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Random and Gene Specific Amplification as Tool for Phylogenetic Assessment: A Study of HIV Subjects From North India

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ABSTRACT

Genetic polymorphisms are frequent variations at a specific location in the genome. They arise from the mutation at a locus. This preliminary work is aimed to assess the application of random and gene specific amplification as tools for phylogenetic study in HIV +ve subjects from two North Indian states (Punjab and Rajasthan). DNA extracted from 44 samples of HIV +ve blood (22 samples from each location) was subjected to random amplified polymorphic DNA – polymerase chain reaction (RAPD-PCR) by using 12 different primers. These include RAPD as well as human gene specific primers for liver function parameters. The results were subjected to phylogenetic assessment as well as analysis of molecular variance (AMOVA) for estimating population differentiation. When combined primers were used, AMOVA indicated that, higher percentage of variation (84 %) was attributed to within the population variation than among the population variation (16 %) of region under study. Our results confirm the authenticity of molecular marker analysis as the cluster pattern generated by combined primer analysis seems to be an extended pattern of analysis based on different individual primer sets. A combination of all primer sets will therefore help to provide whole genome coverage and reduce the errors in genetic similarity estimation based on any one marker system alone. These results can be of significant importance in future studies on assessment of genetic polymorphisms by using RAPD as well as gene specific primers.

Keywords: *Gene specific primers, AST, ALT, ALP, ALB, liver function, HIV/AIDS, RAPD-PCR, North India, molecular marker*

1. INTRODUCTION

Characterization of genomic DNA through identification and determination of random polymorphic markers has proved to be a powerful application of DNA technology. Random amplified polymorphic DNA – polymerase chain reaction (RAPD-PCR) is a DNA fingerprinting technique used to detect genomic polymorphisms [1]. It can also be used for genomic analysis of HIV-1-infected individuals and for the identification of human molecular markers that may correlate with susceptibility to HIV-1-infection, or differences in disease progression among HIV-1-infected individuals [2]. The genomic DNA profiling by RAPD analysis have applications as wide ranging as paternity testing and forensic science, through investigation of genetic disease genes and identification of the sources of plant and animal products, to ecological population studies [3]. A large number of highly informative DNA markers have been developed for the identification of genetic polymorphism and RAPD technique based on the PCR is among the most commonly used molecular technique to develop DNA markers [4]. The individual electrophoretic patterns obtained by RAPD-PCR can offer a simple and reliable approach to DNA analysis [5]. Surrogate markers in HIV disease can be used as an attractive method of assessing the efficacy of new treatments more quickly than by using clinical end-points [6]. The study of molecular variation within a single species can be used to analyse molecular variance (AMOVA) which produces estimates of variance components and *F*-statistic analogs, reflecting the correlation of haplotypic diversity at different levels of hierarchical subdivision [7].

The aim of this study was to assess the application of random and gene specific amplification as tools for phylogenetic study in HIV+ve subjects from North India.

2. MATERIALS AND METHODS

2.1 Blood Samples

Human blood samples from newly diagnosed HIV +ve persons were obtained from two locations in North India; Ludhiana (Punjab state) and Jodhpur (Rajasthan state) between September, 2010 to June, 2011, with due permission from the concerned authorities. DNA was extracted from 44 samples of HIV +ve blood (22 samples from each location). The study was conducted at Department of Biotechnology, Meerut Institute of Engineering and Technology (MIET), Meerut and YPL Diagnostics, Meerut. All chemicals, reagents, enzymes

and buffers used in DNA isolation from human blood, gel electrophoresis and RAPD-PCR were of analytical or molecular biology grade.

2.2 Primers

A total of 12 primers were used for the molecular marker studies. The first set consisting of four primers of unknown genes was customized as reported by Aikhionbare et al. [2]. The rest four sets (two primers each) were human gene specific for liver function parameters [aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and serum albumin (ALB)] and were designed by using Primer3 and BLAST online tools available on National Center for Biotechnology Information (NCBI) web-site [8]. All primers were custom synthesized by Bioserve, Hyderabad, India. The characteristics of the molecular primers and the amplification results are shown in table 1.

2.3 Blood DNA Extraction

The protocol for DNA extraction was modified from the procedure given by Hirata et al. [9]. 400 µl of blood sample and 600 µl of 0.154 M NaCl were mixed (homogenized) in an eppendorf tube by gently inverting the tube for 30 seconds and then centrifuged for 5 minutes at 4300 rpm. The pellet was dissolved in 1 ml of Tris Buffer – I [10 mmol/L Tris-HCl (pH adjusted to 8.0), 10 mmol/L KCl, 10 mmol/L $MgCl_2$, 2 mmol/L EDTA (pH adjusted to 8.0 with NaOH pellets) and 25 mL/L Triton X-100] and after 5 minutes incubation, the eppendorf was centrifuged at 5500 rpm for 8 minutes. The supernatant was discarded and the pellet was washed twice with Tris Buffer – I. The left over pellet was incubated in 220 µl of Tris Buffer – II [10 mmol/L Tris-HCl (pH adjusted to 8.0), 10 mmol/L KCl, 10 mmol/L $MgCl_2$, 2 mmol/L EDTA (pH adjusted to 8.0 with NaOH pellets), 0.4 mol/L NaCl and 10 gm/L of SDS] at 56°C for 15 minutes. 100 µl of 5 M NaCl was added to precipitate the cellular proteins and the eppendorf tube was then centrifuged at 4000 rpm for 10 minutes. The supernatant was carefully taken in a fresh 1.5 ml eppendorf tube and DNA precipitated by using chilled absolute ethanol. The pellet was dissolved in 100 µl of Tris – EDTA Buffer (10 mmol/L Tris-HCl (pH adjusted to 8.0) and 1 mmol/L EDTA) after centrifugation and the DNA samples were then stored at 4°C for further analysis (or at – 20°C for later uses). The isolated DNA was then quantified at ultra violet absorbance of 260 nm using UV-VIS scanning spectrophotometer (Jasco).

Table 1. Results of Primer Amplification

Primer Code	Sequence (5'→3')	Annealing Temp.(°C)	Molecular weight range (bp)	No. of Poly bands	No. of Mono band	Diversity in value of PIC	Resolving Power
FA-1	GGTGCACGTT	31	200 - 1800	9	4	0.4311	1.3787
FA-2	GTTTCGCTCC	28	200 - 1500	4	3	0.2654	1.4692
FA-3	CCACGGGAAG	31	100 - 1600	3	7	0.2065	1.5871
FA-4	ACGGCGTATG	33	250 - 1100	2	3	0.2437	1.5126
AST-1	CGGACCCCCGCAAG	52	200 - 1300	4	5	0.3091	1.3818
AST-2	CGCGGGTTCGCAGT	52	200 - 1400	6	1	0.6415	0.7171
ALT-1	TACGCCGGGCAGCA	59	300 - 1500	0	9	0	2.0
ALT-2	CGCGGGTTCGCAGT	53	100 - 1200	2	5	0.2480	1.5041
ALP-1	CCCGCCGTGGGTCT	55	300 - 1300	2	4	0.2774	1.4452
ALP-2	ACGGCGGGGAGGAC	53	200 - 2000	3	6	0.2905	1.4191
ALB-1	GCAGCGGCACAGCA	62	100 - 2200	3	5	0.3620	1.2761
ALB-2	TGCAGCGGCACAGC	61	150 - 1400	3	4	0.3724	1.2552

Poly: Polymorphic, Mono: Monomorphic, PIC: Polymorphic Information Content

2.4 PCR Reaction for RAPD

DNA amplification for RAPD was performed by using following components: Taq Assay Buffer (10 X), dNTPs Mix (10mM), DNA (25ng/µl), Primer (25 ng/µl), Taq DNA Polymerase (3U/µl) and double-distilled water, in 25 µl volume. The PCR tubes were transferred to the DNA thermal cycler (Corbett) and amplification reactions were performed with the following thermal profile: initial denaturation for 4 minutes, 40 repeats of [denaturation (30 seconds) – annealing (30 seconds) – extension (1 minute)] and a final extension of 3 minutes.

2.5 Agarose Gel Electrophoresis

15 µl of RAPD-PCR product from each PCR vial was loaded separately in each individual well in 4% agarose gel (total 44 samples with one individual primer). The corner lanes were loaded with 10 µl of the readymade marker DNA (100 bp DNA ladder). The products were run in the agarose gel at 50 volts [10]. The gel was then observed under UV light in a trans-illuminator (Alpha imager). Consistency of band profiles was assessed by triplicate analysis. Bands were sized and matched directly on the gels and a 0-1 matrix sheet was prepared on the basis of presence and absence of bands. The data was converted into dendogram form (phylogenetic tree) by Numerical Taxonomy System (NTSYS) software and analysed using UPGMA. To describe the partitioning of genetic variation between and within groups, analysis of molecular variance (AMOVA) is used for estimating population differentiation directly from molecular data and testing hypotheses about such differentiation [7].

The study was approved by Institutional Research Board (IRB) and Institutional Ethical Committee (IEC) of MIET, Meerut.

3. RESULTS AND DISCUSSION

The results of PCR amplification of 44 genotypes by four random amplified polymorphic DNA (RAPD) primers and eight gene specific primers are as shown in table 1, while molecular profiling patterns of 44 HIV +ve genomes with some of the primers (FA-1, FA-3, AST-1, ALT-1, ALP-1 and ALP-2) are shown in figures 1-6. The dendogram formed on the basis of 0-1 analysis of all the gel images is shown in figure 7. Table 2 and figure 8 show the pattern and statistical results of AMOVA respectively.

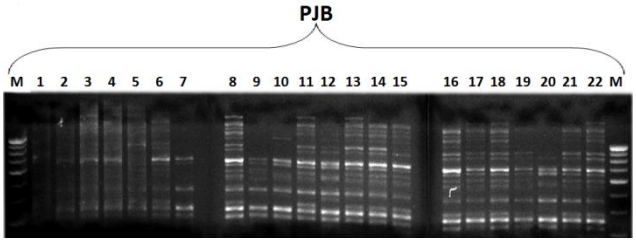

Figure 1. Molecular profiling patterns of 44 HIV +ve genomes with FA-1 primer [State-1 (Punjab)]

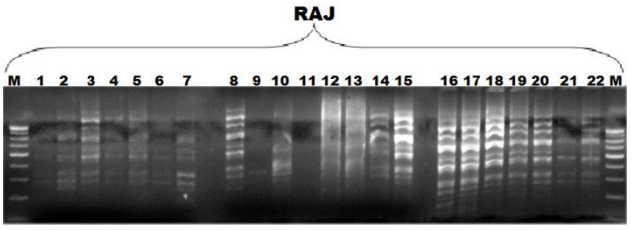

Figure 2. Molecular profiling patterns of 44 HIV +ve genomes with FA-3 primer [State-2 (Rajasthan)]

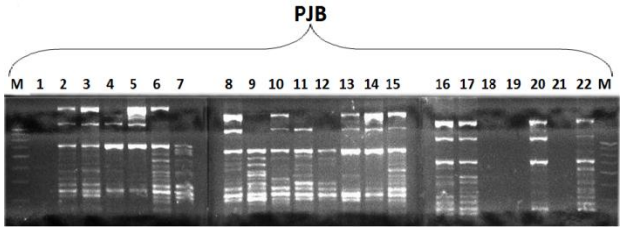

Figure 3. Molecular profiling patterns of 44 HIV +ve genomes with AST-1 primer [State-1 (Punjab)]

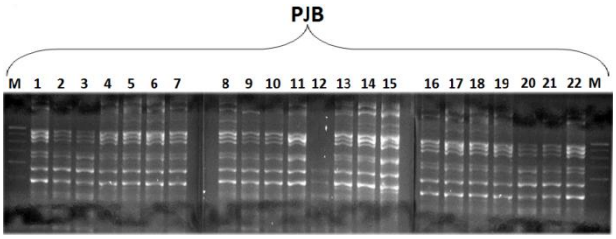

Figure 4. Molecular profiling patterns of 44 HIV +ve genomes with ALT-1 primer [State-1 (Punjab)]

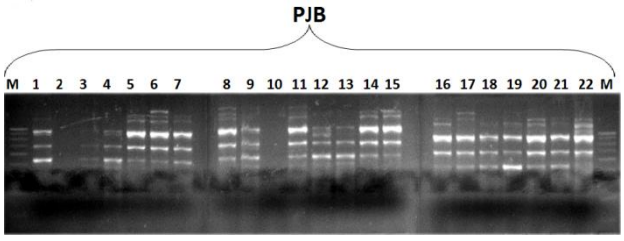

Figure 5. Molecular profiling patterns of 44 HIV +ve genomes with ALP-1 primer [State-1 (Punjab)]

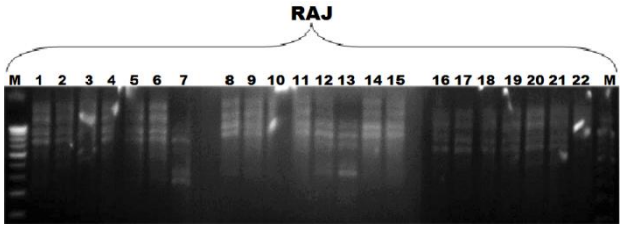

Figure 6. Molecular profiling patterns of 44 HIV +ve genomes with ALP-2 primer [State-2 (Rajasthan)]

Table 2. Statistical Results for AMOVA

Primer sets	Source of Variation	Degrees of Freedom (df)	Sum of Squares (SS)	Est. Var.	*F*
All	Among populations	1.000	35.068	1.281	5.089
	Within Population	42.000	289.409	6.891	

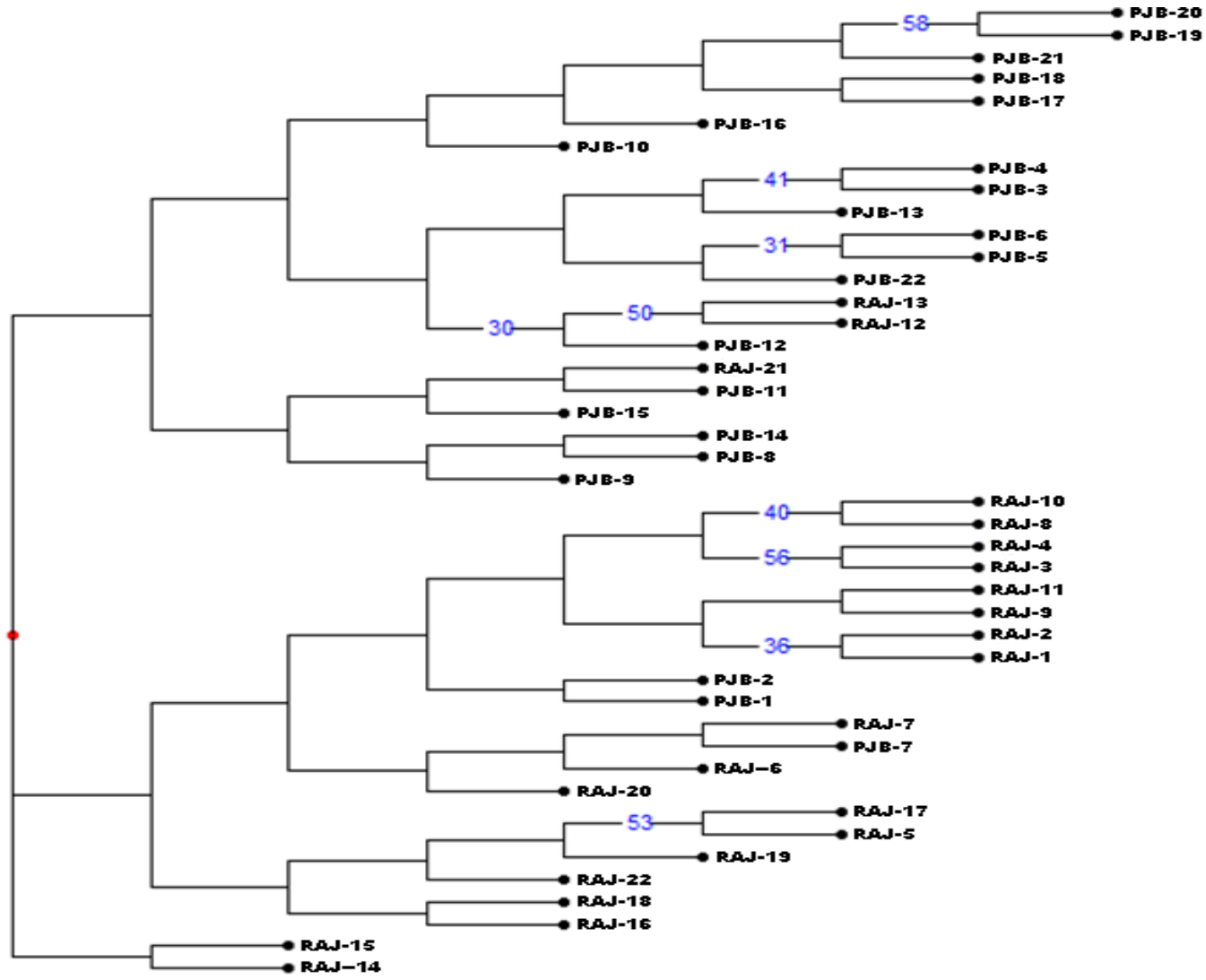

Figure 7. Dendogram for all primers (Combined)

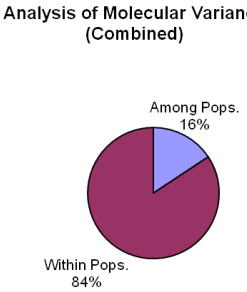

Figure 8. Analysis of Molecular Variance (Combined 12 Primers)

Combined primer profiling of 44 genotypes with the help of all 12 primers follow some of the characteristic profiling pattern of individual primer analysis, as PJB-15 follow the same pattern in individual as well as in combined analysis. PJB-10, PJB-6 and PJB-5 show the same pattern as in AST-ALT group. RAJ-3, RAJ-4, RAJ-1, RAJ-2, RAJ-14 and RAJ-15 follow the pattern as in ALP primer set. PJB-1, PJB-2, PJB-7 and RAJ-7 show the pattern followed by ALB primer set. These results confirm the authenticity of the molecular marker analysis, as the cluster pattern generated by combined primer analysis seems to be an extended pattern of analysis based on different individual primer sets. When combined primers were used, AMOVA indicated that, higher percentage of variation (84 %) was attributed to within the population variation than among the population variation (16 %) of region under study (Figure 3). The estimated variance within the population (6.89) was higher as compared to among the populations (1.28) as shown in table 2.

Primers with high polymorphic information content (PIC) values are useful for estimating relationships between genotypes. Primers with low PIC values on the other hand can be used to analyse chromosome regions of special interest. The PIC value depends on the diversity of the genotype tested [11-13]. The results of hierarchical cluster analysis carried out with the four marker types together reflected those obtained using each marker type separately. The relationships between the genotypes were also clearly demonstrable. The joint use of various marker types allow the tested genotypes to be reliably distinguished, while also providing a clear picture of how they were related. Our results are in good agreement with those previously obtained using markers [2], which provide a good estimation of genetic correlation [14-16]. RAPD primers also distinguish genotypes under study remarkably [17]. RAPD analysis could be adapted for the rapid and efficient screening of DNA from large cohort groups to identify unknown genomic polymorphisms that may have clinical relevance to pathology of HIV-1 or other diseases. Furthermore, RAPD analysis may be a useful tool in exploiting single nucleotide polymorphism within human genes, both as related to genotype–phenotype correlations and as a tool for linkage and association studies in complex diseases [2]. RAPD primers bind randomly to different parts of the genome, so theoretically they cover the genome uniformly. Williams et al. [1, 18] suggested that all data from a given set of RAPD-PCR profiles are not equally usable as molecular markers and fact that most polymorphic RAPD markers are dominant [19]. A combination of all primer sets will therefore help to provide whole genome coverage and reduce the errors in genetic similarity estimation based on any one marker system alone. Also if we assume that by increasing the number of molecular primers, the variance of individual genetic similarity estimation is decreasing, the large number of polymorphic bands obtained in our analysis should provide more precise genetic similarity estimates.

The F-statistics and the analysis of genetic diversity in sub-divided population have been attempted by several workers [20-22]. Long et al. [23] attempted the correlation for genetic distance between two population sub-divisions, while Cockerham [24] analysed different gene frequencies. Recently analysis of genetic diversity among encet populations from two locations in southwestern part of Ethiopia shows higher percentage of variation between the two populations of enset as compared to within population variation [25]. Our results of high variation within the population are similar to the study on genetic divergence of two populations by Watterson [26].

4. CONCLUSION

The molecular marker analysis leading to hierarchical cluster analysis with four marker types together reflect the authenticity of the procedure. The joint use of various marker types shows the relationship patterns between various genotypes within the same population. However it is felt that without knowing the location of various products (enzymes) on the chromosome, the results may be considerably distorted. The phylogenetic clustering of HIV +ve subjects in two populations shows that the relationship was pronounced within the same population rather than among the two populations. The high degree of variability among the subjects of the same population may possibly be attributed to the degree of HIV infection, thereby viral load, in a subject. However more specific and extensive studies require microarray analysis of genomic DNA of HIV +ve subjects. A combination of all primer sets will therefore help to provide whole genome coverage and reduce the errors in genetic similarity estimation based on any one marker system alone.

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