Nucleic acid amplification technology screening for hepatitis C virus and human immunodeficiency virus for blood donations

Mohammad S. Bamaga, MSc, PhD, Fawzi F. Bokhari, BSc, AbdulRahaman M. Aboud, BSc, Meshal Al-Malki, BSc, Faris Q. Alenzi, MSc, PhD.

ABSTRACT

Objectives: To investigate the performance of the commercial Roche COBAS AmpliScreen™ assay, and demonstrate whether the COBAS AmpliScreen™ human immunodeficiency virus-1 (HIV-1) test, v1.5, and COBAS AmpliScreen™ hepatitis C virus (HCV) v 2.0 for screening for HIV-1 and HCV RNA in the donated blood units from which plasma mini pools were collected, by nucleic acid amplification technology (NAT), could detect the positive pools and reduce the risk of transmission of infections for those routinely tested by serological assays.

Methods: The study was performed on 3288 plasma samples collected from blood donors in a period of 13 months, from August 2004 to August 2005, at Al-Hada Armed Forces Hospital, Molecular Pathology Laboratory, Taif, Kingdom of Saudi Arabia. The samples were tested by the reverse transcriptase polymerase chain reaction (RT-PCR) after RNA extraction (this represents the major method in NAT assays), in parallel with the routine serological testing to detect qualitatively for HIV-1 and HCV.

Results: The NAT assays that include an automated COBAS AmpliPrep™ system for RNA extraction and COBAS Amplicor™ Analyzer using AmpliScreen™ kits for RT-PCR assays, and the routine serological screening assays for the detection of the HIV-1 and HCV RNA in the plasma samples from the blood donors have shown to be a reliable combination that would meet our requirements. The collected data further confirms the results from the serological assays and enables us to decrease the residual risk of transmission to a minimum with the finding of no seronegative window period donation. The results demonstrate that out of 3288 samples, the percentages of RT-PCR (NAT) negative blood donations that were also confirmed as seronegative were 99% for HCV, and 99.1% for HIV-1.

Conclusion: The modified combined systems (automated COBAS AmpliPrep™ system for RNA extraction and COBAS Amplicor™ Analyzer using AmpliScreen™ kits for RT-PCR assays) for NAT screening assays has allowed the release of all blood donations supplied in the specified period of the study with no seronegative window period donations. This facilitates keeping the residual risk of transmission of HIV-1 and HCV to its minimum through blood transfusion.

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There is increased public concern for the safety of blood transfusion in regard to transfusion-transmitted infections especially infections caused by hepatitis C virus (HCV), human immunodeficiency virus (HIV) and hepatitis B virus (HBV). The prevalence of HIV-1 was shown to be 0.15% and HCV 0.63% among our blood donors in the population group served by our hospital. This was

From the Department of Molecular Pathology (Bamaga, Bokhari, Aboud, Al-Malki), Al-Hada Armed Forces Hospital, Taif, and the Department of Medical Laboratory Sciences (Alenzi), College of Applied Medical Sciences, King Faisal University, Dammam, *Kingdom of Saudi Arabia*.

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Address correspondence and reprint request to: Dr. Mohammad S. Bamaga, Department of Molecular Pathology, Al-Hada Armed Forces Hospital, PO Box 1347, HHRC 479, Taif, *Kingdom of Saudi Arabia*. Tel. +966 (2) 7541610/1028. Fax. +966 (2) 7541238. E-mail: mbamaga@hotmail.com

based on the screening performed by serological testing only. In addition, it was shown by another investigator in another region to be 0.4% for HCV, and zero for HIV.[1] The prevalence of HCV and HIV-1 varies among the local regions and more studies are needed for an overall estimation. However, the risk of transmitting infection to transfusion recipients has been now reduced drastically, and this is due to the careful selection of the donors and the continuous improvement of the serologic screening assays. The problem of the serologic screening assay is the inability to detect donors with very recent infections during the pre-seroconversion, in other words, the "window periods" of the viral infections. Furthermore, some donors may exhibit negative antibody testing against HCV and HIV-1 that may allow them to escape the routine serological screening of blood. In 1999, the estimated risk for transmission of HCV and HIV-1 by a single antibody negative component was quoted as 1/150,000 for HCV and 1/676,000 for HIV-1.[2] Viremia in HIV-1 infection precedes the antibody response in the symptomatic primary infection,[3] and tests employing nucleic acid technologies were used to detect the infecting viruses RNA or DNA. The aim of using such testing was to reduce the window of infectivity that is not possible with the current serological screening tests. The window period can be reduced by as much as 60 days for HCV and 11 days for HIV infections. However, the introduction of nucleic acid testing (NAT), for HCV and HIV-1 RNA, has considerably reduced the risk of window period transmission.[4] The recent development of commercially available assays using NAT assays has allowed many blood donation facility centers to consider applying such tests to screen blood donors. Individual blood unit testing by the current NAT does not have sufficient reasonable costs. Testing of mini-pools made up of small aliquots from several individual units has been proposed. Numerous studies have shown that mini-pool testing can be incorporated into existing blood donor screening programs, and that it should be able to detect window period infections in blood donations.[5-7] Availability of the ready to use, quality-controlled (QC) commercial PCR tests might be used for mini-pools testing to provide blood and plasma centers the need to devote inordinate resources to the design, manufacture, validation, and QC of their own reagents and methods of in-house developed reverse transcriptase polymerase chain reaction (RT-PCR) screening assays.[7] The number of samples used in the NAT testing per each mini-pool may vary, which usually accommodates the requirements of the blood donation centers for overall sensitivity of the testing as well as for the financial expenditures. The number of samples used in the mini-pools for screening of HIV-1 and HCV has been suggested to be of critical importance to minimize false-negative in the NAT testing.[8,9] In this study, we record the performance of the commercially available Roche COBAS AmpliScreen™ assays, and demonstrate that the COBAS AmpliScreen™ HIV-1 test, v1.5, and COBAS AmpliScreen™ HCV v 2.0 meets our specifications for screening donated plasma mini-pools, and could detect the positive pools and reduce the risk of transmission of infections for those tested routinely by serological assays.

Methods. ***Specimens.*** To detect qualitatively for HCV and HIV-1, and to assess our newly adapted NAT method by using 2 COBAS AmpliScreen™ systems for HCV test, version 2.0, and HIV-1 test, version 1.5, 3288 individual plasma samples (from August 2004 to August 2005) were collected, drawn at the same time of collection of the blood donation.

Plasma-pooling preparations. The plasma samples were all put into 1.5 mL (at least 1 mL for each plasma sample) microcentrifuge tubes then mini-pooled, consisting of 2 donor's samples, and were prepared by mixing the 250 μl of each donor's plasma to make up the final volume of 500 μl. The original donor's plasma sample was then archived for storage at -24^{o}C for a minimum of 6 months before being discarded. If there is a need to test an individual plasma sample again, the archived donor's sample is used and tested individually.

RNA extraction from plasma samples. A volume of 500 μl of the mini-pools consisting of 2 donors' samples were loaded into specialized tubes, and checked for the correct volume against standardized volume tubes of the same type. All mini-pools were then loaded into the COBAS AmpliPrep™ system using a modified protocol COBAS AmpliPrep™ Total Nucleic Acid Isolation (TNAI) kit, Roche. The modification was in the Multiprep Internal Control (MP IC) addition of the COBAS AmpliScreen MultiPrep™ Specimen Preparation and Control kit, Roche. One vial of MP IC was added to the Multi-Reagent cassette (TNAI CS3) used in the TNAI kit and approximately 950 μl of the Internal Control diluent was added and mixed gently. The remainder of the extraction of the RNA for HCV and HIV-1 was according to the manufacturer's protocol, as stated in the insert of the COBAS AmpliPrep™ TNAI kit and the COBAS AmpliPrep™ operation manual. At the end of the RNA extraction steps, a total volume of 70 μl was used to prepare for the HCV and HIV-1 RT-PCR mix for each mini-pool tested.

RT-PCR Amplification and detection. Both RT-PCR assays were carried out as instructed by the COBAS AmpliScreen™ HCV, version 2.0, and COBAS AmpliScreen™ HIV-1, version 1.5, Roche, as stated in the inserts accompanying the kits.

Hybridization Reaction. Following PCR amplification, the COBAS Amplicor™ Analyzer automatically adds denaturation solution to the reaction tubes to chemically denature the HCV or HIV-1 amplicon in each test of AmpliScreen™ and the HCV or HIV-1 Internal Control amplicon to form single-stranded DNA. Aliquots of denatured amplicon are then transferred to 2 detection cups. A suspension of magnetic particles coated with an oligonucleotide probe specific for HCV or HIV-1 amplicon as well as HCV or HIV-1 Internal Control amplicon is added to the individual reaction tube. The biotin-labeled HCV target and HCV Internal Control amplicon or HIV-1 target and HIV-1 Internal Control amplicon are hybridized to the target-specific oligonucleotide probes bound to the magnetic particles. This hybridization of amplicon to the target-specific probe increases the overall specificity of the 2 AmpliScreen™ assays for amplification and detection of HCV and HIV-1 RNA.

Detection Reaction. Following the hybridization reaction, the COBAS Amplico™ Analyzer washes the magnetic particles in the detection cups to remove unbound material, and then adds avidin-horseradish peroxidase conjugate. The avidin-horseradish peroxidase conjugate binds to the hybridized biotin-labeled amplicon. The COBAS Amplicor™ Analyzer removes unbound conjugate by washing the magnetic particles and then adds a substrate solution containing hydrogen peroxide and 3,3',5,5'-tetramethylbenzidine (TMB) to each detection-cup. In the presence of hydrogen peroxide, the particle-bound horseradish peroxidase catalyzes the oxidation of TMB to form a colored complex. The absorbance is measured by the COBAS Amplicor™ Analyzer at a wavelength of 660 nm. Hence, the 4 amplification products generated from HCV and IC or HIV-1 and IC target RNA, were detected calorimetrically after hybridization to HCV-specific and IC-specific oligonucleotide probes or HIV-1-specific and IC-specific oligonucleotide probes bound to magnetic particles.

Serological assays screening for HCV and HIV. All donations were screened as follows: for HCV antibody detection a HCV version 3.0, Abbot, AXSYM® system, kit was used and it is a microparticle enzyme immunoassay for the qualitative detection of antibody to hepatitis C virus (anti-HCV) in human serum or plasma. The kit was used exactly as instructed in the manufacturer's assay manual. For HIV immunological testing an Enzygnost® HIV Integral, Dade Behring, was employed and it is an enzyme immunoassay for the qualitative detection of HIV infection based on the determination of HIV p24 antigen, antibodies to HIV of types 1 and 2 (HIV-1 and HIV-2), as well as against antibodies to HIV-1, subtype O, in serum and plasma. The enzyme immunoassay is processed on a special ELISA processor for testing single samples and not pooled or diluted samples. The protocol used followed the manufacturer's instructions.

Determination of specificity and limit of detection for the NAT assay using the modified RNA extraction protocol. The modified RNA extraction protocol was validated by using VeriSure™ Triplex Controls, AcroMetrix, Benicia, California, USA. The VeriSure™ Triplex HIV-1/HCV/HBV External Quality Control was developed for use with the COBAS AmpliScreen™ HIV-1 Test, the COBAS AmpliScreen™ HCV Test and the COBAS AmpliScreen™ HBV Test. The controls are intended to provide a means of estimating accuracy, and has the potential for detecting systematic deviations of nucleic acid test procedures for the qualitative determination of HIV-1 RNA, HCV RNA and HBV DNA (HBV has not been studied in this setting). The controls were used following the manufacturer's recommendations in the accompanying insert. The detection limit was determined by using pre-determined quantitative PCR with a high viral load of patients' serum specimens sent to our laboratory for quantitative RT-PCR by using COBAS Amplicor™ HCV Monitor test, version 2.0 and consequently several serial dilutions were prepared from each specimen mixed with negative human plasma. These dilutions were prepared for NAT assay, similarly to those collected from blood donations. A similar process was used for the determination of detection limit for HIV-1 using COBAS AmpliScreen™ HIV-1 test with high viral load patients' specimens that have been tested already by COBAS Amplicor HIV-1 Monitor™ test kit. All the positive specimens with high viral load used for the determination of detection limit had been tested prior to the NAT testing and were stored for 2-4 months -70^{O}C prior to the preparation of serial dilutions.

Results. In our study we have applied the RT-PCR testing for the detection of HCV and HIV-1 RNA in the plasma collected from the donors in a mini-pool of 2 individual samples, this is the least in the mini-pools tested so far worldwide, for the period from August 2004 to August 2005 before the release of labile components. This allowed the screening by RT-PCR

Table 1 - Hepatitis C virus (HCV) screening results using reverse transcriptase polymerase chain reaction and antibodies by microparticle enzyme immunoassay.

Month	Year	No. of screened blood units	Inhibition in PCR	HCV Ab	No. of PCR results	
					Positive	Negative
August	2004	216	0	3	1	215
September	2004	328	0	4	3	325
October	2004	166	0	0	0	166
November	2004	136	0	1	0	136
December	2004	236	8	2	2	226
January	2005	292	7	6	1	284
February	2005	246	7	0	0	239
March	2005	258	0	0	0	258
April	2005	306	2	0	0	304
May	2005	354	1	9	0	353
June	2005	252	0	0	0	252
July	2005	244	0	6	0	244
August	2005	254	0	4	0	254
Total		**3288**	**25**	**35**	**7**	**3256**

PCR - polymerase chain reaction

of a total of 3288 donations using the above mentioned Roche systems for HCV and HIV-1 RNA. The average of the units tested per months was approximately 252, and the mean time needed for the screening by PCR was 7.6 hours that included preparations of the plasma sample, mini-pooling (manual), RNA extraction by COBA AmpliPrep™ and performing of NAT by COBAS Amplicor™ Analyzer system, Roche. The entire procedure was easily managed by one skilled technologist, valid runs of NAT testing were explained by testing of the external controls in both kits of the COBAS AmpliScreen™ system for HCV and HIV-1 and per individual test for each mini-pool when the internal controls in each reaction tube were positive. Each invalid run of external controls per run was repeated, this usually consists of 10 mini-pools and one positive and one negative control supplied with the kits. In this manner, the inhibition of the test for each mini-pool was repeated and tested individually per donor (no pooling). If the individual blood unit of each donor tested again showed inhibition, the blood unit will not be released (**Tables 1 & 2**).

HCV and HIV antibody serological and RT-PCR screening assays. The antibody screening for the donors' samples was carried out in the immunochemistry laboratory on the same day NAT assays were performed and in some instances, the NAT assays testing were giving an earlier turnaround time of 2 hours. The results obtained during the 13 months period of the HCV and HIV-1 NAT mini-pools screening by using the modified procedure AmpliPrep™/AmpliScreen™ method, are summarized in **Tables 1 & 2**. Invalid (inhibitions) results of the mini-pools tested were 0.76% for HCV and 0.79% of HIV-1 and HCV RT-PCR and HIV-1 RT-PCR positive were 0.21% and 0.0% of the mini-pools tested.

Antibody negative and RT-PCR positive. During the period of the study, there were no RT-PCR positive mini-pools that have been tested and found anti-HCV negative. This represents that there is no seronegative window period donation during the study period. The HIV-1 mini-pools screening also did not show any seronegative window period donation with RT-PCR positive in the specified period. The total number of donors tested is 3288 in the specified period (see discussion).

Antibody positive and RT-PCR positive. The total number of anti-HCV positive donors plasma samples that were confirmed by our RT-PCR method during the period of the study was 7 (0.20%). There were inconsistencies between the results of 28 donors' plasma specimens with anti-HCV positive and negative by our RT-PCR method. This represents 80% of the 35 anti-HCV positive by the method used (see discussion section). Additionally, there were inconsistencies between the result of anti-HIV screening and the RT-PCR. Six donors' plasma specimens tested positive with the commercially available HIV immunological testing (Enzygnost®

Table 2 – Human immunodeficiency virus-1 (HIV-1) screening results using reverse transcriptase polymerase chain reaction and antibodies by the enzyme immunoassay for the qualitative detection of HIV-1 and 2.

Month	Year	No. of screened blood units	Inhibition in PCR	HIV Ab	No. of PCR results	
					Positive	Negative
August	2004	216	0	2	0	216
September	2004	328	0	0	0	328
October	2004	166	0	0	0	166
November	2004	136	0	0	0	136
December	2004	236	8	0	0	226
January	2005	292	7	0	0	285
February	2005	246	7	0	0	239
March	2005	258	0	3	0	258
April	2005	306	2	0	0	304
May	2005	354	1	0	0	353
June	2005	252	0	0	0	252
July	2005	244	0	1	0	244
August	2005	254	1	0	0	253
Total		**3288**	**26**	6	**0**	**3260**

PCR - polymerase chain reaction

HIV Integral kit, Dade Behring, Germany). None of these 6 samples tested positive with our RT-PCR assay; the results are presented in **Table 2**. It was not shown to be a false-positive since the assay was repeatedly used to confirm the result of the mini-pools on the individual donor's plasma specimens of the 6 positive anti-HIV results found.

Antibody negative RT-PCR negative. For anti-HCV screening, it was observed from the obtained result during the period of the study that 3219 (97.9%) of the total number of tested plasma samples were proven negative by serological assay screening. In turn, the RT-PCR assay screening for plasma samples that were negative were 3256 (99%), suggesting more non-viremic, (negative) HCV RNA, infection. While negative anti-HIV screening was 3253 (98.9%), the negative for RT-PCR assay screening was 3260 (99.2%) of the total donors' plasmas samples (**Tables 1 & 2**). Both serological assays and RT-PCR assays screening results were collected separately at the same time of testing.

Inhibitions of the RT-PCR screening assays. The results of the RT-PCR screening assays for HCV and HIV-1 was delayed by one extra day as some donors' plasma may contain inhibitory substances and the percentage was as low as 0.76% and 0.79% for HCV and HIV-1 (**Tables 1 & 2**). It was noticed that most of the inhibitions were during December through February.

Specificity and limit of detection of the NAT assay and the modified RNA extraction protocol. The results obtained from the modified extraction protocol using COBAS AmpliPrep™ TNAI kit (modified protocol), Roche, were in agreement with COBAS AmpliScreen™ Multiprep Specimen Preparation and Control kit (standard protocol), data are not shown. The limit of detection was determined by the comparison of the data collected from COBAS Amplicor™ HCV Monitor test, version 2.0 kit, and we found that the data were in correlation with the COBAS AmpliScreen™ HCV Test. Similarly, the data from the COBAS Amplicor HIV-1 Monitor™ test. No data are shown (see Methods section).

Discussion. In this paper we demonstrate that the combination of the AmpliPrep™ RNA extraction, amplification and then detection by using AmpliScreen™ kits operated on the COBAS Amplicor system, is reliable and robust for blood donor screening for HCV and HIV-1 RNA. The assay was monitored for the results verification on daily runs to assure the accuracy of the results obtained from each mini-pool against the internal controls and the positive and negative controls for HCV and HIV-1. These controls are included in each daily run to take up to 16.7% of all runs for the tested mini-pools during the study. The invalid result for the runs was insignificant, 3% for HCV and HIV-1 screening. Most of the invalid runs were at the early stage of the implementation of the NAT screening and later they were all valid, possibly due to technicality of

the whole system, namely, AmpliPrep™ extraction, AmpliScreen™ kits and COBAS Amplicor™ Analyzer system. Furthermore, the performance of this combination for NAT screening has shown a low percentage of inhibitions (invalid) mini-pools. In many instances (approximately 73%), the inhibition of the mini-pools tested negative when repeated individually. The reason is unknown, and further investigations may be needed, this may not be due to the presence of PCR inhibitors in the donors' samples. In the present study, this combination was not in the original protocol of the AmpliScreen™ kits and perhaps there is a need for approval by Roche and the Food and Drug Administration, USA, before it can be launched to the users. However, the combination did not show any significant decrease in the limit of detection in our primary validation compared with the original protocol for extraction by MultiPrep™ kit. This confirms the previous findings by others.[7,10,11] The implementation of commercial NAT assays screening for HIV-1 and HCV RNA in the mini-pools samples has not contributed in the delay of the results release of each unit of donated blood. The results were however, independent of the serological testing and were collected sometimes earlier than the release of HIV-Ab and HCV-Ab. The detection limit was in accordance with the WHO standards in our primary evaluations of the combination protocol and our observations of these standards did not show any unsatisfactory results as compared with other studies.[5] Moreover, the sensitivity of the 95% detection limit was maintained as standardized the WHO by using the VeriSure™ Triplex Controls, AcroMetrix, Benicia, California, USA (see Methods section). The residual risk for HCV or HIV-1 transmission by blood transfusion after NAT implementation is currently estimated to be extremely low in many countries, and one was shown to be the lowest among all that have been reported from Italy recently.[11] However, during the 13 months study period there was no anti-HCV negative RNA (RT-PCR) positive identified out of the 3288 donation analyzed. This might be due to the small number of the donations tested by NAT assays, comparable to the high number of donations analyzed and reported in other studies.[5,12] For this reason, the finding of pre-seroconversion for HIV-1 and HCV infections among the donors of blood would be at its early stage for our study period. Additionally, the statistically calculated risk of transmission of HIV-1 and HCV through blood donation based on the serological testing and prior to NAT assays that are implemented now is not available yet in our area. It is not statistically clear if the residual risk of transmission would be lowered after our implementation of NAT assays screening for HIV-1 and HCV among our blood donations, as this will be the results of gathering the data from an ongoing project in our area. The use of mini-pools consisting of 2 plasma samples was decided since we require the NAT testing to be performed on a daily basis without interrupting the flow of the blood units' supply in the hospital. Furthermore, the total number of donors donating annually here is considered very low comparable to the number reported by other blood bank laboratories around the world.[9] Increasing the mini-pools to 2, 4, 8, 12, 24 and 48 will approximately cost 98, 48, 24, 16, 8 and 4 SR per donor's sample, respectively, for one parameter only, HIV-1 or HCV. The mini-pool size, therefore, has a financial impact on the NAT testing per individual donor. Among the important findings here, the anti-HCV positive cases tested by the serological screening assay and negative by our RT-PCR assay, is the finding of the high percentage (80%) of the inconsistency, namely, false-positive. This is extremely important justification for implementing the NAT testing for blood bank and donation centers. At present, it is our assignment to confirm the positive cases of anti-HCV by testing them individually by NAT assay and if it is shown to be negative the blood unit should be quarantined. So far, it is recommended not to use such units and we await further recommendations by WHO and the American Association for Blood Bank. There is, however, a limitation in the availability of commercial NAT assay kits with the ability to detect HIV-1 only, and the chance for the detection of HIV-2 is not possible so far, especially using the AmpliScreen™ kits. This might justify the continuation of the routine serological testing for the blood donation. There are no data available now to indicate the prevalence of HIV-2 infection in our region to minimize the transmission risk of such infection. Regardless of the HIV-2 prevalence, it is extremely important to consider implementation of NAT screening for HCV and HIV-1 in the blood donations for maximum safety of the blood supply.

In summary, the commercially available RT-PCR assays (COBAS AmpliScreen™ kits) in combination with automated extractions for RNA (COBAS AmpliPrep™ system and using a modified protocol COBAS AmpliPrep™ TNAI kit, Roche) for HIV-1 and HCV screening of blood donations can be easily applied to the routine daily work of all blood bank laboratories or blood supply centers. During the period of the study, the blood supply flow was not interrupted by the implementation of NAT assays, and confirmation of the serologically routine assays for HIV-1 and HCV screening negativity were more

effective in lowering the peculiar residual risk of transmission of HIV-1 and HCV through blood transfusion.

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