Rapid Detection of Human Immunodeficiency Virus Type 1 Subtype E Infection by PCR

Mao-Yuan Chen,1 Wei-Kung Wang,2 Ming-Cheng Lee,3 Shing-Jer Twu,4 Shiow-Ing Wu,5 and Chun-Nan Lee3,6*

Department of Internal Medicine,1 Institute of Microbiology,2 and School and Graduate Institute of Medical Technology,3 College of Medicine, and College of Public Health,4 National Taiwan University, and Department of Laboratory Medicine, National Taiwan University Hospital,6 and Taipei Municipal Venereal Disease Control Institute,5 Taipei, Taiwan

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The CRF01_AE (subtype E) strain of human immunodeficiency virus type 1 (HIV-1), originally reported in Thailand, spread rapidly to and showed prevalence in several countries in Southeast Asia, including Taiwan. This strain was also found in other regions of the world. Based on sequence analysis of the vpu gene, a nested PCR assay including an outer primer pair and a subtype E-specific inner primer pair was developed in this study for rapid detection of subtype E viruses. It was tested with 397 HIV-1-positive samples of known subtypes. For these samples, the sensitivity of detection of subtype E viruses was 100% (127 of 127), and the specificity was 97.8% (264 of 270). Although six samples of either subtype A or G showed a positive PCR, most of the cross-reactivity could be reduced by raising the annealing temperature from 54°C to 63°C. When tested with 195 HIV-positive samples of unknown subtypes, the assay had a sensitivity of 98.0% and a specificity of 98.6%. This is a simple, convenient, and sensitive method for rapid detection of subtype E viruses, especially in regions in which viruses of subtypes B and E are predominant.
of genomic DNA was subjected to the first-round PCR with the outer primers (TAT-1 and EN70), and 1 μl of the first-round PCR product was subjected to the second-round PCR with the inner primers (154.1 and KPN) (23). Another microliter of the first-round PCR product was subjected to the subtype E PCR assay with the primers VPUEN1 and VPUEN2. The amplification conditions were 30 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 30 s, followed by a final extension at 72°C for 10 min. Standard precautions recommended for PCR were taken to avoid contamination (20).

One hundred twenty-seven subtype E and 259 subtype B samples, which were previously determined by nucleotide sequence analysis (23), were subjected to the subtype E PCR assay first. As exemplified in Fig. 1, strong bands of the expected size of 187 bp were seen in all of the subtype E samples, but in none of the subtype B samples. As the control, the conserved primers 154.1 and KPN were used in the second-round PCR, and the 398-bp PCR products were detected in all of these samples (Fig. 1). We next tested samples of subtypes A, C, and G by this method. Four out of five subtype G samples and two out of two subtype A samples gave positive results, although one of the subtype A samples showed a weak band (data not shown). All four subtype C samples were negative. These results are summarized in Table 1. For these samples of known subtypes, the sensitivity of detection of subtype E strains was 100%, and the specificity was 97.8% (264 of 270).

The nucleotide sequences corresponding to the regions of VPUEN1 and VPUEN2 for these subtype A, C, and G strains were then aligned (Table 2). Compared with the VPUEN1 and VPUEN2 sequences, the subtype C strains differ by at least 18 nucleotides, and the subtype G strains differ by 7 to 10 nucleotides. One of the subtype A strains differs at 12 positions, and the other differs at 8 positions (Table 2).

To test whether the cross-reactivity of subtype A and G strains could be reduced by raising the annealing temperature in our subtype E PCR assay, the annealing temperature was increased (in increments of 2 to 3°C) empirically. It was found that an annealing temperature of 63°C could greatly reduce the cross-reactivity, while still preserving the reactivity of subtype E strains (data not shown). The results are summarized in Table 1. All 127 subtype E strains showed positive results, and only 1 subtype A strain (A1) was still reactive at an annealing temperature of 63°C.

To examine the applicability of this assay for detection of subtype E viruses from samples of unknown subtypes, 195
and the specificity of these unknown samples revealed that the sensitivity was 98.0%.

Subtype E virus has been reported to be associated with a higher risk of heterosexual transmission than subtype B virus (19). Soto-Ramirez et al. demonstrated that subtype E virus grew more efficiently in Langerhans cells than subtype B virus, suggesting that Langerhans cell tropism is associated with the efficiency of heterosexual transmission of HIV-1 (39), although other studies did not support this hypothesis (10, 35). A recent study reported that the levels of plasma HIV RNA at the earliest time within 3 months of seroconversion were more than three times higher for persons infected with subtype E virus than for those infected with subtype B virus and suggested that initial viral load may be related to the biological difference between these two viruses (15). Whatever the mechanism, the observations that subtype E virus spread rapidly and became the predominant virus have been documented in many countries, especially those in Southeast Asia. In Thailand, approximately 96% of HIV-1-infected Thais carried subtype E virus (36). Subtype E infection also constituted a significant portion of the heterosexually transmitted group in Taiwan (23). Heterosexual men, mostly businessmen traveling back and forth, contracted subtype E virus in Thailand and brought it to Taiwan in the early 1990s.

In this study, a Vpu gene-based PCR assay was developed for rapid detection of subtype E strains. Vpu has an amphipathic nature and consists of a hydrophobic N-terminal membrane anchor and a polar C-terminal cytoplasmic domain (12). A highly conserved region in the cytoplasmic domain has been shown to be critical for the biological function of Vpu (6). We have analyzed 114 Vpu sequences, including 62 subtype B strains and 52 subtype E strains, to study the frequency of nucleotide substitution at each position. While comparison of the full-length Vpu genes of the subtype E consensus and the subtype B consensus revealed a diversity of 20.3%, a higher degree of variation was found in the regions between nucleotides 18 to 45 and 181 to 204 of the Vpu gene. Furthermore, the sequences in these two regions are relatively conserved in the subtype E viruses. We therefore designed the subtype E-specific primers on the basis of the sequences of these two regions in our PCR assay. When applied to 397 samples of known subtypes, this assay could successfully differentiate subtype E strains from subtype B strains.

The subtype E Vpu PCR assay was also tested with 195 clinical samples of unknown subtypes. The samples with positive results were considered as subtype E, and those with negative results were considered as subtype B. Compared with the subtypes determined by Vpu sequence analysis, the accuracy of the assay was 97.4% (190 of 195) (Table 3). Taking together the results from a total of 592 samples, all but 1 of the 176 subtype E samples could be detected. The one subtype E sample that gave a negative result was positive after the assay had been repeated. This could have been due to inadequate treatment of the sample or a technical error. On the other hand, the specificity was found to be 98.1% (408 of 416). Since

### Table 2. Comparison of the nucleotide sequences in the Vpu gene corresponding to the region of subtype E-specific primers

<table>
<thead>
<tr>
<th>Sample</th>
<th>Nucleotide sequence corresponding to VPUEN1</th>
<th>Nucleotide sequence corresponding to VPUEN2</th>
<th>PCR result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Consensus E</td>
<td>TAG. GCAAT. AAGTGACTGATAGTGCCG</td>
<td>ACAGATGAAATTGGCCAAATGTG</td>
<td>+</td>
</tr>
<tr>
<td>A1</td>
<td>CAG-GGATTT-GGCT-</td>
<td>GACGATGAAATTGGCCAAATGTG</td>
<td>+</td>
</tr>
<tr>
<td>A2</td>
<td>CAG-GGATTT-GGCT-</td>
<td>GACGATGAAATTGGCCAAATGTG</td>
<td>+</td>
</tr>
<tr>
<td>C1</td>
<td>GAGTTGAGCTGATAGTGCCG</td>
<td>CAG-GGATTT-GGCT-</td>
<td>-</td>
</tr>
<tr>
<td>C2</td>
<td>GAGTTGAGCTGATAGTGCCG</td>
<td>CAG-GGATTT-GGCT-</td>
<td>-</td>
</tr>
<tr>
<td>C3</td>
<td>GAGTTGAGCTGATAGTGCCG</td>
<td>CAG-GGATTT-GGCT-</td>
<td>-</td>
</tr>
<tr>
<td>C4</td>
<td>GAGTTGAGCTGATAGTGCCG</td>
<td>CAG-GGATTT-GGCT-</td>
<td>-</td>
</tr>
<tr>
<td>G1</td>
<td>GAGTTGAGCTGATAGTGCCG</td>
<td>CAG-GGATTT-GGCT-</td>
<td>+</td>
</tr>
<tr>
<td>G2</td>
<td>GAGTTGAGCTGATAGTGCCG</td>
<td>CAG-GGATTT-GGCT-</td>
<td>+</td>
</tr>
<tr>
<td>G3</td>
<td>GAGTTGAGCTGATAGTGCCG</td>
<td>CAG-GGATTT-GGCT-</td>
<td>+</td>
</tr>
<tr>
<td>G4</td>
<td>GAGTTGAGCTGATAGTGCCG</td>
<td>CAG-GGATTT-GGCT-</td>
<td>+</td>
</tr>
<tr>
<td>G5</td>
<td>GAGTTGAGCTGATAGTGCCG</td>
<td>CAG-GGATTT-GGCT-</td>
<td>+</td>
</tr>
</tbody>
</table>

a A dash indicates that the nucleotide is the same as the consensus subtype E sequence; a dot represents a space (no nucleotide at that position).
b Subtype E-specific PCR at annealing temperature of 54°C.

### Table 3. Correlation of the results obtained with the subtype E PCR assay and the subtypes determined by nucleotide sequence analysis

<table>
<thead>
<tr>
<th>Subtype determined by sequence analysis</th>
<th>No. of samples with subtype E-specific PCR resulta</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>B</td>
<td>0</td>
<td>142</td>
</tr>
<tr>
<td>E</td>
<td>48</td>
<td>1</td>
</tr>
<tr>
<td>F</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>G</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>50</td>
<td>145</td>
</tr>
</tbody>
</table>

a The annealing temperature for the PCR was 54°C.
the high specificity was based on samples of predominant subtypes B and E, an additional seven non-B/non-E samples, including three subtype C and four subtype G, were also examined by this assay. All three subtype C samples were negative. While the four subtype G samples were positive, they became negative after the annealing temperature had been raised to 63°C (data not shown). The specificity remained high (97.2% [411 of 423]).

Similar to other Southeast Asian countries, subtypes A, C, and F have also been found in Taiwan occasionally (5, 21, 23). In addition, subtype G has been identified in a few patients (22). The application of the vpu PCR assay in a population infected with multiple HIV subtype needs to be addressed. Based on the vpu sequence analyses, subtypes A and G seem to have less nucleotide variation in the regions of so-called subtype E-specific primers (Table 2). Consistent with this, subtype A and G samples were positive in the subtype E PCR assay at an annealing temperature of 54°C. However, when the annealing temperature was raised to 63°C, most of the cross-reactivity could be reduced (Table 1). These findings suggest that further optimization of the PCR conditions can increase the specificity of this assay. Moreover, by the same approach, it is possible to design primers specific for other subtypes and develop subtype-specific PCR assays for rapid detection of other subtypes, especially in regions in which multiple HIV-1 subtypes cocirculate.

Subtypes B and E are the predominant subtypes in Taiwan and in many Southeast Asian countries as well (5, 23). The subtype E PCR assay developed in this study could rapidly and quite accurately differentiate subtype E from subtype B strains with high sensitivity and specificity. This assay is easy to perform and inexpensive. The method could be applied in large-scale studies. It would be especially useful in regions in which viruses of subtypes B and E are the predominant subtypes.

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REFERENCES


