

Magnetic bead technology for viral RNA extraction from serum in blood bank screening

ABSTRACT

Nucleic acid amplification testing (NAT) was recently recommended by Brazilian legislation and has been implemented at some blood banks in the city of São Paulo, Brazil, in an attempt to reduce blood-borne transmission of human immunodeficiency virus (HIV) and hepatitis C virus. **Objective:** Manual magnetic particle-based extraction methods for HIV and HCV viral nucleic acids were evaluated in combination with detection by reverse transcriptase - polymerase chain reaction (RT-PCR) one-step. **Methods:** Blood donor samples were collected from January 2010 to September 2010, and minipools of them were submitted to testing. ELISA was used for the analysis of anti-HCV/HIV antibodies. Detection and amplification of viral RNA was performed using real-time PCR. **Results:** Out of 20.808 samples screened, 53 samples (29 for HCV and 24 for HIV) were confirmed as positive by serological and NAT methods. **Conclusion:** The manual magnetic bead-based extraction in combination with real-time PCR detection can be used to routinely screen blood donation for viremic donors to further increase the safety of blood products.

Keywords: HCV; HIV; infection; reverse transcriptase polymerase chain reaction; blood banks.

INTRODUCTION

The risk of transfusion-transmitted hepatitis C virus (HCV) and human immunodeficiency virus (HIV) has progressively decreased during the past few decades as a consequence of the introduction of several preventive measures. These include careful donor selection, repeat donors, enhanced donor questioning for eligibility and refinement of blood donation screening.¹⁻³

The implementation of nucleic acid amplification test (NAT), together with the existing antigen/antibody-based assays for donor screening, has further reduced the residual risk of infection in the recipient by shortening the window phase. During this phase, an infected donor may harbor large amounts of infectious viral particles in the absence of serological markers and/or signs and symptoms of an ongoing infection.²⁻⁶

At present, single-donation testing is expensive,⁷ and in larger blood banks, it is not feasible due to the intensive workload. Dilution due to pool sampling and the need for the highest sensitivity has led to various methodological protocols for improvement

of extraction efficiency, such as centrifugation^{8,9} or the addition of agents that support precipitation of viral particles.

Allain¹⁰ described a residual risk of viral transmission of less than 1:250,000 for HCV and 1:1.3 million for HIV in Europe and United States in the year 2000. After introduction of NAT for HCV and HIV, the residual risk was reduced to 1:2-4 million.

Stramer et al.² reported NAT yield data from the first 4 years of NAT screening in the United States and concluded that the use of NAT prevented the transmission of approximately 5 cases of HIV-1 infections and 56 cases of HCV infections annually.

In Brazil, the residual risk for HIV-1 markers in blood donors is higher than that in the United States. Sabino et al.¹¹ and Wendel et al.¹² described an estimate of 1.56 per 100,000 donations following HIV antibody screening in repeat donors. Barreto et al.¹³ reported a residual risk for HIV of 4.1 per 1 million based on the incidence among first-time donors in São Paulo, Brazil. Using a less sensitive EIA has an NAT yield of 10.8 per 1 million donations based on published window periods.

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Brazilian legislation does not yet recommend the implementation of NAT screening for HIV and HCV. The Brazilian government has decided to create a commission to analyze the “advantages and disadvantages” before implementation of NAT,¹⁴ discussing the high cost of implementation of this test and the possible ways it could be used in the blood banks.

NAT technology has been implemented in our service since October 2008, using minipool samples ($n = 6$, 1.5 mL) for HIV/HCV, in a real-time PCR one-step test. In our laboratory (Colsan, São Paulo, Brazil), in the period of 2005 to 2010, the median point estimates of residual risk of transfusion-transmitted infections were 1 per 93,227 for HCV and 1 per 97,940 for HIV.

The introduction of real-time PCR analysis has led to considerable progress in automating the amplification and detection steps, but nucleic acid isolation remains laborious when performed manually. Compared with standard separation procedures, magnetic-separation techniques have several advantages, including simplicity in handling, a high automation potential and low cost.¹⁵

METHODS

Samples and standards

Samples from Colsan Blood Bank in São Paulo, Brazil, were tested for HIV and HCV with a NAT assay (DNA Technology of Brazil) by real-time PCR (Applied Biosystems) according to the manufacturer's instructions. In parallel, anti-HIV and anti-HCV screening were performed by enzyme-linked immunosorbent assay (ELISA) testing, as determined by Brazilian legislation.

From January 1st, 2010 to September 30th, 2010, 20,808 donations were enrolled in this study. Two hundred-fifty microliters of each serum sample were mixed in a serum pool of 6 samples. If a minipool was reactive for HIV and/or HCV, it was re-analyzed in individual reactions to determine the identity of the reactive donor. For individual tests, 300 μ L were used as a sample.

Working reagents for nucleic acid amplification techniques from DNA Technology of Brazil were used to establish the analytical sensitivity of the multiplex assay.

Control reagents contained 100 IU/mL of the RNA HCV (genotypes 1-6) and HIV-1/2. Amplification of an internal control was included in every assay to exclude false negative results due to interference of inhibitors and to ensure the performance of the nucleic acid extraction procedure.

RNA isolation by magnetic beads

Total RNA was extracted and purified using magnetic bead technology with the appropriate kit (Chemagic Viral DNA/RNA kit). Pools of 0.250 mL of serum or plasma of each sample were centrifuged for 1 hour at 25,000 rcf to form the viral pellet, and then 0.100 mL of lysis buffer was added

to each tube. The samples were vortexed and incubated at room temperature for 10 minutes.

Subsequently, 0.150 mL of the magnetic particles (magnetic beads) were added and incubated at room temperature for 20 minutes with agitation every 10 minutes (Figure 1A). The tubes were placed in a magnet specific to the separation of the magnetic beads containing the viral RNA, and the supernatant was removed (Figure 1B); 0.200 mL of wash solution was then added, and again the tubes were homogenized to wash the magnetic beads. The tubes were again transferred to the magnet to separate the beads containing the captured viral RNA. A second wash was performed with 0.150 mL, after which the beads were resuspended with 0.050 mL of elution buffer (Figure 1C). The isolated RNA (0.015 mL) was used in the real-time PCR for the detection of viral RNA (HIV or HCV).

After lysis, in a first step, magnetic beads with a specific functionalization are added to the biosuspension in order to bind the RNA target. Because of the small size of the particles, thorough mixing in the next step achieves very fast binding of the product. Subsequently, the loaded magnetic beads are retained selectively by magnetic separation and separated from the supernatant. Final washing and elution steps deliver the target product free from solids in a purified, often also concentrated form.

Reverse transcription

RNA was reverse transcribed into cDNA by the addition of Superscript III Platinum[®] One-Step in the mix containing all necessary reagents for amplification. Real-time amplification was performed using a 7500 Real-Time Sequence Detection System (SDS; ABI Prism 7500; Applied Biosystems, CA, USA). Real-time PCR product accumulation was monitored using a TaqMan probe.

Quantitative real-time PCR

Relative gene expression was calculated using conditions at the early stages of PCR. At this point, amplification is logarithmic and can thus be correlated to the initial copy number of gene transcripts. Reactions were cycled 40 times under the conditions previously determined by conventional PCR. At the end of PCR reaction, the temperature was increased from 60°C to

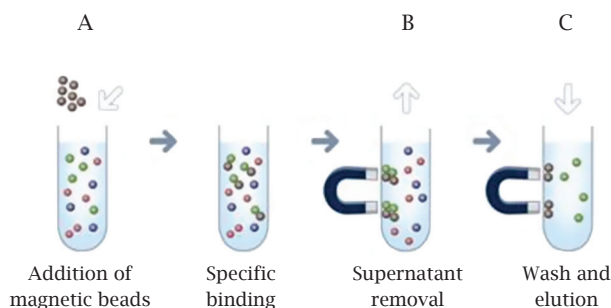


Figure 1: The basic principle of viral isolation by magnetic beads.

95°C at a rate of 2°C/min. During this time, fluorescence was measured every 15 s to construct a melting curve. A non-templated control was run with each assay.

PCR was performed with commercial primers that are selective for HIV, HCV and an internal control (DNA Technology of Brazil). PCR was performed with primers HIV sense (5' - TAA AGC TTG CCT TGA GTG CT - 3'), HIV antisense (5' - GTC TGA GGG ATC CTA GTT ACC AG - 3'), and probe HIV P (5' - FAM - AGT AGT GTG GCC GTC TGT TGT GTG - 3') are located in the LTR region of HIV-1 genome Accession Number - HM583638. HCV sense (5' - CGG GAG AGC CAT AGT GGT 3'), HCV antisense (5' - CGC GAC CCA ACA CTA CTC - 3') and probe HCV P (5' - FAM - TGC GGA ACC GGT GAG TAC ACC - 3') Accession Number - HM594184.1

Enzyme-linked immunosorbent assay (ELISA)

Testing for HCV antibodies was performed with a third generation enzyme immunoassay (EIA-3; bioMérieux Co.) following the manufacturer's instructions. S/CO ratio (S, sample ratio and CO, cutoff ratio) was determined for all the blood samples and based on this we created two groups: Group A in which S/CO ratio was < 3, and group B in which S/CO ratio was > 3.^{16,17} That algorithm was developed in order to establish the relationship between S/CO ratio and the probability RT-PCR been positive.¹⁸

Amplification and detection

Fifteen microliters of eluate were used as a template for HCV/HIV RNA reverse transcription polymerase chain reaction (RT-PCR), by a 7500 real-time PCR system (Applied Biosystems). The PCR amplification and detection was performed according to the manufacturer's instructions (DNA

technology of Brazil). Internal controls monitored the validity of the PCR in each reaction.

Statistical method

Kappa statistic was used to evaluate the agreement between tests.¹⁶ Kappa is calculated from the observed and expected frequencies on the diagonal of a square table of frequencies, with a 95% interval, used to study the reliability of nominal or categorical variables, where:

$$K = (po - pe) / (1 - pe)$$

k = kappa statistic

po = proportion of observed agreement of diagnoses.

pe = expected proportion of diagnostic agreement due to chance

In this study, the kappa statistic should be interpreted as the proportion of agreement between the two tests used beyond chance. In the case of perfect agreement, the value of Kappa = 1 (tests are consistent in all cases); Kappa is zero if the correlation is entirely due to chance, and negative when the correlation is lower than expected by chance.

RESULTS

A total of 3,468 minipools, representing 20,808 blood donations were tested simultaneously by ELISA and NAT. All pools that tested negative for the presence of these viral sequences were in accordance with results obtained from serological routine screening. In the period studied, were obtained 29 samples positive for anti-HCV and 24 samples positive for anti-HIV. All samples were also detected by NAT. No samples were positive to anti-HCV/HIV and negative by NAT (Table 1). All of the

Table 1. Samples tested for HCV/HIV NAT - Colsan (Associação Beneficente de Coleta de Sangue)

Period	Number of samples tested in minipool	Number of positive samples to HIV RNA and anti-HIV	Number of positive samples to HCV RNA and anti-HCV
January 2010	2.421	0	4
February 2010	2.193	1	3
March 2010	4.472	2	4
April 2010	1.838	0	0
May 2010	1.628	3	2
June 2010	2.033	3	5
July 2010	2.153	6	4
August 2010	2.240	4	2
September 2010	1.830	5	5
Total	20.808	24	29

Kappa = 0.99.

results were validated by the internal positive control (IPC). There was almost perfect agreement ($Kappa > 0.99$) according to the classification of Landis et al.¹⁷

DISCUSSION

Transfusion safety is one of the major concerns in the blood bank setting. In the past 30 years, great progress towards safer transfusions was made, especially concerning transmission of viruses. Such strides were made due to the adoption of good manufacturing practices, better donor screening policies, an increase of repeat donors, and new technologies used in determining the biological qualification of donors.

The NAT was first described in 1985 and began to be used in the screening of plasma products. In the late 1990s, Europe and the USA started screening blood donations for HIV and HCV. Now, it is well established that implementation of NAT is a crucial step to further reduce the residual risk of transfusion-transmitted diseases, notably HIV and HCV, to produce a safer blood inventory.

Although the introduction of real-time PCR has led to considerable progress in automating the amplification and detection steps of NAT, nucleic acid isolation remains very labor-intensive when performed manually. Traditional phenol-chloroform extraction and ethanol precipitation methods are complicated, time-consuming, hazardous, and unsuitable for processing high numbers of samples.

Several methods of nucleic acid isolation have been developed, i.e., extraction based on the method described by Boom,¹⁹ which uses the principle of adsorption of nucleic acids to silica matrices in the presence of “chemotropic salts” and alcohol.²⁰

According to the literature, the salt guanidine thiocyanate is one of the most powerful chemotropic agents and is commonly used in the Boom method.²⁰ Guanidine thiocyanate has both RNase-inactivating and lysing properties for viral particles and cellular structures^{21,22} and promotes binding of nucleic acids to silica surfaces.²³ The Boom method is most commonly implemented in conjunction with spin columns.

Table 2. Advantages and disadvantages between extraction methods RNA

	Silica Column	Magnetics beads
Sample volume	0.140 mL	0.250 mL
Assay time	30 min	3 hours
Pool	No	Yes
Limit detection	310 IU/mL	100 IU/mL
Cost/test	US\$ 13.00	US\$ 6.00

In this study, we used magnetic beads that bind nucleic acids to their proprietary magnetic polyvinyl alcohol-based particles. The bound nucleic acids are then transferred through the steps of the extraction process. This extraction technology has successfully been evaluated to screen for viral nucleic acids in routine blood donations.

Pichl et al.²⁴ used a total of 102 minipools, representing approximately 612 blood donations that were replicated from routine NAT pools, to analyze for B19 (parvovirus) DNA, HBV DNA, and HAV (hepatitis A virus) RNA. In our study, 3,468 minipools representing 20,808 blood donations replicated from routine NAT pools were analyzed for HIV and HCV RNA.

Nine minipools were positive for HIV RNA, and 18 were positive for HCV RNA. These minipools were broken out to determine which sample was reactive. All pools, positive and negative, were in agreement with results obtained from routine screening. All were valid due to a negative result for the IPC RNA, indicating that the tests are highly specific and reliable.

The limit of detection determined for Parvovirus B19 by Pichl et al.²⁴ was 875 IU/mL, 260 IU/mL for HAV and 1274 IU/mL for HBV. In our study, the detection limit for HCV and HIV was 100 IU/mL.

Hourfar et al.²⁵ compared two methods of extraction: using a kit for extraction by silica column (QIAamp) and another by magnetic beads. The detection limit was 23.1 IU for HIV-1 and 10.7 IU of HCV per PCR for the bead extraction and 21.6 IU for HIV-1 per PCR and 5.4 IU for HCV per PCR for the QIAamp extraction (Qiagen).

Jarvis et al.²⁶ uses the “decant” method on NucliSens automated extractor systems (bioMérieux UK Ltd., Basingstoke, UK) to extract nucleic acid from 2 mL plasma minipools as described by the manufacturer. The assays have 95% detection levels of 7.25 IU/mL for HCV and 39.8 IU/mL for HIV.

In our study, we observed that magnetic bead technology was very effective. The detection limits were 100 UI/mL, using minipools and duplex PCR reactions (Table 2).

EIA results are reported as “reactive” or “nonreactive,” and the EIA signal to cutoff (S/CO) ratio may also be reported as “high” or “low.” The EIA S/CO ratio is a comparison of the optical density of the patient’s positive EIA result to the optical density of the laboratory’s positive EIA control. If the ratio is high (> 3.0 using the most widely employed ELISA diagnostic kits), the positive predictive value (that the patient really has HCV antibodies in the blood) of the patient’s result is high.

Albertoni et al.²⁷ observed that 90% of the samples with an S/CO ratio of > 3 were positive in the RT-PCR test, confirming the serological tests; in contrast, 13% samples with S/CO ratio < 3 were confirmed as positive by RT-PCR. It was suggested that another 87% patients were false-positive by ELISA. In our study, we could see that all samples with S/CO < 3 were negative by RT-PCR, and samples with S/CO > 3 were con-

firmed positive by RT-PCR, indicating that there is indeed a strong relationship between the levels of HCV antibodies and HCV RNA. From the data obtained, it can be concluded that using magnetic beads for viral nucleic acid extraction from minipool samples is feasible as a manual process, shows high sensitivity, and offers the possibility to perform different PCR assays from one sample eluate.

It is important to note that, on average, the cost for the extraction of viral RNA by the column method is about \$13.00 per test. However, when the magnetic beads are used for extraction, the cost is reduced by half. Therefore, it can be concluded that the magnetic bead technology for extraction of viral RNA is trustworthy, easy to perform, and cost-effective.

CONCLUSION

The manual magnetic bead-based extraction in combination with RT-PCR detection can be used routinely to screen blood donations for HIV and HCV viruses to further increase the safety of blood products.

In this study we noticed that the centrifugation for 1 hour of biological material to be analyzed has been crucial for the formation of the pellet for extraction of viral RNA. Moreover, it was extremely difficult to standardize the concentration of primers and probes in the multiplex assay (HIV + IPC) and (HCV + IPC)

Author's contribution: All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript; GAA, CPA, PRBA, SSA, FOC, MJBGC, NS, and JAB conducted the experiments, and GAA, CPA, SSA and FOC wrote the manuscript.

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REFERENCES

- Alter HJ, Houghton M. Hepatitis C virus and eliminating post transfusion hepatitis. *Nat Med.* 2000;6:1082-86.
- Stramer SL, Glynn SA, Kleinman SH, et al. Detection of HIV-1 and HCV infections among antibody-negative blood donors by nucleic acid-amplification testing. *N Engl J Med.* 2004;351:760-68.
- Roth WK, Weber M, Seifried E. Feasibility and efficacy of routine PCR screening of blood donations for hepatitis C virus, hepatitis B virus, and HIV-1 in a blood-bank setting. *Lancet.* 1999;353:359-63.
- Laperche S. Blood safety and nucleic acid testing in Europe. *Euro Survey.* 2005;10:3-4.
- Coste J, Reesink HW, Engelfriet CP, Laperche S. Implementation of donor screening for infectious agents transmitted by blood by nucleic acid technology. *Vox Sang.* 2005;88:289-303.
- Velati C, Fomiatti L, Baruffi L, Romano L, Zanetti A. Impact of nucleic acid amplification technology (NAT) in Italy in the three years following implementation (2001-2003). *Euro Surveill.* 2005;10:12-4.
- Jackson BR, Busch MO, Stramer SL, AuBuchon JP. The cost-effectiveness of NAT for HIV, HCV and HBV in whole-blood donations. *Transfusion.* 2003; 43:721-9.
- Roth WK, Buhr S, Drosten C, Seifried E. NAT and viral safety in blood transfusion. *Vox Sang* 2000;78(Suppl):257-9.
- Braham S, Gandhi J, Beard S, Cohen B. Evaluation of the Roche lightCycler parvovirus B19 quantification kit for the diagnosis of parvovirus B19 infections. *J Clin Virol.* 2004;31:5-10.
- Allain JP. Transfusion risks of yesterday and of today. *Transfus Clin Biol.* 2003;10:1-5.
- Sabino EC, Salles NA, Sáez-Alquezar A, Santos GR, Chamone DF, Busch MP. Estimated risk of transfusion-transmitted HIV infection in São Paulo, Brazil. *Transfusion.* 1999;39:1152-53.
- Wendel S, Fachini RM, Levi JE, et al. A single window-period donation detected by human immunodeficiency virus p24 antigen after 5 years of routine screening in a group of Brazilian blood banks. *Vox Sang.* 2002;83:309-12.
- Barreto CC, Sabino EC, Gonçalves TT, et al. Prevalence, incidence and residual risk of human immunodeficiency virus among community and replacement first-time blood donors in São Paulo, Brazil. *Transfusion.* 2005;45:1709-14.
- Ministério da Saúde, Secretaria de Atenção à Saúde, Departamento de Atenção Especializada, Coordenação Geral de Sangue e Hemoderivados. Estágio Atual da Implantação dos Testes de Ácido Nucléico para HIV e HCV - NAT HIV/HCV na rede de Serviços de Hemoterapia do Brasil. Nota técnica, 2010.
- Hourfar MK, Schimidt M, Seifried E, Roth WK. Evaluation of an automated high-volume extraction method for viral nucleic acids in comparison to a manual procedure with preceding enrichment. *Vox Sang.* 2005;89:71-76.
- Fleiss JL. Statistical methods for rates and proportion. 2nd ed. New York, John Wiley & Sons 1981; 212-25.
- Landis JR, Koch GG. The measurement of observer agreement for categorical data. *Biometrics.* 1977;33:159-174.
- Gerberding JL, Fleming DW, Snider DE. Guidelines for laboratory testing and result reporting of antibody to hepatitis C virus. Epidemiology Program Office, Centers for Disease control and Prevention (CDC), Department of Health and Human Services 2003; 52:1-15.
- Boom R, Sol JCR, Salimans MM, Jansen CL, Wertheim-van Dillen PM, Van Der Noordaa J. Rapid and simple method for purification of nucleic acids. *J Clin Microbiol.* 1990;28:495-503.
- Vogelstein B, Gillespie D. Preparative and purification of DNA from agarose. *Proc Natl Acad Sci USA.* 1979;76:615-69.
- Castellino FJ, Barker R. The denaturing effectiveness of guanidinium, carbamoylguanidinium, and guanylguanidinium salts. *Biochemistry.* 1968;7:4135-38.
- Chirgwin JM, Przbyla AE, MacDonald RJ, Rutter WJ. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry.* 1979;8:5294-99.
- Melzak KA, Sherwood CS, Turner RFB, Haynes CA. Driving forces for DNA adsorption to silica in perchlorate solutions. *J Colloid Interface Sci.* 1996;181:635-44.
- Pichl L, Heitmann A, Herzog P, Oster J, Smets H, Schottstedt V. Magnetic bead technology in viral RNA and DNA extraction from plasma minipools. *Transfusion.* 2005;45:1106-10.

25. Hourfar MK, Michelsen U, Schmidt M, Berger A, Seifried E, Roth WK. High-throughput purification of viral RNA based on novel aqueous chemistry for nucleic acid isolation. *Clin Chemistry*. 2005;7:1217-22.
26. Jarvis L, Becker J, Tender A, et al. Evaluation of the Roche cobas 201 system and cobas TaqScreen multiplex test for blood screening: European multicenter study. *Transfusion*. 2008;48:1853-61.
27. Albertoni GA, Arnoni CP, Araújo PRB, Carvalho FO, Barreto JA. Signal to-cut-off (S/CO) ratio and detection of HCV genotype 1 by real time PCR one-step method: is there any direct relationship? *Braz J Infect Dis*. 2010; 14:147-52.