versus temperature graph depicting melting curves. The

Fig. 2. The *rt* gene from sample Mnp97 was amplified and used in the MHAbce v.2 assay using probes designed from subtypes C, B and AE. The specific fluorescence (ΔRn) obtained was plotted against the number of cycles. ΔRn was observed with both the probes, subtype C (1) and subtype B (2). No specific fluorescence was detected with the probe for subtype AE (3). The horizontal line represents a threshold value based on negative controls.

Table 1. Determination of HIV-1 subtypes using MHAbce v.2 and HMA

Sample No.	MHAbce								HMA		Sample No.	MHAbce								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		<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		
Subtype C										Dual-probe reactivity													
1	Mnp6	C	C	C	C	C	C	C	NR	C	C	1	Mnp24	C	C	B/C	B/C	C	C	C	C	C	C
2	Mnp27	C	C	C	C	C	C	C	C	C	C	2	Mnp92	C	C	B/C	C	C	C	C	C	C	C
3	Mnp44	C	C	C	C	C	C	C	C	C	C	3	Mnp97	C	C	B/C	C	C	NR	C	C	C	B
4	Mnp47	C	C	C	C	C	C	NR	C	C	C	4	Mnp153	B	NR	B/C	B	NR	B	NR	NR	B	B
5	Mnp61	C	C	C	C	NR	C	C	C	C	C	5	Mnp54	C	NR	NR	NR	C	B/C	NR	NR	B	B
6	Mnp91	C	C	NR	C	C	C	C	C	C	B	Multigenomic recombinants											
7	Mnp96	C	C	C	C	C	C	C	C	C	C											1	Mnp40
8	Mnp98	C	C	C	C	C	NR	C	C	C	B	2	Mnp68	C	NR	NR	C	NR	C	B	C	C	C
9	Mnp112	C	C	C	C	C	C	C	C	C	C	3	Mnp86	C	C	B	C	NR	NR	B	B	C	C
10	Mnp126	C	NR	C	C	NR	C	C	C	C	C	4	Mnp89	C	C	C	B	C	NR	C	C	C	C
11	Mnp129	C	NR	C	C	C	C	C	C	C	C	5	Mnp90	C	AE	C	C	C	NR	C	C	C	C
12	Mnp137	C	C	C	C	C	NR	C	C	C	C	6	Mnp128	C	C	NR	B	B	B	B	C	C	B
13	Mnp165	NR	C	C	C	C	C	C	C	C	C	7	Mnp146	C	C	B	C	NR	C	B	B	C	C
14	Mnp175	C	C	C	C	C	NR	C	C	C	C	8	Mnp154	C	C	C	C	C	B	C	C	C	B
15	Mnp176	C	C	C	C	C	C	C	C	C	C	Multigenomic recombinants and dual-probe reactivity											
Subtype B										Multigenomic recombinants and dual-probe reactivity													
1	Mnp180	B	B	B	B	NR	B	B	B	B	B	1	Mnp166	C	C	B	B/C	C	C	C	C	C	B

Thirty samples were subjected to MHA using different probes, as depicted in figure 1. The subtypes were determined on the basis of specific probe reactivity. Fifteen samples reacted with subtype C probes only; all the probes for subtype B reacted with one sample; 5 samples reacted with both the probes for subtypes B and C; 8 samples

reacted with different subtype-specific probes, B, C and AE, indicating multigenomic variations; dual-probe reactivity and multigenomic variation were observed with one sample. The results of HMA for all the samples were also shown. NR = No probe reactivity.

highest peak indicated the PCR product generated, with the lowest one pointing towards the primer dimers (data not shown). In order to validate this assay, the samples of known subtypes determined from DNA sequence analysis were included in MHA and no discrepancy was observed. Furthermore, assay performance was verified by sequencing of the samples after the MHA analysis. To rule out the possibility of cross-contamination, each Taq-Man reaction was tested twice with the same primers and probes in separate plates at different times.

As revealed from MHAbce v.2 results, 15 samples were of subtype C (Mnp6, Mnp27, Mnp44, Mnp47, Mnp61, Mnp91, Mnp96, Mnp98, Mnp112, Mnp126, Mnp129, Mnp137, Mnp 165, Mnp175 and Mnp176; table 1) and 1 sample was of subtype B (Mnp180; table 1). Dual-probe reactivity was found in 5 samples (Mnp24, Mnp92, Mnp97, Mnp153 and Mnp54), and a multigenomic recombination pattern in 8 samples (Mnp40, Mnp68, Mnp86, Mnp89, Mnp90, Mnp128, Mnp 146 and Mnp154), whereas 1 sample (Mnp166) showed both dual and multigenomic reactivity. Dual-probe reactivity occurred in reverse transcriptase and integrase genes. Here, the probes belonging to subtypes B and C for these genomic regions reacted equally in separate reactions. The dual-probe reactivity pattern of one such representative sample (Mnp97) in the *rt* gene is shown in figure 2, where both the probes C and B had reacted equally, while the AE probe had no reactivity.

In summary, our data provide evidence for the possibility of new emerging strains of HIV-1 along with their dual infection among the IDU population from Manipur. The increasing number of recombinants documented so far shows that subtype distribution is a dynamic process and viruses that are already mosaic are likely to be transformed into further recombination events. In many samples, the occurrence of a B/C recombination in *gag*, *pol* and *env* genes emphasized the presence of intragenomic recombinants. The sequence analysis of the full-length HIV-1 genome of these samples is in process to find out the recombination break points. In a few cases probe hybridization failed, which might be due to genetic diversity of HIV-1 within the clade, thus causing the hybridization of probes to heterotypic strains and decreasing the specificity, as described by others [12]. In conclusion, continuous monitoring of HIV-1 strains in this part of the country as well as characterization of emerging new recombinant forms is warranted, which might help in designing appropriate candidate HIV-1 vaccine.

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