Failure to Quantify Viral Load with Two of the Three Commercial Methods in a Pregnant Woman Harboring an HIV Type 1 Subtype G Strain

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ABSTRACT

The level of HIV-1 RNA in plasma has become one of the most important markers in the follow-up of HIV-infected patients. Three techniques are commercially available: both the Amplicor HIV Monitor and the NASBA HIV-1 RNA QT are target amplification methods, whereas the Quantiplex HIV RNA assay is a branched DNA signal amplification technique. Detection in both target amplification techniques is based on a single primer pair and a single probe in the *gag* region, whereas multiple probes capture the *pol* region of the viral RNA in the branched DNA assay. We investigated the discrepant observation of an undetectable viral load in an immunodeficient pregnant HIV-1-infected patient of African origin with no prior antiretroviral treatment. Although clinical progression was present in this patient with tuberculosis and a low CD4 cell count, viral load determinations with both the Amplicor Monitor and NASBA assays revealed no detectable RNA levels. The presence of HIV-1 RNA in the plasma of the patient was demonstrated by an in-house RNA-PCR. Subsequent HIV-1 RNA quantification with the branched DNA method revealed a high viremia (460,000 copies/ml). DNA sequence analysis of the *gag* gene identified a subtype G HIV-1 strain (HIV-1$_{BL}$). To our knowledge this is the first report of a patient harboring an HIV-1 genotype of the main group with a high viral load as quantified by the branched DNA assay, but undetectable with the two commercial HIV RNA amplification techniques because of genetic divergence. In the case of discrepant low viral loads determined by one amplification technique in patients with advanced clinical stage one should use an alternative quantification technique for confirmation.

INTRODUCTION

QUANTIFICATION OF THE virion-associated RNA in plasma has become a parameter of choice in the follow-up of HIV-1 infected patients.[1] Clinical studies have demonstrated a clear correlation between the viral load and the clinical outcome of the patients. Patients with more than 100,000 HIV RNA copies/ml of plasma within 6 months of seroconversion were 10-fold more likely to progress to AIDS over a 5-year period than those with fewer than 100,000 copies/ml, whereas patients with an HIV RNA copy number less than 10,000/ml did not progress to AIDS during the next 5 years.[2] In another study, subjects who had been followed for more than 10 years were stratified according to their plasma HIV RNA level. For patients with CD4 cell counts above 500/μl, more than 70% progressed to AIDS and died within 10 years if their baseline plasma viral RNA level was above 10,000 copies/ml. In contrast, less than 30% of those with plasma viral loads below 10,000 copies/ml died within 10 years.[3] Recently, it has been reported that the prognosis of HIV-infected persons is most accurately defined by combined measurement of plasma HIV-1 RNA and CD4+ lymphocytes.[4] Therefore the decision to initiate antiviral drug therapy is now generally guided by a viral load determination. Highly effective antiviral combination therapies consisting of protease inhibitors and nucleoside reverse transcriptase inhibitors result in a 2 log reduction of the viral

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load.[5] Decreases of viral load are generally accompanied by an increase in the CD4 cell counts. A return to baseline of the HIV-1 RNA level, which points to treatment failure, is associated with the emergence of drug-resistant virus strains.[6] A consensus has been reached that viral load measurements, in addition to CD4^{+} lymphocyte counts, are highly informative for evaluating the antiretroviral effect of drugs.[7,8] Finally, a direct correlation has been found between the risk of mother-to-child transmission and the HIV RNA level in the maternal plasma, although according to two reports high maternal viral load appears insufficient to fully explain vertical transmission.[9,10]

Three commercial techniques have been developed for the quantification of HIV-1 viral RNA in plasma. The Amplicor HIV Monitor test (Roche Diagnostics, Basel, Switzerland) uses the polymerase chain reaction to amplify a fragment of the *gag* gene. The fragment is detected using a probe and a colorimetric reaction. The first-generation test used in this work has a detection limit of 400 copies of HIV-1 RNA per milliliter and is performed on 200 μl of plasma. NASBA (Organon Teknika, Boxtel, The Netherlands) is an isothermal amplification method with a detection limit of 4000 copies of HIV-1 RNA per milliliter and uses a sample value of 100 μl in the first-generation test. A fragment of the *gag* gene is amplified with primers that are almost entirely overlapping the primers used by Amplicor. A similar probe is used in a chemiluminescent detection assay. In the second-generation branched DNA (bDNA) method (Quantiplex HIV 2.0 assay; Chiron, Emeryville, CA) the viral RNA is captured by multiple probes complementary to the *pol* region, and subsequently hybridized to branched DNA molecules and enzyme-labeled probes. The current version of the assay has a quantification limit of 500 HIV-1 RNA copies per milliliter but it requires duplicate 1-ml samples. Comparative evaluations of the first generations of the three techniques revealed no major differences in performance.[11–13] The presence of 45 target probes in the bDNA assay, which were designed to hybridize with all known group M subtypes, increases the probability of also detecting and quantifying genetically divergent HIV-1 subtypes.

MATERIALS AND METHODS

Sample collection and storage

Serology was performed on fresh serum samples. Blood samples for viral load determination and sequencing were collected in tubes containing EDTA as anticoagulant. After centrifugation, plasma was aliquoted and stored at −80°C. The viral load determination with bDNA on the pretreatment sample was done on plasma stored at −20°C. Blood was also collected in cell preparation tubes (CPT; Becton Dickinson, Franklin Lakes, NJ) containing sodium citrate as anticoagulant for cell culture or DNA polymerase chain reaction (PCR). Lymphocytes were separated, counted, pelleted, and stored at −80°C.

HIV-1 serology

HIV-1 serology was determined with both the Abbott AxSYM HIV-1/2 MEIA test and the ELISA Genscreen HIV 1/2 (Sanofi Pasteur, Marnes la Coquette, France). The p24 antigen test used was the Innotest HIV antigen MAb (Innogenet-

ics, Ghent, Belgium). Immunoblotting was performed with the HIV blot 2.2 from Genelabs Diagnostics (Singapore) using the criteria from the manufacturer.

HIV RNA quantification

RNA quantification using the NASBA HIV-1 RNA QT (Organon Teknika), the Amplicor HIV Monitor test (Roche Diagnostics), and the Quantiplex HIV 2.0 assay (Chiron) were performed according to the instructions of the manufacturer. In the NASBA test HIV-1 RNA is extracted from 100 μl of plasma. The RNA derived from 10 μl of plasma is amplified isothermally using avian myeloblastosis virus (AMV) reverse transcriptase, RNase H, and T7 RNA polymerase using the *gag*-1 primer set.[14] The HIV-1 amplicons are detected by an electrochemiluminescent *gag* probe.[14] The detection limit is 4000 copies/ml. In the Amplicor Monitor test HIV-1 RNA is extracted from 200 μl of plasma. A single-step RNA PCR is performed on the RNA from 25 μl of plasma using the biotinylated SK462/431 *gag* primer set, which is almost identical to the NASBA primer set and the same *gag* probe is in the NASBA test (see Fig. 1). Detection is based on an avidin–horseradish peroxidase enzyme-linked immunosorbent assay (ELISA)-type colorimetric reaction. The detection limit of the Amplicor Monitor test varies around 200 c/ml and the quantification limit is 400 c/ml. RNA quantification with bDNA was carried out at the Chiron Reference Testing Laboratory in Amsterdam, using the Quantiplex HIV 2.0 assay on two 1-ml plasma samples stored at −80°C. The viral load in the pretreatment sample was performed on two 50-μl plasma samples stored at −20°C according to a modified protocol.[15] HIV-1 RNA from 1 ml (or 50 μl) of plasma is extracted and immobilized using 10 different HIV-1 *pol* probes. Chemiluminescent detection is performed using 35 different HIV-1 *pol* probes, each labeled with excess amounts of alkaline phosphatase according to the branched DNA principle. The quantification limit is 500 c/ml.

PCR detection of proviral DNA and viral RNA

Detection of proviral HIV-1 DNA or viral HIV-1 RNA was based on our diagnostic HIV-1 PCR assay.[16,17] Briefly, genomic DNA was extracted from 10[6] pelleted lymphocytes (stored at −70°C) using the QIAamp blood kit (Qiagen, Chatsworth, CA) and dissolved in 100 μl of MilliQ water. Ten microliters was used for each PCR. Viral RNA was extracted from 100 μl of plasma with Trizol (GIBCO-Life Technologies, Gaithersburg, MD) and dissolved in 20 μl of diethyl pyrocarbonate (DEPC)-treated water. Six microliters of RNA was reverse transcribed into 40 μl of cDNA using the GeneAmp RNA-PCR core kit (Perkin-Elmer, Norwalk, CT) and the random hexamers provided. Ten microliters of cDNA was used in each PCR. Three nested PCR reactions amplifying the *pol*, LTR-*gag*, or *env* gene fragment were run as described previously.[16,17] Amplicons were detected by ethidium bromide staining after electrophoretic separation in a 6% polyacrylamide gel. A dilution series of a positive control was included (HIV-1-infected ACH2 cells) to ensure a detection limit between 1 and 10 copies. For the proviral DNA samples, amplification of human globin gene using the KM38/PC03 primers was run in parallel to assess the quality of the DNA used.

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HXB2       CACAGTGGGG GGACATCAAG CAGCCATGCA AATGTTAAAA GAGACCATCA ATGAGGAAGC TGCAGAATGG
BL         TACAGTGGGG GGGCATCAAG CAGCTATGCA AATGCTAAAG GAGACTATCA ATGATGAAGC TGCAGAATGG
NASBA       AGTGGGG GGACATCAAG CAGCCATGCA AA
AMPLICOR    AGTTGGA GGACATCAAG CAGCCATGCA AAT
GAG-PROBE                                         GAGACCATCA ATGAGGAAGC TGCAGAATGG


HXB2       GATAGAGTGC ATCCAGTGCA TGCAGGGCCT ATTGCACCAG GCCAGATGAG AGAACCAAGG GGAAGTGACA
BL         GACAGGCTAC ATCCACARCA GGCAGGGCCT ATTGCACCAG GCCAGATAAG GGACCCAACG GGAAGTGATA
NASBA                                                                TGAG AGAACCAAGG GGAAGTGACA
AMPLICOR                                                              AG AGAACCAAGG GGAACTGACA
GAG-PROBE  GAT


HXB2       TAGCAGGAAC TACTAGTACC CTTCAGGAAC
BL         TAGCAGGARC TACTAGTACC CTGCAGGAAC
NASBA      TAGCAcccta tagtgagtcg tattagaatt
AMPLICOR   TAGCA
GAG-PROBE
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FIG. 1. DNA sequence analysis of a *gag* gene fragment of the HIV-1$_{BL}$ strain in the region around the primers and probe of both RNA amplification techniques. The alignment shows the sequence of the HIV-1 strain HIV-1$_{BL}$ with the sense (5' → 3') sequences of the primers and probe used for amplification with Amplicor Monitor and NASBA. Amplicor and NASBA use identical probes. The T7 promoter sequence, at the 5' end of the downstream NASBA primer, is in lower case. The HXB2 sequence on top represents the *gag* fragment of the prototype HIV-1$_{IIIB}$ sequence spanning nt 1355 to 1524 (EMBL Accession Number K03455). The observed mismatches with the primers or probe are indicated in boldface.

Sequencing of the viral gag *gene fragment*

For the sequencing reactions viral RNA and cDNA synthesis was as described above but using as specific cDNA primers AV104 (5' GACCAACAAGGTTTCTGTC 3', antisense, nucleotides [nt] 1739–1757 of HXB2, EMBL accession number K03455) when sequencing the 329-bp *gag* fragment or AV152 (5' CCAAAGAGTGATTTGAGG 3', antisense, nt 2952–2269 of HXB2) when sequencing the 882-bp *gag* fragment.

For the 329-bp *gag* fragment, 10 µl of cDNA was amplified in a nested PCR and sequenced as described.[13] Briefly, primers AV103 (5' GCCATATCACCTAGAACTTT 3', sense, nt 1224–1243 of HXB2) and AV104 were used in the outer PCR, whereas the inner PCR was performed with primers tagged with the M13 universal (USP) and reverse sequencing primers (RSP): AV105 (5' *GTTGTAAAACGACGGCCAGT*GCCACC-CCACAAGATTTAAA 3', sense, nt 1323–1342 of HXB2 [tags in italics]) and AV106 (5' biotin-*CAGGAAACAGCTATGAC*TCC-TTGTCTTATGTCCAGAATGC 3', antisense, NT 1630–1652 of HXB2) using PCR buffers and AmpliTaq from Perkin-Elmer.

For the 882-nt *gag* sequence cDNA was amplified using the following PCRs. The outer PCR reaction mixture (50 µl) (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 2 mM MgCl$_2$, 200 µM dNTPs) contained 10 µl of cDNA, AmpliTaq (0.025 U/µl; Perkin-Elmer), and a 0.2 µM concentration of the primers AV11 (5' TCTAGCAGTGGCGCC 3', sense, nt 628–642 of HXB2) and AV152. The following cycling conditions on a Perkin-Elmer PE 9600 were used: 95°C for 45 sec; 55°C for 30 sec and 72°C for 1 min for 40 cycles, followed by a terminal extension for 10 min at 72°C and cooling to 4°C. Two microliters of outer PCR product was amplified in two separate inner PCR reactions. The reaction conditions were identical to the outer PCR reaction except for primer concentration of 0.5 µM. In one inner PCR the primer pair AV103–AV104 was used; in the second the primer pair AV159 (5' GGGGT-TAAATAAAATAGTAAG 3', sense, nt 1592–1612 of HXB2)–AV160 (5' CTGTTCTGAAGAAAATTCC 3', antisense, nt 2125–2143 of HXB2) was used. The cycling condi-

tions of the Triothermobloc of Biometra (Westburg, Heusden, The Netherlands) were as follows: 95°C for 30 sec; 45°C for 30 sec and 72°C for 1 min for 30 cycles, followed by a terminal extension for 10 min at 72°C and cooling to 4°C.

The inner PCR product was purified on a 1% SeaKem (FMC, Life Sciences International, Zellik, Belgium) agarose gel, excised, and extracted from the gel with the QIAquick gel extraction kit (Westburg). Solid-phase sequencing of the small *gag* gene fragment was done using streptavidin-coated Dynabeads (Dynal, Lake Success, NY) and the Auto Read T7 sequencing kit (Pharmacia Biotech, Piscataway, NJ) with the fluorescein isothiocyanate (FITC)-labeled ALF M13USP for the bead-bound fragment, and FITC-labeled ALF M13RSP for the eluate. Sequencing fragments were separated on an ALF (automated laser fluorescent sequencer; Pharmacia Biotech). For the large *gag* gene fragment sequencing of both inner PCR products was done with the ABI 310 sequencer, using the ABI PRISM dye terminator cycle sequencing core kit (Perkin-Elmer) and following the procedures of the manufacturer, using the following cycling conditions on the Biometra Triothermobloc: 96°C for 30 sec; 50°C for 15 sec; 60°C for 4 min during 25 cycles followed by cooling to 4°C.

Phylogenetic analysis

The 882-nt sequenced *gag* fragment was used for a BLAST search on the daily update of the EMBL/GB/DDBJ/PDB databases. The fragment represents the sequence homologous to nt 1243–2125 of HIVHXB2. From the sequences with a high score, of each subtype the three sequences with the highest score were retrieved from the database. Only two sequences of subtype G and of subtype H could be retrieved. The sequences were aligned using the GeneWorks software package, resulting in an 885-nt consensus fragment. Phylogeny construction and evaluation were done using the Phylip (3.56)[18] and MacClade (3.0) software packages.[19] A nucleotide state changes chart was made from the alignment with MacClade, using as guide tree a neighbor-joining (NJ) tree obtained with standard parameters in

Phylip. For the analyzed alignments, the chart can be described as symmetrical, with a transition/transversion bias of 2.7. Next, the neighbor-joining method implemented in the Phylip package was used. Distances were calculated with the Felsenstein model employing the empirical base frequencies, and the empirically determined transitions/transversions bias of 2.7 was used. The NJ tree was statistically evaluated using 1000 bootstrap samples.[20] The values on the branches represent the percentage of trees for which the sequences at one end of the branch are a monophyletic group. The graphical presentation of the tree was done with the Treeview program (RDM Page, University of Glasgow, UK).

RESULTS

Clinical case

A 26-year-old woman was referred to our outpatient clinic in the summer of 1996. She was born and lived in the Equateur province of Zaire until the late 1980s, then lived in Kinshasa before moving to Belgium in 1993. She was pregnant for 23 weeks and was on therapy with rifampin, isoniazide, and ethambutol for tuberculosis of the lung, larynx, and cervical lymph nodes, diagnosed 3 weeks before. On that occasion a diagnosis of HIV-1 infection and AIDS had been made.

She was counseled and blood samples for viral load monitoring and CD4 counting were obtained. The number of CD4-positive lymphocytes was $73/\mu l$. Plasma viral load determined with the Amplicor Monitor assay was below the detection limit of this method (160 c/ml). Because of the pregnancy, zidovudine monotherapy was initiated in addition to Pneumocystis carinii pneumonia (PCP) prophylaxis. Owing to the discrepancy between a profound immunodeficiency and a low viral load in the absence of antiretroviral treatment, viral load determination was repeated using both Amplicor Monitor and NASBA assays on a follow-up sample. Viral load was slightly above the detection limit of the Amplicor Monitor assay (500–600 c/ml) and negative using the NASBA assay (below 4000 c/ml). Viremia was detectable using an in-house RNA PCR. At that time an intrinsic technical problem for the determination of viral load in this African patient was suspected. Just after the mother gave birth to a healthy girl, more plasma was obtained for additional viral load testing using the bDNA assay technology. After delivery the baby was immediately treated with zidovudine. Lymphocytes obtained from the baby on two occasions (at birth and after 1 month) were negative in HIV DNA PCR and cell culture. Two subsequent HIV DNA PCR tests on lymphocytes of the baby, obtained at 2 and 3 months after birth, remained negative as well. After birth lamivudine was added to zidovudine in the mother's antiviral treatment. A protease inhibitor has since been added to the antiviral therapy regimens.

Laboratory diagnosis

The diagnosis of HIV-1 infection in this patient was based on a positive HIV-1 serology that was confirmed on a sample 2 weeks later (data not shown). Both screening tests (microparticle enzyme immunoassay [MEIA] and ELISA) were strongly positive and a complete pattern of seroreactivity was observed in the immunoblots. p24 antigen was negative and the CD4 cell count ranged between 73 and 121 cells/μl.

Quantification of viral load has been instituted as a routine procedure for newly diagnosed cases of HIV infection at the AIDS Reference Laboratory (Leuven, Belgium). In this instance, the Amplicor Monitor RT-PCR assay method was used to quantify the patient's plasma viral RNA (Table 1). The result for this assay was below the quantification limit (i.e., 160 copies/ml) for the Amplicor Monitor assay. The undetectable level of virus appeared in conflict with the clinical presentation. Therefore an attempt to quantify the viral load was repeated on consecutive samples with both the Amplicor Monitor and NASBA assays. Results slightly above the limit of quantification were obtained with the Amplicor Monitor assay and the NASBA assay results remained negative. Plasma viremia was evaluated using a qualitative in-house RNA PCR. This technique consists of three nested PCR reactions amplifying LTR-gag, pol, and env gene fragments and is highly sensitive (detection limit, 1 to 10 copies of cDNA). Amplification was obtained in all three gene fragments, ensuring that viral RNA was circulating in the plasma. The HIV-1 diagnostic proviral DNA PCR of patient lymphocytes, using the same three nested PCRs, was also positive, confirming HIV infection.

Finally, 2 ml of a fresh plasma sample obtained from the patient was sent to the Chiron Reference Testing Laboratory in Amsterdam for testing by the bDNA assay. A viral load of 4.8 $\log_{10}$ copies/ml was obtained. As a result of this high viral load, the bDNA assay was used to quantify the level of virus in a stored pretreatment plasma sample (result, 5.66 $\log_{10}$ copies/ml), as well as a plasma sample obtained 1 month after initiation of a combination therapy consisting of zidovudine and lamivudine (result, 3.37 $\log_{10}$ copies/ml).

Genetic analysis

We investigated the discrepancy in the viral load results obtained with bDNA versus Amplicor Monitor and NASBA assays, sequencing the gag gene fragment that is amplified with both of the latter techniques. In a first step, a 329-bp gag fragment was amplified from viral RNA in plasma obtained from the patient on October 9, 1996 (Table 1) and sequenced. The DNA sequence of the relevant region around primers and probe is shown in Fig. 1. It revealed several mismatches with the Amplicor Monitor and NASBA primers and probe, especially at the 3' end of the downstream primers. With the Amplicor Monitor primers and probe 12 mismatches were observed, and 10 were observed with the NASBA primers and probe. This explains why the primer pairs used in the Amplicor Monitor assay and the NASBA assay were not able to detect this strain reliably. The 329-nt fragment was divergent from all known subtypes (results not shown), which did not allow subtyping of the virus strain. Therefore we sequenced 882 nt of the gag gene, overlapping entirely the 329-bp fragment and including almost the entire p24 and p7 genes. Phylogenetic analysis showed a clear separation between the seven subtypes within group M. The strain HIV-1 ANT70, belonging to subtype O, was used as outgroup. In the tree shown in Fig. 2,[20a–g] the actual branch length toward ANT70 is 3.5 times longer than presented. High bootstrap values (>96%) were obtained for all subtypes except

TABLE 1. VIRAL LOAD DETERMINATIONS AND CD4 COUNTS

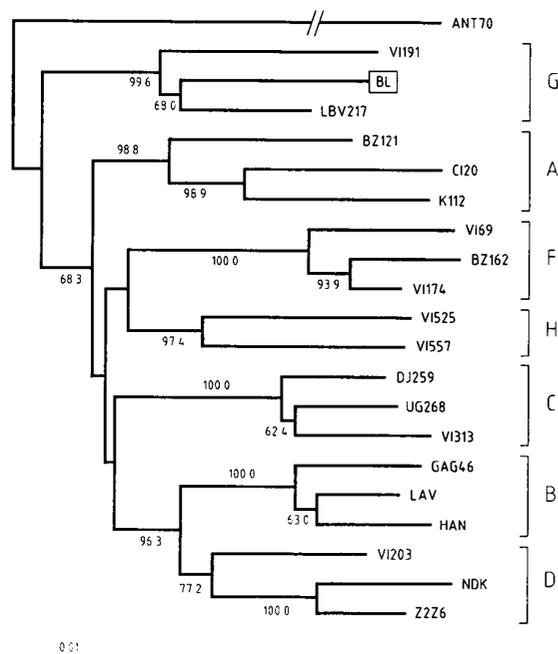| Date of sample	Viral load (log copies/ml)			CD4 count (cells/μl)	Antiviral therapy
	Amplicor	NASBA	bDNA		
June 12, 1996	<2.2	<3.6	5.66	73	None
June 17, 1996	ND	<3.6	ND	121	AZT
August 16, 1996	2.77	<3.6	ND	ND	AZT
October 3, 1996	2.69	ND	ND	ND	AZT
October 9, 1996	<1.83	<3.6	4.8	75	AZT
November 6, 1996	ND	ND	ND	134	AZT/3TC
December 2, 1996	ND	ND	3.37	ND	AZT/3TC

Abbreviation: ND, not determined.

FIG. 2. Phylogenetic analysis. Phylogenetic analysis of an 882-nt *gag* sequence of HIV-1$_{BL}$, using the neighbor-joining (NJ) approach described in Materials and Methods. The fragment represents the sequence homologous to nt 1243–2125 of HIVHXB2 (AC K03455). Two to three strains of each subtype were included (see Materials and Methods) and ANT70, a group O strain (AC L20587[20a]), was used as outgroup. The values on the branches represent the percentage of trees for which the sequences at one end of the branch are a monophyletic group. The main clusters correspond to the subtypes within group M containing the following strains: subtype A (BZ121, AC L22083[20b]; CI20, AC L11755[20c]; K112, AC L11768[20c]), subtype B (GAG46[20h], AC U29413[20d]; HAN, AC U43141[20e]; LAV, AC A10447[20c]), subtype C (DJ259, AC L11764[20e]; UG2687, AC L11799[20e]; VI313 AC L11787[20e]), subtype D (NDK, AC M27323[20f]; Z2Z6, AC M22639[20g]; VI203, AC L11784[20c]), subtype F (BZ162, AC L11751[20c]; VI69, AC L11796[20c]; VI174, AC L11782[20c]), subtype G (LBV217, AC L11778[20c]; VI191, AC L11796[20c]), subtype H (VI525, AC L11792[20c]; VI557, AC L11793[20c]).

for subtype D, with a value of 77%. The subtype of the strains used was identified according to the HIV-1 Database of Los Alamos.[21] The presently sequenced strain, referred to as HIV-1$_{BL}$, clearly clusters within subtype G.

DISCUSSION

We report on the identification of an HIV-1 strain (HIV-1$_{BL}$) that could not be quantified correctly using either the Amplicor Monitor or NASBA amplification techniques. The branched DNA method, however, quantified a high viremia in the patient. The isolate was obtained from a woman coming from Zaire. The patient was pregnant at the time of diagnosis of HIV infection. The initial viral load determination had no influence on the clinical management of the patient since monotherapy with zidovudine was initiated because of pregnancy. No complications during pregnancy occurred and she gave birth to a healthy girl. After delivery the newborn was immediately treated with zidovudine. Lymphocytes obtained from the newborn until 3 months after birth were negative by HIV DNA PCR and cell culture. Postpartum, lamivudine was associated with zidovudine in the mother's antiviral treatment. The antiviral therapy of the mother resulted in an overall 2.3 log$_{10}$ reduction in viral RNA (Table 1).

HIV is characterized by a high degree of genetic variation. According to phylogenetic studies of *env* and *gag* gene sequences, the majority of HIV-1 sequences appear to cluster into one large group (group M [major]) with a few outliers (group O). The sequences of the major group have been further subdivided into 8–10 genetic subtypes, A through J. Whereas all genetic subtypes known to date have also been found in Africa, the distribution of the various strains varies geographically. Whereas HIV-1 subtype B is predominant in North America, there is a greater heterogeneity of HIV-1 strains in European patients. For instance, more than 30% of HIV-infected Belgians carry non-B HIV-1 subtypes, which were introduced into the population at least 10 years ago.[22] It is known that HIV-1 subtype O and HIV-2 strains cannot be correctly quantified by either of the three commercial assays. Yet HIV-1 subtype O and HIV-2 are detectable by the bDNA assay, but quantification is not accurate. It was anticipated that both the Amplicor Monitor and NASBA assays are not entirely reliable in quantifying non-B subtypes of group M.[23] In a study the quantification of

HIV-1 RNA in culture supernatants from HIV-1 subtype A to H strains by the three commercial assays was compared. Whereas quantification results were comparable for all these methods when analyzing subtypes B, C, D, E, F, and H, with the Amplicor Monitor test lower values were obtained with some subtype A strains and with the NASBA assay lower values were obtained for some subtype G strains.[24] To our knowledge this HIV-1 strain (HIV-1$_{BL}$) is the first reported clinical isolate present at high titers that is of the main subgroup and is not detected by Amplicor Monitor or NASBA assay.

On the basis of the initial DNA sequence of a 329-nt *gag* gene fragment from HIV-1$_{BL}$, spanning the primer and probe sites used in the NASBA and Amplicor Monitor assays, a high sequence divergence with known HIV-1 strains was observed. The mismatches observed explain why both Amplicor Monitor and NASBA assays had difficulties in detecting the strain. On the basis of a phylogenetic analysis of a larger 882-nt *gag* gene sequence the strain HIV-1$_{BL}$ was found to belong to the subtype G. In the study by Coste *et al.* viral loads of two HIV-1 subtype G strains were considerably lower when determined with NASBA compared with bDNA and Amplicor Monitor.[24] Apparently, not the genotype per se, but the sequence at the primer and probe binding sites determines whether the target amplification techniques will be able to quantify accurately a specific HIV-1 isolate. Even some subtype B strains are not equally quantified by the Amplicor Monitor or NASBA assays.[13] These findings question the usefulness of genotyping isolates before viral load determination in order to identify all non-B genotypes at risk for erroneous viral load determination.

The pretreatment or baseline viral load of an HIV-infected patient provides the most important information regarding the prognosis for progression to disease and death as well as for measuring the effectiveness of an initiated antiviral therapeutic regimen. This case report highlights potential problems associated with assay methods that are incapable of adequately quantifying genetically divergent HIV-1 strains. It illustrates the potential limitations of using single primer pairs in PCR target amplification methods that can only inadequately accommodate the genetic diversity observed in HIV-1. In addition, potential shifts in virus populations induced by the selective pressure of antiretroviral therapies may influence viral load determination by "single primer pair" techniques. These considerations would argue for selection of an assay method (e.g., bDNA) that is capable of quantifying genetically diverse strains of HIV-1. Because of the potential adverse consequences of insensitive viral load determinations in clinical patient management, we intend to perform follow-up testing using the bDNA assay method on patients suspected of being infected with subtypes of HIV-1 that cannot be quantified adequately by RT-PCR. Therefore we are currently comparing viral load results obtained with the three commercial quantification techniques on a large series of samples obtained from patients that are suspected of being infected with non-B subtype strains. Preliminary data indicate that in some samples the viral load quantified with bDNA is lower than that determined with the Amplicor Monitor or NASBA assays. It might thus be a good strategy to perform baseline determinations with two alternative techniques (target amplification versus signal amplification) and to choose for follow-up of each patient that assay that provides the highest viral load, assuming that this is the most accurate one. True ac-

curacy can be determined only when calibrated standards (for instance, after viral particle counting by electron microscopy) would be available for all subtypes. But even then, individual sequence variation might not be accounted for. Overall, this raises questions about stratifying patients according to viral load, especially when dealing with divergent strains.

Roche and Organon Teknika are developing new versions of their respective Amplicor Monitor and NASBA assays to correct current shortcomings to properly quantify various HIV-1 subtypes. Which improvements could be made? It has been shown, using RNA transcripts, that lowering of the annealing temperature, and introduction of a reverse transcription step that gradually increases in temperature, improve the amplification efficiency of RT-PCR for HIV *gag* subtype A. Further improvement was obtained by substitution of 5-methylcytosine for cytosine throughout or the substitution of inosine at positions of variable bases.[25] Inclusion of other primer pairs may also be envisaged. In fact, addition of a new *gag* primer pair (HIV-1 Monitor primer mix 1) to the Amplicor Monitor reaction mixture, as recommended by the manufacturer for divergent HIV-1 strains, did not result in a better quantification of HIV-1$_{BL}$. Therefore it will be useful to evaluate the new prototype assays with various, genetically diverse HIV-1 isolates including the HIV-1$_{BL}$ strain.

ACKNOWLEDGMENTS

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