First evidence of transmission of an HIV-1 M/O intergroup recombinant virus

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Objective: Despite the genetic divergence between HIV-1 groups M and O, HIV-1 M/O intergroup recombinants were reported. Actually, there is no data on the transmissibility of such recombinant forms. During a surveillance of HIV genetic diversity in Cameroon, we investigated the possible direct transmission of an HIV-1 M/O recombinant virus in an HIV-infected couple.

Methods: Consecutive samples obtained from the couple were analysed for detection of dual HIV-1 groups M and O infections, and HIV-1 M/O recombinant forms. Analyses were performed using a serological and molecular algorithm based on HIV serotyping and group-specific PCRs targeting the polymerase and envelope genes. Pattern characterization of the strains found in both patients was based on complete genome sequencing. Phylogenetic and similarity profile analyses were performed to investigate the genetic relationship between viruses from both spouses and the previously described recombinant forms.

Results: The sero-molecular algorithm data showed a group O serotype confirmed by molecular analysis in the envelope regions, whereas molecular tests identified HIV-1 group M in the polymerase. Phylogenetic analyses and similarity profiles of the full-length genome sequences showed that both spouses were infected with a unique recombinant virus having two recombination breakpoints in the *vpr* gene and LTR region. No phylogenetic link was found with the previous M/O recombinants.

Conclusion: We provide, for the first time, molecular evidence of direct transmission of an HIV-1 M/O recombinant, highlighting the potential spread of these divergent viruses. The importance of HIV-1 recombination on genetic evolution and public health when implying divergent strains as group O has to be carefully considered.

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Introduction

The human immunodeficiency virus type 1 (HIV-1) genetic diversity includes four groups: M (major), O (outlier), N (non-M, non-O) and P [1–3], whereas HIV-2 includes nine groups: A-I [4]. In the Central African sub-region, all HIV-1 groups as well as HIV-2 viruses have been shown to circulate. HIV-1 group M

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(HIV-1/M) and HIV-1 group O (HIV-1/O) have been particularly shown to co-circulate in Cameroon [5,6]. This co-circulation provides an ideal environment for HIV-1/M and HIV-1/O dual infections as previously described in some patients [5–7]. Despite the great genetic divergence between the two groups, the emergence of HIV-1 M/O intergroup recombinants was described in 1999 and 2004 [5,8,9]. In these three cases, the recombinant HIV-1 M/O was associated with dual infections HIV-1/M + HIV-1/O. Recently in 2010, Vessiere *et al*. described for the first time an HIV-1 M/O that was not associated with dual infections in a Cameroonian woman living in France suggesting a direct transmission of the recombinant virus [10].

Recombination is a common phenomenon of HIV-1 genetic evolution. This contributes significantly to the genetic diversity of circulating HIV strains and may have important implications for diagnosis, development of therapeutic strategies and vaccine design especially when implying group O fragments. Indeed, previous findings have shown that HIV-1 group O strains divergence in the Env region resulted in misdiagnosis using serological screening tests [11–14]. As concern with follow-up of HIV patients, viral load quantification could be problematic according to the group (M or O) of the target region if commercial kits are specific to HIV-1/M strains. In addition, HIV-1 group O strains are considered to be naturally resistant to non-nucleoside reverse transcriptase inhibitors (NNRTIs) which are mostly used in first line ART in resource-limited settings [15]. Thus, mosaic genomes including one or several regions or fragments of HIV-1/O can lead to an erroneous diagnosis and/or a nonadapted management with an increased risk of virological failure using cART.

To date, there is no data on the viability and transmissibility of such recombinant forms. In the three first reports, the recombinant forms were always associated with at least one of the parental strains, indicating that recombinants can emerge in a patient but without knowledge on their transmissibility potentials [5,8,9]. The characterization of the fourth recombinant in the absence of parental strains was an indirect evidence that these forms can become a predominant population and directly transmissible [10]. In this article, we present for the first time, molecular evidence of an HIV-1 M/O recombinant transmission in the absence of dual infections between a man and his wife living in Cameroon.

Patients and methods

Patients and samples

Blood sample from a 40-year-old man (REC003) diagnosed elsewhere as HIV positive in 2005, was received in the Virology laboratory of Centre Pasteur du Cameroun (CPC) in October 2012 for HIV viral load monitoring. In January 2013, he began cART consisting of lamivudine+zidovudine+lopinavir/ritonavir. In March 2013, his $CD4^+$ cell counts were 161 cells/μl, with no overt clinical symptoms. REC003 was married for 12 years with his wife, REC024, a 37-year-old woman diagnosed HIV-1 positive in 2012 during pregnancy. At the time of blood collection in March 2013, her $CD4^+$ cell counts were 301 cells/μl and she too was not experiencing any overt clinical manifestations.

Investigation of these two cases was performed on sequential samples available for REC003 (October 2012, March and September 2013) and for REC024 (March and September 2013). Viral load monitoring was performed on these samples with the Abbott Real-Time HIV-1 assay (Abbott Laboratories, Wiesbaden, Germany) according to the manufacturer's recommendations.

Detection and characterization of the HIV-1 M/O forms

Due to the co-circulation of HIV-1/M and HIV-1/O in Cameroon, the CPC has developed a serological and molecular algorithm to detect HIV-1/M + HIV-1/O dual infections and HIV-1 M/O recombinant forms (supplementary figure 1, http://links.lww.com/QAD/A776).

First, HIV-1 serotyping is performed as a routine test for diagnosis and prior to all viral load analysis to determine HIV type (HIV-1 or 2) and HIV-1 groups (M-N-O-P), with an in-house ELISA assay as previously described [16,17].

Secondly, all samples reactive to HIV-1/O or both HIV-1/M and /O as well as those that are not reactive to HIV-1/M or /O were investigated with molecular techniques. Conventional nested PCRs were performed for the detection of HIV-1/M + HIV-1/O dual infections from RNA and proviral DNA, using group M and O specific primer sets targeting the Polymerase [protease (PROT), reverse transcriptase (RT) and integrase (INT)] and Envelope (GP41) regions followed by sequencing as previously described [10,18–21].

Genetic distances were calculated on the sub-genomic sequences and phylogenetic characterization was performed using reference sequences downloaded from the LANL HIV database (http://www.hiv.lanl.gov) and representative of HIV-1/M subtypes and HIV-1/O clades (supplementary methods, http://links.lww.com/QAD/A776).

When discordant results were observed (not all PCR positive for both groups M and O in each genomic region), a recombinant form of the two viruses was suspected. Following previous results highlighting the *vpr* gene as a possible recombination hotspot [22], the

Table 1. Serotyping and molecular results.

		REC003			REC024	
		October 2012[a] None[b] 5.4 log[c]	March 2013 3 months 3.1 log	September 2013 9 months 2.7 log	March 2013 None 3.1 log	September 2013 None 3.0 log
Group M specific PCR	PROT	+	+	+	+	+
	RT	+	+	+	+	+
	INT	+	+	+	+	+
	GP41	−	−	−	−	−
Group O specific PCR	PROT	−	−	−	−	−
	RT	−	−	−	−	−
	INT	−	−	−	−	−
	GP41	+	+	+	+	+
vpr PCR	MM	−	−	−	−	−
	OO	−	−	−	−	−
	MO	+	+	+	+	+
	OM	−	−	−	−	−

+, positive; −, negative.
[a]Sample collection date.
[b]cART (combination antiretroviral therapy).
[c]Viral load results using Abbott Real-time HIV-1.

presence of a recombination breakpoint in this gene was investigated using homologous and heterologous combinations of HIV-1/M and HIV-1/O primers as described elsewhere [9] with slight modifications to the outer primers (sense VIF-1mod 5′ GGGTYTATTACAGRGACAGCAGAG 3′; antisense VPU-1mod 5′ GGGTCTGTGGGWACACAGGC 3′). These primers amplify accessory genes of HIV-1 groups M and O in the first round PCR.

Finally, near full-length genomes characterization was obtained from RNA extracted from samples of the first time point of each patient, by the amplification of seven overlapping fragments using group-specific RT-PCR followed by nested PCR and sequencing (supplementary methods, http://links.lww.com/QAD/A776). The similarity plots were generated by the SimPlot software with 350 nucleotide (nt) windows, 20 nt increments, and the Kimura 2-parameter method with a transition–transversion (Ts/Tv) ratio of 2.0. The genome maps were obtained using the Recombinant HIV Drawing Tool (http://www.hiv.lanl.gov/content/sequence/DRAW_CRF/recom_mapper.html). Near full-length genome of the previously described HIV-1 M/O recombinant RBF208 (accession number, GQ351296) [10] was included for comparison.

The newly determined near full-length nucleotide sequences have been submitted to the GenBank database with accession numbers KM438031 and KM438032.

Results

HIV serotyping indicated that REC003 and REC024 (husband and wife) were both sero-reactive with only peptides of HIV-1 group O. Plasma viral loads obtained with Abbott HIV-1 assay for the three REC003 and the two REC024 samples are summarized in Table 1. Due to the nonspecific amplification of both HIV-1/M and HIV-1/O by this technique, the results only indicated the global replication level and not the presence of one or both viruses.

Using the group-specific RT-nested PCRs on plasma RNAs, we obtained HIV-1/M positive results in the Pol (PROT, RT and INT) region and HIV-1 O-positive results in the Env (GP41) region from both REC003 and REC024 (Table 1). These results were consistent for all consecutive blood samples collected from the two patients and confirmed on proviral DNA. Altogether, nucleotide BLAST search and phylogenetic analyses using sequences generated from all amplicons confirmed that the Pol region of the viruses from both REC003 and REC024 was derived from an HIV-1/M (subtype F2), whereas the GP41 portion was derived from an HIV-1/O clade A (Fig. 1). Both Pol (PROT-RT) and Env (GP41)-based phylogenetic trees uncovered a close relationship between the viral sequences from REC003 and REC024 with all samples of both patients collected overtime (Fig. 1). Estimates of evolutionary divergence between sequences obtained from intra-patient samples showed low levels of divergence associated to inter-patient values indicating the own evolution in the two patients (supplementary table 2, http://links.lww.com/QAD/A776).

These data indicated the likelihood of a transmitted recombinant form in both persons. Following previous results highlighting the *vpr* as a possible recombination hotspot, exploration of a *vpr* breakpoint was carried out using homologous (MM and OO) and heterologous (MO and OM) primers of the *vpr* gene. Only the heterologous MO (5′M and 3′O) nested PCRs yielded a

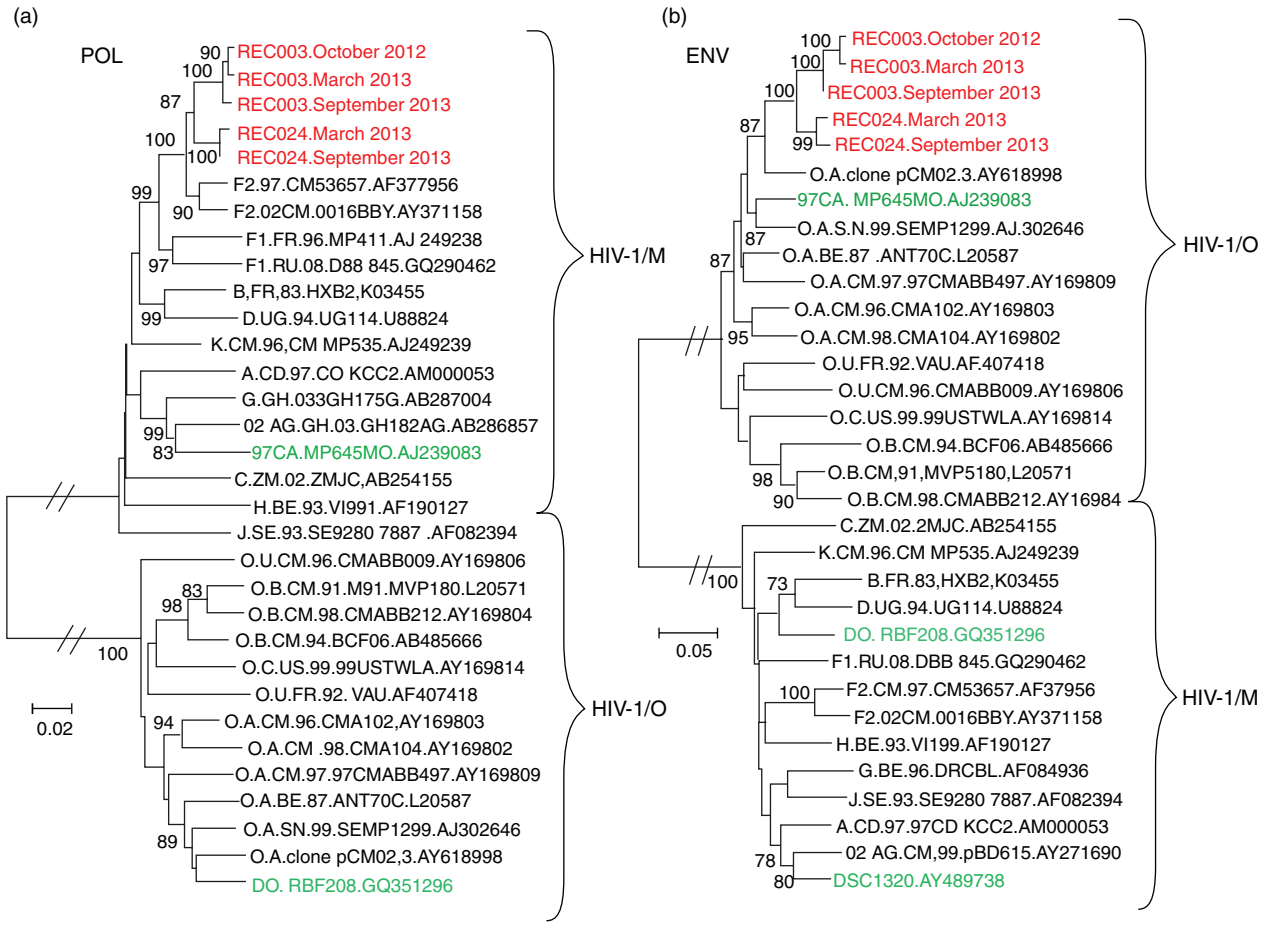

Fig. 1. Phylogenetic relationships of the sequences derived from the Pol and Env genomic regions of the viruses from REC003 and REC024. The neighbour-joining tree is based on the alignment of the *pol* (PROT-RT) sequences (a) and *env* (GP41) sequences (b) regions of the viral genome. The new sequences obtained from all the samples analysed for both patients (REC003 and REC024) are highlighted in red. All available databases reference strains downloaded from Los Alamos database (http://www.hiv.lanl.gov) are named by the subtypes or clades, isolate names and GenBank accession numbers. Previously reported recombinants: (i) DSC1320 described by Yamaguchi *et al.* [5] (ii) 97CA.MP645MO described by Peeters *et al.* [9] and (iii) DO.RBF208 described by Vessiere *et al.* [10] are further highlighted in green. The reliability of the tree topologies was estimated by bootstrap analysis with 1000 pseudoreplicate data sets. For clarity, all bootstrap less than 70 have been omitted. The scale is shown at the bottom as substitutions per site.

positive result whereas others remained negative (Table 1). These results were further confirmed by sequencing and recombination analysis showing a breakpoint in the *vpr* gene with no parental viruses (HIV-1 groups M or O) present.

Further characterization of the near complete sequences of the first available sample of REC003 and REC024 viruses displayed an M-O mosaic structure with the Pol region belonging to HIV-1/M and the Env region belonging to HIV-1/O (Fig. 2a). Analyses of the LTR sequences obtained from proviral DNA showed a second recombination breakpoint in both REC003 and REC024 viruses. Both near complete genomes featured a close relatedness across the entire genome, with the least similarity in the Env region, and displayed the same breaking points located within the *vpr* gene and the LTR region (Fig. 2a). Comparison to each other and to the near full-length genome of HIV-1 M/O recombinant, DO.RBF208, previously described [10] showed no relation with the latter (Fig. 2a). Compared to the other recombinants previously reported (Fig. 2b), these two viruses defined a new M/O recombinant strain.

Discussion

Recombination is a common phenomenon of HIV genetic evolution that has led to numerous URF and CRF among HIV-1 group M strains and even among

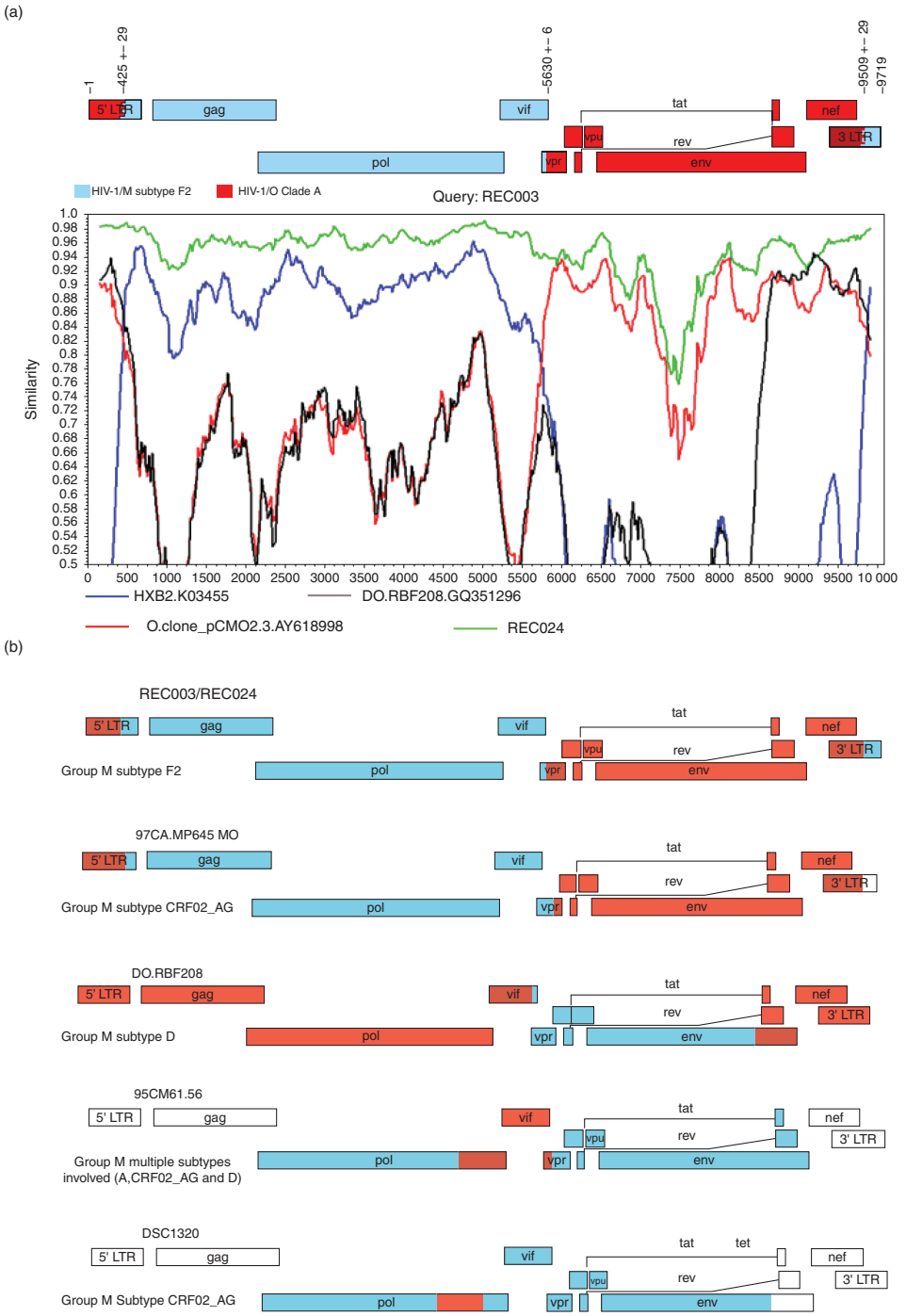

Fig. 2. Recombination analysis of the full-length genomes of REC003 and REC024 viruses. (a) 'Virtual' complete genomes were constructed by assembling LTR sequence (obtained from proviral DNA) to the corresponding near full-length genome (from RNA extracted from the October 2012 and March 2013 samples of REC003 and REC024, respectively). These virtual complete genomes sequences (9806 pb for REC003 and 9839 pb for REC024) were further aligned with HIV-1 group M isolate HXB2 (blue) and HIV-1 group O isolate clone pCMO2.3 (red) as well as the near full-length genome of the previously described recombinant form DO.RBF208 (black). In the similarity plot, REC003 was queried using a window/overlap of 350/20 nucleotides and the Kimura 2-parameter method with a transition–transversion (Ts/Tv) ratio of 2.0. Mapping of the *vpr* and LTR breakpoints on the full-length genomes was done using HIV Recombinant Drawing Tool (http://www.hiv.lanl.gov/content/sequence/DRAW_CRF/recom_mapper.html). (b) Genomic structures of the new form described here and the previously reported recombinants – 97CA.MP645MO by Peeters *et al.* [9], DO.RBF208 by Vessiere *et al.* [10], 95CM61.56 by Takehisa *et al.* [8] and DSC1320 by Yamaguchi *et al.* [5] – are represented on the figure. Group M derived portions of the genome are represented in blue colour (subtype involved is indicating) and group O derived portions in the red.

HIV-2 (http://www.hiv.lanl.gov). It occurs during the reverse transcription stage of viral replication and requires the presence of the viral genomes of the parental strains in the same cell [23–25]. Although genetically distant, three HIV-1 M/O recombinant viruses have been previously reported in patients co-infected with groups M and O strains [5,8,9]. The fourth HIV-1 M/O recombinant virus described in 2010 was not associated with dual infections. The latter suggested for the first time the transmission of an HIV-1 M/O recombinant form without the HIV-1/M and HIV-1/O 'parental' viruses [10]. More recently, we reported the circulation of multiple putative recombinant forms in Cameroun [22]. However, no documented data about the viability and transmissibility of such recombinant forms was available. We described here for the first time the presence of the same HIV-1 M/O recombinant strain in the absence of dual infections in a couple.

The near full-length genome characterization showed the identical pattern of the strains found in the couple with two breakpoints (Fig. 2). A breakpoint located in the *vpr* gene was first detected and together with previous reports, the result supports the hypothesis that this region could be a hotspot for recombination between HIV-1/M and /O. However, when a recombination breakpoint is determined, another one must exist due to the similarity of the two LTR regions. So, once the *vpr* breakpoint was identified, a second breakpoint had to be investigated. We were not able to find it across the near full-length genome obtained from RNA, for which the entire LTR regions were not amplified. Using proviral DNA, we identified the second breakpoint in the LTR region. These genetic profiles associated to the phylogenic analyses, proved the transmission of the recombinant virus between the spouses. The genetic distances and the less similarity in the envelope observed between the two strains, a region under strong immune pressure, showed that these forms could have been transmitted a few years ago and that they continued to evolve independently in the individuals. Though the husband was diagnosed earlier and put on ART earlier than the wife, no data was available on who was first infected, leading to the absence of evolution chronology.

Genetic comparison with the few already published recombinant strains showed that the recombinant HIV1-M/O strain described here was not genetically related to others. This indicates that the co-circulation of HIV-1/M and O is associated with the genesis of multiple recombinant patterns that are viable and transmissible.

Little is known about the virological properties and consequences of these forms. Only one in-vitro study on HIV-1 M/O recombinant has shown that it was highly replicative compared to its 'parental' strain HIV-1/M [9]. This high fitness combined with the transmissibility demonstrates that there could be a high diffusion potential of these recombinant forms. The characterization of these new strains will give us the opportunity to further analyse the phenotypic properties of these forms compared to groups M and O strains. Considering the stable prevalence of HIV-1/O in Cameroon during the last decade whereas group M has increased exponentially [18,26], recombination between the two virus groups could be a potential evolving mechanism that group O is using to efficiently spread in the population.

Moreover, this intergroup recombination could have important consequences in HIV diagnosis and follow-up. First, HIV-1 group O has hitherto been shown to present false negative results with some serological tests [11,27,28]. So, a recombinant form having a group O envelope can give negative result with some tests. Second, another related consequence could be on the viral load quantification assays, with some techniques being optimal with HIV-1/M viruses and less suitable with HIV-1/O [29,30]. In our routine analyses, HIV-1 group determination is based on serotyping assay which targets the Env region using specific peptides. Our results indicated that both REC003 and REC024 were reactive only with HIV-1/O peptides; this led to an incorrect interpretation of a group O infection since the Gag and Polymerase regions were from HIV-1/M (Fig. 2). Viral load assays generally use LTR, Gag and Integrase regions that can be group-specific; so false quantification could occur in the presence of an HIV-1 M/O recombinant virus following the mosaic pattern.

Finally, treatment difficulties could be observed in patients infected with such recombinant forms especially if the Polymerase region of the recombinant virus originates from HIV-1/O. Most HIV-1/O strains are known to be naturally resistant to NNRTIs due to the presence of Y181C mutation in the RT gene [10,15]. This drug class is widely used in Central Africa, including Cameroon as the first line treatment; this could lead to rapid virological failure if RT gene belongs to group O virus. Thus, it could be useful to identify recombinant viruses before putting patients on first line therapy consisting of NNRTIs. Furthermore, another challenge that could be faced in such areas of high viral diversity is the use of NNRTIs for the prevention of mother-to-child transmission of HIV.

In conclusion, our results demonstrate the viability and transmissibility of an HIV-1 M/O intergroup recombinant in Cameroon. These results reinforce evidence of the implication of recombination in the dynamic evolution of HIV-1 viruses in an environment with great genetic diversity like Cameroon. However, future studies should focus on the extent of their circulation in west and central Africa as well as the phenotypic

properties of such intergroup recombinants. Considering the shape of the viral diversity through the emergence of diverse viral recombinants with an unpredictable potential of widespread diffusion, potential impacts on diagnosis, monitoring, therapeutic management and vaccine design have to be carefully considered. In particular, routinely used tests and algorithms have to be set up in reference centres so as to diagnose these cases of HIV-1 M/O intergroup recombinants in circulation.

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Conflicts of interest

There are no conflicts of interest.

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