AIDS RESEARCH AND HUMAN RETROVIRUSES
Volume 14, Number 11, 1998, pp. 973–977
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Short Communication

Differential Diagnosis of HIV Type 1 Group O and M Infection by Polymerase Chain Reaction and *Pst*I Restriction Analysis of the *pol* Gene Fragment

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ABSTRACT

HIV-1 group O serological screening or confirmation strategies so far have not proved 100% sensitive and specific, indicating a lack of antibody reactivity or cross-reactivity with group O antigens. Therefore, genetic analysis currently represents the only method by which confirm presumed HIV-1 group O or group O/M infections. We have optimized the sensitivity (100%) and specificity (100%) of an HIV-1 group O/M-specific PCR of a *pol* gene fragment. In addition, we report on a highly sensitive (97.2%) and specific (100%) method for differentiation between HIV-1 group O and group M viruses, using PCR and *Pst*I enzyme restriction fragment analysis of a *pol* fragment. Compared with sequencing, these methods are fast, inexpensive, and simple.

On the basis of phylogenetic analysis, HIV-1 isolates have been subdivided into groups O and M.[1] For polymerase chain reaction (PCR)-based diagnosis of HIV-1 we previously described highly sensitive (93%) and specific (100%) nested primers in the *pol* gene, which may be used to detect group O as well as group M infections, starting with HIV-1 infected primary peripheral blood mononuclear cells (PBMCs).[2] On the basis of the signature pattern of the aligned *pol* fragments representing the fragment amplified in the first round of the diagnostic PCR, we previously reported on an HIV-1 group O/M-specific PCR[3] assay for the confirmation of group O infection results obtained by HIV-1 ANT-70 V3 peptide enzyme-linked immunosorbent assay (ELISA).[4] Here we report on the use of a newly designed group O-specific primer to increase the specificity of the group O/M-specific PCR, as well as on *Pst*I restriction analysis of the nested *pol* fragment, to distinguish HIV-1 group O from HIV-1 group M infections.

Group O/M-specific PCR

As reported previously,[3] group O/M-specific PCR was performed using diagnostic outer primers H1P4235 and H1P4538 for the first-round PCR (Fig. 1). For nested PCR, the diagnostic inner antisense primer H1P4481 was combined with either sense primer H1P4241O or H1P4241M to differentiate among group O and group M infections (Fig. 1). False-positive results were obtained for a few group M isolates in the group O/M-specific PCR. To increase the specificity of the group O/M-spe-

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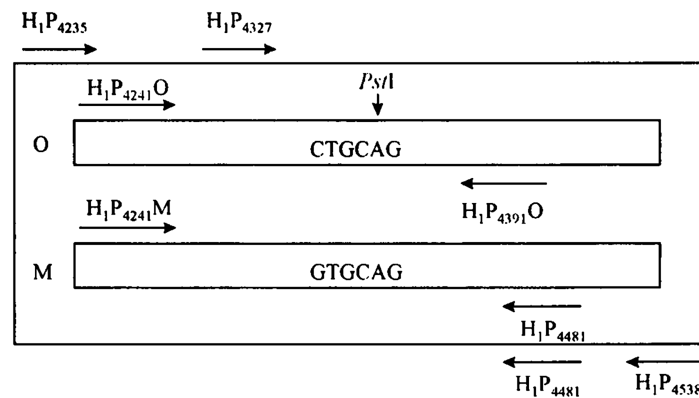
Localisation of HIV-1 *pol* primers



PCR products and *Pst*I restriction length

1st round PCR $H_1P_{4235} + H_1P_{4538} \rightarrow$ 324 bp

		group M	group O	*Pst*I treatment M	*Pst*I treatment O
2nd round PCR	$H_1P_{4327} + H_1P_{4481}$	175	175	175	107+ 68
	$H_1P_{4241}M + H_1P_{4481}$	239	-	239	-
	$H_1P_{4241}O + H_1P_{4391}O$	-	192	-	132 + 60
	$H_1P_{4235} + H_1P_{4481}$	267	267	267	107 + 160

Primer sequences of HIV-1 for nested *pol* PCR

Primers	sequence (5'→ 3')
H1P4235*	ccctacaatccccaaagtcaagg
H1P4241M*	tagaatctatgaataaagaattaaagaa
H1P4241O*	tagaagccatgaataaggaattaaaatc
H1P4327*	taagacagcagtacaaatggcag
H1P4391O*	tttgtaattctgttgtttgtatttgtga
H1P4481*	gctgtccctgtaataaacccg
H1P4538*	tactgccccttcacctttcca

FIG. 1. *Top*: Localization of HIV-1 *pol* primers. *Middle*: PCR products and *Pst*I restriction length. *Bottom*: Primer sequences of HIV-1 for nested *pol* PCR. (*) Numbering according to the sequence of HIV-1 LAI.

cific PCR, a new inner antisense primer, H1P4391O (Fig. 1), was designed to replace the shared diagnostic inner antisense primer H1P4481 for group O-specific amplification, and evaluated by testing with genetically confirmed group O and group M isolates.

PCR fragments were obtained by starting with DNA isolated from cultured PBMCs of 35 genetically confirmed HIV-1 group M isolates (subtype A, $n = 6$; B, $n = 5$; C, $n = 5$; D, $n = 3$; E, $n = 2$; F, $n = 6$; G, $n = 4$; H, $n = 4$),[5] and 21 reported[3,6] and 4 unpublished (L. Gürtler, personal communication, 1997) genetically confirmed HIV-1 group O isolates (Table 1). Using this new group O primer, the group O/M-specific PCR attained 100% sensitivity and specificity.

Sensitivity and specificity are calculated by the following formulas:

$$\text{Sensitivity} = \text{true positives}/(\text{true positives} + \text{false negatives}) \times 100$$

with true positives being the number of genetically confirmed group O samples scoring positive with the group O-specific primer in PCR, and the number of genetically confirmed group M samples scoring positive with the group M-specific primer in PCR; and false negatives being the number of genetically confirmed group O samples scoring negative with the group O-specific primer in PCR, and the number of genetically confirmed group M samples scoring negative with the group M-specific primer in PCR.

$$\text{Specificity} = \text{true negatives}/(\text{true negatives} + \text{false positives}) \times 100$$

with true negatives being the number of genetically confirmed group O samples scoring negative with the group M-specific primer in PCR, and the number of genetically confirmed group M samples scoring negative with the group O-specific primer in PCR; and false positives being the number of genetically

confirmed group O samples scoring positive with the group M-specific primer in PCR, and the number of genetically confirmed group M samples scoring positive with the group O-specific primer in PCR.

TABLE 1. STRAINS ANALYZED FOR PRESENCE OF *PstI* RESTRICTION SITE AND GROUP O/M-SPECIFIC AMPLIFICATION

1. *PstI* restriction signature pattern in HIV-1 group M diagnostic *pol* fragment sequences in Los Alamos Database 1995 and GenBank (n = 33)

Subtype A (n = 1)	U455
Subtype B (n = 21)	SF2, LAI, HXB2R, NL43, NY5CG, LW123, MN, JRCSF, JRFL, OYI, CAM1, HAN, D31, RF, YU2, BCSG3C, P896, 3202A12, 3202A21, MANC, WEAU160
Subtype C (n = 1)	ETH2220
Subtype D (n = 3)	ELI, Z2Z6, NDK
Intersubtype hybrids (n = 7)	MAL, IBNG, 93TH253, CM240, 90CR102, Z321, ZAM184

2. HIV-1 group O/M-specific PCR and *PstI* restriction analysis

HIV-1 group M samples

Cocultured lymphocytes (n = 35)

Subtype A (n = 6)	VI897, VI1198, VI1199, VI1240, VI1243, VI1280
Subtype B (n = 5)	VI810, VI811, VI819, VI833, VI835
Subtype C (n = 5)	VI849, VI882, VI1052, VI1144, VI1206
Subtype D (n = 3)	VI1308, VI1309, VI1234
Subtype E (n = 2)	VI1249, CA10
Subtype F (n = 6)	VI850, VI961, VI1267, CA4, CA16, CA20
Subtype G (n = 4)	VI525, VI526, VI1197, LBV21-7
Subtype H (n = 4)	VI557, VI991, VI997, CA13

Plasma (n = 4)

Subtype A (n = 4)	U454, EB046, KNH712, NJ223

HIV-1 group O samples

Cocultured lymphocytes (n = 25)

MVP2171, MVP2901, MVP2902, MVP4354, MVP6104, MVP6245, MVP6405, MVP6599, MVP6778, MVP7851, MVP8161, MVP5180 (kindly provided by L. Gürtler)

BCF01, BCF02, BCF03, BCF06, BCF07, BCF08, BCF11, BCF13, BCFXX (kindly provided by F. Simon)

Ant70, partner Ant70, CA9, VI686 (ITM)

plasma (n = 11)

D830, U377, U459, CNPS468 (kindly provided by L. Zekeng)

EB040, EB050 (kindly provided by P. Ndumbe)

Five coded samples send by UNAIDS, including one sample from which we could not extract RNA (kindly provided by G. Vercauteren).

BJ3/7/115 (ITM).

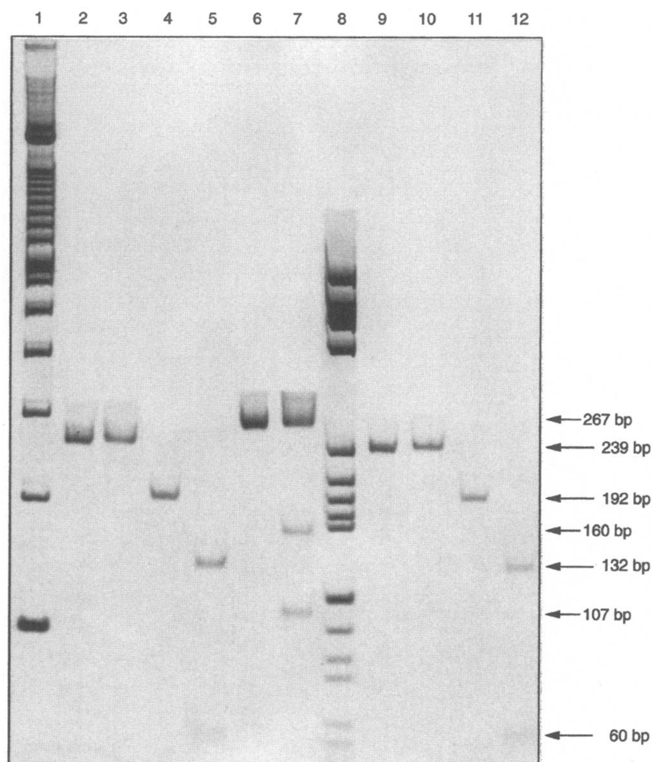


FIG. 2. Restriction analysis of the *pol* fragment. For each isolate, the first and second lanes contain the amplification products that are not treated and treated with *PstI* enzyme, respectively. The positions and lengths of the fragments of interest, obtained after 7.5% polyacrylamide gel electrophoresis, ethidium bromide staining, and visualization under UV, are indicated. DNA was extracted from cocultured lymphocytes as previously described,[2] and RNA extraction and RT-PCR were carried out as described earlier.[10] The first-round PCR amplification for 35 cycles was performed in a 50-μl reaction mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, a 0.2 mM concentration of each dNTP, 1.25 units of *Taq* polymerase (Perkin-Elmer, Zaventem, Belgium), and 0.4 μM concentrations of primers H1P4235 and H1P4538. Cycling conditions were as follows: 0.5 min at 94°C, 0.5 min at 50°C, and 0.5 min at 72°C in a thermal DNA cycler machine (Perkin-Elmer Cetus). In the last round, the extension was elongated to 7 min. Two microliters of the amplified DNA was removed and nested PCR was performed for 25 cycles under the conditions described above, using either 2.5 mM $MgCl_2$ and group M primers H1P4241M and H1P4481, or 1.5 mM $MgCl_2$ and group O primers H1P4241O and H1P4391O. X, Molecular weight marker V (Boehringer Mannheim, Brussels, Belgium). After a 1-hr incubation at 37°C with *PstI* enzyme in the appropriate buffer (Boehringer Mannheim), the PCR fragment is electrophoresed on a polyacrylamide gel (7.5%), stained with ethidium bromide, and visualized under UV. Lane 1, 100 base-pair ladder (Pharmacia, Roosendaal, The Netherlands); lane 8, molecular weight marker V (Boehringer Mannheim); lanes 2 and 3, nested group M-specific (H1P4241M–H1P4481) PCR product of a group M sample and the *PstI* digest (Fig. 1), respectively; lanes 4 and 5, nested group O-specific (H1P4241O–H1P4391O) PCR product of a group O sample and the *PstI* digest (Fig. 1), respectively; lanes 6 and 7, heminested (H1P4235–H1P4481) PCR product of a group O/M dual infection[12] and the *PstI* digest (Fig. 1), respectively; lanes 9 and 10, group M-specific (H1P4241M–H1P4481) PCR product of a group O/M dual infection and the *PstI* digest (Fig. 1), respectively; lanes 11 and 12, group O-specific (H1P4241O–H1P4391O) PCR product of a group O/M dual infection and the *PstI* digest (Fig. 1), respectively.

*Pst*I *restriction analysis*

On the basis of sequence information in the Los Alamos Database[1] and GenBank available for the nested *pol* fragment (175 bp) as obtained by diagnostic PCR,[2,7] a unique *Pst*I restriction site (CTGCAG) was found to be present in group O (n = 4), but not in group M (GTGCAG) viruses (n = 33; A, n = 1; B, n = 21; C, n = 1; D, n = 3; intersubtype hybrids, n = 7). PCR fragments were obtained starting with DNA isolated from cultured PBMCs of an additional 35 genetically confirmed HIV-1 group M isolates (subtype A, n = 6; B, n = 5; C, n = 5; D, n = 3; E, n = 2; F, n = 6; G, n = 4; H, n = 4)[5] and 21 reported[3] and 4 unpublished (L. Gürtler, personal communication, 1997) genetically confirmed HIV-1 group O isolates (Table 1).

None of the 68 HIV-1 group M isolates, representing subtypes A–H, showed evidence of *Pst*I cleavage of the group M 239-bp *pol* fragment, but most (i.e., all but BCF08) HIV-1 group O isolates showed evidence of *Pst*I digestion of the 192-bp group O *pol* fragment (Figs. 1 and 2). Sequence analysis of the 192-bp *pol* fragment of BCF08 confirmed the absence of the *Pst*I restriction site (CTCCAG). HIV-1 group O isolates were identified with a sensitivity of 96% (24 of 25) and a specificity of 100%.

Sensitivity and specificity are calculated by the following formulas:

Sensitivity = true positives/(true positives + false negatives) × 100

with true positives being the number of genetically confirmed group O samples having the *pst*I restriction site, and false negatives being the number of genetically confirmed group O samples not having the *Pst*I restriction site.

Specificity = true negatives/(true negatives + false positives) × 100

with true negatives being the number of genetically confirmed group M samples not having the *Pst*I restriction site; and false positives being the number of genetically confirmed group M samples having the *Pst*I restriction site.

The *Pst*I site in the group O 192-bp *pol* fragment is highly conserved as was observed in five consecutive isolates taken between March 1987 and July 1996 from a group O HIV-$1_{ANT\text{-}70}$-infected couple (data not shown).[8,9] An example of the characteristic *Pst*I restriction pattern of an HIV-1 group M, group O, and group O/M dually infected individual is shown in Fig. 2.

Evaluation of group O/M-specific PCR and PstI *restriction analysis starting with plasma of HIV-1-infected individuals*

RNA extraction and RT-PCR[10] were performed on the plasma of 16 HIV-1-infected individuals whose sera were reactive in a group O V3 peptide ELISA[4] (data not shown). Fourteen of 16 sera were confirmed as group O positive by line immunoassay (INNO-LIA HIV-1 type O, second version; Innogenetics, Ghent, Belgium), as previously described[11]; 3 of these samples indicated group O/M dual infection. Two other sera were identified as being group M infected. Ten of 11 HIV-1 group O infections identified by group O V3 peptide ELISA and INNO-LIA HIV-1 type O reactivity were confirmed by group O/M-specific PCR and *Pst*I restriction analysis. From the remaining samples no RNA could be extracted, thereby preventing any determination by group O/M-specific PCR. Concerning the three sera that were group O/M dual reactive by INNO-LIA HIV-1 type O, only one was confirmed as a group O/M dual infection by sequencing, PCR, and *Pst*I restriction analysis.[12] The two remaining INNO-LIA HIV-1 type O dual-reactive samples were found to be group M infections by both PCR and *Pst*I restriction analysis. Both samples were classified as subtype A by heteroduplex mobility assay (HMA) analysis.[13] The two samples classified as group M infections on the basis of the INNO-LIA HIV-1 type O serology were confirmed as group M infections by group O/M-specific PCR and *Pst*I restriction analysis.

Group O/M-specific PCR and PstI *restriction analysis of plasma from a group O/M dual-infected individual*

We reanalyzed a previously documented group O/M dual infection in an individual of Bénin,[12] by the improved group O/M-specific PCR, as well as by *Pst*I restriction analysis (Fig. 2). RT-PCR using diagnostic primers H1P4235 and H1P4538, and heminested PCR using H1P4235 and H1P4481 (Fig. 1), resulted in a PCR product obtained for both group M and O variants in the plasma sample (Fig. 2, lane 6). *Pst*I restriction analysis of this fragment revealed the intact group M PCR product, whereas the group O PCR product was digested (Fig. 2, lane 7). Group O/M-specific PCR resulted in amplification of both HIV-1 group O and group M variants, using the group O-specific (Fig. 2, lane 11) and group M-specific (Fig. 2, lane 9) primers, respectively. The group O-specific PCR fragment was digested by *Pst*I treatment (Fig. 2, lane 12), whereas the group M-specific amplification product was not (Fig. 2, lane 10).

In conclusion, combining the results staged above, *pol* HIV-1 group O/M-specific PCR was optimized to a sensitivity and specificity of 100% on genetically well-characterized samples (n = 75). The *Pst*I enzyme restriction fragment analysis of the *pol* fragment allowed us to identify HIV-1 group O isolates with a sensitivity of 97.2% (35 of 36) and a specificity of 100% (72 of 72). As compared with sequencing, this is a fast, inexpensive, and simple method for the genetic differentiation of HIV-1 group O and group M viruses. In combination with the optimized group O/M-specific PCR, *Pst*I enzyme restriction fragment analysis has proved to be an additional confirmation method by which to identify and distinguish group O, group M, and dual group O/M infections.

ACKNOWLEDGMENTS

This work was supported by Grants G.3301.96 and G.0134.97 of the Fonds voor Wetenschappelijk Onderzoek, Brussels, and by EC Project IC 18-CT96-0110.

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