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研究報告の概要

○ヒト免疫不全ウイルス1型(HIV-1)に対する血液スクリーニング核酸増幅検査(NAT)における二つの異なる増幅対象の必要性

背景: HIV-1 RNA陽性供血血液5件が、3つの異なるCEマーク取得済みNATスクリーニングアッセイで検出されなかった。これらの事象は2件の輸血関連HIV-1 感染に関係していた。関連するNATアッセイはモノターゲットアッセイで、異なるウイルスゲノム領域(グループ特異的抗原またはロングターミナルリピート)を増幅するものである。偽陰性結果の原因調査が始められた。

研究デザインと方法: NAT偽陰性の5つの血漿検体を、異なる設計のCEマーク取得済みHIV-1 NATシステム12種類において比較調査した。それぞれのアッセイでHIV-1変異の相対的な増幅効率を調査した。偽陰性NATアッセイの対象領域における配列の変異によってプライマーとプローブの比較を行った。

結果: 偽陰性結果のモノターゲットNATと同様の方法で設計されたいくつかのNATアッセイにも、ウイルス変異の検出において欠陥が見られた。それぞれの例において、増幅対象領域の配列が変異し、アッセイに用いるプライマー及びプローブとの不一致を起こした。いくつかのデュアルターゲットアッセイは増幅効果が減少したが、偽陰性結果は示さなかった。

結論: HIVは新しいウイルス変異の急速な進化によって特徴付けられるが、新しい配列の進化は予測できない。モノターゲット領域のNATスクリーニングアッセイはデュアルターゲットアッセイよりも配列の変異に対してより脆弱のように見える。モノターゲットNATアッセイによる偽陰性結果に基づき、ポール・エーリッヒ研究所はドイツのHIV-1供血スクリーニングにデュアルターゲットNATシステムを要請することを考慮している。

使用上の注意記載状況・その他参考事項等

赤十字アルブミン20
赤十字アルブミン25
赤十字アルブミン5%静注12.5g/250mL
赤十字アルブミン20%静注4g/20mL
赤十字アルブミン20%静注10g/50mL
赤十字アルブミン25%静注12.5g/20mL

血液を原料とすることに由来する感染症伝播等

報告企業の意見	今後の対応
CEマーク取得済みのHIV-1 NATスクリーニングアッセイでHIV-1陽性供血血液が検出出来なかった件について調査した。その結果、デュアルターゲットNATの必要性が示唆されたとの報告である。 これまで、本製剤によるHIV感染の報告はない。また本製剤の製造工程には、平成11年8月30日付医薬発第1047号に沿ったウイルス・プロセスバリデーションによって検証された2つの異なるウイルス除去・不活化工程が含まれている。さらに最終製品についてHIV-NAT陰性であることを確認している事から本製剤の安全性は確保されていると考える。	日本赤十字社では、化学発光酵素免疫測定法(CLEIA)による抗体検査を行い、陰性の検体について20プールNATを実施している。2008年よりさらに感度を上げHIV-1/2及びHIVグループOの検出が可能なNATを実施している。20プールNAT導入の2004年以降、輸血によるHIV感染例は発生していない。今後、次世代NATシステム導入の際には、本報告のようなウイルス変異にも対応できるよう検討を進める。HIVに関する新たな知見等について今後も情報の収集に努める。なお、アルブミン製剤は70年も昔、HIV、HBV、HCVが発見されるはるか以前から、スクリーニングも施されていない血漿を原料に製造されても、いかなるウイルス感染を起こしたとの報告はない。極めて安全で特別な対応を必要としない。

参考3

Blood screening nucleic acid amplification tests for human immunodeficiency virus Type 1 may require two different amplification targets

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BACKGROUND: Five cases of human immunodeficiency virus Type 1 (HIV-1) RNA–positive blood donations are described that escaped detection by three different CE-marked nucleic acid amplification technique (NAT) screening assays. These events were associated with two HIV-1 transmissions to recipients of blood components. The implicated NAT assays are monotarget assays and amplify in different viral genome regions (group-specific antigen or long terminal repeat). Investigations into the cause of the false-negative test results were initiated.

STUDY DESIGN AND METHODS: Plasma specimens of the five NAT false-negative cases were comparatively investigated in 12 CE-marked HIV-1 NAT systems of differing design. The relative amplification efficiency for the HIV-1 variant was determined for each assay. Sequencing of the variants in the region targeted by each false-negative NAT assay allowed comparison with the respective primers and probes.

RESULTS: Some of the NAT assays designed in a similar way to false-negative monotarget NATs also revealed deficiencies in detecting the viral variants. In each case sequencing of the assay target region in the variants demonstrated mismatches with primers and probes used by the assays. Some dual-target assays showed decreased amplification efficiency, but not false-negative results.

CONCLUSION: HIV is characterized by its rapid evolution of new viral variants. The evolution of new sequences is unpredictable; NAT screening assays with a single target region appear to be more vulnerable to sequence variations than dual-target assays. Based on this experience with false-negative tests results by monotarget NAT assays, the Paul-Ehrlich-Institut is considering requesting dual-target NAT assays for HIV-1 blood donation screening in Germany.

The most recent report of the Joint United Nations Program on HIV/AIDS (UNAIDS) and the World Health Organization (WHO) calculated that there were 33.4 million human immunodeficiency virus (HIV)-infected people worldwide by the end of 2008, with 2.7 million new infections alone in 2008.[1] These data reflect a continuing increase in the number of people living with HIV. Despite improved access to antiretroviral therapy in low- and middle-income countries, it is estimated that 2 million AIDS-related deaths occurred worldwide in 2008. Compared to the global HIV situation, Germany is among those countries with low prevalence and incidence rates in the general population as well as in blood donors. In Germany, between 2000 and 2007 the prevalence of HIV-1 infection in first-time donors was less than 10 HIV-1–infected donors per 100,000 applicant donors.[2] The HIV-1 infection rate (incidence) is also very low with less than 1 new infection per 100,000 repeat donors during the same period. Nevertheless, immense efforts have been made to prevent transfusion-associated HIV transmissions, including the use of highly developed technologies for blood screening. When nucleic

ABBREVIATIONS: *gag* = group-specific antigen; ID(s) = individual donation(s); IVD = in vitro diagnostic medical device; LTR = long terminal repeat; NAT = nucleic acid amplification technique; PEI = Paul-Ehrlich-Institut; *pol* = polymerase.

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acid amplification techniques (NAT) became available, Germany was one of the first countries in the world to implement this technology as a mandatory tool for blood screening in addition to serologic assays. NAT screening was introduced for hepatitis C virus (HCV) in 1999 followed by HIV-1 in 2004.[3,4] During the introduction of NAT, commercial assays designed for blood screening were scarce, and therefore flexible approaches were necessary. Compromises were made with regard to both test performance (e.g., sample pooling) and acceptance of different validated assay types for blood screening, including CE-marked diagnostic assays of high sensitivity or in-house–developed screening assays.[5] More recently, a variety of different HIV-1 NATs have become available on the common market in Europe following the CE marking process defined by the "in vitro diagnostic medical device (IVD) Directive."[6] In Germany, a minimal individual-donation (ID) sensitivity of 10,000 international units (IU) HIV-1 RNA/mL (based on the WHO international standard for HIV-1 RNA) was defined for HIV-1 NAT used for blood screening.[4,7] After several years of NAT in Germany the NAT yield (donations from the diagnostic window phase: NAT positive, anti-HIV negative) was determined.[8] From 2004 to 2010, a total of 23 HIV-1 NAT yield cases were found in more than 31 million NAT-screened donations, while two HIV-1 transmissions despite NAT were observed for this period. The first HIV-1 transmission was associated with a false-negative test result in the routine NAT assay. The assay targeted a region of the HIV-1 group-specific antigen (*gag*) gene and was shown to underestimate the viral load of the HIV-1 variant in this case.[9] Four additional cases (Cases 2-5) of nondetection of HIV-1 RNA by CE-marked assays were reported more recently to the Paul-Ehrlich-Institut (PEI), one of which resulted in another virus transmission to a recipient of a blood component. In this study we compare the detection efficiencies of different HIV-1 NAT assays for each of these cases and analyze the underlying HIV-1 sequences targeted by the assays. The suitability of monotarget NATs for the screening of strains of HIV is discussed. In our opinion, these cases demonstrate the need for at least two different amplification targets in "state-of-the-art" NAT blood screening systems for HIV-1 Group M.

MATERIALS AND METHODS

Plasma specimens

Samples of the cases, testing falsely negative in CE-marked NAT assays, were provided to the PEI by the respective blood collection centers. The cases were numbered chronologically from 1 to 5, as reported to the PEI. The viral loads were determined using quantitative HIV-1 NATs. Based on the mean value obtained by different proficient quantitative assays, replicate plasma samples, either neat or serially diluted (in pooled negative human plasma) were subsequently used for comparative testing of a variety of different HIV-1 NATs. Furthermore, sequence analysis of NAT target regions in the viral genome was performed. An overview of the cases including the specimens analyzed is provided in Table 1.

HIV-1 NAT assays used in comparative study

Samples of the five false-negative cases were analyzed in 12 CE-marked NAT assays for HIV-1 RNA detection (six qualitative, six quantitative assays). The qualitative assays are CE-marked intended for use in blood screening, while the quantitative assays have been designed for diagnostic use, for example, patient monitoring. After validation, some of the highly sensitive quantitative assays are also used in blood screening in Germany. All assays were performed strictly following the instructions for use.

Comparative testing of the five plasma samples was performed by serial dilution of each of the HIV-1 RNA–positive specimens, and test results were assessed in comparison to the sensitivity claimed by the manufacturer. All investigations were performed in parallel with serial dilutions of the well-characterized PEI HIV-1 RNA reference preparation (3441/04, Subtype B, 80,000 IU/mL) which has been calibrated against the WHO international standard.[7] Assay performance characteristics described in the package inserts had to be reconfirmed using this reference material. Detection efficiency of individual assays for Cases 1 through 5 materials was determined by assessment of the obtained test results (expressed as IU/mL, Ct values, sample-to-cutoff values, or relative light units) in comparison to the results obtained with the PEI HIV-1 RNA reference preparation. Repeat experiments were performed to account for technology-intrinsic variation of results. Assay results for Cases 1 through 5 are expressed using symbols in Table 2 relative to the reported HIV-1 concentrations (quantitative assays) or to the detection efficiency of qualitative assays, when compared both to the 95% limit of detection claimed in the instructions for use, and to the performance with the PEI reference material. In Table 2 we used the following assessment scheme to report NAT assay efficiency: no detection, highly reduced detection (by a factor of >10), moderately reduced detection (by a factor of <10), and consistent detection.

Twelve CE-marked HIV-1 NAT assays were included as follows.

1. CAP CTM v1: COBAS AmpliPrep/COBAS TaqMan HIV-1 Test (quantitative; Roche Molecular Systems, Pleasanton, CA).
2. HPS CTM v1: HPS viral nucleic acid kit/COBAS TaqMan HIV-1 Test (quantitative; Roche Molecular Systems).
3. CAS v1.5: COBAS AmpliScreen HIV-1 Test, Version 1.5 (qualitative; Roche Molecular Systems).

TABLE 1. Summary of HIV-1 NAT nondetection cases

Case	Donor type, sex, age (years)	Donation dates	Screening NAT	HIV screening results	Viral load (IU/mL)	Donor status	HIV-1 transmission by
1	RD, male, 44	January 2007	CAP CTM v1	Ab neg RNA neg	10,000	WP	RBCs
		April 2007		Ab pos RNA neg	650	SC	
2	RD, male, 26	July 2007	CAP CTM v1	Ab neg RNA neg	0		
		October 2007		Ab pos RNA neg	80,000	SC	
3	RD, male, 26	May 2009	CTS MPX	Ab neg RNA neg	0		
		August 2009	CTS MPX	Ab neg RNA neg	20,000	WP	RBCs
		July 2010	VSPK v1.1	Ab pos RNA pos	260,000	SC	
4	RD, male, 42	March 2010	VSPK v1.1	Ab neg RNA neg	0		
		June 2010		Ab neg RNA neg	0		
		October 2010		Ab pos RNA neg	200,000	SC	
5	FTD, male, 18	October 2010	VSPK v1.1	Ab pos RNA neg	2,000	SC	

Ab = anti-HIV-1/2; FTD = first-time donor; neg = negative; pos = positive; RD = repeat donor; SC = seroconversion; WP = window period.

TABLE 2. Comparative testing of CE-marked HIV-1 NAT systems using Cases 1-5

Case	FN routine NAT	HIV-1 target regions:	CAP CTM v1 *gag*	HPS CTM v1 *gag*	CAS v1.5 *gag*	CAM v1.5 *gag*	CTS MPX LTR	VSPK v1.1 LTR	artus LTR	DRK LTR	Abbott RT *pol*	VSPK v1.2 LTR	CAP CTM v2 *gag* + *LTR*	Ultrio plus *pol* + *LTR*
1, 2	CAP CTM v1		((+))	–	+	+	+	+	+	+	+	+	(+)	+
3	CTS MPX		*(wd)*	+	+	+	–	+	+	+	+	+	(+)	+
4, 5	VSPK v1.1		*(wd)*	+	+	+	+	–	((+))	+	+	+	+	(+)

– = no detection; ((+)) = highly reduced (factor >10) detection efficiency; (+) = moderately reduced (factor <10) detection efficiency; + = consistent detection efficiency; FN = false-negative; *(wd)* = test version Version 1 has been withdrawn by the manufacturer.

4. CAM v1.5: COBAS Amplicor HIV-1 Monitor Test, Version 1.5 (quantitative; Roche Molecular Systems).
5. cobas TaqScreen MPX Test for use with cobas S201 system (qualitative; Roche Molecular Systems).
6. VSPK v1.1: Virus Screening PCR Kit, Version 1.1 (qualitative; GFE Blut mbH, Frankfurt, Germany).
7. artus: artus HIV-1 RG RT-PCR Kit (quantitative; Qiagen GmbH, Hilden, Germany).
8. DRK: DRK HIV-1 PCR kit (qualitative; DRK BSD Baden Württemberg-Hessen, Frankfurt, Germany).
9. Abbott RT: Abbott RealTime HIV-1 assay (quantitative; Abbott Molecular, Des Plaines, IL).
10. VSPK v1.2: Virus Screening PCR Kit, Version 1.2 (qualitative; GFE Blut mbH)
11. CAP CTM v2: COBAS AmpliPrep/COBAS TaqMan HIV-1 Test, Version 2.0 (quantitative; Roche Molecular Systems).
12. Ultrio Plus: Procleix Ultrio Plus Assay (qualitative; Gen-Probe, San Diego, CA).

The CAM v1.5 assay is regarded as representative for the related Amplicor HIV-1 Monitor Test Version 1.5 and COBAS AmpliPrep/COBAS Amplicor HIV-1 Monitor Test, Version 1.5 because of the common amplification module; the Ultrio Plus assay is regarded as representative for the related assay Procleix Ultrio sharing the same HIV-1 amplification.

Sequence analysis

Viral RNA was extracted from 500 µL of plasma using the QIAamp DSP virus kit (Qiagen); amplicons of the 5′-long terminal repeat (LTR) were generated by cDNA synthesis and nested PCR using the outer antisense primer LTR-oa1 (5′-TAATACCGCTCTCGCACC-3′) and outer sense primer LTR-os1 (5′-CTTTTTGCCTGTACTGGGTCTC-3′). For the nested PCR procedure, the primers LTR-is1 (5′-CTGGGAGCTCTCTGGCTAACTA-3′) combined with LTR-ia1 (5′-TCCTTCTAGCCTCCGCTAGTC-3′) were used as sense or antisense primers, respectively. Direct sequencing of the amplification products was performed on both strands using a cycle sequencing kit (BigDye Terminator Version 3.1, Applied Biosystems, Foster City, CA) on a genetic analyzer (ABI 3730xl, Applied Biosystems). For all amplification and sequencing reactions the PEI HIV-1 RNA reference preparation (3441/04, Subtype B, 80,000 IU/mL) was analyzed in parallel as a positive control and to control for potential contamination.

Sequences representing 243 bp of the 5′-LTR region (Nucleotides 483-725) were aligned and compared with the corresponding sequence of the HIV-1 prototype HBX2 (Accession Number K03455). This part of the 5′-LTR region covers the target region of the VSPK, artus, and CTS MPX assays. Although we do not disclose the primer-probe sequences used in the different assays, data are reported concerning the frequency of mismatches and their relative position for several of the assays studied.

RESULTS

Different German blood establishments have recently reported five cases of HIV-1 RNA–positive blood donations missed by NAT screening. The five donors (all male) had not reported any HIV risk factors in the routine eligibility screening questionnaire. The NAT assays concerned were three different CE-marked screening tests (CAP CTM Version 1, CTS MPX, and VSPK Version 1.1) from two manufacturers (Roche Molecular Systems and GFE Blut mbH). Two of these cases resulted in HIV transmission to the recipients by transfusion of the corresponding red blood cells (RBCs).

The five cases were missed by the routine NAT assay despite viral RNA at a concentration level estimated to be sufficient for detection by the NAT screening system in place (Table 1). Samples from these cases were investigated using a variety of different HIV-1 NAT assays. The results are summarized in a semiquantitative manner describing the relative detection efficiency of the assays (Table 2).

Sequence analysis of the LTR target region was performed for the cases concerned (Fig. 1). There is high homology between sequences of primers and probes used in NAT assays and the prototype HXB2 sequence. When compared with the HIV-1 prototype HXB2, the sequence of the PEI HIV-1 RNA reference preparation exhibits five nucleotide changes in this in the part of the LTR analyzed (Nucleotides 483-725); these changes do not affect the efficiencies of the different NAT assays studied.

Case 1

Some details of the first case have already been published.[9] In April 2007, a male repeat donor was found to be anti-HIV-1 positive and HIV-1 NAT negative (CAP CTM v1, minipool and ID). The previous donation from January 2007 tested negative both by the serologic and the NAT assay (CAP CTM v1, pool of 96). The RBCs from the January donation infected the recipient with HIV-1, which was confirmed by molecular analysis. Retrospectively, it was shown that the CAP CTM v1 assay underquantified the HIV variant in both the donor and the recipient samples by a factor of up to 100.

Comparative tests

Performance of different NAT assays with the follow-up plasma from the April 2007 donation (650 IU HIV-1 RNA/mL; artus) confirmed the underquantitation performed by the CAP CTM v1 assay and the closely related HPS CTM v1 assay. Spurred on by reports of underquantitation for the CAP CTM v1, the manufacturer brought out a new

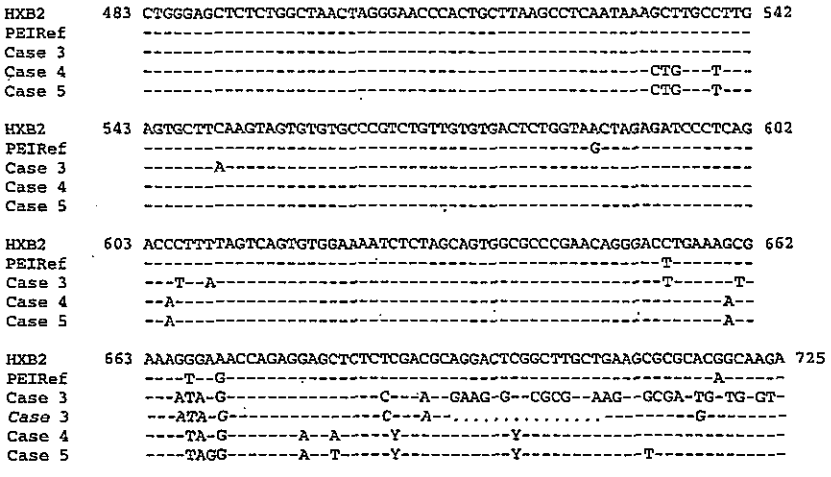

Fig. 1. Alignment of HIV-1 LTR sequences. Alignment of partial 5'LTR sequences (nucleotides 483-725) derived from amplified genomes of the "PEI HIV-1 RNA reference preparation" (#3441/04; subtype B), samples from non-detection Cases 3-5 and the HIV-1 prototype HXB2 (accession number K03455). Dashes represent homology. Differences are shown by the appropriate base letter or points (deletion). The alignment revealed a gap of 15 nucleotides (693-707) for the 3' part of the Case 3 sequence (see characters in italic).

version of the assay (CAP CTM v2) by incorporating a second target region, that is, the LTR, in addition to the *gag* region already amplified in the assay.[10,11] Although detection and quantification of Case 1 samples was clearly improved with the new version of the assay, its efficiency was still lower than that of other quantitative assays and is most probably due to the continued failure of the *gag* amplification by the improved assay.

Several qualitative or quantitative assays, when investigated with serial dilutions of the viremic plasma, showed no evidence of underestimation of this HIV variant, including the CAS v1.5 and CAM v1.5 assays as related monotarget *gag* amplification systems, when compared with the CAP CTM v1 assay.

Sequence analysis

Sequence analysis of the *gag* region of the HIV variant and alignment with primer and probe sequences used in the CAP CTM v1 assay has already been published.[9] It revealed three mismatches (one with the probe and two with the antisense primer). Subsequently, the mismatch at Position –3 at the 3' end of the antisense primer was shown to be responsible for the underquantitation of both this and a series of related HIV-1 variants (e.g., Case 2) by the implicated Roche CTM v1 assays (CAP CTM v1, HPS CTM v1).

Case 2

In October 2007 a repeat donor was tested positive for anti-HIV-1/2 but was negative by NAT screening (CAP CTM v1, pool of 96). Further investigations revealed positive test results with several other HIV-1 NATs. The screening results of the previous donation from July 2007 had been negative, and the RBCs were transfused. The backup sample from July 2007 was tested HIV-1 RNA negative by HIV-1 NATs (Abbott RT, Ultrio Plus), which picked up the HIV-1 variant of the October donation. The recipient of the RBCs had died in the meantime, without causal relationship to the transfusion or an infection. Both sequence analysis of the *gag* region and comparative testing in different NAT assays confirmed the HIV-1 variant of Case 2 as analogous to the Case 1 HIV-1 variant.

Case 3

In July 2010 an HIV-1 seroconversion was observed in a repeat blood donor whose HIV-1–positive status became obvious by a reactive test result in the HIV antigen and antibody combination assay confirmed in Western blot analysis (index donation). Furthermore, a reactive test result was obtained in the minipool (pool size of 96) with the multiplex PCR test system VSPK v1.1, which had been introduced as the routine NAT screening system at the blood collection site in May 2010. A donor-initiated lookback was initiated.

The previous donations by the donor were made in August and May 2009. These donations tested negative both in the serologic screening assay and in the previously used multiplex NAT system, the CTS MPX test. At the time this multiplex assay for HIV, HCV, and hepatitis B virus (HBV) had been used for minipools of 96.

However, reinvestigation of the backup samples from August 2009 resulted in a positive test result in the VSPK v1.1 assay both in ID-NAT and in a simulated minipool NAT of 96 donations. With the CTS MPX assay, however, the respective results were repeatedly negative for both kinds of sample. The HIV-1 RNA concentration was 20,000 IU/mL.

Backup samples of the previous donation from May 2009 tested negative in all HIV-1 screening assays. The data suggest HIV-1 infection of the donor during summer 2009, with the donor still being in the serologic window phase at the time of his donation in August 2009. The RBCs of the August 2009 donation were transfused in an 81-year-old male patient resulting in infection with HIV-1, which was confirmed by serology and NAT. The August 2009 donor plasma was still held in quarantine and available for further investigation; fortunately, the corresponding platelets had not been transfused.

Comparative tests

Comparative testing with the August 2009 plasma confirmed complete failure of the monotarget LTR assay CTS MPX, even with the neat plasma. Furthermore, there was an underquantitation of the viral load observed with the dual-target CAP CTM v2 (LTR, *gag*) when compared to the unmodified, original *gag* monotarget assay, HPS CTM v1, or other quantitative assays. This may be explained by reduced detection efficiency in the dual-target assay with the failure of one of the target regions (the LTR amplification systems may be related in both assays from the manufacturer, CAP CTM v2 and cobas TaqScreen MPX Test).

Sequence analysis

Sequence alignment of the LTR region of the Case 3 HIV-1 variant with primer and probes of the cobas TaqScreen MPX test showed good homology with one primer and the probe; however, it was highly divergent compared to the second Group M primer used in the test system. By contrast, comparison of the LTR from Case 3 with the oligonucleotides used in the VSPK v1.1 or artus assays resulted in good matches.

Case 4

An anti-HIV–positive test result (anti-HIV-1 enzyme-linked immunosorbent assay [ELISA] and Western blot positive) was obtained in a male repeat donor in October 2010. The screening NAT test VSPK v1.1 performed in pools of 96 was negative for this donation. By contrast, further analysis of the backup sample revealed a positive result with another NAT assay of different design indicating a false-negative test result by the NAT screening system used at this blood collection site. The two previous whole blood donations from June 2010 and March 2010 gave negative test results in the same routine serologic and NAT assays used in October 2010. Retesting with another sensitive NAT of different design (Abbott RT; shown to be positive with the index donation) did not detect HIV-1 RNA in the backup samples from June 2010 and March 2010. The most likely explanation for these findings is a seroconversion between June and October.

Comparative testing

Comparative testing with the backup sample of the index donation from October 2010 (200,000 IU/mL) was performed using serial dilutions of the material. The failure of the monotarget LTR assay VSPK v1.1 assay was confirmed while the monotarget LTR assay CTS MPX provided positive results. The sample was underquantified by a factor of more than 50 by the artus monotarget LTR assay. Although the dual-target (LTR, polymerase [*pol*]) qualitative Ultrio Plus assay detected the sample, a reduced efficiency (by a factor of 6) was observed with this assay compared to the relative detection efficiency of serially diluted PEI reference materials.

Sequence analysis

The sequence analysis of the LTR region revealed mismatches with one of the primers used in the VSPK v1.1 assay, apparently resulting in the complete failure of the assay to amplify this HIV-1 variant. Other assays based on LTR sequences are also affected by the sequence variations observed in this variant. The artus assay also shows mismatches with one of its primers, although these are situated toward the 5′ end of the primer. This presumably explains the severe rate of underquantitation of the sample while still showing low residual amplification efficiency. The amplification of this variant by the artus assay might also be hampered by further mismatches in the binding region for the second primer.

Relative underestimation was observed for the Ultrio Plus assay (Table 2). The assay uses both the LTR and the *pol* as targets and might be affected by mismatches in the LTR. Although there are mismatches of this HIV-1 variant with one of the Ultrio LTR primers (J. Linnen, Gen-Probe, personal communication, 2011), additional studies (e.g., sequencing of the *pol* region of the HIV-1 variant) are needed to explain the lower efficiency of the assay. The CTS MPX assay amplifies this variant consistently, a finding substantiated by high homology between the viral variant and the primer-probe system of this assay with only a few mismatches at noncritical positions.

Case 5

The most recent case of a false-negative NAT result is a first-time blood donor (male, 18 years) who was tested anti-HIV-1/2 positive by ELISA and confirmatory Western blot in October 2010. NAT was negative, both in pools of 96 and in ID testing, using the VSPK v1.1 assay. To further investigate the possibility of an elite controller (i.e., an untreated HIV-infected individual without measurable viremia), the specimens were tested using another sensitive NAT assay of different design and shown to be reactive. The plasma from this whole blood donation was investigated using the panel of NAT assays. The overall reactivity pattern with the different assays for Case 5 is very similar to that obtained with Case 4. Sequence analysis revealed nucleotide changes very similar to those of Case 4, leading to the same explanation of differential reactivity in the different assays. However, there are few differences between these two related variants, with changes at three positions in the 243-bp region analyzed for the different primer-probe target sites of the different assays (Fig. 1).

DISCUSSION

The five HIV-1–positive blood donors described in this study, although positive by serologic screening (anti-HIV-1/2 ELISA), all showed false-negative NAT results in the respective screening assays (Table 1). The cases concern four recently seroconverted repeat donors (Cases 1-4) and one first-time donor with unknown infection history (Case 5). The cases occurred at different blood collection sites in Germany, and three different NAT systems from two manufacturers gave rise to false-negative test results. Two diagnostic window-phase donations, missed by the routine screening NAT assay, led to HIV-1 transmission to the RBC recipients.

For Case 1 the sequence analysis of the NAT target region (*gag*) has already been published.[9] Only a small number of entries in the public databases matched this specific sequence. Case 2 is of very similar *gag* sequence pattern and appeared at another blood donation center in Germany. Both sequence pattern and reactivity in different assays were highly similar between these cases confirming the urgent need for improvement of the CAP CTM v1 assay and withdrawal of the previous version from blood screening.

For Cases 3, 4, and 5, we analyzed the viral sequence of the 5′-LTR region targeted by the different NAT assays by comparison to the PEI HIV-1 RNA reference material and to the reference sequence HXB2 (Fig. 1). The three cases revealed unique sequence patterns not present in public HIV sequence databases.

The LTR region of the Case 3 HIV-1 variant differs at 13.6% of positions (33/243) compared to HXB2, with the lowest level of homology in the 3′ part of this LTR fragment. The low homology is mainly caused by a deletion of 15 nucleotides (Fig. 1, Case 3 sequence in italics). This deletion was not present in any public HIV sequence database. The deletion has no obvious inhibitory effect on the replication of HIV-1 as shown by the transmission event. Further investigations on this transmission event included HIV-1 sequence analysis in the index donation (July 2010) and in the RBC recipient (August 2010, 1 year after transfusion). Briefly, comparison of different regions of the HIV-1 genome (5′-LTR, *pol*) derived from donor (August 2009, July 2010) and recipient (August 2010) showed greater than 99.5% nucleotide identity, clustering with Subtype B sequences (data not shown). These results confirm the HIV transmission via RBCs from the HIV-infected blood donor and demonstrate sequence stability for this HIV variant in both donor and recipient for more than 1 year.

The HIV-1 variants of Cases 4 and 5 were missed by the same routine NAT system and furthermore exhibit similar reactivity in the different NAT assays studied for detection of this variant. These two variants exhibit a very similar (although not identical) pattern and rate of nucleotide exchanges in the 5′-LTR target region (243 bp) of the NAT assays analyzed, when compared to reference sequence HXB2 (differences at 13 [5.3%] or 15 [6.2%] positions) in Case 4 and Case 5, respectively. These variants show common patterns, with neighboring nucleotide exchanges. Several assays target one of the affected regions potentially explaining why different assays of related design show reduced efficiency for these two HIV-1 variants.[12]

Cases 4 and 5 appeared in different blood collection centers in different regions of Germany. The particular similarity in the LTR region is not reflected by nucleotide sequences in *env* (V3, V4). This region is of much lower homology between the two HIV-1 variants (data not shown). Therefore, there is currently no indication for a common infection source of the two donors.

Even if certain mismatches with primers or probes were prognosticated for viral variants, the extent of underquantitation or nondetection to be expected is often not predictable. While genetic change is inevitable, and may affect primer and probe regions, the extent to which viral loads are underestimated or viruses are simply not detected is often unpredictable. Although general rules have been established for the design of primers and probes to tolerate mismatches, experimental studies should be performed to measure the effect of individual mismatches and mismatch combinations on assay performance, exactly matching specified assay conditions. Besides the number and position of mismatches, several factors have been shown to contribute to the effect, including mismatch and primer type, neighboring sequences, and design of the amplification reaction.[13,14]

Monotarget versus dual-target screening NATs

There are now several actual examples of HIV-1 variants escaping detection by monotarget NATs targeting different regions of the HIV-1 genome. A recent study from Italy describes false-negative or underquantified results with the COBAS AmpliScreen HIV-1 Test, Version 1.5 and COBAS Amplicor HIV-1 Monitor Test, Version 1.5, which are monotarget assays.[15] These assays use *gag* primers and probes different from those of the CAP CTM v1 and are affected by different HIV-1 *gag* variants compared to Cases 1 and 2 described above.[9,15] Furthermore, the Italian case also does not seem to affect the *gag* portion in the dual-target CAP CTM v2 assay as the published quantitative data suggest. Therefore, the recent Italian case appears to be due to different *gag* sequence variation affecting different *gag* monotarget assays when compared to Cases 1 and 2 of our study. Another recent publication from Germany described new genetic polymorphisms in the LTR region affecting a real-time PCR blood-screening test developed in house.[16] Two unrelated HIV-1-positive blood donations

were missed by NAT, whereas parallel serologic testing was positive for both donations. The observed LTR sequence polymorphisms are different from those described in our study. The implicated in-house assay was subsequently improved to pick up the LTR polymorphisms. The CE-marked VSPK v1.1 assay was also modified to detect the viral variants from Cases 4 and 5 and was replaced by the new CE-marked assay VSPK v1.2. Modification of monotarget assays may provide an interim solution to deal with such deficiencies. However, from a conceptual point of view the inclusion of a second target region clearly appears to be the preferable approach long term.

The false-negative results were always obtained with screening NATs comprising a single amplification system designed for HIV-1 Group M sequences. The risk of a false-negative test results appears to be proportionally lower with more than one amplification target region in a screening NAT assay, especially for detection of a highly variable virus like HIV-1. We recognize that there are monotarget assays without reported mismatch-based failure in blood screening; however, evolution of HIV-1 sequence variants appears unpredictable and may therefore affect any NAT assay. The frequency of use in blood screening impacts the probability for the observation of adverse events for NAT assays. The risk of a false-negative screening test result may be directly translated into the risk of HIV-1 transmission by transfusion if the donor is in the diagnostic window phase at the time of donation. We show indirect evidence that reactivity of dual-target assays may be compromised in the case of failure of one of the targets; nevertheless, a significantly lower risk is expected for dual-target assays with the a second amplification system being able to widely compensate for the complete failure of the other component when affected by an emerging HIV-1 variant. There is currently no evidence of a dual-target assay missing one of the cases described in this study or in related reports. A recent publication describes a transfusion-associated transmission despite dual-target HIV-1 NAT.[17] However, the authors interpret this incident as being due to the low HIV-1 RNA concentration during the eclipse period of early HIV-1 infection. Nondetection of low-level viremia may still occur with any sensitive NAT system. This constitutes the nonpreventable residual risk in the era of NAT.

The detection failures, due to mismatches in monotarget HIV-1 NAT assays occurred during routine blood screening in two European countries with low HIV-1 rates in their blood donor populations. We therefore conclude from our study and from related cases published recently that dual (or more)-target HIV-1 Group M NAT assays should be considered for use in blood screening. We would define a dual-target assay for HIV-1 Group M screening NATs as a system where two different amplicons are generated with different primer sets, with the amplicons being detected by different probes. It might be of secondary importance if the different targets are located within one viral genomic region (e.g., the LTR) or in separate regions.

Critical assessment of blood screening laboratory data

In our view, both blood donation testing laboratories and NAT kit manufacturers should become aware of and regularly examine those test results that are not easily interpretable. Such results should include negative NAT tests for HIV-1, HCV, or HBV with simultaneous confirmed positive anti-HIV-1, anti-HCV, or hepatitis B surface antigen results, respectively. This constellation of diagnostic markers can occur, with higher probabilities for HBV (29%) and HCV (26%) compared to HIV-1 (11%), as recently published for US blood donors.[18] The review should include NAT assays of different design, to be in a position to identify false-negative results in the screening NAT. The PEI would be able to provide support by performing more extended analysis of discrepant test results, with the inclusion of a variety of assays (serologic, NAT). However, the availability of sufficient specimen volumes of important cases is often an issue. In our experience, specimens of great scientific value, identified by screening of blood donations, have often already been destroyed, after strictly following respective standard operating procedures, thus preventing follow-up investigations.

Viral sequences used for assay design

The treatment management of HIV patients is often based on sequence information of the *pol* (i.e., domains of protease and reverse transcriptase) and *env* regions. The use of these sequences for NAT assay design is limited. There is an urgent need to share newly identified virus sequences, particularly in the genomic regions targeted by blood screening NAT assays, for example, by making these sequences available in public databases. IVD manufacturers may gain knowledge about emerging viral sequences with respect to their own assays by investigating customer complaints. In our experience, individual IVD manufacturers are sometimes well aware of circulating variants potentially causing problems in their assays. Manufacturers may feel uncomfortable making this sequence information of viral variants publicly available. Despite commercial competition, it might be of mutual benefit to share new sequence data, especially for those manufacturers using similar viral genome regions as amplification targets. An IVD-specific sequence database at an independent institution may be helpful both for the design of new assays and for the surveillance of existing assays.

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CONFLICT OF INTEREST

The authors state no conflict of interest relevant to this manuscript submitted to **TRANSFUSION**.

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