The Using of Multiplex RT-qPCR for Pooled Samples to Detect Hepatitis B Virus, Hepatitis C Virus, Human Immunodeficiency Virus within Iraqi Blood Donors

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Abstract

Background: The blood of blood donors is screening for hepatitis B virus (HBV), hepatitis C virus (HCV), and human immunodeficiency virus (HIV) infection by enzyme immunoassay (EIA) in the National Center of Blood Bank in Baghdad. The residual risk of EIA negative samples is not estimated till now in Iraq.

Objective: To detect HBV, HCV and HIV viruses within seronegative plasma of blood donors by a commercially available multiplex nucleic acid amplification tests (NAT) with mini-pooling system.

Methods: One thousand (1000) blood donors were screened by EIA revealed negative results then NAT was performed on pools of samples, each pool contain ten seronegative plasmas (MP10) i.e., 100 minipools.

Results: The detected positive minipools 10 seronegative plasmas by NAT for HBV, HCV and HIV were 3 MP10.

Conclusion: The use of NAT appeared to be sensitive and reliable to detect occult HBV and overcome the seroconversion problem related with HCV and HIV within seronegative plasmas of blood donors. Therefore, the implementation of NAT with mini-pooling in addition to serological tests for routine blood donor screening will improve and ensure the safety of blood transfusion in Iraq.

Keywords: Blood transfusion, widow period, multiplexes PCR

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List of abbreviations: EIA = Enzyme immune assay, ELISA = Enzyme-linked immunosorbent assay, MP = Minippools, NAT = Nucleic acid amplification technique, PCR = Polymerase chain reaction, vDWP= Viral diagnostic window period

Introduction

Serological tests (detection of antigens or antibodies) had historically been the foundation of blood screening for transfusion transmitted infections [1], but it remains limited and problematic because of 1) viral pre-seroconversion window period (PWP) i.e., the time period that needed for specific antibodies development and become detectable in the blood after a recent virus infection, 2) donors infected with genetic and immune variants viral strains and 3) cases of immunosilent infections (carriers), while the serological tests only provide positive results once the donor's immune system reacts against the respective pathogens [2].

Over two decades ago, advanced and newer tests like nucleic acid amplification tests (NAT) have helped in shorting the viral "window

period" [1]. NAT is a molecular technique with high sensitivity and specificity. It is an amplification technique of targeted regions of viral RNA or DNA and could detect the presence of viruses earlier than the serological screening methods, so it will narrow the window period of hepatitis B virus (HBV), hepatitis C virus (HCV), and human immunodeficiency virus (HIV) infection [3].

International survey on NAT testing of blood donations implemented from 1999-2009 to screen blood donations for HCV and HIV-1 on more than 300 million blood donors and about 100 million blood donors screened for HBV. The results revealed there were over 2000 NAT-reactive with serology negative donations that would otherwise have been transfused [4] also the results have shown that all over infection rates for HCV 1:447000, for HIV 1:111000 and for HBV 1:66000 [5]. But NATs are expensive technique with about 5-10-fold greater than that of the most expensive enzyme immunoassay. To overcome the cost problem that related to NATs, two strategies have been suggested, the using of pooled plasma samples so that fewer tests are required to screen large numbers of samples with using of multiplex polymerase chain reaction (PCR) assays that can detect simultaneously several viruses in one reaction tube [6]. World health organization (WHO) mentioned in their guidelines in 2017 that the length of viral diagnostic window period (vDWP) for different assays categories is clearly different, the using of mini-pooling of 16 samples with multiplex NAT test is shortening the vDWP 7-11 days, 27-37 days, 5-7 days for HIV, HBV and HCV respectively while the enzyme immunoassay (EIA) is shortening the vDWP 16-21 days, 42 days, 60 days for HIV, HBV and HCV respectively [7].

In Iraq, till now serological tests are the reliable standard methods for screening donated blood. The question here, are these tests enough to ensure the safety of blood or plasma for transfusion? Therefore, this study was designed as an attempt to make insight on many important points related to blood transfusion in Iraq.

The study aimed to determine the presence of positive HBV, HCV and HIV in seronegative donated blood within Iraqi blood donors, also to elucidate sensitivity and specificity of the currently used serological tests in blood screening in comparison with NAT assay, and the rate of transmitted HBV, HCV, and HIV during the seroconversion window.

Methods

Subjects and samples collection

Blood volunteers attended to National Center of Blood Bank in Bab-AlMuadham, Baghdad, during the period from July 2018 to January 2019.

The serological assays were performed on plasma samples by using most recent serological kits to detect the HBsAg by enzyme-linked immunosorbent assay (ELISA) (Lot no. Bs-1904-4, fortress, UK), Advanced diagnostic (Lot no. 201704120, USA); advanced kit for detection of antibody to hepatitis C virus by ELISA and detect the presence of HIV-1/2 antibodies and/ or HIV-1 p24 antigen in plasma by EIA through Fourth generation (Lot no. 2017091201, Advanced USA) kit.

One thousand (1000) blood donors who revealed seronegative results for HBV, HCV, and HIV were enrolled in this study and about 1.5 ml of seronegative plasmas were collected in microcentrifuge tube then stored at -80°C until use. The samples were categorized into 500 of plasma samples collected after 6 h from blood withdrawal and the rest (another 500) were collected after 12 h from blood withdrawal (according to the blood bank system).

Pooling of plasma samples

Pooling of seronegative plasma from blood donors for nucleic acid extraction and NAT was carried out as shown in table (1); pre-estimated positive samples 10^3 copies/ml for HBV and HCV and 10^4 copies/ml for HIV were used for qualifying the pooling system to obtain the most appropriate numbers and volume of

samples that could be used in pooling. The pooling validation was done by mixing positive samples with negative samples, for example: One HIV positive plasma (150 µl) + one HCV positive plasma (150 µl) + one HBV positive plasma (150 µl) + 150 µl from 7 individual negative plasma samples resulting in totally 1500 µl pool for 10 individual samples i.e., mini pool 10 (MP10).

Table 1. Pooling system validation used in this study

Sample's volume	No. of pooling	Results for HIV, HCV and HBV
100 µl from each sample	5 samples pooled	Failure (+-+)
100 µl from each sample	6 samples pooled	Failure (--+)
100 µl from each sample	10 samples pooled	Failure (---)
150 µl from each sample	5 samples pooled	Success (+++)
150 µl from each sample	6 samples pooled	Success (+++)
150 µl from each sample	10 samples pooled	Success (+++)

Nucleic acid extraction (RNA and DNA)

QIAamp® MinElute® Virus Spin kit 50 (Lot no. 163029117 Qiagen/Germany), was used according to the manufacturer's instruction for simultaneous extraction and purification of viral RNA and DNA from mini- pooled 10 seronegative plasmas of blood donors. The concentration and the purity of all extracted samples were measured using Quantus Florometer (promega/USA); then, DNA extracts (eluent) were stored at -20°C.

Detection of HBV, HCV, and HIV in minipooled plasmas of seronegative blood donors using multiplex real-time RT PCR

The HCV/HBV/HIV Real-TM (Lot no.10H18H705 sacace /Italy) [8] kit was used; it is a qualitative Real-Time RT PCR test for detection of HIV RNA, HCV RNA and HBV DNA in human plasma. The extracted RNA/DNA from plasma is amplified by using RT-amplification then detection of fluorescent reporter dye probes specific for HCV, HBV, HIV and internal control (IC). This kit is intended for use for individual donors or could be used to test pools comprised of equal aliquots. The recommended samples numbers in one pool must be not more than 5-10 (100-200 µl of the plasma for each sample). The extracted RNA/DNA sample (15 µl) were added to tube with prepared Reaction Mix according to manufacturer's instructions and mixed. The tubes (Samples, positive and negative controls) were transferred to real-time PCR thermalcycler ABI® 7500 (Applied Biosystem /USA). Thermo-cycling profile on Real-time instrument (plate type) was as follows: 1 cycle at 50°C for 20 min, 1 cycle for 15 min at 95°C, 4 cycles for 20 sec at 95°C, 40 sec at 46°C, then 42 cycles for 5 sec at 95°C, 40 sec at 60°C, 40 sec at 45°C. Results were accepted if the positive amplification and negative amplification controls along with negative and positive controls of extraction are passed. Sample was considered to be positive for HCV or HIV or HBV if the value of Ct was lower than 33 according to the manufacturer's instructions [8].

Statistical analysis

The data were processed using SPSS version 16.0.0, Microsoft Excel 2010, and Graphpad Prism version 7.04. The data of the current study were scrutinized carefully in terms of being parametric or non-parametric using normality tests. Accordingly, the proper statistical tests were used. Student t-test and ANOVA test were used for parametric data and Mann-Whitney u test was used for non-parametric data to measure the significance of

difference in means taking into account whether variables of analysis sharing different or equal variance.

Results

The collected 1000 seronegative plasma samples from blood donors were pooled in 100 mini-pools (MP10). The pooled samples were screened by NAT tests with commercial multiplex PCR kit for HBV, HCV and HIV detection (Figures 1 and 2). The results of multiplex PCR revealed that about 3/100 of MP10 seronegative plasmas were HBV, HCV, and HIV positive, as shown in figure 3.
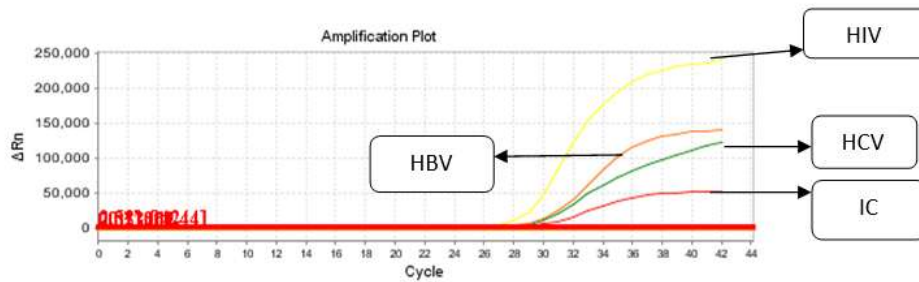

Figure 1. Demonstrate the simultaneous detection of HBV, HCV and HIV with internal control (IC) by multiplexRT- qpcr. on the Fam (Green) channel HCV cDNA was detected, while the Joe (Yellow)/HEX/TET/Cy3 channel detected the HIV cDNA, on the Rox (Orange)/TexasRed channel the HBV DNA was detected and IC on the Cy5 (Red) channel
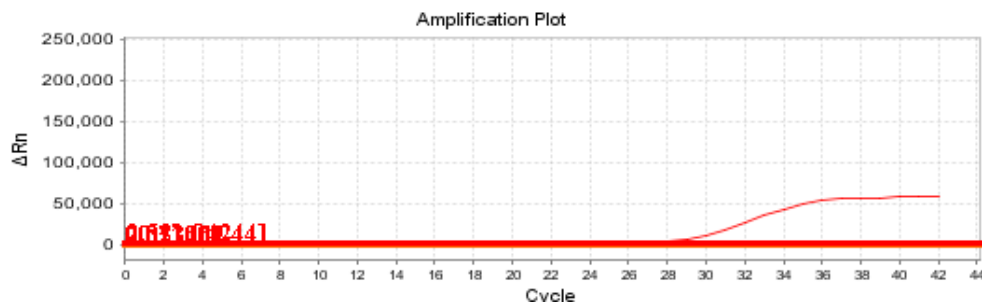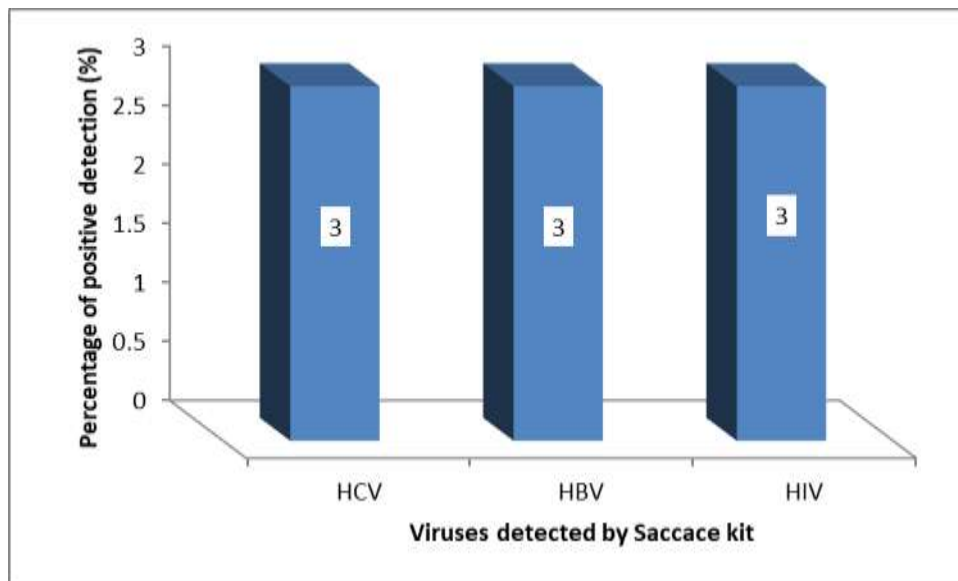

Figure 2. Represent the NC (negative control of extraction) of multiplex RT-qPCR, when the IC only is amplified

Figure 3. The percentage of positive mini- pooled 10 seronegative plasma samples of blood donors that detected by Sacace commercial kit multiplex PCR for HBV, HCV, and HIV

The detection rate of HBV, HCV, and HIV within 6 h versus 12 h after being withdrawn was shown to be borderline different for HIV and HCV. All the three positive minipools, 3/50 (6%) samples for HIV RNA and HCV RNA were found in plasma from the group of 6 h blood after withdrawal while zero positive sample was found in 12 h group (P=0.06), as shown in figure 4. On the other hand, the positive HBV DNA was found in 2/50 (4%) in 12 h blood group versus 1/50 (2%) in 6 h blood group; however, there was no significant difference in the rate of positive HBV DNA between 6h and 12h blood groups (P>0.05), as shown in figure 4.
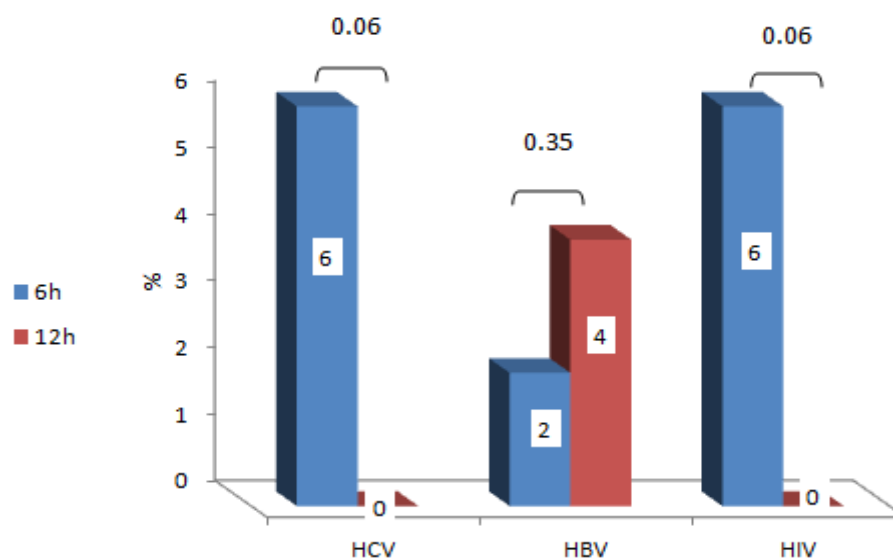
Figure 4. Percentage of positive detection of HCV, HBV, and HIV by Saccace commercial kit within the first 6h versus 12h h from withdrawing blood. The difference in detection rate of HCV and HIV is borderline significantly better in 6h compared to 12h withdrawal of blood. (P value < 0.05)

Discussion

In Iraq, serological screening for blood donors is a standard dependable method for blood screening assay in absence of NAT. There is no previous study found to estimate the residual risk of HIV, HCV, and HBV transmission through blood transfusion during viral seroconversion. The current study estimated the rate of positive HBV, HCV, and HIV within blood donor's seronegative plasma by using multiplex real-time RT-PCR, for mini-pooling samples. Hence, 100 mini-pools (MP10) of seronegative plasma were screened by multiplex PCR revealed that 3 of MP10 were positive for HBV, 3 for HCV, and 3 for HIV. Accordingly, if each positive MP10 contained at minimum one reactive virus, the percentage of transmitted HBV, HCV and HIV during seroconversion will be 0.3% or slightly more. The current research findings are comparable to the results of the research done by donor's database of Dubai Blood Donation Centre (DBDC) from 2008-2009; after introducing of multiplex assay in UAE through 2 years (19%) of blood donors were HBV NAT positive and HBsAg negative. A study applied on 59,283 samples, the potential HBV-positive donors were 187 screened by NAT and serologic assays. Up to 50 HCV-infected donors (12.3%) were reactive for HCV RNA by NAT with negative anti-HCV but only two HIV-infected donors who they were HIV RNA and anti-HIV reactive [9].

Another study that was conducted in Turkey in 2017, screening of 3000 seronegative donors by NAT was performed on pools of six blood sera, 9 HBV (0.3%) and 1 HCV (0.03%) and 1 HIV (0.03%) were detected and revealed positive results by NAT [10]. In 2019 Iraqi study by Al Sharifi et al. was conducted on 100 multitransfused thalassemic patients to estimate the prevalence of hepatitis B and C viruses by EIA and PCR in thalassemic patients and its relation with blood transfusion, they didn't have any previous HBV and HCV infection and didn't have vaccine, (12%) of patients had a positive HBcAb, while 3 (3%) had positive HBsAg, and higher percentage of HCV infected patients (91%) who regularly received blood transfusion every month and finally the Iraqi study concluded that the sensitive and

reliable screening tests required for blood transfusion improvement [11]. According to WHO guidelines for blood transfusion in 2017, NAT screening method reduces the window period 4-7 days for HIV, 3-5 days for HCV and 17-27 days for HBV while the viral window periods were 14-28 days for HIV, 9-80 days for HCV and 42-55 days for HBV with serological tests [7].

The pooling of 6 to 16 specimens that termed minipool nucleic acid testing (MP-NAT) is done in United State, while in some other countries individual donation testing is performed [12]. At the first time, the majority of countries performed the NAT testing in minipools of 96–16 pooled samples; but recently there was a direction towards smaller pools of 6 to individual donations (ID) in order to increase testing sensitivity [13].

The current study estimated the impact of the time from blood collection to screening assay and observed that the detection of HCV and HIV was higher after 6 h than 12 h without such observation for HBV. Almeida et al. evaluated the HBV DNA in plasma samples stability when it stored at 4°C for up to 7 days and then at -7°C (frozen), their results revealed insignificant decrease in viral load [14]. Gessoni et al. used samples containing different titers of HCV, HIV-1, and HBV to study the stability of viral genomes; they noticed that HCV and HIV-1 RNA can be stored at 4°C for 72h; HBV DNA can be stored until 168 h or about 7 days without reducing in the viral titer [15]. The stability of HBV DNA for longer period than HCV and HIV RNA related to the principle that DNA is more stable than RNA and, hence, more resistant to the effects of storage conditions [16].

Finally, in spite of NAT has the ability to detect the transfusion-transmissible viruses during the window periods or seroconversion too earlier than serological screening assay, hence the blood screening should be done by serological and NAT assay because both NAT and serological assays can complement each other [17].

This study concluded that was an estimated risk of HCV, HIV and HBV transmission through blood transfusion in already-tested seronegative donated blood samples. This can

originate from the sereconversion of HBV, HCV, and HIV and it is obviously that NAT screening assay was sensitive and reliable screening assay in the detection of transfusion-transmissible viruses. Moreover, the use of multiplex qPCR along with mini-pooling of samples was cost effective, time-saving and reduced the cross-contamination problems.

Acknowledgement

Author contribution

Dr. Majid: Collection of samples, performing minipooling and NAT assay. Both Dr. Abdulamir and Dr. Ahmed supervised the study. Aufi: Results interpretation and analysis of NAT assay. Abdullah: Performing the serological assay for all collecting plasma samples.

Conflict of interest

Authors declares that there is no conflict of interest.

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