Risk Minimization Measures for Blood Screening HIV-1 Nucleic Acid Amplification Technique Assays in Germany

Michael Chudy^a Julia Kress^a Jochen Halbauer^b Margarethe Heiden^c Markus B. Funk^b
C. Micha Nübling^a

^a Section of Molecular Virology,

^b Section of Hemovigilance and IVD Vigilance,

^c Section of Transfusion Medicine, Paul-Ehrlich-Institut, Langen, Germany

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Summary

Background: Several publications describe HIV-1 RNA false-negative results or viral load underquantitation associated with Communauté Européenne(CE)-marked qualitative or quantitative nucleic acid amplification technique (NAT) assays. 6 cases occurred during blood screening in Germany, with 2 of them causing HIV-1 transmissions to recipients of blood components. The implicated NAT assays were mono-target assays amplifying in different viral genome regions (*gag* or long terminal repeat). **Methods:** Specimens characterized by HIV-1 NAT underquantitation or false-negative NAT results were comparatively investigated in CE-marked HIV-1 NAT systems of different design to identify potential reasons. The target regions of the viral nucleic acids were sequenced and these sequences compared to primers and probes of the assays. Potential risk minimization measures were considered for quantitative and blood-screening HIV-1 NAT systems. **Results:** Nucleotide sequencing of the viral target region in cases of HIV-1 RNA underquantitation or false-negative test results revealed new HIV-1 variants that were mismatched with primers and probes used in some mono-target assays. So far, dual-target NAT assays have not been associated with mismatch-based false-negative test results. From 2015, the Paul Ehrlich Institute will request HIV-1 NAT assays of dual-target design or an analogous solution for further reducing the risk in blood screening. **Conclusion:** HIV differs from other blood-borne viruses with regard to its fast

evolution of new viral variants. The evolution of new sequences is hardly predictable; therefore, NAT assays with only 1 target region appear to be more vulnerable to sequence variations than dual-target assays. The associated risk may be higher for HIV-1 NAT assays used for blood screening compared to quantitative assays used for monitoring HIV-1-infected patients. In HIV-1 screening NAT assays of dual-target design may adequately address the risk imposed by new HIV-1 variants.

Introduction

As summarized by the European Center for Disease Control (ECDC) in its most recent report [1], HIV infection remains one of the major public health problems in Europe. Nevertheless, on a global scale, the overall HIV epidemiology in Europe appears moderate. The HIV epidemiology in risk groups differs between countries. At present, in Europe, men who have sex with men (MSM) comprise the largest group of cases (38%), followed by those who acquired the virus through heterosexual contact (24%) and injecting drug users (4%). Cases in MSM increased by 39% between 2004 and 2010, while cases acquired by heterosexual transmission or in other risk groups have remained stable or are declining. Based on the introduction of highly sensitive test systems for donor screening, the risk for infection via blood transfusion has become very low in Europe.

In Germany, both the general and the blood donor population are characterized by low prevalence and incidence rates when compared to the global HIV situation. For blood donors

Table 1. CE-marked HIV-1 NAT assays^a

Abbreviation	Name	Type	Manufacturer
CAP CTM v1	COBAS AmpliPrep/COBAS TaqMan HIV-1 test	quantitative	Roche Molecular Systems, Pleasanton, CA
HPS CTM v1	HPS Viral Nucleic Acid Kit/COBAS TaqMan HIV-1 test	quantitative	Roche Molecular Systems, Pleasanton, CA
CAS v1.5	COBAS AmpliScreen HIV-1 test v1.5	qualitative	Roche Molecular Systems, Pleasanton, CA
CAM v1.5	COBAS Amplicor HIV-1 monitor test, v1.5	quantitative	Roche Molecular Systems, Pleasanton, CA
CTS MPX	cobas TaqScreen MPX test for use with cobas S201 system	qualitative	Roche Molecular Systems, Pleasanton, CA
VSPK v1.1	Virus screening PCR kit v1.1	qualitative	GFE Blut, Frankfurt/M.
artus	artus HIV-1: RG RT-PCR kit	quantitative	Qiagen GmbH, Hilden
Abbott RT	Abbott real time HIV-1 assay	quantitative	Abbott Molecular, Des Plaines, IL
VSPK v1.2	Virus screening PCR kit v1.2	qualitative	GFE Blut, Frankfurt/M.
CAP CTM v2	COBAS AmpliPrep/COBAS TaqMan HIV-1 test, v2.0	quantitative	Roche Molecular Systems, Pleasanton, CA
Ultrio Plus	Procleix Ultrio Plus assay	qualitative	Gen-Probe, San Diego, CA

CE = Communauté Européenne, NAT = nucleic acid amplification technique.

^aThe CAM v1.5 assay is regarded as representative for the related assays (Amplicor HIV-1 Monitor Test, v1.5 and COBAS AmpliPrep/ COBAS Amplicor HIV-1 Monitor Test, v1.5) because of the common amplification module; the Ultrio Plus assay is regarded as representative for the related Procleix Ultrio Assay, which has the same HIV-1 amplification features.

in Germany the most recent published data describe, for the period 2008–2010, an HIV prevalence in first-time donors (FTD) of 6.8 per 100,000 applicant donors, and an HIV sero-conversion rate of 2.4 per 100,000 repeat donors [2]. Continuous efforts to prevent transfusion-associated HIV-1 transmissions led to the introduction of NAT tests for blood screening in addition to serological assays. Although some blood donation centers had already introduced HIV-1 NAT in the mid-90s on a voluntary basis [3], the Paul Ehrlich Institute (PEI) required HIV-1 NAT from 2004 on, with a minimal sensitivity limit for the individual donation of 10,000 IU HIV-1 RNA/ml (based on the WHO International Standard for HIV-1 RNA) [4, 5]. In Germany, this NAT requirement facilitates pooling of specimens prior to NAT testing and the use of different validated assay types for blood screening including Communauté Européenne(CE)-marked diagnostic assays of high sensitivity or in-house-developed screening assays [6].

After some years of NAT testing experience in Germany, the NAT yield (donations from the diagnostic window phase: NAT positive, anti-HIV negative) was determined [7] and recently updated. From 2004 to December 2010, 23 HIV-1 NAT yield cases were found in more than 31 million NAT-screened donations, but 2 HIV-1 transmissions, despite NAT testing, were observed during this period. These HIV-1 transmissions should have been interdicted by NAT due to the high viral loads present in the donors. The cases were traceable to false-negative test results in the routine NAT assays due to new variants of the common HIV-1 subtype B missed by the assays' design. Recently, details of these 2 transmission cases and a number of related cases of false-negative HIV-1 NAT screening results have been published [8–12]. In addition, we must emphasize that cases of false-negative HIV-1 NAT based on low viral load combined with suboptimal NAT sensitivity in the screening of mini pools cannot be excluded. Fortunately, until now, respective transmissions have not been

experienced in Germany. However, a residual risk of HIV transmission exists even with NAT performed for each individual donation (ID-NAT).

There have been several reports of underquantitation of the viral load by CE-marked quantitative (diagnostic) HIV-1 NAT, traceable also to the assays' design [13–16]. These false-negative or underquantified test results occurred with screening or diagnostic NAT assays targeting 1 region of the HIV-1 genome in combination with new HIV-1 variants. The nucleic acid sequences of these new HIV-1 subtype B variants were not available at the time of assay design. In some cases, in vitro diagnostic medical device (IVD) manufacturers reacted promptly by adapting the assay design, either by adapting primer/probe sequences for inclusion of new viral variants [9] or by inclusion of a second target region into the assay design [17, 18].

Potential risk minimization measures have been discussed at both the European and national regulatory level. While risk assessment for quantitative diagnostic HIV-1 NAT assays negated the necessity of immediate regulatory consequences at the European level, risk assessment for blood-screening HIV-1 NAT assays resulted in our requirement for dual-target design, or analogous safety solution, at national level. This requirement has been defined in the context of quality control of blood components, which are regulated under the German Drug Law. A similar discussion is currently underway at the European level regarding a potential update of the Common Technical Specifications for qualitative HIV-1 NATs [19].

Material and Methods

Plasma Specimens

Blood collection centers provided samples from blood donations that tested falsely negative in CE-marked NAT assays used for screening (cases 1–6). The viral load was determined using different quantitative HIV-1 NATs targeting different regions of the viral genome. The mean

viral load for cases 1–6 was determined using quantitative NAT assays that were thought to be not affected by the mutations described for the cases. Based on the mean value obtained by the different proficient quantitative assays, neat plasma and dilution series (in pooled negative human plasma) were subsequently used in replicates for comparative testing of a variety of different qualitative HIV-1 NATs. Furthermore, sequence analysis of NAT target regions in the viral genome was performed.

Comparative Study with Different HIV-1 NAT Assays

The comparative study (more details published in [11]) was extended with plasma of the more recent case 6 (published as Case 2 in [12]). Briefly, 11 CE-marked NAT assays for HIV-1 RNA detection (5 qualitative, 6 quantitative assays, see table 1) were included in the comparative study with materials from the 6 NAT false-negative cases. Comparative testing of plasma samples from the cases 1–6 was performed on replicate dilution series of the HIV-1 RNA-positive specimens. Detection efficiency of individual assays for material from the 6 cases was determined by assessment of the test results obtained (e.g. IU/ml, ct values, sample/cut-off values or relative light units) in comparison to the results obtained with the PEI HIV-1 RNA reference preparation.

Sequence Analysis

Details of sequence analysis have already been published [8, 11, 12]. Sequences derived from the amplicons representing 243 bp of the 5' long terminal repeat (LTR) region (nucleotides 483–725) were aligned and compared with the corresponding sequence of the HIV-1 prototype HXB2 (accession number K03455) (fig. 1). This part of the 5'LTR region covers the target region of the assays VSPK, artus and CTS MPX.

Information Exchange with Blood Donor Centers and Experts

A survey was initiated at the end of 2010 in which all German blood donation centers were asked to provide information regarding their experience with underquantitated/false-negative HIV-1 RNA test results for HIV-infected blood donors. Furthermore, an expert meeting was

held at the PEI on 8 June 2011 to exchange information with NAT manufacturers, blood donation centers and virological experts taking care of HIV-infected patients. Potential measures for risk minimization were discussed, e.g. mandatory requirements with regards to assay design or increasing the minimal sensitivity limits for HIV-1 RNA screening.

Results

Laboratory Investigations

The 6 recent cases of HIV-1 RNA-positive blood donations missed by the screening NAT system were reported by various establishments dealing with blood in Germany to the PEI (table 2). Details of these cases have already been published [8, 11, 12]. The 6 donors (1 female, 5 male) had not reported any HIV risk factors on the routine eligibility-screening questionnaire. The NAT assays concerned were 4 different CE-marked screening tests (CAP CTM v1, CTS MPX, VSPK v1.1, VSPKv1.2) from 2 manufacturers (Roche Molecular Systems and GFE Blut mbH). 2 of these cases resulted in HIV transmission to the recipients by transfusion of the corresponding red blood cell (RBC) concentrates.

The 6 cases (table 2) were missed by the routine NAT assay despite viral RNA at a concentration level estimated sufficient for detection by the screening NAT system in place. We investigated material from these cases in a variety of different HIV-1 NAT assays. The results are summarized in table 3 in a semi-quantitative manner describing the relative detection efficiency of the assays. The results show that assays

Table 2. HIV-1 NAT non-detection cases

Case	Donor type, sex, age	Donation dates	Screening NAT	HIV screening results	Viral load, IU/ml	Status	Transmission
1	RD, male, 44	Jan 2007	CAP CTM v1	Ab neg RNA neg	10,000	WP	RBCs
		Apr 2007		Ab pos RNA neg	650	SC	n-t
2	RD, male, 22	Jul 2007	CAP CTM v1	Ab neg RNA neg	0	HIV-neg	no
		Oct 2007		Ab pos RNA neg	80,000	SC	
3	RD, male, 26	May 2009	CTS MPX	Ab neg RNA neg	0	HIV-neg	no
		Aug 2009	CTS MPX	Ab neg RNA neg	20,000	WP	RBCs
		Jul 2010	VSPK v1.1	Ab pos RNA pos	260,000	SC	n-t
4	RD, male, 42	Mar 2010	VSPK v1.1	Ab neg RNA neg	0	HIV-neg	no
		Jun 2010		Ab neg RNA neg	0	HIV-neg	no
		Oct 2010		Ab pos RNA neg	200,000	SC	n-t
5	FTD, male, 18	Oct 2010	VSPK v1.1	Ab pos RNA neg	2,000	SC	n-t.
6	FTD, female, 31	Jul 2012	VSPK v1.2	Ab pos RNA neg	19,000	SC	n-t

RD = repeat donor; FTD = first time donor; Ab = anti-HIV1/2 antibody; WP = window period; SC = seroconversion; RBCs = red blood cells; n-t = not transfused.

of related design may be affected by the same viral variant; furthermore, HIV-1 NATs of dual-target design did not provide false-negative results, although lower reactivity may be observed with the failure of 1 of the target regions. In our definition dual-target design is characterized by the creation and detection of 2 different amplicons based on 2 different sets of primers and probes.

We performed nucleotide sequence analysis of the corresponding LTR target region and aligned sequences derived from amplified viral RNA from cases 3–6 and the PEI HIV-1 RNA reference preparation with the HIV-1 prototype HXB2 sequence (fig. 1). There was high homology between sequences of primers and probes used in NAT assays and the HIV-1 prototype HXB2 sequence. When compared with HXB2, the sequence of the PEI HIV-1 RNA reference preparation exhibited 5 nucleotide exchanges in this LTR part (nucleotides 483–725), without an effect on the efficiencies of the different NAT assays studied. In contrast, the viral variants exhibited remarkable differences to the HXB2 sequence, both in regard to point mutations and major sequence deletions (cases 3 and 6). Case 6 exhibited an additional upstream insertion [12] not covered by the alignment in figure 1.

Results of Survey and Expert Meeting

The data basis at the PEI with regards to blood-screening NAT failures may be incomplete since anti-HIV-1/2-positive blood donations do not need to be further investigated using different NAT assays. To obtain a broader data basis for decision making, the PEI decided to perform a survey among all blood donation centers in Germany, and to ask for further NAT results that differed between various NAT assays or for improbable combinations of test results, e.g. anti-HIV1/2-positive/HIV-1 NAT negative. For the period 2007–2010, 17 cases were reported to have shown negative results in an HIV-1 NAT test for donors who were proven to be HIV infected

(3–5 cases per year). In 14 of the 17 cases, the serological screening test was positive, and the donations were not released for further processing and the donors banned. NAT testing of mini pools may have compromised sensitivity. There is no requirement for in depth analysis of such cases; therefore, we are not aware of so-called elite controllers (anti-HIV-positive, ID-NAT negative) in this group.

The remaining 3 cases were from anti-HIV-negative HIV-infected donors. In 1 case, no transmission of the virus occurred despite the transfusion of a blood component from the HIV-infected donor [7]. Furthermore, there were the 2 cases of negative NAT and negative serological screening with blood products that were released and caused HIV transmissions to the recipients (cases 1 and 3 of this manuscript and of [11]). With regard to the donors tested and the reports for the period 2007–2010, the frequency of HIV transmissions was calculated as 1 per 9.64 million donations.

These results were discussed at a meeting at the PEI with the participation of experts from blood donor centers, virological institutes and IVD manufacturers. The cases 1–5 (case 6 is more recent) led to questions being raised about a potential increase in the risk of HIV-1 variants by newly evolving mutations. The experts from virological institutes confirmed that in HIV patients there was an increase of viral variants that could not be detected or were underquantitated by CE-marked NAT assays. They estimated that it could only be a matter of time before such variants entered the blood donor population. A regular assessment of public sequence databases (such as the “Los Alamos National Laboratory HIV Sequence Database”) was estimated to be of only limited benefit for the determination of future risks since the publication of viral sequence data following current epidemiological developments occurred with considerable delay.

The question of whether increasing the minimal sensitivity defined for blood donations in Germany (10,000 IU/ml)

Table 3. Comparative investigation of CE-marked HIV-1 NAT systems using material from cases 1–6

Case	FN routine NAT	HIV-1 target regions										
		CAP CTM v1 <i>gag</i>	HPS CTM v1 <i>gag</i>	CASv1.5 <i>gag</i>	CAM v1.5 <i>gag</i>	CTSM PX LTR	VSPK v1.1 LTR	artus LTR	Abbott RT <i>pol</i>	VSPK v1.2 LTR	CAP CTM v2 <i>gag</i> + <i>LTR</i>	Ultrio Plus <i>pol</i> + <i>LTR</i>
1, 2	CAP CTM v1	((+))	--	+	+	+	+	+	+	+	(+)	+
3	CTS MPX	(wd)	+	+	+	--	+	+	+	+	(+)	+
4, 5	VSPK v1.1	(wd)	+	+	+	+	--	((+))	+	+	+	(+)
6	VSPK v1.2	(wd)	+	+	+	--	(wd)	--	+	--	(+)	(+)

LTR = long terminal repeat; *gag* = group-specific antigen; *pol* = polymerase; -- = no detection; ((+)) = highly reduced (factor > 10) detection efficiency; (+) = moderately reduced (factor < 10) detection efficiency; + = consistent detection efficiency; FN = false-negative; (wd) = test version withdrawn by the manufacturer.

Fig. 2. Reduction of diagnostic window by increase of minimal sensitivity. Course of viremia (RNA kinetics) prior to seroconversion (anti-HIV). Due to HIV-1 RNA doubling time (t_d) of 17 h a 10-fold increase in minimal individual donation sensitivity (e.g. 10^4 IU/ml to 10^3 IU/ml) is calculated to result in a reduction of the diagnostic window by 2.3 days ($17 \cdot \log_2(10)$).

would be another potential safety measure was raised. However, with an average doubling time for HIV of 17 h during the diagnostic window [20], an exemplary 10-fold increase of minimal sensitivity (10,000 to 1,000 IU/ml) would reduce the diagnostic window period only by 56 h or 2.3 days (fig. 2); an exemplary 100-fold increase would reflect current ID-NAT sensitivity and reduce this phase by 4.6 days.

Regulatory Requirement of the PEI

Based on the cases summarized above and discussions, the PEI decided to require from 1 January 2015, dual target HIV-1 NATs (or an ‘analogous solution’) for the screening in Germany of cellular blood components, therapeutic individual plasma and stem cell preparations for hematopoietic reconstitution [21]. The PEI defines as dual-target assays those HIV-1 NAT tests in which 2 different amplicons are created for the detection of HIV-1 RNA. In this definition, the PEI has refrained consciously from detailed specifications, e.g. with regard to separated or non-separated (multiplex) amplification and detection approaches or with regard to the selection of target regions. In the validation studies proof must be provided that the failure of 1 region can be compensated for by the other. Since respective naturally occurring virus variants will be available only for few cases, validation studies may use in vitro-transcribed RNA fragments or an unlabeled probe (for 1 of the target regions). Adherence to the Common Technical Specifications [19] is not changed with this decision. The validation of tests manufactured and used in-house need to be performed accordingly. Fulfillment of the requirements has to be provided for each target region. The requested minimal sensitivity will not be changed at this time. The IVD manufacturers requested that no exclusive technical requirement (like dual-target design) be stipulated since other

solutions of equivalent impact might appear. Responding to this comment, the PEI requirement is in principle open for 'analogous solutions', i.e. future technical alternatives to the dual-target approach, despite lack of real examples at the time being.

Discussion

The 6 cases of HIV-1-screening NAT failures have already been published separately [8, 11, 12] and are now summarized in this publication. They showed false-negative NAT test results for blood donors whose HIV infection was detected by reactive anti-HIV-1/2 enzyme-linked immunosorbent assay (ELISA) (table 2). The cases concern 4 recently seroconverted repeat donors (cases 1–4) and 2 first time donors with currently unknown temporal infection history (cases 5 and 6). They occurred at different blood collection sites in Germany, and 4 different NAT systems of 2 manufacturers were affected by the false-negative test results. 2 diagnostic window phase donations missed by the routine screening NAT led to HIV-1 transmission to the RBC recipients. These cases were picked up by procedures established in establishments dealing with blood in case of HIV-1-positive donors: Look-back procedures are in place to trigger the re-analysis of specimens from previous donation(s) in cases of seroconversion of a repeat donor. Comparative testing including further assays may be performed if a case of a less probable combination of test results, e.g. anti-HIV1/2 positive, screening NAT negative. A related publication describes false-negative or underquantified results with the mono-target assays COBAS AmpliScreen HIV-1 Test v1.5 and COBAS Amplicor HIV-1 Monitor Test v1.5 with viral variants circulating in Italy [10]. Another publication from Germany described new genetic polymorphisms in the LTR region affecting an in-house developed real-time PCR blood-screening test [9].

However, related test failures also occurred in the diagnostic field, resulting in underquantitation of the viral load in HIV patients or false-negative NAT results [10, 13–16]. This kind of test failures in the diagnostic field may not become obvious as long as there are no discrepancies revealed using comparative NAT assays. There are now several publications describing test failures of diagnostic NAT assays identified by systematic comparative analysis of large numbers of individual patient specimens. These test failures (false-negative, underquantitation of viral load) occurred exclusively with mono-target assays measuring viral variants that exhibited mismatches with primer or probe sequences of the NAT target region. All of these variants were not known at the time of the assay development and, therefore, could not be included into the assay design.

Concerning risk minimization, different scenarios can be considered. The risk associated with a false-negative test result or with underquantitation of the viral load depends on

the field of assay application. This risk is estimated to be significantly lower with the diagnostic compared to the screening application of NAT assays. Quantitative HIV-1 NATs are applied in the diagnostic field to initiate and/or monitor antiretroviral therapy in known HIV-infected patients. In addition, in many patients further markers are determined (e.g. CD4) contributing to the therapeutic decision. Therapy-induced kinetics of viral load are monitored, which may reveal useful information for patients even with an assay underquantifying the viral variant. Furthermore, the manufacturer of the quantitative NAT assay associated with several reports of underquantitation in HIV-positive patient specimens reacted promptly by upgrading the assay with a second target region (dual-target assay) [17]. Specimens missed or underquantitated by the previous mono-target assay version were shown to be diagnosed correctly by the new assay version [18], and, so far, no major deficiencies have been identified with the improved assay design.

In contrast, in blood screening, the HIV-1 NAT is used to identify individuals donating during the diagnostic window phase of their recent infection, which by definition is not yet detected by anti-HIV1/2 assays. There is no second assay performed to detect early HIV-infection-phase donations, and, therefore, false-negative HIV-1 NAT results may allow the direct release of blood component(s), potentially causing infection in the recipient(s). Unfortunately, 2 HIV-1 infection cases of this kind have occurred in RBC recipients.

These latest false-negative HIV-1 NAT results have all occurred with screening NATs comprising a single amplification system designed for HIV-1 group M sequences. The risk of a false-negative test result appears to be proportionally lower with more than 1 amplification target region in a screening NAT assay designed for detection of a highly variable virus like HIV-1. We recognize that there are mono-target 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 PEI decided to require the use of improved screening NATs from the January 1, 2015. The interim period between announcement and mandatory implementation should give IVD manufacturers sufficient time for respective developments. Nevertheless, there are several manufacturers already offering CE-marked dual-target assays. Performance evaluation studies should provide sufficient evidence for compliance with the Common Technical specifications. Validation studies of dual-target approaches should consider both target regions separately to show compensation of failure of 1 target, and thus showing proof of concept. The PEI requirement considers alternative solutions other than dual-target assays as equally acceptable provided that they display a similar safety effect. The published requirement, therefore, does not define the dual-target approach as the only technical solution. However, at the time being technological details of alternative solutions are not so easy to imagine.

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The authors state that there is no conflict of interest relevant to this manuscript.

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