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# Subtyping of HIV-1 BY gag heteroduplex mobility assay

**Santanu Das[1*] and Srikanth Tripathy[2]**

[1]Department of Biotechnology, Seshadripuram First Grade College, Yelahanka New Town, Bangalore, Karnataka [2]Molecular Virology National AIDS Research Institute (ICMR) Pune, Maharashtra, India

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***Abstract***

**Background and Aims**: Surveillance of the different HIV-1 subtypes has important implications for developing candidate vaccines and understanding the dynamics of HIV-1 transmission in various populations. In this study, HIV-1 viral subtypes were determined for homologies in p24 region by the heteroduplex mobility assay (HMA) in 124 patients and by sequencing of '*gag* gene' with sexually transmitted diseases (STD) in selected places all over India. **Methodology:** Proviral DNA from whole blood and from PBMC's from recent sero-converters and seropositive individuals were analyzed. **Results:** Of 121 samples analyzed, 115 (95%) were HIV-1 C subtype, 5(4%) were HIV-1 A subtype and 1(1%) was HIV-1 B subtype. Further analysis revealed that 114 (99%) of the C subtype samples had maximum homology to the C6-Indian reference strain, while 1 (1%) was most homologous to the C3-Zambian strain. **Conclusion:** These findings have important implications for the design and testing of effective candidate HIV-1 vaccines in India.

***Keywords***: HIV-1; heteroduplex mobility assay; AIDS; STD



## *1. Introduction:*

With the beginning of the 3rd millennium after more than 2 decades of the first report of AIDS the world is going to see an epidemic of HIV/AIDS that is despite impressive progress in basic and clinical science, grudgingly affected by these discoveries and the pandemic continues to spread at a rocketing and alarming speed. Five million people have become infected with HIV in year 2002 bringing to a record 42 million the number of individuals lining with AIDS or HIV. And cumulative AIDS death by 2002 since the beginning of epidemic is 27.9 million according to UN report. . The steepest rises are now seen in Asia particularly in south and south east Asia over 6 million people with HIV/ AIDS. Thailand and India show the highest number of reported AIDS cases and HIV-seropositive individuals[1]. In 1986, after the first evidence of HIV infection in India in FSW in Tamil Nadu India has seen a rapidly and explosively spreading of epidemics in populations through heterosexual transmission and intravenous drug users. All the projections about the future impact of HIV in India is based on seroprevalence studies, and Most of these studies focused on convenience sampling of available sera from high risk groups. The Number of AIDS cases in India is doubling every 14 months, and the spread is predominantly by the Heterosexual route. Thus the seroprevalence of HIV infection is increasing alarmingly.

Development of a vaccine against HIV-1 is critical for controlling the global AIDS epidemic. One of the greatest challenges is identifying a vaccine or cocktail of vaccines that will protect against all subtypes of HIV[2]. Much is known about the nine genetic subtype or clades of M group of HIV-1 different clades or strains display distinctive properties. Different strains of the same species of micro organism often display distinctive properties. In a growing number or cases, linkage of phenotypic traits with genetic markers is allowing complicated biological assays to be replaced by genetic typing. The detection of such genetic variation has been revolutionized by the PCR, which allows fragments of even the most complex genomes to be isolated in an

**Corresponding author*
*Full Address :*
*Department of Biotechnology, Seshadripuram First Grade College, Yelahanka New Town, Bangalore, Karnataka, India*
*Phone no. +919886564873*
*E-mail: sans_blue1@yahoo.com*

essentially pure form in a matter of hours. Differences between gene segments can then be determined by direct sequencing of the PCR product. To further expedite and thereby to extend genetic screening assays to greater numbers of samples, Heteroduplex Mobility Assay ( HMA )or an sequencing methods have been developed that are a speedy case of use, high specificity are a simpler, typically require less complex less complex apparatus and are of lower cost than DNA sequencing hetero duplex analysis has been used in the field of medical genetics and for the detection of genetic polymorphisms in human population[3]. These methods have involved use of denaturing as well as non-denaturing gel electrophoresis conditions. Vinyl polymer -gels have also been used for the diagnosis of mutations within proto – oncogenes. Now sequencing genetic analysis methods can also facilitate study of complex and rapidly involving genetic systems such as RNA viruses. Heteroduplex mobility essays, now used to classify HIV-1 strains into genetic subtypes. These assays are also applicable to the analysis of other highly variable microorganisms. HMA used to classify HIV-1 strains into genetic subtypes or variation, which has a tremendous impact on vaccine efficiency, designing, development and production. Difficulties in establishing protection against slightly heterogeneous strains in animal vaccine models suggest that the high level of genetic variation found between the major subtypes of HIV-1 affect vaccine efficacy. It is therefore possible that vaccines will induce levels of protection against challenge strains that at least are in part proportional to their level of genetic relatedness to the vaccine strains. The major purpose of this study was to apply a simple reliable and quick method for the subtype identification of large number of samples. HMA fulfills these objectives by passing laborious colony an sequencing studies. A long awaited preventive vaccine remains an elusive target, seemingly in sight but just over the horizon.

## *2. Materials and methods*

### 2.1 Subject

The patients attending several sexually transmitted disease STD clinics in Pune, Chennai, Maharashtra, Ahmadabad, Calcutta, Manipuri and rest of India operated by the national AIDS research Institute enrolled in an ongoing prospective study of HIV infection were eligible for this study. Informed consent was obtained and pretest and post test counseling was offered to all study patients and participants. Specimens collected from a subset of these individuals were analyzed by hetero duplex mobility Assay (HMA). The 124 individuals found to be HIV seropositive during their visit provided sample of whole blood for viral subtyping collected in EDTA coated tubes.

### 2.2 Specimens preparations

Following proper and informed consent, blood samples were drawn in purple top EDTA coated tubes. DNA was extracted from Whole blood & PBMC's.

a. DNA extraction form whole blood: DNA from whole blood was extracted by using QIA amp DNA Blooming kit (QIAGEIN Gmbh Hidden, Germany)[4]

b. DNA extraction from PBMC`s : SEPERATION OF PBMC'sfrom blood by ficoll Hypaque (Histopaque, Sigma, USA) density gradient centrifugation.

#### *2.2.1 Separated peripheral blood mononuclear cells (PBMCs) from whole blood*

Routinely DNA can be extracted from PBMCs or Whole blood. Other than these two sources DNA can be extracted from filter paper, paraffin embedded tissue, blood clot, swabs or dried blood spots. Before starting with DNA extraction, following points should be considered. Extracted DNA was stored in eppendorf tubes at - 70 °C until further use. Estimation of DNA is done by spectra – photometer at 240nm-260nm

#### *2.2.2 Generation of HIV-1 proviral DNA PCR amplicons*[5]

The methodology for generation of the amplicons required for gag HMA depends on the starting Material, total RNA, genomic DNA or plasmid DNA. Nested PCR is required in order to increase specificity and amplicon yield, when starting material is DNA template extracted from PBMC's sample .The first round pcr primers amplify a 1.1 kbp fragment spanning amino acid 16 of gag p24 upto amino acid 33 of the protease gene with reference to the genome of the HIV-1 group M isolates ELI.

Oligonucleotide primers sequence for conducting 1st round PCR amplification were:

For 1st round ( SENSE ):

H1G777 5'-TCACCTAGAACTTTGAATGCATGGG;

(corresponding to position 777 – 801).

And 1st round ( ANTI- SENSE ):

H1P202 5' – CTAATACTGTATCAT CTGCTCCTGT;

( corresponding to position 1874 – 1898 ).

The 1st round DNA PCR reaction was 50µl reaction containing, Sterile Millipore water, 10x PCR buffer (100mM Tris-HCl pH 8.8, 500mM KCl, 0.8% Nonidet P40), $MgCl_2$ (2mM), dNTP mix (50*M each of the dNTPs), Primer H1G777, Primer H1P202, Taq DNA polymerase, Genomic DNA 1*g per PCR reaction. Programming the eppendorf Master Cycler generated the amplification of the genomic DNA. The cycling

conditions followed for this round of PCR were hot start with 94°C for 2 min, followed by 35 cycles of 94°C for 30 secs, 50°C for 30 secs and 72°C for 90 secs followed by product extension at 72°C for 7 min and was held at 4°C until further processing or storage at –20 °C. The most important part is that all the reaction mix and adding of genomic DNA is done on ice and then put on PCR.

The 2nd round nested PCR primers generate a 460 bp gag gene fragment corresponding to the region coding for amino acid 132 of p24 upto amino acid 40 of p7 in the genome of isolate ELI . Both set of primers can or is used for amplifying HIV-1 group M sequences.

The oligonucleotide primers sequence for nested 2nd round PCR amplification were:

NESTED SENSE:

H1Gag 1584 5' – AAAGAT GGATAA TCCTGGG;

(corresponding to position 1566 – 1589 ).

NESTED ANTISENSE

G17 5' – TCCACATTTCCAACAGCCCTTTT;

(corresponding to position 1566-1589).

The 2nd round or nested PCR is usually a 100 ul reaction .The reaction mix was prepared exclusively on ice by addition of Sterile /autoclaved millipore water,10x PCR buffer, $MgCl_2$, dNTP mix, primer H1Gag 1584, primer g 17, gene Taq DNA polymerase and template was first round PCR product of 2 ul .

The generation or amplification of 460 bp Gag gene fragment was done by using the following thermal cycling program in eppendorf Master Cycler.

Programming the eppendorf Master Cycler generated the amplification of the genomic DNA. The cycling conditions followed for this round of PCR were hot start with 94°C for 2 min, followed by 35 cycles of 94°C for 30 secs, 50°C for 30 secs and 72°C for 60 secs followed by product extension at 72°C for 7 min and was held at 4°C until further processing or storage at –20 °C

The NIH AIDS Research and Reference Reagent Programme supplied the oligonucleotide primers, which amplify only HIV-1 group M fragments and the set of primers were provided at a concentration of 100pmol/ul.

The primers were provided at a concentration of 10 pmol/ul which is 10 times dilution of original stock which is 100 uM/100 µl for setting up of both 1st round and 2nd round PCR.

The reference plasmids were also provided / supplied by NIH AIDS Research and Reference Reagent Programme and the plasmids were cloned with gag genes of HIV-1 group M subtypes. The representative of subtypes are from A to J and counts up to 35 plus 1-C6 constituting different sub groups of A to J.

The reference plasmids were provided at a concentration of 10 ng/ul in TE ( 10 mM Tris. HCl pH 7.4 / 0.1mM EDTA).

The purified reference plasmid DNA was amplified by 2nd round / nested PCR by taking 2ul of it. And followed the same process/protocol of amplifying the 1st round sample DNA PCR product which produces 460 bp gag gene fragment .The oligonucleotide primers used were:

H1Gag 1584

G 17

The PCR 2nd round products of both sample DNA and plasmid were checked by 1% agarose to determine the size of the desired fragment and quantity. 5ul of each sample with 1 ul of 6x loading dye was taken and loaded for 1% agarose gel electrophoresis at 100 volts in 1x TAE ( 89 mM Tris, 89 mM Acetate, 2 mM EDTA pH 8.0 ) and gel containing 2ul of ethidium bromide at 1ug/ ul.

### *2.3 gag* heteroduplex mobility assay[5]

gag HMA is performed by PAGE which is a vertical gel electrophoresis and protocol followed according to :

Heyndrick L, Van der Auwera G,Guido van der Groen . Heteroduplex Mobility Analysis HIV – 1 group M gag Subtyping Kit Protocol Version 3,December 2000.Catalogue of NIH AIDS Research & Reference Reagent Program 2000,Rockville, MD,USA.

The Gag HMA was performed by taking 6.5 ul of sample DNA and 5 ul of reference plasmid both 2nd round PCR products representing :

A1- Rwanda, A2 –Cote d'Ivoire, A3 - DRC, A4 - Kenya

B1 – Thailand, B2 – Uganda, B3 – Belgium,

C1 –Djibouti, C2 – DRC, C3 – Uganda , C4 -Zambia , C5 – Zambia , C6 - India

D1 – Kenya,

E1 - Thailand, E2 – Cameroon, E3 - Thailand

In each eppendorf tubes ( 0.5ml / 0.2ml ) 6.5ul of sample DNA to which individual reference plasmid and 1.1 ul of HMA annealing buffer ( 100 mM NaCl , 10 mM Tris, pH 7.8 and 2 mM EDTA ) was added then short spin ,vortexed and short spin .

Denatured at 94 degree C for 2 min in thermal cycler and quick chilled on ice for reannealling. This critical step of denaturation and quick chilling is done to obtain the homoduplexes and heteroduplexes.

Homoduplexes were formed between sample –sample as well as between reference plasmids. Whereas the heteroduplexes were formed only between sample DNA and reference plasmid strains.

The mixture of homoduplex and heteroduplex along with 3ul of 6x loading dye was loaded on PAGE.

PAGE was performed in 5% polyacrylamide 20% urea gel solution at 250 volts for 2.5 hours. Before loading mix to well pre run of PAGE was done for 10 – 15 min wells were washed with buffer to remove urea . PAGE was performed using V 15 –17 vertical gel electrophoresis apparatus and buffer compartments filled with 1x TBE. After 2.5 hours the gels were taken and stained for 30 min in 0.6x TBE buffer 500 ml containing 35 ul of ethidium bromide (1 ug/ul ) before visualizing in Image master VDS.

The mobility of homoduplexes are fastest in gel as the show 100% complementation or homology. Whereas the mobility of heteroduplexes vary according to nucleotide mismatches, Insertions and deletions formed between the sample DNA and the individual reference plasmid strain. The subtype is determined by visualizing the heteroduplex, which had migrated or moved to the closest corresponding homoduplex (Fig I).

## *3. Results*

The DNA specimens amplified yielded the expected 460 bp following the two rounds of PCR 1st round PCR and nested PCR. Samples could not be amplified in sufficient qualities to determine the HIV-1 subtypes mobility of the heteroduplexes formed between 121 amplified DNA products and references strains A1, A2, A3, A4, B1,B2,B3,B4, C1,C2,C3, C4, C5, C6, D1, D2, E1, E2, E3 were analyzed. Patients sample PCR product in the absence of any reference strain served as a control for the homoduplex formation and for heteroduplexes that will indicate multiple variants in the samples these assays determined that 5 (4%) samples belonged to subtype A; 1 (1%) subtypes B, and the rest 1, 5 95% were most homologous to one of the subtype C strains.

### 3.1 HIV subtype C strains variation[7]

The Samples classified as A, B, C subtype were analyzed further to determine their maximum homoglogies with the 6 subtypes C references strains C1, C2, C3, C4, C5, and C6 . C6 shows maximum mobility in aerylamide gel for the heteroduplexes formed between the sample and the C6 reference strain. All such samples were classified as B subtype were also analyzed to determine their maximum homologies with the subtype 4-A reference strains A1, A2, A3, A, and 4-B reference strains B1, B2, B3,B4, respectively show maximum homology of the heteroduplex formed between sample and A1 reference strain and samples were classified as A1 genotype shows maximum homology of the heteroduplex formed between sample and B1 reference strain and thus samples were classified as B1 genotype . classified C6 genotype maximum homology of the heterodyplexes formed between sample and C3 reference stains and these samples were classified as C3 Genotype likewise the 5 sample classified as 4 subtype and the 1 samples.

Based on these criteria 114 (94%) samples were classified as C6 genotype, 1 (1%) as C3, 5 (7%) as A1 and 1 (2%) as B1 genotypes . C6 genotype (94%) was more commonly observed than the other genotype like C3(1%), A1 (4%) and B 1 (1%).

Analysis of nucleotide homologies among the C6 and C3 genotype:

Each of the C6 samples showed a unique pattern of heteroduplex mobility with the reference C6 strain. Though they were all classified as C6 genotype each exhibited a variable degree of homology with the C6 reference strain. To address the homologies within the C^ genotype several fair wise combinations were

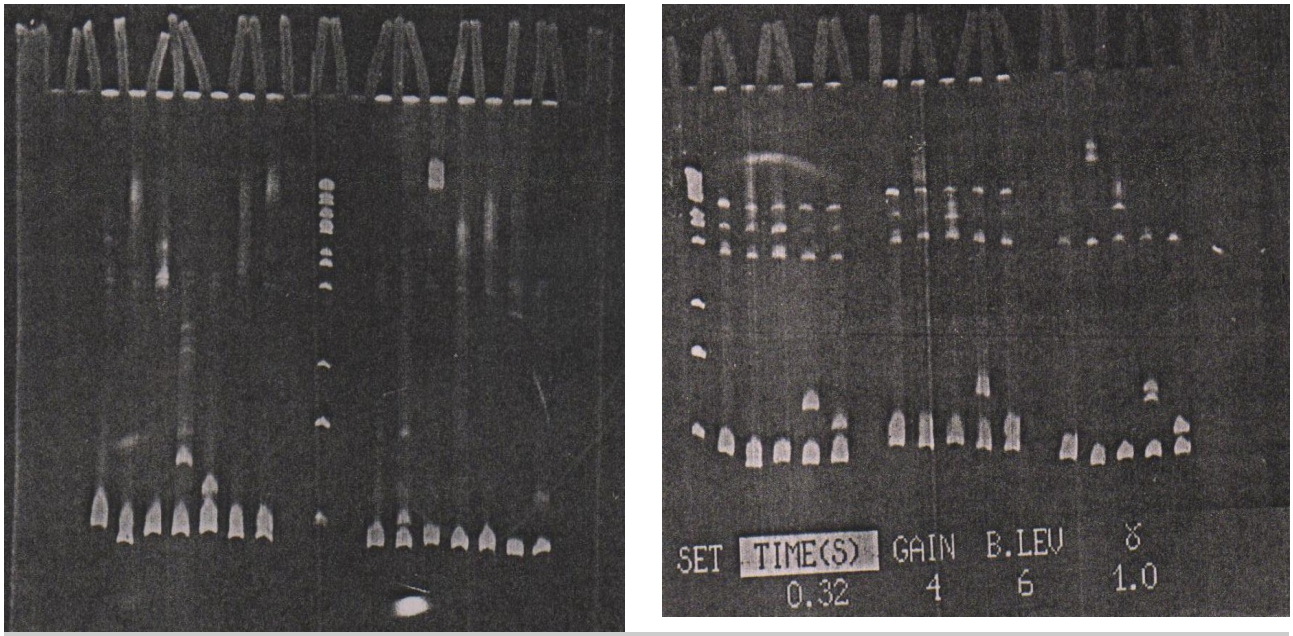


**Figure I: HIV-1 subtyping**

analyzed by HMA. Results of HMA studies between reference C6 and other responsibilities C6 samples showed a high degree (95%) of homology with C^int eh p24 region of the HIV-1 gag gene.

Among some samples showed comparatively less (85% homology some samples tested were found to be close related to reference C6 while some samples showed comparatively more divergence with reference. Additional analysis of hetero duplexes formed with in the members of the C6 group in various pair-wise combinations revealed that the majority of samples were not only closely related to reference but eh not only closely related to one another.

When similar studies were extended to the members of the C3 genotype, no prototype candidate could be ascertained for the C3 cluster. Except for the relatedness between reference C3 and one sample having C3 genotype. As expected crosses between the members of C6 and C3 also revealed considerable retardation of a of the heteroduplex in mobilities.

### 3.2 Analysis of nucleotide diversity with individuals subjects

To determine the extent of viral quasispecies diversity within individual subjects p24 ( 460bp) PCR products were analyzed for formation for intra sample alone sere used as control in HMA and served heteroduplex from heteroplex between the sample and prototype or reference strain.

Formation of the single homoduplex DNA and suggested the presence of a single dominant HIV strain. Most of the samples collected from seroconverter and seropositive showed the present of one major dominant strain they showed the presence of major heteroduplex[8].

Other displayed many heteroduplexes suggesting the presence of quasicpecies. Sero positive or serocommerted individuals with unknown duration of infection showed a higher degree of quarispecies complexities as revealed by HMA. Where as some individuals studies had a single dominant HIV strains.

## *4. Discussion*

HIV-1 is one if the unique viruses since genetically divergent stains can exist int eh same population the epidemic in Thailand for example includes both HIV-1 subtypes E and B. although their distribution is generally geographyically restricted through out the world most of the subtypes have been shown to exist in African continent. In a very short time, India, has experienced a rapid explosive HIV epidemic. Early in the epidemic these was evidence for the introduction of both HIV –1 and HIV-2 in India. The first small studies of HIV-1 subtypes described subtype in India. The first manor evidence of subtype other than C was the 1994 description of 4 HIV-1 subtype B viral isolates from STD patients in Hyderabad recently identified in Punjab and Delhi and one subtype A from STD clinic in Mumbai thus India is the only Asian country with evidence offer the simultaneous introduction and transmission of 3 subtypes of HIV –1 as well as HIV-2.

During the study period 124 HIV infected patients were screened and with their proper consent blood samples were taken. PBMC sample sand whole blood samples of the patients were available and taken for HMA studies. Analysis of degraphic data suggested that the samples studied from the groups were representative with respect to age, gender occupation commercial sex work a exposure to commercial sex workers. This study presents HIV –1 subtype analysis on the largest number of samples collected from different sites India to date and confirms that most of the HIV-1 infections in western India continue to be subtype C. in addition to isolated samples of subtype A and B these samples have also demonstrated evidence for 2 diverse HIV-1 subtype in India name analyzed samples from HIV seropositive individuals with unknown duration of infection. As with the seroprevalent samples we found evidence for the recent transmissions of both C6 and C3 genotype strains, as well as infected subtype and Subtype B.

Earlier studies of nucleotide divergence of subtype C viruses from Mumbai and Punjab and subtype B viruses from Hyderabad have reported a high degree of homology between small number of samples suggesting restricted. However these studies were limited by their size and may be geographically restricted. However these studies were limited byt heir size and may not represent an adequate sampling of the circulating viral strains to permit generalization on the relationship between sequence divergence and the timing of the introduction of the strains. In our analysis of the 121 samples from several city, we found evidence 5 for a higher degree of nycleotide divergence (by HMA) in C3 strains than in those samples most homologous with C6 reference strains from India. This suggests that C6 may represent a strain variation unique to the Indian epidemic. The distinctions between C6 and C3 genotypes was solvely made on the heteroduplexes DNA mobility's relative to the references strains homoduplex mobility's. The reference C6 and C3 strains have 10 percent nucleotide mismatches in the genomic region analysed. The approximate percent homologies mentioned in the text are based on the distance between homo and hetero duplex bands. However it may be mentioned that deletions or insertions compared to point mutations may reflect on over estimation of percent divergence. Nucleotide true distinction between C6 and C3. however the major purpose of this study was to apply a simple reliable and quick method for the subtype identification

of large number of samples. HMA fulfills these objectives by passing laborious colony an sequencing studies[9].

The efficiency of transmission of one genotype over the other cannot be ascertained with the present data. However some of the C6 related. Strained analysis in this study is indicated divergence from th main C6 cluster. If the degree of divergence is assumed to be related to time (number or replication cycles ad immune selection) then one would expect to see appearance of more divergent C6 strains over a period of time. Continuation of such studies therefore are justified in detecting more divergent strains an appearance of new subtype or recombinant strains. Quasispecies completely was observed at right proportion (61.5%) in samples collected from individuals apparently infected for longer duration. HMA may have limitations in detecting quasispecifics with very low copy number and the observed duplexes mainly represent major viral variants Recent reports describe that the HIV infected individuals with multiple qurispecies exhibit a moderate loss of CD4 cells compared with a rapid rate of CD4 cells loss in patients with less viral variants[10].

HMA can also be used to map the genomic region of homology or diversity by amplifying a specific region and comparing the same with references strains. Analysis of samples which was shown to be varying in p24 genomic region was compared to the reference C6 subtype, was shown to be closely homologous to the same reference strain when p24 6 regions of sample and reference strain were apparently in major homology r3gion p24 N-terminus region. Limited nucleotide sequencing data available on Indian c type HIV-1 strains have also suggested that h ep24 gag region was more consumed and nucleotide sequence variations were observed in major homology region N terminus of gag this study has demonstrated prior infection and recent transmission of multiple subtypes (A,B,and C) and multiple genotypes (C3, C6) of HIV –1 in patients attending several STD clinics serving the same urban area of Pune India. Although subtype C remains the predominant strain, transmission of multiple HIV-1 within the same comities in India design of effective candidate HIV-1vaccines for India. The HIV pandemic has been the scourge & the medical field since it seems to have no remissions since it cam on the scene[11].

A global pandemic with geographical variations genotype changes has ,made the test of Indians/ developing a individual vaccine that much more difficult. Subtyping the virus has given more teeth to the right against the line and may be also a great help to develop the ideal global/ candidate vaccine.

Immunology of HIV is very difficult from most the viruses and HIV has changed the tunds & the immunologist. The old school of through that we can suppress HIV multiplication has simmered down an faded away instead we are trying to take the virus by the horns. The most important aspect of subtyping is the inst that the epitome I virus that has into can be determined and vaccine that may combine these properties can be developed.

The HIV horizon is a never ending one but the battle continuous it remains to be seen who wins the war is it the science or medicine or a triumph for HIV.

## *Conflict of interest*

The author's declares none.

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