

**DIVISION OF
MOLECULAR VIROLOGY**

Division of Molecular Virology

The Division is engaged in three fold research activities: Laboratory Research, Clinical Research and Support services to Clinical Trials. Molecular epidemiology and characterization of HIV-1 and HIV-2 circulating in India is a major area where the division is putting lots of efforts, especially identification of emerging and re-emerging recombinant forms of HIV-1. In addition, different fundamental and basic research studies are ongoing towards understanding the mode of transmission and pathogenesis; thrust areas being HIV-1 clade C envelope function in cellular tropism, sensitivity of envelope proteins to entry inhibitors and neutralizing antibodies at different disease stages, natural products as candidate microbicides for HIV-1 and antiretroviral drug resistance. There are 5 Junior Research Fellows (JRF) working in the division on their Ph.D. thesis. The research activities are being supported by extramural funding such as from Department of Biotechnology (DBT), World Health Organization (WHO), National Institutes of Health (NIH), USA, and National AIDS Control Organization as well through intramural grants.

A. Full-Length Genome Analysis of HIV-1 isolates from India

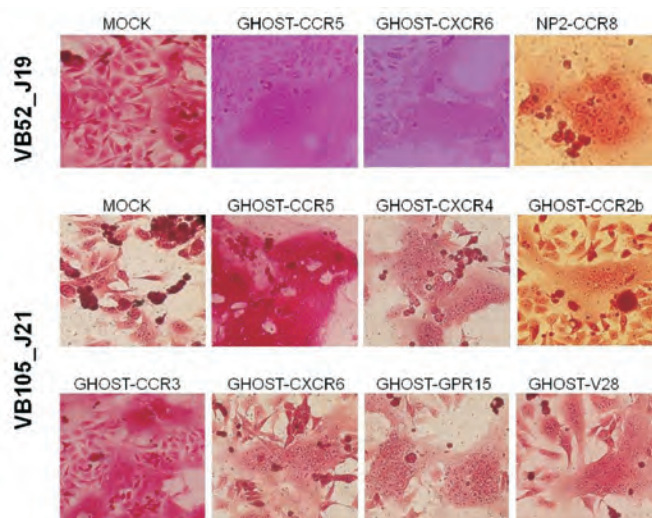
[Lead Investigators: Dr. R. S. Paranjape]

This study is funded by DBT, Government of India. In the last reporting period (2006-2007), we documented and published novel forms of B/C recombinants in India. In continuation of the same project, we have screened additional HIV-1 strains for disseminating their near full length genetic properties. Virus samples obtained from Viral Bank and infected PBMCs from other projects of NARI have been co-cultured with donor PBMCs respectively that were isolated from donor blood by Density Grade centrifugation method. Genomic DNA was isolated by using Qia Amp DNA Blood Mini kit (Qiagen, Inc.) and near full length genomes of HIV (9 KB) were amplified using Expand Long Template Polymerase (Roche, Inc.). Purified amplicons were cloned in pCR 2.1 vector (Invitrogen, Inc.). Plasmid DNA was isolated and sequenced by using ABI 3100 genetic analyzer using Big Dye Terminator method. We have cloned near full length genome of 37 viruses (at least 2 clones for each virus) and sequencing has been completed for 26 viruses. Interestingly, though all the clones have been found as subtype C, we have found one clone exhibiting novel form of A1/C recombinant and one subtype A virus. Further characterization is being carried out.

B. Cellular tropism of HIV-1 of India clade and characterization of their biological properties in T cells and monocyte-derived macrophages.

[Ph.D. student: Ms. Lavina Gharu, Guide: Dr. J. Bhattacharya]

The principal aim of the study is two fold; to study the co-receptor usage and tropism of HIV-1 envelope of Indian clade C in different disease stages and to identify and characterize HIV-1 R5 variants that modulate broader and narrow tropism resulting in modulation in virus entry, transmission and pathogenesis. Our objectives are:

Figure 4.2: Co-receptor usage by two envelope clones as determined by cell

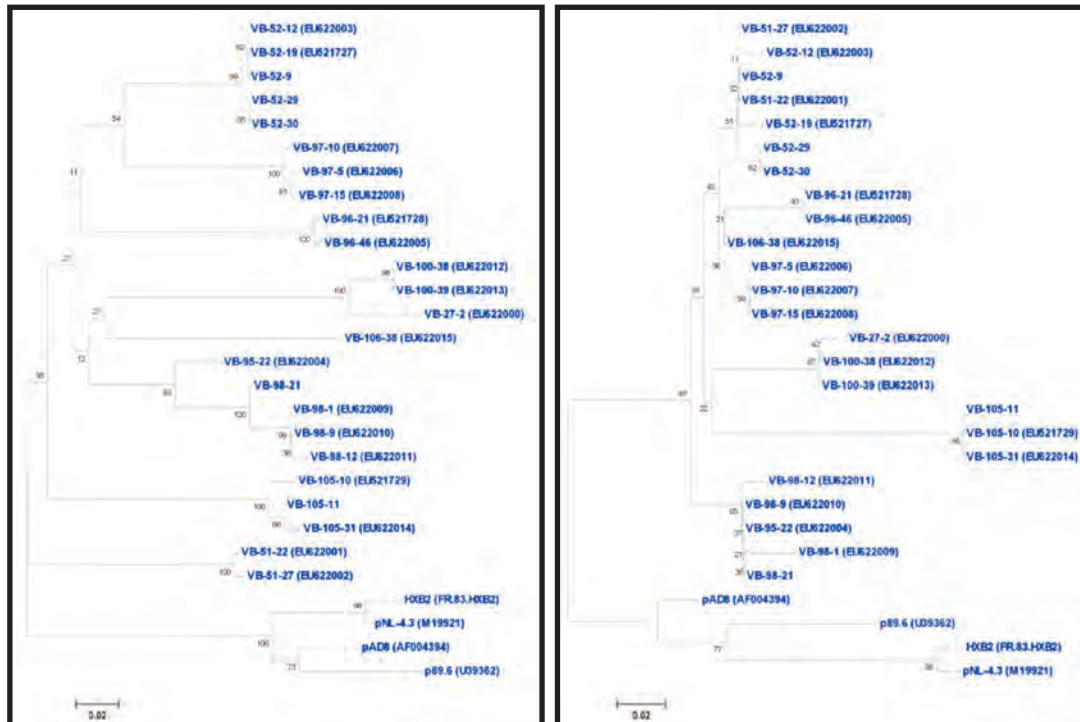
Co-receptor usage by two envelope clones as determined by cell: cell fusion assay. Env+293T cells were co-cultivated with CD4+ GHOST and NP2 cells expressing different co-receptors and multi-nucleated giant cells were scored as a function of co-receptor usages by a specific envelope clone.

Table 4.1: Coreceptor usages of multi-tropic India Clade C Envelope in PBMCs in presence/absence of CCR5 (TAK-779) and CXCR4 (AMD-3100) antagonists of entry

Virus/Clone	Inhibitor	CCR5	CXCR4	CCR2b	CCR3	CCR8	GPR15	CXCR6	CX3CR1
NARI-VB52	No drug	+	-	-	-	+	-	+	-
	TAK-779	-	-	-	-	+	-	+	-
	AMD-3100	+	-	-	-	+	-	+	-
NARI-VB105	No drug	+	+	+	+	-	+	+	+
	TAK-779	-	+	+	+	-	+	+	+
	AMD3100	+	-	+	+	-	+	+	+
	TAK-779+AMD-3100	-	-	+	+	-	+	+	+
NARI-VB52.J19 (Clonal)	No drug	+	-	-	-	+	-	+	-
	TAK-779	-	-	-	-	+	-	+	-
	AMD-3100	+	-	-	-	+	-	+	-
NARI-VB105.J21 (Clonal)	No drug	+	+	+	+	-	+	+	+
	TAK-779	-	+	+	+	-	+	+	+
	AMD-3100	+	-	+	+	-	+	+	+
	TAK-779+AMD-3100	-	-	+	+	-	+	+	+

All the functional clones were sequenced and phylogenetic analyses done (Figure 4.3). The DNA sequences were submitted into GenBank and can be obtained from NCBI or Los Alamos HIV-1 database. We are currently assessing the stoichiometric usage of CD4 and CCR5 by these envelope clones and their relevance in HIV-1 entry and transmission. We also aim to amplify more envelope clones from asymptomatic cases and characterize further.

Figure 4.3: Phylogenetic tree of V1V2 (A) and V3 (B) loops of *gp120* of HIV-1 India clade C envclones prepared with MEGA 3.1 using kumura-2 parameter model.



C. Studies on the role of gag and vpu on HIV-1 envelope assembly in T cells and macrophages.

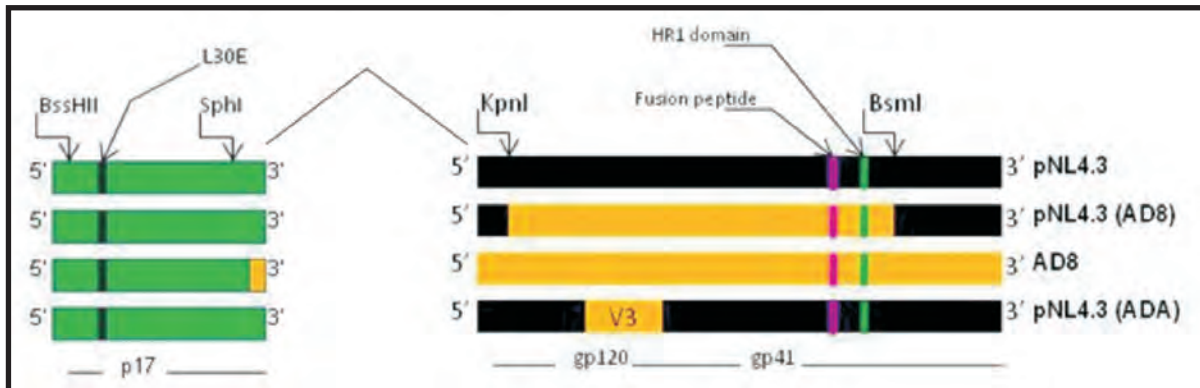
[Ph.D. student: Ms. Archana Gautam, Guide: Dr. J. Bhattacharya]

The primary objectives of the study were:

- (i) To study the effect of mutations in Gag region(s) on HIV-1 envelope assembly and to map functional domains within gag and env that are critical for viral assembly and budding.
- (ii) To study the role of specific gag and envelope domain in AD8 envelope trafficking in specialized cellular compartment/s supporting envelope assembly.
- (iii) To study the role of *Vpu* on Envelope incorporation during budding and viral infectivity.

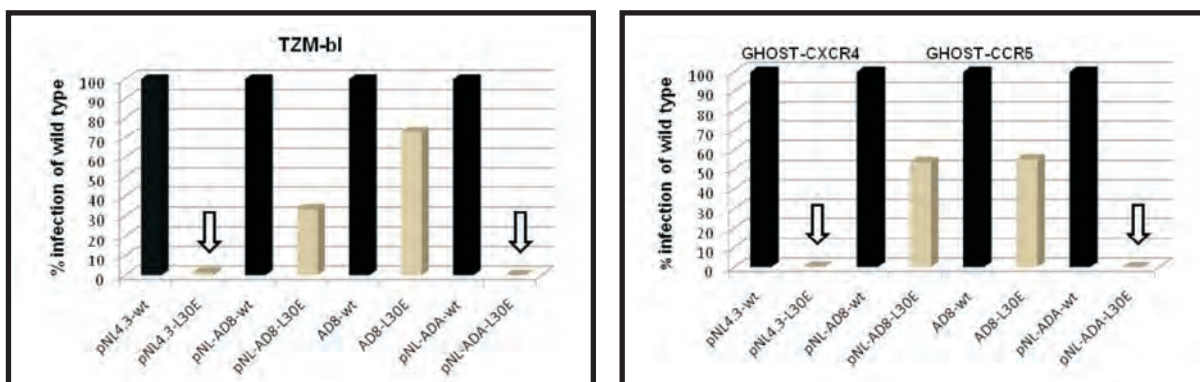
We reported in our last annual report (2006-2007) that p17 gag modulates envelope association with lipid rafts by co-localization experiment. Further, we investigated whether this phenomenon is universal or it requires specific domains in envelope and gag for strong interaction and assembly. To address this, same substitution in p17 (L30E) were constructed in different backbone of R5/macrophage tropic molecular clones, such as AD8, pNL43 (AD8) and pNL43 (ADA) (Figure 4.4).

Figure 4.4: Organization of engineered HIV-1 molecular chimeric clones. Changes in color denote dissimilar DNA sequences.



All the engineered HIV-1 clones were transfected to 293T cells to obtain progeny virions and infectivity assays were carried out in TZM-bl, GHOST-CD4-CCR5 and GHOST-CD4-CXCR4 cells. Interestingly, our results showed that a single amino acid substitution (L30E) that had drastic effects on envelop assembly onto virus particles in pNL43 is reversed significantly by pNL43 (AD8) and AD8 molecular clones (Figure 4.5). Sequencing and gene mapping revealed that specific protein motif upstream of gp41 transmembrane domain (TM) between fusion peptide and HR1 domains of envelope plays vital role in strong interaction between p17 gag and envelope required for efficient envelope assembly and virus transmission. Currently we are assessing amino acid motif/s in this region that is/are critical for HIV-1 envelope assembly and whether our *in vitro* data corroborates with primary cell types that are exploited by HIV-1 *in vivo*. To our knowledge this is the first evidence of a region in gp41 outside cytoplasmic domain modulating gag-env interaction.

Figure 4.5: Infectivity assay of HIV-1 molecular clones with and without single amino acid substitution (L30E) in p17 gag in TZM-bl, GHOST-CXCR4 and GHOST-CCR5 cells.



D. Neutralization epitope mapping in HIV-1 envelope of Indian origin and construction of envelope-based peptide Immunogen for generation of broadly cross-reactive neutralizing antibodies

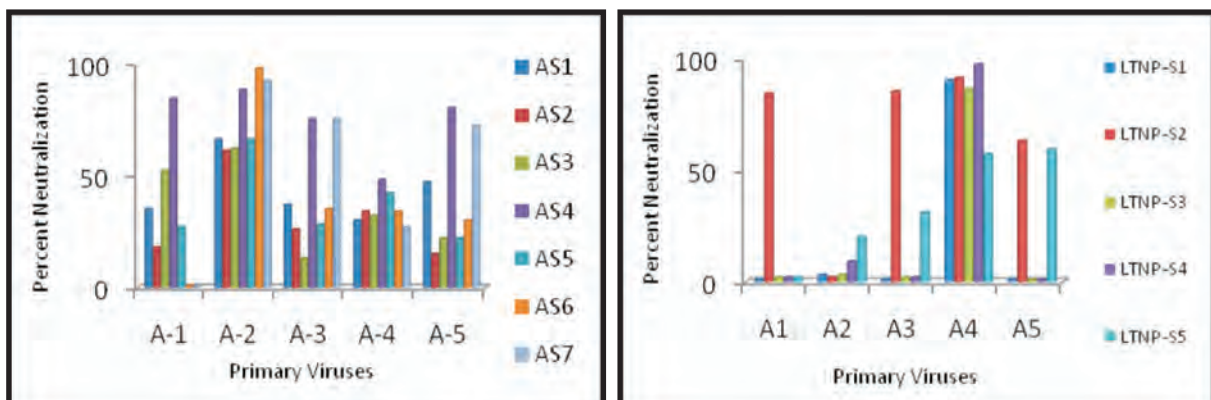
[Ph.D. student: Mr. Rajesh Ringe, Guide: Dr. J. Bhattacharya]

This study is funded jointly by DBT-ICMR. The main objectives of the study are:

- (i) To isolate and amplify complete envelope genes from primary HIV-1 viruses from acutely infected/recent seroconverters and to generate panel of pseudotype reporter viruses carrying envelopes from early and late phases.
- (ii) To test in a single round infectivity assay the sensitivity of envelope to serum/plasma neutralizing antibodies obtained from different disease stages.
- (iii) To generate escape variants in primary cell types and to map motifs conferring neutralization resistance.

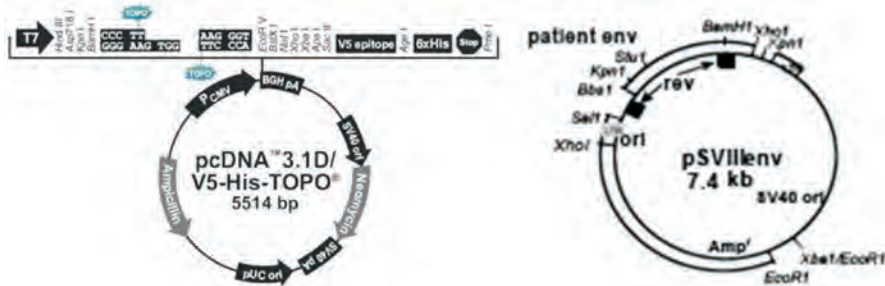
Our goal is to identify sensitive and insensitive envelopes which would be further characterized for identification of motifs conferring neutralization phenotype. The motifs conferring significant sensitivity would be exploited to design construct for candidate vaccineeliciting broadly neutralizing antibodies in the long term goal. Our first approach was to collect specimens from ART naïve acutely infected or from patients with recent infection. We obtained blood and plasma specimens of seven HIV+ patients with recent infection from the Department of Immunology. In addition, we have obtained plasma and serum specimens of five asymptomatic patients infected for a period of 6-8 years with a CD4 count between 400-500 per cu.mm from NARI clinic. We could grow only 5 primary viruses from PBMCs of 5 recent infected patients. The serum and plasma specimens were heat inactivated (in order to neutralize complement function) and stored for neutralization assays. Our initial approach was to characterize the primary viruses in order to select neutralization sensitive and insensitive viruses before amplifying envelope (gp160) protein. Neutralization assays were carried out in genetically engineered HeLa cell line (TZM-bl) that expressed high amounts of cell surface CCR5 and CXCR4 in addition to CD4. These cells were infected with primary isolates with appropriate amounts TCID₅₀ dosages in presence of various dilutions of sera/plasma. The results obtained were shown in Figure 4.6.

Figure 4.6. Neutralization sensitivity of primary isolates of HIV-1 derived from recent infection to autologous and heterologous plasma/serum antibodies.



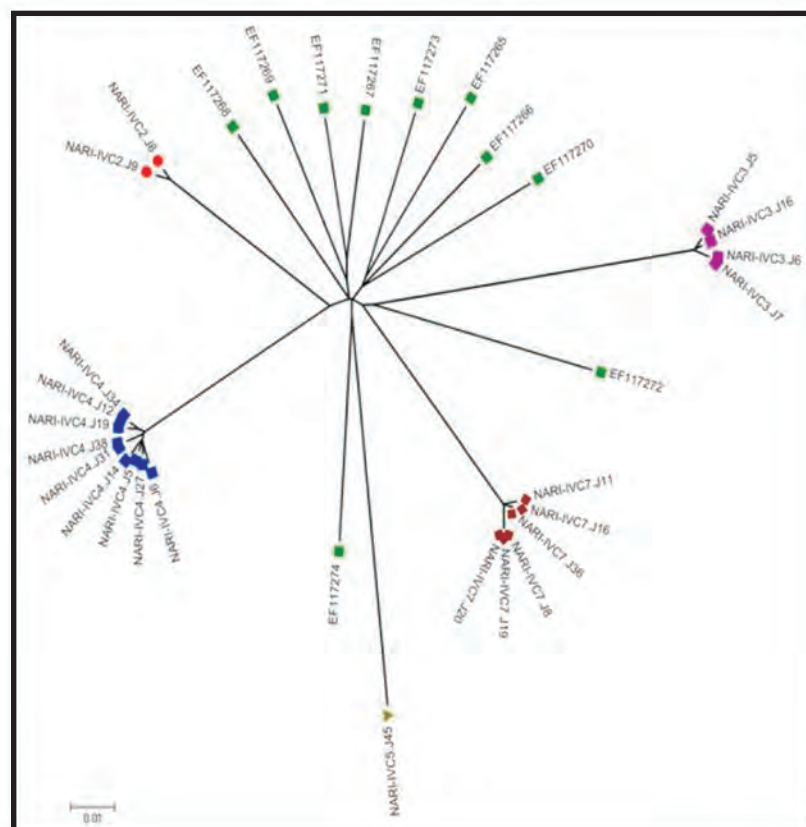
To understand whether these effects are due to specific fitness of the virus or a combined effect of viral quasispecies, we further amplified and cloned complete functional *gp160* clones from co-cultivated PBMC to mammalian expression vectors either by blunt-end ligation in pcDNA3.1TOPO (Invitrogen) or by staggered end ligation in pSVIIenv (Figure 4.7).

Figure 4.7. Mammalian expression vectors used for cloning functional *gp160* genes.



We have obtained 12 genetically distinct clones from 6 patients diagnosed with recent HIV-1 infection and found that they are dispersed in phylogenetic analysis (Figure 4.8). All the clones belong to clade C and uses CCR5 strictly for entry. The GenBank accession numbers of the *gp160* clones are EU908214 to EU908225. We are currently assessing the neutralization sensitivity of these molecular envelope clones both against plasma/serum antibodies as well as known broadly cross reactive antibodies against gp120 and gp41.

Figure 4.8. Phylogenetic tree of *gp160* clones obtained from recently infected patients. Green blocks represent known *gp160* of India clade C clones obtained from acutely infected patients available in GenBank.



E. Comprehensive molecular analysis of HIV incidence, genetic diversity and anti-retroviral drug-resistance mutations in diverse risk groups across Western India

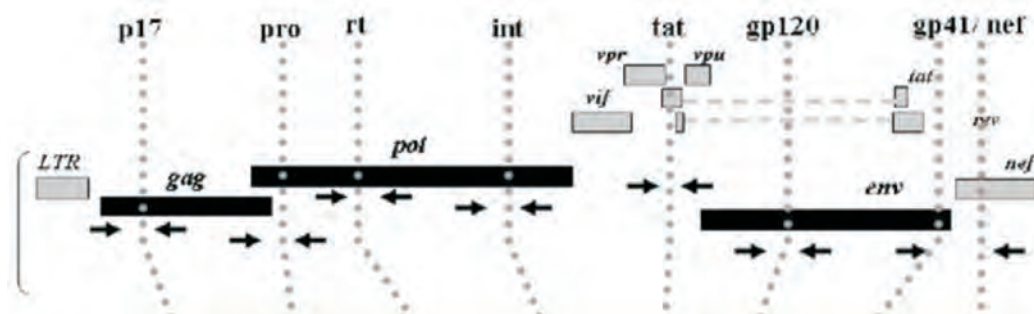
[Ph.D. student: Ms. Sampurna Mukhopadhyay, Guide: Dr. J. Bhattacharya]

This study is funded jointly by DBT-ICMR. The primary objectives of the study are:

- (i) Surveillance for circulating recombinants, thus to generate data recent infection in different risk groups and geographical locations using detuned assays in cross-sectional studies
- (ii) Screening of the isolates from different geographical locales for identification of strains with into subtype recombination/mutations.
- (iii) Characterization of recombinants through full length sequencing and determination of the breakpoints.
- (iv) To generate database for presence of drug-resistance mutations in drug-naive and treated persons from various risk groups and geographic regions of India.

Our primary goal was to obtain blood/plasma/serum specimens from high HIV-1 prevalent regions of Western India. Specimens were collected and being collected from diverse risk groups such as intravenous drug users (IDU), Men having sex with Men (MSM), and Female Sex Workers (FSW). The first step towards screening of genetically divergent HIV-1 strains was to optimize conditions in 7900HT Fast Real Time PCR system for standardization of identification of different subtypes as well as recombinants by a necessary known as Multiregion Hybridization Assay or MHA. The MHA assay required two step PCR reaction; first with universal pairs of primer sets and amplification of 7 distinct HIV genes and second round involving Real Time PCR with gene specific universal primers against each of first round amplicons and subtype (B, C and A/E)-specific fluorescent probes (Figure 4.9).

Figure 4.9: Multiregion hybridization assay (MHA V.2).

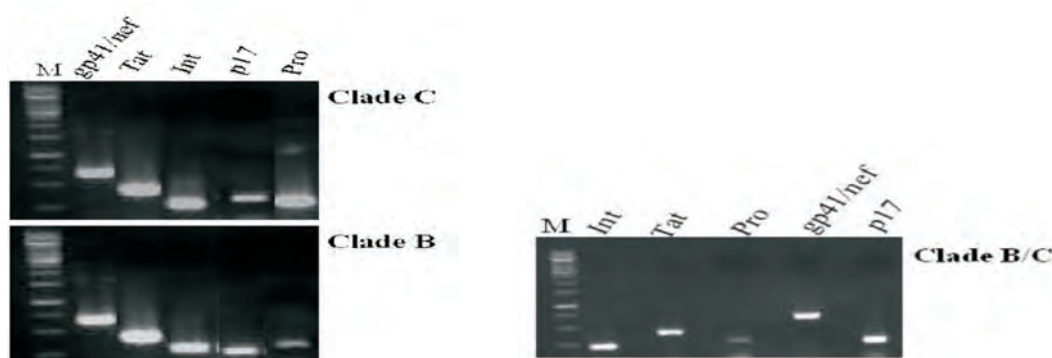


Multiregion hybridization assay (MHA V.2). In the first round, 7 distinct regions are amplified (*p17*, *pro*, *RT*, *int*, *tat*, *gp120*, *gp41/nef*). In the second round each of the amplicons are subjected to amplification in Real Time PCR (in ABI7900HT) with gene-specific universal primer pair and subtype specific probe. Amplification in the second round would be an indicative of such subtype and will be further characterized by sequencing.

We have successfully optimized first round PCR conditions for amplifications from DNA (subtype B, C, B/C) as well as from plasma and serum (Figure 4.9). The second round involving Real Time

PCR of subtype specific gene amplification is currently being standardized. Once standardized and MHA_{bce} V.2 optimized we aim to screen for HIV-1 variants in different risk groups. The assay will help in rapid diagnosis of gene specific recombinants in MHA assay which will further be taken for full length sequencing to precisely map for breakpoints. Once we identify genetically distinct strain, we will also amplify *pol* and assess ART resistant gene.

Figure 4.10: Amplification of 5 distinct HIV-1 genes from clade C (A), clade B (B) and B/C recombinant (C) in the first round of PCR using same cycling condition as a part of standardization.



F. In vitro testing of indigenously developed active principles for identification and characterization of candidate anti-retroviral microbicides:

[PI: Dr. Smita Kulkarni, Co-Investigators: Dr. A. R. Risbud, Dr. J. Bhattacharya, Dr. K. Walia]

This project is expected to set a mechanism for screening of indigenous drugs for anti-HIV and anti-STI activity. The project is being carried out with four research institutes: National Chemical Laboratory (NCL), Pune; National Institute for Research in Reproductive Health (NIRRH), Mumbai; SNDT College of Pharmacy, Mumbai and Talwar Research Foundation, New Delhi; who have expertise in indigenous drug development. The anti-HIV and anti-STI testing will be conducted at NARI using fixed algorithm, under GLP compliance following strict quality control/quality assurance procedures.

This is an exploratory approach to promote development of indigenous candidate microbicides. Hence in order to achieve screening of large number of test products in least possible time, a high throughput assay that uses TZM-bl cells (genetically engineered HeLa cells that express CD4, CXCR4 and CCR5 genes and contain *tat* inducible luciferase and β -gal reporter genes) will be used. After primary screening, the lead compounds will be assessed for activity against CCR5 and CXCR4 tropic primary HIV-1 isolates using PM-1 (T cells expressing CXCR4 and CCR5 coreceptors) or activated peripheral blood mononuclear cells (PBMC). Preparations showing reduction of HIV growth in TZM-bl assay and PM-1/PBMC assay will be tested for their capacity to inhibit HIV transmission from epithelial cells to PBMCs and activity against HIV-1 using primary target cells (cervical, vaginal and rectal explants). The broad spectrum compounds that have shown anti-HIV activity in all assays, will be further tested for activity against other STI pathogens. Products showing anti-HIV and anti-STI activity will be tested in combination to check whether they act additively. The salient features of the developments that have occurred during the last year are as follows:

Product Selection Committee (PSC) has been formed by involving experts in various related fields like Toxicology, Pharmacology, and medicinal plant chemistry to pre-screen compounds/material for testing. The committee has formulated stringent criteria which will be of national use. Arrangements for determining chemical profile of the developed products have been made with NCL, Pune.

The TZM-bl cells and PM-1 cells have been obtained from NIH AIDS Research and Reference Reagent Programme (NIHARRRP), expanded and stored at appropriate temperatures. The viral stocks of HIV-1 IIB and the Indian isolates have been developed in activated PBMC/H9 cells and titrated. Standardization of the cytotoxicity and anti-HIV assays using known inhibitors is in progress. Essential cell lines, viruses, bacterial strains for anti-STI testing have been obtained from NIH American Type Culture Collection (ATCC), expanded and stored at appropriate temperatures. The standardization of assay for testing anti-STI activity is ongoing.

II. Viral load testing:

As a service activity, the Virology Section is providing support to institutional projects and NIH funded anti-retroviral trials by estimating the HIV-1 viral load. During April 07 to March 08, a total of 1660 samples were tested for HIV-1 viral load using Roche COBAS Amplicor kit. The break up of samples, processed for viral load as per the project is given in the Table below.

Sr. No.	Name of project	Samples tested (April 07 to Mar 08)
1.	AIDS Clinical Trail Group (ACTG) 5175	909
2.	HIV Prevention Trial Network (HPTN) 052	434
3.	Mother to child transmission (MIT)	103
4.	Intramural projects samples referred by physicians	214
	Total	1660

During this period, as a part of external quality assurance, we participated and successfully passed in six panels sent by Virology Quality Assurance (VQA) laboratory, Rush University Medical Centre, USA.