# Correspondence

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## Subtype-specific polymerase chain reaction for the identification of HIV-1 genetic subtypes circulating in Africa

Phylogenetic analysis of many isolates of HIV from Africa and from other regions in the world has revealed two groups of HIV-1 isolates: group M, the major group with at least 10 different genetic subtypes (A–J), and group O [1].The global HIV pandemic is extremely heterogeneous and dynamic in nature [2]. The current distribution of subtypes needs to be better established. Studying HIV-1 genetic variation at the global level is important in identifying the emergence of subtypes that may be more readily transmitted, that have an altered virulence, that escape detection by commercial diagnostic assays, and in ensuring that vaccine antigens are directed against contemporary strains of the virus circulating within specific populations [3–6].

The broadest capability for the surveillance of genetic subtypes is by V3-loop peptide enzyme-linked immunosorbent assay. Nevertheless, the feasibility of V3 serotyping for the determination of genetic subtypes is limited because a substantial proportion of mismatches between serological and genetic subtypes occurs in areas where different subtypes have been cocirculating for a longer period of time [7]. Another commonly used technique is the heteroduplex mobility assay (HMA), which is a polymerase chain reaction (PCR)-based procedure allowing rapid HIV subtyping using a set of reference reagents representing different subtypes. Subtyping by HMA has shown excellent concordance with DNA sequencing, the golden standard technique, in determining HIV-1 group M subtypes [8,9]. HMA is less expensive and easier to perform than sequencing, but is still laborious when large series of samples have to be analysed.

There is an urgent need to develop less expensive and easy techniques to allow subtyping on a large scale. Preliminary data suggest that subtype A is predominantly found in West and Central Africa, representing 60–90% of the viral subtypes circulating in these regions [2]. We have therefore developed the following subtyping strategy to rapidly identify subtype A samples by a subtype A-specific PCR followed by HMA for samples not recognised by this PCR.

A total of 424 HIV-positive samples, genetically characterized in the *env* region by HMA or sequencing, or both, were tested with subtype A-specific primers. A total of 276 samples were identified as subtype A, and 148 were identified as non-A representing the seven remaining subtypes from B to H (31 subtype B, 30 subtype C, 13 subtype D, eight subtype E, eight subtype F, 49 subtype G, and nine subtype H). The geographic origins of these samples are shown in Table 1.

The genetic subtypes of the different samples were determined in the envelope region by HMA or sequencing, or both. For HMA, we used ED5–ED12 as outer primers and ES7–ES8 as inner primers; heteroduplex molecules were obtained by mixing two divergent PCR-amplified DNA fragments (the unknown patient strain with a plasmid from typed reference strains) as described by Delwart *et al.* [7]. Samples undetermined by HMA were sequenced either with ES7 and ES8 or with ED31 and ED33 primers. Alignment of the sequences with known sequences from reference strains representing the different genetic subtypes and phylogenetic analysis to determine the genetic subtype was performed using CLUSTAL W.

For the subtype A-specific PCR, a nested PCR reaction was performed to obtain a 350 base-pair fragment using outer primers ED5–ED12 (the same primers as used for HMA), and inner primers LRM1B (5′-CAAGCACATTGTAATGTC-3′) and LRM3B (5′-CACYTTATTWYTCCTKDRATGG-3′, where Y = C + T, W = A + T, K = G + T, D = G + A + T, and R = A + G), which were specific for subtype A. PCR reactions for the first round (ED5–ED12) were performed as previously described by Delwart *et al.* [7]. A first denaturation step for 5 min at 94°C was followed by 30 cycles of 94°C for 15 sec, 55°C for 30 sec, and 72°C for 2 min, with a final extension for 7 min at 72°C for the first round. Five microlitres from this amplification were used for the second round with the inner primers using the following cycling conditions for 35 cycles: 94°C for 20 sec, 55°C for 30 sec, 72°C for 1 min, with a final extension of 5 min at 72°C. The reaction mixture comprised 50 mmol/l KCl, 10 mmol/l Tris-HCl (pH 9), 0.1% Triton X100, 1.25 mmol/l $MgCl_2$ for the first round or 1.5 mmol/l $MgCl_2$ for the second round, 10 pmol of each primer for the first round and 20 pmol for the second round, 0.2 mmol/l of each dNTP for the first round and 0.04 mmol/l for the second round, and 2.5 U *Taq* polymerase. The PCR amplification products were detected by electrophoresis on a 1% agarose gel and visualized by ethidium bromide staining.

**Table 1.** Number and percentage of the subtype A samples from different African countries and non-A samples reactive with subtype A-specific primers.

| Genetic subtype | Origin of samples (n) | No. tested | No. (%) reactive |
|---|---|---|---|
| A | Burkina Faso | 8 | 7 (87.5) |
| | Mali | 80 | 64 (80.0) |
| | Senegal | 33 | 28 (84.8) |
| | Nigeria | 75 | 60 (80.0) |
| | Cameroon | 40 | 20 (50.0) |
| | DRC | 29 | 15 (51.7) |
| | Unknown | 11 | 7 (63.6) |
| Total A | | 276 | 201 (72.8) |
| B | France (27), Cameroon (4) | 31 | 0 (0) |
| C | Djibouti (23), Mali (3), Senegal (2), Nigeria (1), unknown (1) | 30 | 2 (6.6) |
| D | Cameroon (2), Mali (1), Nigeria (1), Senegal (2), DRC (1), unknown (6) | 13 | 0 (0) |
| E | Cambodia (5), Djibouti (1) DRC (1), unknown (1) | 8 | 0 (0) |
| F | Cameroon (5), DRC (1) CAR (1), Chad (1), unknown (1) | 8 | 0 (0) |
| G | Nigeria (28), Burkina Faso (8) Mali (9), DRC (2), Senegal (1) | 49 | 0 (0) |
| H | DRC (8), unknown (1) | 9 | 0 (0) |
| Total non-A | | 148 | 2 (1.35) |

DRC, Democratic Republic of Congo (formerly Zaïre); CAR, Central African Republic.

Table 1 summarizes the results obtained on subtype A samples from different African countries, and the non-A samples. Overall, 201 out of the 276 subtype A samples were detected, resulting in an overall sensitivity of 72.8%. The sensitivity changed according to the geographical origin of the samples and ranged from 50 to 87.5%. The highest numbers of subtype A strains were detected in samples from West African countries with 87.5% in Burkina Faso, 84.8% in Senegal, and 80.0% in Mali and Nigeria, whereas the lowest values were seen in samples of Central African origin with only 50.0 and 51.7% of the subtype A strains detected in samples from Cameroon and the Democratic Republic of Congo (formerly Zaïre), respectively. Amongst the 148 non-A samples, only two (1.35%) were reactive with the subtype A-specific primers, resulting in an overall specificity 98.6%. The two samples reactive were identified as subtype C by HMA and by the sequence of the C2-V3 region.

Preliminary data indicate a very heterogeneous distribution and dominance of different genetic subtypes depending on the country analysed [2]. To date, there have been relatively few systematic large-scale attempts to characterize HIV isolates. Those viruses actually characterized were obtained from convenience samples rather than from random sampling and so their representativeness is uncertain, one of the obstacles being the complexity and limitations of the techniques actually available for genetic subtyping. Less expensive and easier techniques that allow subtyping on a large scale are needed. In Africa, in regions where subtype A predominates but other subtypes are also present we propose the following strategy for subtyping in the envelope region: first a subtype A-specific PCR to eliminate subtype A samples, identify the non-A samples by HMA and further characterize by sequencing those samples indeterminate by HMA. Based on our results, this strategy will allow a rapid and efficient screening for HIV subtypes in West African countries. The different results obtained for subtype A strains from West and Central Africa confirm the large diversity within this subtype.

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## Quantitative techniques and HIV-1 load in plasma and semen

Vernazza *et al.* [1] recently questioned the techniques that we used to measure the HIV-1 RNA copy number in semen [2]. They stated that we did not comment on the RNA extraction protocol used in our competitive reverse transcriptase polymerase chain reaction (cRT-PCR) assay, and implied that inhibitory factors were present in our samples.

First, although we did not comment on our RNA extraction protocol, we cited a reference for the procedure, following convention, which was the guanidinium thiocyanate method described by Chomczynski and Sacchi [3]. This method can be considered the standard procedure for RNA extraction from biological samples.

Second, cRT-PCR is a reliable procedure with which to quantify mRNA species, and any inhibition present in the samples to be quantified by cRT-PCR does not affect the results. The general concept of cRT-PCR consists of co-amplifying two different templates with similar lengths bearing the same primer recognition sequences in the same tube, thus ensuring identical thermodynamics and amplification efficiency for both template species. The quantity of one of the templates introduced must be known, and after PCR, the amplification products are clearly distinguishable by gel electrophoresis analysis, thus allowing the densitometric comparison of the relative intensities of the bands for both species. The ratio of PCR products is related to the initial template concentration [4–6].

This method, which we used in our study, appears to be the most sensitive procedure currently available. In fact, this procedure detects a copy number as low as 10 copies/ml compared with 1000 copies/ml by the nucleic acid sequence-based amplification method used by Vernazza *et al.* [7].

Technical considerations apart, Vernazza and colleagues are concerned about the different percentage of patients with HIV RNA levels above 1000 copies/ml (62% in their study versus 46% in ours). In actual fact, when their figures are broken down into populations, the percentage of patients with HIV RNA levels above 1000 copies/ml varies widely: 70% of their Swiss patients and 52% of their US patients, the latter being comparable with the 46% reported in our study.

Vernazza and coworkers are mistaken when they affirm that the HIV-infected men in their study and in ours had similar characteristics. There were major differences between the two populations, and it is these differences that could explain the discrepancies between the results of the two studies. First, 68.3% of our patients were injecting drug users [2] versus 12.8% in their study [7]. In addition, our patients had a better immunological status: 34.5% with CD4 cell counts $> 500 \times 10^6/l$ [2] versus only 14.8% of those reported by Vernazza *et al.* [7].

In summary, the cRT-PCR assay that we used to quantify the HIV RNA copy number in semen and plasma is a sensitive and reliable procedure, and differences between our study and that reported by Vernazza *et al.* [7] can be attributed to differences in the populations studied.

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