Identification of Subtype C Human Immunodeficiency Virus Type 1 by Subtype-Specific PCR and Its Use in the Characterization of Viruses Circulating in the Southern Parts of India


Molecular Virology Laboratory, Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Department of Neuropathology, Department of Neurology, and Department of Neurovirology, National Institute of Mental Health and Neurosciences, Microtest Innovations Pvt. Ltd., Seva Free Clinic, Osmania Medical College, Hyderabad, Andhra Pradesh, Allegheny-Singer Research Institute, and Thomas E. Starzl Transplantation Institute, Department of Surgery, and Department of Infectious Diseases and Microbiology, University of Pittsburgh, Pittsburgh, Pennsylvania

Received 25 September 2003/Returned for modification 29 October 2003/Accepted 21 February 2004

Human immunodeficiency virus type 1 (HIV-1) subtype C viruses are associated with nearly half of worldwide HIV-1 infections and are most predominant in India and the southern and eastern parts of Africa. Earlier reports from India identified the preponderance of subtype C and a small proportion of subtype A viruses. Subsequent reports identifying multiple subtypes suggest new introductions and/or their detection due to extended screening. The southern parts of India constitute emerging areas of the epidemic, but it is not known whether HIV-1 infection in these areas is associated with subtype C viruses or is due to the potential new introduction of non-subtype C viruses. Here, we describe the development of a specific and sensitive PCR-based strategy to identify subtype C-viruses (C-PCR). The strategy is based on amplifying a region encompassing a long terminal repeat and gag in the first round, followed by two sets of nested primers; one amplifies multiple subtypes, while the other is specific to subtype C. The common HIV and subtype C-specific fragments are distinguishable by length differences in agarose gels and by the difference in the numbers of NF-κB sites encoded in the subtype C-specific fragment. We implemented this method to screen 256 HIV-1-infected individuals from 35 towns and cities in four states in the south and a city in the east. With the exception of single samples of subtypes A and B and a B/C recombinant, we found all to be infected with subtype C viruses, and the subtype assignments were confirmed in a subset by using heteroduplex mobility assays and phylogenetic analysis of sequences. We propose the use of C-PCR to facilitate rapid molecular epidemiologic characterization to aid vaccine and therapeutic strategies.

The development of a vaccine against human immunodeficiency virus type 1 (HIV-1) has been affected by the ability of HIV to acquire high levels of genetic heterogeneity, which is also expected to pose problems in expanding therapeutic intervention to resource-poor countries. HIV-1 is divided into distinct groups and subtypes or clades based on genetic differences (22) that are unevenly distributed in different parts of the world. Group M HIV-1, which is most prevalent, has been divided into at least 10 different subtypes and a number of intersubtype circulating recombinant forms. Among the various subtypes, subtype C is predominant and is associated with >50% of all HIV-1 infections (10, 12, 38) and with the establishment and rapid growth of epidemics in India, China, Brazil, and the southern and eastern parts of Africa. Although the preponderance of HIV infections in India appear to be associated with subtype C viruses, several studies have reported the prevalences of subtypes A, B, D, AE, and Thai B (2, 13, 19, 53), as well as the recombinant forms (27), at rates varying from 0 to 100% (5, 8, 15, 28, 45, 49). It is not clear if the extensive proliferation of subtype C viruses might be attributed to demographic or genetic factors unique to these populations or to the presence of intrinsic viral biologic properties that offer potential advantages (11, 40, 41). For example, subtypes C and A have been documented since early stages of the epidemic in India, but the reason why subtype C viruses have come to predominate is not clear.

Most studies in India have involved a small number of samples and/or were restricted to city or local levels and hence do not offer reliable estimates of subtype prevalence. Further-
more, these studies have also left out the newly emerging high-prevalence areas. For example, characterization of HIV subtypes from the southern states has not been reported, even though a high population prevalence of HIV infection has been predicted on the basis of >1% prevalence among antenatal clinic subjects and a high prevalence among high-risk groups (http://www.naco.nic.in). Recent reports of the potential spread of subtype B infections (15) illustrate a need to distinguish whether this is an artifact of the examination of an isolated cluster or the nucleus of a change in the epidemic.

Among the strategies used for subtyping viruses, sequencing viral genomes followed by phylogenetic analyses is the de facto standard but is an expensive and labor-intensive option. Heteroduplex mobility assays (HMA) (6) are the most often used cost-effective alternative and have excellent concordance with phylogeny-based subtyping (1). In spite of HMA being a technically simpler technique, processing a large number of samples requires running parallel electrophoresis gels with multiple subtypes for each sample and is labor-intensive. Therefore, approaches and methods that allow rapid answers to straightforward questions, such as scoring a given sample for subtype assignment, will facilitate processing large numbers of samples and provide greater coverage of the infected population. Subtype sequence-specific PCR is one such method that targets sequences differentially conserved among subtypes. Analogous to sequence-specific (or allele-specific) PCR that has been used extensively in genomic studies (9, 34, 35), this method exploits genetic differences at the level of primer sequences to differentially amplify fragments specific to a given subtype and has been used with other subtypes of HIV-1 (4, 39). Here, we document a PCR strategy (C-PCR) that exploits differences in the long terminal repeat (LTR) region between subtype C and non-subtype C sequences to generate common HIV-1 and subtype C-specific fragments with mobilities distinguishable in an agarose gel. We have optimized the method and implemented it to screen 256 HIV-infected individuals from 35 cities and towns in five states in the southern and eastern parts of India. We found a preponderance of these individuals to be infected with subtype C viruses.

**MATERIALS AND METHODS**

**Clinical samples and DNA extraction.** Blood samples from individuals who were identified as HIV seropositive by multiple enzyme-linked immunosorbent assays and/or Western blots following high-risk exposure and/or with symptoms characteristic of HIV infection were collected in EDTA vacutainers (Becton Dickinson, San Diego, Calif.). The study subjects were voluntary participants under the care of government hospitals, private clinics, and referral centers dedicated to the service of HIV/AIDS in the southern Indian states of Karnataka, Tamil Nadu, Andhra Pradesh, and Kerala. Subjects from the eastern state of West Bengal were part of a high-risk HIV study group at the School of Tropical Medicine in Kolkata, India (14). Blood samples were collected from the patients after informed consent was obtained and under the approval of institutional biosafety and ethics committees at participating institutions. Out of the 256 seropositive samples collected for this study, clinical information was available for 238; 152 subjects were male, 82 were female. The mean ages for the males and the females were 35.2 (range, 17 to 60) and 28.6 (range, 16 to 51) years, respectively. The subjects represented a heterogeneous community of social and demographic groups. The blood samples were transported at room temperature to the Virology Laboratory at the Jawaharlal Nehru Centre for Advanced Scientific Research for processing within 4 days of collection. Genomic DNA was extracted from 0.2 ml of whole blood by using a commercial DNA extraction kit (NucleoSpin Blood; Macherey-Nagel GmbH & Co. KG, Duren, Germany), eluted in a 100-μl volume, and stored at 4°C until it was used. Reference plasmids containing DNAs from various subtypes were obtained from the National Institutes of Health (NIH) AIDS Repository Program. Carryover contamination of PCR products was prevented by adherence to strict procedural and physical safeguards that included reagent preparation and PCR setup, amplification, and post-PCR processing of samples in separate rooms (24).

**Primer design.** We targeted a genomic region extending from the LTR into gag, containing sequences highly conserved among multiple subtypes, as well as stretches of sequences differentially conserved between subtype C and other subtypes. We designed three sets of primers (Table 1). One set of outer primers was designed to amplify a 973-bp LTR-gag fragment from multiple subtypes. A second set of internal primers was designed to amplify a 138-bp LTR fragment specific to subtype C sequences while being refractory to amplification from non-subtype C sequences. A third set of highly conserved internal primers was designed to amplify a 232-bp LTR leader-gag region fragment from all subtypes (Fig. 1).

**TABLE 1. Sequences for primers used in C-PCR**

<table>
<thead>
<tr>
<th>Nature of PCR</th>
<th>Primer name</th>
<th>HXB2 genome coordinates</th>
<th>Sequence*</th>
</tr>
</thead>
<tbody>
<tr>
<td>First round</td>
<td>N419F</td>
<td>130–157</td>
<td>5′-GAT GGT GCT TCA AGC TAG TRC CAG TGG A</td>
</tr>
<tr>
<td></td>
<td>N424R</td>
<td>1075–1104</td>
<td>5′-CTC TAT YTT RTC TAA RGC TTT YTT GGT GTC</td>
</tr>
<tr>
<td>Common-HIV</td>
<td>N420F</td>
<td>683–709</td>
<td>5′-CTC TCG AGC CAG GAC TCG GCT TGC TGA</td>
</tr>
<tr>
<td></td>
<td>N423R</td>
<td>890–915</td>
<td>5′-TTC YAG CCT CCT GCT TGC CCA TAC TA</td>
</tr>
<tr>
<td>Subtype C specific</td>
<td>N415F</td>
<td>256–284</td>
<td>5′-AGT GGA AGT TTT ACA GTC AMC TAG CAC RC</td>
</tr>
<tr>
<td></td>
<td>N417R</td>
<td>367–393</td>
<td>5′-CGC CCA GAC CAC WCC TCC TGR AMC GC</td>
</tr>
</tbody>
</table>

*International Union of Pure and Applied Chemistry codes were used for positions with redundant nucleotides. The sequences for all the reverse primers are presented as the inverse of antisense.
ment of subtype C-specific primers in the 3' end. However, this does not appear to be a hurdle, since none of the subtype c samples tested thus far have failed to amplify and the few that did not amplify were found to be non-subtype C. This is the first subtype-specific PCR report involving a comprehensive characterization of region-encoding primer sequences. The lack of similar characterizations may account for the less than widespread use of previously published primers.

**PCR and cloning.** PCR amplifications were performed using 500 ng of genomic DNA in a 25-μl volume containing 3 mM MgCl₂, 200 μM (each) deoxynucleoside triphosphates, 25 pmol of each primer, and 0.625 U of Taq DNA polymerase (Amersham, Piscataway, N.J.). The first-round PCR conditions were as follows: 94°C for 1 min, 60°C for 1 min, and 72°C for a total of 25 cycles on a thermocycler (Minicycler; M.J. Research). Two microliters of first-round PCR products was transferred to a second-round PCR that contained the four internal primers. The PCR conditions were as follows: 94°C for 1 min, 65°C for 40 s, and 72°C for 40 s for 35 cycles. After the second round, the PCR products were resolved by a 1.5% agarose gel or a 3% NuSieve agarose gel (FMC Bioproducts, Rockland, Maine), and the ethidium bromide-stained DNA bands were captured using a gel documentation system (Alpha Innotech, San Leandro, Calif.). The quality control measures included the negative controls containing template DNAs from uninfected individuals and blind analyses of samples from different known subtypes.

Annealing temperatures and Mg concentrations were optimized for sensitivity and specificity, individually and in multiplex PCR amplifications using DNA templates from two unrelated molecular clones of subtype C (pNDIE [30] and pMJ4 [33]) and one of subtype B (pYU-2 [25]). Defined concentrations of plasmid DNA were serially diluted in the presence of 500 ng of salmon sperm DNA to derive precise template copy numbers ranging from <1 to 10,000 copies in each reaction. We found the subtype C-specific and common HIV primers to consistently amplify 1 to 10 copies of the template DNA in uniplex PCR experiments (Fig. 2). Conditions optimized in uniplex PCR amplifications were found to work in multiplex PCR with no decrease in sensitivity and/or specificity. These findings suggest consistent and reliable detection of small numbers of copies of HIV using the primer pairs and amplification conditions outlined here.

The env C2-V5 region was amplified, cloned, and sequenced from a subset of 45 samples using a nested-PCR amplification strategy as described previously (48). In addition, sequences spanning env V3 regions were obtained from eight additional subjects and used for subtyping but were not included in the phylogenies due to their dissimilar lengths and ambiguous base calls. The LTR region for sequencing studies was amplified using a nested-PCR amplification with primers N299F (1-TGGAWGGGYTAATTTACTCCMARAAA) and N306R (763-CTCTCTCTTCTAGCCTCCGCTAGTCA) to amplify a 789-bp fragment in the first round and primers N300F (106-GGGTCAGATATCCACGAGCTC) and N306R (763-CTCTCTCTTCTAGCCTCCGCTAGTCA) to amplify a 638-bp fragment. The numbers in the primer sequences represent positions corresponding to HXB2 (accession no. K03455). The PCR conditions used for LTR amplification were the same as those for the first round of C-PCR except that the annealing temperature in the first-round PCR for LTR was 50°C. The PCR products were purified using a Spinprep PCR clean-up kit (Novagen, Madison, Wis.) and directly sequenced using internal primers. env PCR fragments were cloned into a TA cloning vector (Invitrogen, Carlsbad, Calif.) and sequenced using dye terminator chemistry. Chromatograms from ABI377 and Beckman CEQ8000 sequencers were edited using Sequencher (GeneCodes, Ann Arbor, Mich.).

**HMA and sequence analysis.** HMA was performed according to methods outlined by Delwart et al. (6), using the HMA-subtyping kit supplied by the NIH AIDS Reference and Reagent Program. An ~700-bp fragment of the env gene, spanning the C2 to V5 domains, was amplified using the primers ES7 and ES8. Heteroduplexes were formed by mixing equal amounts of amplified DNA (~500 ng of each) in a volume of 35 μl and boiling the mixture in the presence of 100 mM NaCl and 2 mM EDTA for 5 min, followed by a quick chill and incubation on ice for 90 min. The heteroduplexes were separated on 5% polyacrylamide gels, stained with ethidium bromide, and scored for subtype assignment. Forty-eight of the 256 clinical samples were tested for concordance among HMA, C-PCR, and phylogenetic analysis.

Sequences corresponding to the LTR and env C2-V5 regions used for phylogenetic reconstructions were collected from multiple subtypes from the Los Alamos database (22), aligned at the codon level, and gap stripped. The rates of nucleotide substitution and gamma distribution parameters were estimated using Pampy+ (51) as described earlier (47). Subtype assignments and depiction of phylogenetic relationships were accomplished using the codeml (with discrete NS site model) and baseml programs for the em and LTR sequences, respectively, in PAML (56).

**Nucleotide sequence accession numbers.** The GenBank accession numbers for the sequences in this study are AY567495 to AY567539 and AY567474 to AY567486.

---

**RESULTS**

**Standardization of C-PCR.** We optimized C-PCR conditions to detect 1 to 10 copies of plasmid templates containing full genomes of single and/or multiple subtypes in individual and multiplex PCR. As illustrated in Fig. 2, the 232-bp Leader/gag common HIV region was amplified from all the subtypes tested thus far, while the 138-bp fragment specific to subtype C was restricted to subtype C (Fig. 2A). All of the C2, C3, and C4 subtype standard sequences supported C-PCR amplification to similar levels. The presence of an additional NF-κB site in clone C4 was reflected in the increased size of the subtype C-specific fragment. In addition to these prototype clones, C-PCR accurately subtype DNAs extracted from primary cul-

---

**FIG. 2.** Specificity and sensitivity of C-PCR. (A) The Specificity of C-PCR was assessed using molecular clones derived from different subtypes of HIV-1 in the following order (from left): A (p92UG037.1), A (p90CF0402.1), B (pBH10), B (p92YU2), B (p90CF), C (pMJ4), C (pNDIE), C (p92BR025.8), D (p94UG114.1), D (p94UG114.1.6), D (p84ZR085.1), E (p90CF0402.1), F (p93BR020.1), G (p92NG003.1), G (p92NG083.2), and H (p90CF056.1). Lane M, DNA size standard; −, control lane. The extra NF-κB site present in the subtype C4 molecular clone can be seen to lead to increased size of the subtype C-specific fragment. (B) Sensitivity of C-PCR. The subtype C molecular clone pNDIE was used at the copy numbers shown above the lanes. Optimization of uniplex PCR conditions and their subsequent use in multiplex PCR with no loss of sensitivity are seen. −, control lane.
resembling NF-κB motif is common in subtype C strains and results in the formation of subtype C by C-PCR. These representative samples demonstrate the typical HMA profiles of subtype C viruses. The labeling with subtype standards used in the analysis, only profiles with subtype A and C standards are shown. –, control lanes that did not include subtype standards. (C) A non-C virus identified as subtype B by HMA. C-PCR failed to amplify the LTR fragment from this sample. (D) A single recombinant virus identified in this study was subtype C in the LTR and subtype B in env. (E) Insertion of additional sequences resembling NF-κB motif is common in subtype C strains and results in bands with lower mobility than expected (Fig. 1A). The insertion of a 15-bp sequence between two authentic NF-κB motifs of one such clinical sample has been confirmed by sequencing the LTR. An NF-κB-like motif is highlighted.

Subtyping clinical samples using C-PCR. Subtype-specific C-PCR was implemented to screen DNAs isolated from 256 samples obtained from 35 towns or cities spread over four states in the south and one city in the east of India. These samples were derived from individuals previously diagnosed as having been HIV infected using serologic assays. Among the five states, Karnataka, Andhra Pradesh, and Tamil Nadu have been considered high-prevalence states based on >1% prevalence among women attending antenatal clinics and a high prevalence among sexually transmitted disease patients. Kerala is considered a low-prevalence state based on low prevalence among sexually transmitted disease patients and <0.1% prevalence among antenatal-clinic patients. Subtype C viruses have been identified as the major subtype circulating in West Bengal State in earlier studies (28, 29, 47), and these samples were included to allow comparison with previous findings.

By randomly selecting samples from each of the states (35, 6, 4, and 3 samples from Karnataka, Andhra Pradesh, Tamil Nadu, and Kerala, respectively) for subtyping by HMA. To confirm the subtype assignment using phylogenetic approaches, additional subsets of 45 and 13 samples were chosen to derive env and LTR sequence data, respectively. As illustrated in Table 2, subtyping by HMA and sequencing was representative because it included a number of different towns. The heteroduplex mobility patterns of representative samples are illustrated below the corresponding subtype C-PCR lanes in Fig. 3B. With the exception of one subtype B/C recombinant shown, env HMA correctly subtyped all samples that were classified as subtype C by C-PCR. Two samples in this study, one from the state of Andhra Pradesh (Fig. 3C) and the other from West Bengal (not shown), failed to amplify the subtype C-specific fragment while yielding correctly sized common HIV-1 fragments. The sample from Andhra Pradesh was found to belong to subtype B by analysis of HMA and LTR sequences, while the other sample from West Bengal was found to be subtype A by HMA and analysis of the env sequence.

We analyzed env C2V5 and LTR sequences from subsets of 45 and 13 samples, respectively (Table 2 and Fig. 4). In phy-
logenetic reconstructions, env sequences sampled from each of the 45 individuals drawn from the four southern states clustered unambiguously with other subtype C sequences from India and other parts of the world. Similarly, all 13 LTR sequences sampled in this study also clustered with other subtype C sequences. These analyses showed that subtyping viruses based on subtype-specific PCR of the LTR region is consistent with the subtype assignments for the env genes and LTRs in these samples. As illustrated in Fig. 5, the study subjects were from different areas in the southern part of the country. The places where non-subtype C viruses were identified are indicated.

### DISCUSSION

We have documented the development of a sensitive and specific PCR-based strategy to identify the presence of subtype C viruses. Using this C-PCR, we document a predominance of subtype C viruses in samples derived from different parts of four southern states and from an eastern city in India. We confirmed C-PCR findings, using HMA and sequence analysis in a subset of samples, as well as the consistency of previous reports of subtype prevalence in Kolkata. This is the first report of molecular epidemiologic characterization of viruses.
from the southern part of India. Additionally, to date this is the largest study of molecular characterization of the HIV-1 subtype distribution in India. While early studies suggested a preponderance of subtype C with a small number of subtype A viruses in India, recent studies have indicated a potentially increasing prevalence of non-subtype C viruses (2, 13, 15, 19, 29, 45, 53). Based on our studies, we conclude that newly emerging areas of epidemic HIV disease in the south continue to be associated with a preponderance of subtype C viruses. However, it is necessary to examine more samples drawn from wider geographic areas to further validate our findings. In addition, we propose designing primers specific to other subtypes in the use of a subtype identification strategy similar to C-PCR to confirm the absence of non-subtype C viruses.

Even though we designed both of our primers to contain subtype-specific mismatches, it is possible to discriminate subtypes by using a single subtype-specific primer. With the exception of subtype A viruses, which show high variability in the 3’ end of N415F (Fig. 1), the region we have targeted appears to be highly suited for designing primers corresponding to each
of the other subtypes. However, we focused this study solely on identifying the presence of subtype C sequences because no information on molecular epidemiology is available from the southern parts of India. These regions represent major epicenters of viral infection, and in view of recent reports documenting the presence of non-subtype C viruses, the need to identify subtype profiles in the emerging epidemics in the southern parts of India is of utmost importance and urgency.

In contrast to most earlier studies that have characterized HIV from major urban epicenters, this study was specifically targeted to explore the natures of viruses circulating in rural parts of India that are geographically distant from major epicenters in each state. While demographic and other information from nonurban areas of the country is slowly becoming available, the proper design of vaccine and antiretroviral therapy programs also requires molecular characterization. Even though we have examined a small and disproportionate number of samples, the study design incorporating a wide geographic area makes up for this deficiency. Regardless of the small number of non-subtype C viruses identified, this study indicates a strong association between subtype C viruses and the epidemic in the southern parts of India. Documentation of the presence of intersubtype recombinants in this study and in others makes it necessary to design strategies specifically targeted to identify them. This can be accomplished by implementing a strategy analogous to C-PCR in a more distant region of the genome. Previous studies have documented recombinants using a similar strategy but involving testing for sequence in the targeted region, supports a wider use of C-PCR targeting different regions of the virus (Fig. 3D). C-PCR, by design, is a multiplexing of two individual regions of the subtype-C virus, thus offering the advantage of reducing false-negative results due to genetic diversity. The Leader/gag region is one of the most conserved regions of the virus. C-PCR, therefore, is highly sensitive to detect all infections by HIV-1 regardless of the subtype. In our experience with hundreds of primary clinical samples, amplification of the LTR/Leader/gag region failed on very rare occasions. This is in contrast to frequent failures encountered in the amplification of the env region for HMA. LTR/Leader/gag amplification occurred even in a few cases where we could not amplify the env region for HMA analysis. A large number of samples could not be genotyped using HMA due to ambiguous band patterns (18, 45). In one study, 17 out of 52 samples were untypeable by HMA (28).

We also encountered several samples that could not be characterized by HMA; however, C-PCR identified all of these samples without ambiguity.

Identifying subtype prevalence, especially in regions where multiple subtypes circulate, is necessary for a number of reasons. Subtype characterization impacts strongly on our understanding of the epidemic and approaches to tackle it. No clear evidence linking genetic subtypes with differences in disease progression is available, but the link cannot be ruled out. Subtype differences are also expected to impact strongly on the efficacy of a vaccine and are most relevant to resource-poor countries. The presence of differences in the pattern of cytotoxic-T-lymphocyte epitope recognition (36, 37) suggests that the high level of sequence differences among subtypes is an important variable in the design of a vaccine. Pending the availability of a potent vaccine, emphasis is being placed on making antiretroviral therapies available in resource-poor settings (42). However, the impact of subtype differences on the outcome following therapy is not clear, since nearly all the available antiretroviral agents were designed in a subtype B setting. Even though most of these agents appear to be effective in suppressing non-subtype B viruses to similar extents, significant differences in enzyme kinetics and mutations associated with reduced sensitivity have been documented (50). For example, subtype C and A polymerases appear to exhibit reduced affinities to a panel of antiretroviral agents (54) and to amplify the effect of mutations associated with reduced susceptibility (55). In addition, Diallo et al. and Loemba et al. have documented distinct differences between subtypes in the rates at which mutations associated with reduced sensitivity to antiretroviral agents evolve and the differences in mutations within subtypes C and B that are associated with reduced sensitivity to antiretroviral agents (7, 26). These studies suggest...
that identifying subtypes at individual and population levels is an important step in monitoring and managing the epidemic.

In summary, we have optimized the conditions for specific amplification of subtype C sequences using C-PCR and implemented it in the analysis of 256 samples derived from a wide geographic area in the southern part of India. With the exception of single samples containing subtypes A and B and a B/C recombinant, the rest contained subtype C viruses. This is the first report of subtype prevalence in the emerging areas of high prevalence in the southern part of India. This study addresses the need for identifying the proportion of infections caused by different subtypes in the design and implementation of vaccine and antiretroviral therapy programs.

ACKNOWLEDGMENTS

U.R. acknowledges institutional financial support from JNCSRR and the National Training and Research Program (NID-43-TW-01403) of the Albert Einstein College of Medicine. N.B.S. and P.K.D. are recipients of CSIR fellowships from the Government of India. R.S. was supported by NIH grant AI 41870 and by the Center for Genomic Sciences, Allegheny Singer Research Institute.

The HMA kit and other reagents were received from the NIH AIDS Research and Reference Reagent Program and the Centralised Facility for AIDS Reagents, National Institute for Biological Standards and Control, UNAIDS. Help from the following individuals in collecting samples from HIV-seropositive individuals is gratefully acknowledged: A. O. Saroja, Consultant Neurologist, KLES Hospital, Belgaum, Karnataka, India; M. N. Balamurugan, Consultant Neurologist, Salem, Tamil Nadu, India; E. Srikant Reddy, Consultant Neurologist, Vijayawada, Andhra Pradesh, India; James Joseph, Seva Free Clinic, Bangalore, India; and Phalguni Gupta, Department of Infectious Diseases and Microbiology, University of Pittsburgh, Pittsburgh, Pa. R.S. acknowledges G. Ehrlich and C. Post for support and encouragement. The Human Brain Tissue Repository for Neurobiological Studies at NIMHANS, Bangalore, India, is acknowledged for providing samples from the Body Fluid Bank.

REFERENCES


