JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 2009, p. 2906–2911 Vol. 47, No. 9
0095-1137/09/$08.00+0 doi:10.1128/JCM.00602-09

Census and Analysis of Persistent False-Negative Results in Serological Diagnosis of Human Immunodeficiency Virus Type 1 Group O Infections▽

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Received 25 March 2009/Returned for modification 6 May 2009/Accepted 15 July 2009

Human immunodeficiency viruses (HIV) have a high level of genetic diversity. The outlier variants of HIV type 1 (HIV-1) group O are distantly related to HIV-1 group M. Their divergence has an impact on serological diagnosis, with a risk of false-negative results. In this study, we report 20 failure cases, involving patients with primary or chronic infection, in France and Cameroon between 2001 and 2008. Our results indicate that some assays detected group O infection much less efficiently than others. Two major reasons for these false-negative results were identified: the presence or absence of a group O-specific antigen (and the designed sequence) for the detection of antibodies and the greater envelope variability of group O than of group M strains. This study highlights the complexity of screening for these divergent variants and the need to evaluate test performance with a large panel of strains, due to the extensive diversity of group O variants.

Human immunodeficiency viruses (HIV) have a high level of genetic diversity. HIV type 1 (HIV-1) has been classified into three groups, M (major), N (non-M and non-O), and O (outlier), whereas HIV-2 has been classified into eight groups. This diversity has an impact on serological diagnosis, virological follow-up, and therapeutic management (6, 7, 22).

HIV-1 group O (HIV-O) viruses are distantly related to HIV-1 group M (HIV-M) (8) and are classified into at least three major clades (A to C) (24). HIV-O remains endemic in central Africa, particularly in Cameroon, where it is thought to account for about 1% of all cases of HIV-1 infection (about 10,000 to 20,000 people) (2, 28). It has spread in a very limited manner in other parts of the world (14, 16, 26, 27). In France, the establishment of the RES-O network for the surveillance of HIV-O infections has led to the identification of 119 patients since the description of the first case in 1992 (1; our unpublished data). The prevalence of HIV-O infections among new diagnoses of HIV in France is currently estimated at 0.1% (3).

The genetic divergence of HIV-O makes virological follow-up by commercially available viral load assays difficult, due to mismatches with primers and probes initially designed for HIV-M variants (15, 23). This may also account for the inefficacy or low efficacy of several antiretroviral agents for the treatment of HIV-O-infected patients (10, 11). It also has implications for serological diagnosis, because HIV-O infections are frequently identified on the basis of atypical Western blot profiles and/or immunovirological discrepancies (1, 9). Indeed soon after HIV-O identification, serological screening assays were found to give rise to false-negative results (19, 25). These diagnostic problems led to changes in most of the available assays, with the incorporation of a peptide representative of the immunodominant region (IDR) of the gp41 transmembrane glycoprotein specific to group O variants. Nonetheless, false-negative results continue to be reported for some patients infected with HIV-O (17, 29). In this study, we aimed to describe all the false-negative cases we have been faced with in France and Cameroon, with a view to analyzing the causes of these false-negative results.

MATERIALS AND METHODS

We collected data concerning false-negative results for HIV-O infection obtained between 2001 and 2008, on the basis of (i) notifications of failures reported to the French Health Products Safety Agency (AFSSAPS; Saint-Denis, France), responsible for the monitoring and control of in vitro diagnostic medical devices; (ii) investigations of difficult diagnoses referred to the French national reference center for HIV (Tours, France) (17, 29); (iii) observations over a 3-year collaboration with the Pasteur Centre in Cameroon (CPC; Yaoundé, Cameroon); and (iv) an evaluation of the clinical sensitivity for HIV-O of non-automated rapid diagnostic tests (NARTs) (Paris and Rouen, France) (13).

French guidelines currently recommend the use of two screening assays for the diagnosis of HIV infection and specific p24 detection in cases of suspected primary infection. In our census, suspected false negatives were initial identified on the basis of discrepancies (i) between the results obtained with different screening assays, (ii) between the test results and clinical status (primary infection), or (iii) between positive serological results and an absence of virus detection during virological monitoring. At the CPC, HIV infection is diagnosed with an algorithm based on three consecutive assays. False-negative results were

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▽ Published ahead of print on 22 July 2009.

suspected when discrepancies were found between the results of these three tests.

All suspected false-negative samples were then explored by complementary analysis with various serological tests from different companies, making use of different antigens and detection formats. The group-specific diagnosis of HIV-O infection was confirmed by serotyping in serum or plasma samples, as previously described (4). This process is based on antibodies reacting with both the IDR of gp41 and the V3 loop gp120 of HIV-1 groups M and O and HIV-2 in a homemade enzyme-linked immunosorbent assay (ELISA).

We then carried out molecular confirmation and phylogenetic characterization by amplifying and sequencing the gp41 region and/or the Pol region of the viral RNA in the plasma, as previously described (5, 24). We focused in particular on the sequences of the gp41 region, because this region contains the IDR, which is used as the HIV-O-specific antigen in commercial assays. The sequences obtained were compared with a consensus HIV-O sequence defined on the basis of an analysis of 208 sequences available at the Los Alamos database (HIV Sequence Database [http://www.hiv.lanl.gov/content/index]) and through the French network RES-O. We defined a consensus sequence for HIV-M strains based on the 208 sequences from the Los Alamos database to ensure that all calculations of the frequency of residue conservation were performed similarly and that the results were compatible for comparison with the HIV-O consensus. We calculated the genetic distances between IDR sequences and the group O consensus sequence using a PAM matrix, with MEGA 4 software (26a).

RESULTS

The cases and results obtained are summarized in Table 1. The table shows the complete data obtained for the various serological assays. These assays were designed to detect p24 antigen of HIV-1 only, anti-HIV antibodies only (second- and third-generation enzyme immunoassays and NARTs), or both antibodies and p24 antigen (fourth-generation tests).

We identified 20 cases of false-negative results among our cases from France and Cameroon. False-negative results were found in both patients with primary infection and patients with established chronic infections.

Patients with primary infections. We identified two cases of primary infection on the basis of clinical symptoms combined with a Western blot profile indicative of seroconversion. P1 was a woman with symptomatic primary infection contracted following heterosexual intercourse with a Caucasian HIV-O-infected partner (for details see reference 17). Sequential samples were obtained over a period of 2 months following the onset of symptoms. A false-negative result for the Vidas HIV P24 II test (which detects p24 antigen only) was first suspected on the basis of clinical stage and HIV-positive results in screening tests (Table 1). Sequential samples demonstrated deficiencies in the Vidas HIV P24 II test, because the initial sample was positive with the Modular HIV Antigen test. In this primary infection context, combined fourth-generation tests also performed inconsistently, with a lack of early detection for the first two samples with the Genscreen HIV Ag-Ab Ultra assay. This inconsistency may due to lower sensitivity for the detection of p24 antigen from HIV-O variants (20) or a lower sensitivity for antibody detection, because third-generation tests gave positive results (Table 1). The presence of antibodies in these early samples was confirmed by the results of the Vidas HIV DUO Ultra, the only test capable of discriminating reactivities between antibodies (positive for each sample from P1) and antigen (negative). This result confirmed the deficiency of the Vidas HIV P24 II assay for antigen detection.

P2 was a man at the end of the primary infection stage, whose sexual partner was an HIV-O-positive woman from Cameroon (18). Samples taken during primary infection and 8 months later were available for this patient. By contrast to what was observed for P1, the Vidas HIV P24 II test correctly detected p24 antigen in the early samples and fourth-generation tests were positive. The third-generation assay AxSYM HIV1/2 gO gave a strong positive result, whereas the Genscreen HIV1/2 version 2 test gave a weakly positive result, at the limit of detection, for the earliest sample and a negative result for the next sample. The lower sensitivity of NARTs than of ELISA-based tests may be responsible for the negative results obtained with the Determine assay for the earliest sample. However, the persistently negative result obtained for the sample collected 8 months after primary infection and the very weakly positive signal obtained with the ImmunoComb II HIV1&2 Bispot test for the same sample are more suggestive of deficiencies in detection.

Patients with established chronic infection. Infections in patients P3 to P20 were identified by diagnosis during the chronic stage, as suggested by Western blot profiles and/or clinical symptoms. Six patients were living in France (P3 to P8), and 12 were identified in Cameroon (P9 to P20).

Major discrepancies were noted for P3, the patient for whom the largest number of assays were carried out; 14 assays were used on sequential samples over a period of 10 years (Table 1) (29). This case was unusual in that infection was initially correctly detected in 1994 with the second-generation tests AxSYM HIV1/HIV2 and Wellcozyme HIV 1 Recombinant but negative results were obtained in 2003 with the screening tests Access HIV 1/2 (third generation) and Vidas HIV DUO (fourth generation) during a checkup after childbirth (Table 1). A NART (Determine; Inverness) and a fourth-generation assay (Genscreen Plus HIV Ag-Ab; Bio-Rad) also gave negative results (Table 1).

For patients P4 to P10 (Table 1), we observed deficiencies in detection (complete failure to detect or results at the threshold of detection) for the Genscreen HIV1/2 version 2 kit ($n = 4$) and the NARTs Determine ($n = 2$), Immunoflow HIV1/2 ($n = 1$), and Retrocheck HIV ($n = 2$). The serum sample from P5 was used to check two different batches of the Determine test kit. A negative result was obtained for the first batch, whereas a borderline, very weakly positive result was obtained with the second batch.

P11 to P20 were patients undergoing screening at the CPC, according to an algorithm based on an initial fourth-generation assay for screening (AxSYM HIV Ag/Ab Combo; Abbott), followed by a third-generation assay (Genscreen HIV1/2 version 2; Bio-Rad) and a NART (Genie II HIV-1/HIV-2; Bio-Rad). A major deficiency was identified for this rapid assay, for which 10 false-negative results were obtained for patients with HIV-O infections (of 30 samples tested in total) (data not shown).

Variability of the IDR and phylogenetic analysis. Samples from seven patients (P1, P3 to P6, P9, and P10) were available for further molecular analysis. The IDRs of samples testing negative with NARTs were not sequenced due to strong reactivity in other tests, highlighting deficiencies in kit performance rather than an effect of antigenic diversity. We aligned the available IDR sequences for the seven cases (Table 2) and compared these sequences with consensus sequences for HIV-O and HIV-M strains. The frequency of the residues found in each group was also calculated (Table 2).

TABLE 1. Results of serological tests used for all 20 patients

Test[a]	Test type[b]	Result[c] for patient(s):										
		P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11–P20
Vidas HIV P24 II (bioMérieux)	p24	**Negative**[1, 2, 3, 6]	Positive[7, 8]									
Modular HIV Antigen (Roche Diagnostics)	p24	Positive[2], negative[4]										
Genetic System HIV1 Ag (Bio-Rad)	p24		Positive[8]									
AxSYM HIV1/HIV2 (Abbott)	2			Positive[9, 11]								
Wellcozyme HIV 1 Recombinant (Murex Diagnostic)	2			Positive[9]								
Murex HIV-1.2 (Murex Diagnostic)	2			Positive[9, 11]								
ImmunoComb II HIV1&2 BiSpot (Inverness)	2*		Weakly positive[8]			Positive[16]		Positive	Positive			
Genie II HIV1/HIV2 (Bio-Rad)	3*									Indeterminate	**Negative**	**Negative**
Immunoflow HIV1/2 (Core Diagnostics)	3*							Positive	**Negative**			
Determine HIV 1/2 (Inverness)	3*		**Negative**[7, 8]	**Negative**[11]	Positive[14]	**Negative**/weakly positive[17]		Positive	Positive	**Negative**		
Retrocheck HIV (Qualpro Diagnostics)	3*							**Negative**	**Negative**			
SD Bioline HIV 1/2 3.0 (Standard Diagnostics)	3*							Positive	Positive			
AxSYM HIV 1/2 gO (Abbott)	3		Positive[7, 8]			Positive[16]						
Prism HIV 1/2 O Plus (Abbott)	3			Positive[12]								
Centaur HIV −1/O/2 (Bayer/Siemens)	3			Positive[11, 13]								
Access HIV 1/2 (Bio-Rad/Beckman Coulter)	3			**Negative**[9, 11, 12, 13]								
Genscreen HIV 1/2 version 2 (Bio-Rad)	3		Weakly positive[7], **Negative**[8]	Positive[12]	**Negative**[14, 15]		**Negative**			Weakly positive	Weakly positive	Positive
Anti-HIV TETRA ELISA (Biotest)	3	Positive[2, 3, 6]		Positive[12]			Positive					
Ortho HIV1/HIV2 Antibody Capture (Ortho Diagnostics)	3			Positive[10]								
Ortho Vitros anti-VIH1 + 2 (Ortho Diagnostics)	3	Positive[2, 3, 6], negative[5]										
Cobas Core anti-HIV 1 + 2+O(Roche Diagnostics)	3						Positive					
Architect HIV Combo (Abbott)	4	Positive[2, 3, 5, 6]					Positive					
AxSYM HIV Ag/Ab Combo (Abbott)	4	Positive[1, 2, 5, 6]	Positive[8]	Positive[10]						Positive	Positive	Positive
Vidas HIV DUO (bioMérieux)	4		Positive[7, 8]	**Negative**[9, 11, 12, 13]	Positive[14, 15]							
Vidas HIV DUO Quick (bioMérieux)	4	Positive[5, 6]										
Vidas HIV DUO Ultra (bioMérieux)	4	**Negative** (for antigen)[1, 2], positive (for antibodies)[1, 2, 5, 6]										

Genscreen Plus HIV Ag-Ab (Bio-Rad)	4		Positive[8]	**Negative**[9]	Positive[14]	Positive[16]
Genscreen HIV Ag-Ab Ultra (Bio-Rad)	4	**Negative**[2, 3] Positive[6]				
Enzygnost HIV Integral (Dade Behring/Siemens)	4			Positive[12]		
Cobas Core HIV Combi (Roche Diagnostics)	4				Positive	

[a] Tests with at least one false-negative result are in boldface.
[b] p24, p24 antigen test. Numbers 2 to 4 indicate the generation of the test. *, NART.
[c] Superscript numbers in parentheses indicate test dates as follows: 1, 15 February 2007; 2, 16 February 2007; 3, 22 February 2007; 4, 3 March 2007; 5, 16 March 2007; 6, 17 April 2007; 7, August 2004; 8, April 2005; 9, 1994; 10, 1995; 11, 1996; 12, 2003; 13, 2004; 14, 3 July 2001; 15, 16 July 2001; 16, July 2003; 17, March 2004. Results for tests used for the initial screening are underlined (for P7 and P8, no initial screening assay was reported; samples were tested in a NART evaluation).

Comparison of the IDR consensus sequences for group M and O strains identified 14 residues common to both groups: L_1A_2, E_4, $Q_{10}Q_{11}L_{12}L_{13}$, $W_{16}G_{17}C_{18}$, G_{20}, and $L_{22}I_{23}C_{24}$ (Table 2). Nevertheless, these residues were not always conserved within each group, particularly L_1 and I_{23}, found in only 73 and 59% of the 208 group O sequences analyzed, respectively. In addition, only 5 of these 14 residues, A_2, E_4, Q_{10}, G_{17}, and C_{24}, were common to the HIV-O and -M sequences investigated. For some positions, 100% conservation for HIV-M but considerable variability among HIV-O strains, whether the consensus residue was the same as for HIV-M (L_1 for 73% of HIV-O strains) or not (I_7 for 75% of HIV-O strains rather than the L_7 in HIV-M) was observed. The numbers of highly conserved positions (frequency of conservation of 99 or 100%) also differed between HIV-M ($n = 14$) and HIV-O ($n = 11$) strains. High levels of variability were observed, in particular, for the antigenic loop between the two cysteine residues (C-C loop), with the consecutive residues in positions 21, 22, and 23 conserved in only 62%, 74%, and 59%, respectively, of the group O sequences, whereas the corresponding residues in group M displayed 97%, 82%, and 100% conservation, respectively. Overall, the level of heterogeneity was higher for HIV-O (mean conservation rates for the consensus residues of 0.94 for the 208 M group sequences and 0.89 for the O group sequences).

The seven group O IDR sequences obtained displayed one to six mutations with respect to the group O consensus sequence. These mutations occurred at the following eight positions in the IDR (numbers of sequences in which the amino acid was altered are in parentheses): L_1 (three), L_6 (two), I_7 (two), N_{14} (one), L_{15} (three), R_{21} (three), L_{22} (four), and I_{23} (four). Interestingly, the $R_{21}L_{22}I_{23}$ motif was altered in all but one IDR sequence (P4); this IDR sequence had a single substitution, $N_{14}S$, not observed in the other sequences from viruses for which false-negative results were obtained. Nevertheless, the assay that failed to detect infection in this patient (Genscreen HIV 1/2 version 2) also gave negative results for patients with other mutation profiles (Table 2).

The PAM matrix-based distances to the consensus group O IDR were calculated for the seven sequences. They ranged from 0.069 to 0.231, and the median value for the 208 sequences from the database was 0.129 (Table 2). Based on these findings, viruses from patients P1 and P9 had the most divergent sequences. However, the sequences of variants from the other five patients differed relatively little from the group O consensus IDR sequence, suggesting that specific mutations at some positions are sufficient to account for the failure of several kits to detect these HIV-O infections. Phylogenetic analysis of the seven samples showed that all belonged to clade A and that none was an atypical outlier form.

Despite the diversity of the IDR observed for the seven viruses concerned, particularly at positions located within the C-C loop, no correlation or link between the failure of a given assay and a common specific motif was identified (Table 2).

DISCUSSION

This analysis of recent cases of false-negative results for the detection of HIV-O infection demonstrates the persistence of problems associated with screening for this group of variants. It includes two cases already reported in France by Henquell

TABLE 2. Frequency of conservation of the residues and genetic distances between IDR sequences

Group or patient	Clade	Amino acid(s) at position[a]:											
		1	2	3	4	5	6	7	8	9	10	11	12
M		***L*** (100)	***A*** (100)	V (66)	***E*** (100)	R (93)	Y (98)	**L** (100)	K (65)	**D** (100)	***Q*** (100)	*Q* (94)	*L* (91)
O		*L* (73)	***A*** (100)	L (99)	***E*** (100)	T (99)	L (69)	I (75)	Q (98)	N (97)	***Q*** (100)	***Q*** (100)	*L* (90)
P1	A	L	A	L	E	T	L	V	Q	N	Q	Q	L
P3	A	L	A	L	E	T	L	L	Q	N	Q	Q	L
P4	A	L	A	L	E	T	L	I	Q	N	Q	Q	L
P5	A	L	A	L	E	T	L	I	Q	N	Q	Q	L
P6	A3	Q	A	L	E	T	F	I	Q	N	Q	Q	L
P9	A3	Q	A	L	E	T	F	I	Q	N	Q	Q	L
P10	A	LQ	A	L	E	T	L	I	Q	N	Q	Q	L

[a] For groups M and O, residues are those of the consensus sequences. Frequencies of conservation (percentages) of the residues among the 208 HIV-M and 208 HIV-O sequences are in parentheses. Residues that are 100% conserved within a group are in boldface. Conserved residues identical in the HIV-O and HIV-M groups are in italics. Residues differing from the HIV-O consensus sequence are underlined.

[b] Genetic distance of sequences from the HIV-O consensus sequence; see Materials and Methods for details.

et al. and Zouhair et al. (17, 29). These two cases and the six additional French cases reported here were identified and explored on the basis of discrepancy between infection stage and tests results or between the two screening assays. Indeed, although the algorithm is likely to be modified in the near future, French guidelines currently recommend the use of two screening assays for the diagnosis of HIV infection. This procedure makes it possible to identify such discrepancies. A few cases of false-negative results involving HIV-M variants (subtypes B, C, and F, for example), resulting from major mutations of the epitope in the IDR, have been described (12, 20, 21). However, although it remains difficult to determine the frequency of false negatives for each HIV group, a large number of the reported failures involve HIV-O. HIV-O infection has a much lower prevalence than HIV-M infection and is restricted to zones of endemicity in central Africa, so this observation highlights the need for further efforts to develop a fully efficient screening assay for this group. It therefore appears to be of prime importance to validate carefully all the HIV screening assays, assessing their real ability to detect HIV-O infection.

It was not possible to test all the samples with all the kits, making complete and objective comparison difficult, but it was nonetheless clear that some assays detected HIV-O infection much less efficiently than others. Analysis of the cases reported in this census suggests two major reasons for false-negative results. First, the presence or absence of a group O-specific antigen for the detection of antibodies against HIV-O is probably a key element. Different amounts of information were provided by different kits or manufacturers, but it was clear that several assays contained no peptide specific for HIV-O. For instance, the Genscreen HIV 1/2 version 2 test was based solely on cross-reactivity between M and O variants for the screening of these samples (information supplied by the manufacturer). For this assay, the false-negative results observed for different samples here and elsewhere (21) suggest that the absence of a specific peptide may well be responsible for the defect. Second, we confirm previous findings (24) suggesting that HIV-O strains are much more diverse than HIV-M strains. This diversity includes the IDR of gp41, a major target for antibodies in HIV-infected patients. This particularly high level of variability of the IDR probably results in differences between screening assays for the detection of HIV-O infection.

The sequence of the antigen used for the detection of antibodies against HIV-O probably also affects reactivity. This is difficult to evaluate in the absence of information concerning the sequences of the peptides used in the various assays. The fourth-generation tests of Bio-Rad use an artificial group O consensus peptide, which failed to detect infection in P3 and in the early samples from P1 but correctly detected infection in samples from P2, P4, and P5. bioMérieux uses a representative sequence of a clade A strain, which failed to detect infection in one case of five. The NART Retrocheck contains a peptide specific for group O HIV-1 strains but failed to detect infection in two cases during our evaluation (13).

Additional difficulties emerge when the subjective nature of interpretation of NARTs influences the results. The discrepancies may be attributed to the operator, as already reported (13), but also to the kit batch. For instance, in our study, a sample at the limit of detection, such as that from P5, tested negative with one batch of the Determine kit but weakly positive with a subsequent batch. In addition to the subjective elements inherent to this type of format, a major defect was observed with the Genie II HIV-1/HIV-2 test, for which 10 false-negative results were obtained. Another NART, the Retrocheck HIV test, which is approved for use in the European Community, was found to be much less effective for HIV-O detection than some other NARTs (13). The common technical specifications for European Community registration do not clearly establish the performance requirement for HIV-O, focusing instead on non-B subtypes of HIV-M. This may cause confusion, and, although manufacturers generally evaluate performance for more than three group O-positive samples, they are only required to test three samples. Given the tremendous diversity of HIV-O, this would seem to be inadequate.

A few of the false negatives identified in this study concerned patients with primary infections. This finding suggests that improvements are also required in the performance of tests for the detection of HIV-O p24.

In conclusion, this study shows the complexity of screening for divergent and rare variants. The broad diversity of HIV-O strains necessitates evaluation of the performance of tests with a large panel, as representative as possible of the diversity of group O variants. Our findings have important implications for recommendations of HIV screening tools in central Africa and also for countries with close links to this region.

TABLE 2—*Continued*

Amino acid(s) at position[a]:												Distance[b]
13	14	15	16	17	18	19	20	21	22	23	24	
L (99)	**G** (100)	I (81)	*W* (99)	***G*** (100)	*C* (99)	S (98)	*G* (99)	K (97)	*L* (82)	***I*** (100)	***C*** (100)	
L (100)	N (84)	L (81)	***W*** (100)	***G*** (100)	***C*** (100)	K (82)	*G* (96)	R (62)	*L* (74)	*I* (59)	***C*** (100)	0.129
L	N	S	W	G	C	K	G	R	S	V	C	0.17
L	N	L	W	G	C	K	G	T	R	I	C	0.073
L	S	L	W	G	C	K	G	R	L	I	C	0.07
L	N	S	W	G	C	K	G	R	L	V	C	0.076
L	N	L	W	G	C	K	G	H	L	V	C	0.069
L	N	R	W	G	C	K	G	Q	P	V	C	0.231
L	N	L	W	G	C	K	G	R	T	IV	C	0.072

ACKNOWLEDGMENTS

We thank Didier Rimpici (CH Montluçon, France) for field assistance.

The French national reference center for HIV and the associated laboratory of Rouen University Hospital are supported by funds from the Institut de Veille Sanitaire (Saint Maurice, France).

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