NUCLEIC ACID TESTING FOR HEPATITIS C VIRAL DETECTION IN ANTI HCV NEGATIVE BLOOD DONORS

JAMIL R.,[1] AZIZ M.[2] AND ANWAR M.J.[3]
[1]Department of Hematology and [3]Chemical Pathology, Amna Inayat medical College Sheikhupura University of Health Sciences and [2]Sheikh Zayed Hospital, Lahore

ABSTRACT

Background and Objectives: Transfusion associated hepatitis C is quite common world over. In developing centuries like Pakistan blood transfusion, needle pricks and use of contaminated instruments are responsible for transmission of virus. The present study was designed to find out the frequency of HCV positivity in anti HCV negative blood donors by Nucleic acid testing (NAT), to detect those cases of HCV which are in their window period and are not identified by routine serological screening. It will be rather appropriate to combine NAT&ELISA screening of blood donors.

Methods: It was a cross sectional study. One thousand healthy, volunteer blood donors with negative anti-HCV status on screening were reconfirmed to be anti-HCV negative by third generation ELISA. NAT testing of these anti-HCV negative donors was done by RT – PCR. In order to economize the cost, RT – PCR was done on 5 samples mini-pool.

Results: Donors had a mean age 29.0 ± 5.8 years with male predominance (99.8%). On PCR testing initially 4 pools were found to be reactive for HCV, these pools were segregated and PCR testing of the individual sample of the reactive pools revealed two HCV positive samples in one pool and one HCV positive sample in each of the other three. Thus 5 donors (0.5%) who were screened to be anti-HCV negative, were found to have HCV – RNA on RT – PCR.

Conclusion: NAT screening of blood donations reduces the transmission risk of HCV which results in greater safety of blood components. Mini-pooling may be used to substantially reduce the cost of NAT without affecting the sensitivity.

Key words: Nucleic acid testing, polymerase chain reaction, hepatitis-C, donor screening, blood transfusion, sero-prevalence.

INTRODUCTION

Blood transfusion (BT) saves millions of lives each year worldwide.[1] However; it carries risks such as transfusion transmitted infections (TTIs)[2] which pose a major challenge for the blood transfusion services worldwide. Since the early 1960's blood banks as well as plasma manufacturing industries have implemented certain strategies to reduce the risk of TTIs but even today donor evaluation, laboratory screening tests and pathogen inactivation procedures fail to achieve zero risk for BT.[3]

Transfusion associated hepatitis (TAH) C virus infection is the most common TTI[4] and accounts for more than 75% cases of TAH around the world.[5] TAH was first recognized in 1940s, with the introduction of HBsAg led to substantial reduction in PTH (post transfusion hepatitis) cases however it was noticed that up to 10% of cases continued to develop PTH, most of which were attributed to an unknown non-A, non-B (NANB) viral agent which was later named as hepatitis C after the cloning of HCV genome, and was established to be the causative agent of more than 90% cases of NANBH.[6]

Nucleic acid testing (NAT) is a molecular technique which is highly sensitive and specific for viral genome detection in a variety of clinical settings.[7] It has been introduced around 2000 for screening blood in order to minimize the chances of TTIs in the recipients where it provides an additional layer of blood safety by detecting those recent infections which are currently in window period and can't be detected by routinely performed screening methods.[8] NAT technique also adds the benefit of resolving false reactive donations on serological methods hence further improves blood safety.[9]

Current study was performed to find out the frequency of HCV infection in anti-HCV negative blood donors by Nucleic acid testing (NAT), to detect those cases of HCV which are in their window period and are not identified by routine serological screening assays in our setup.

Corresponding Author: Dr. Rabiya Jamil, Assistant Professor Hematology
Amna Inayat Medical College, Sheikhupura, University of Health Sciences, Lahore
Address: 151-C Gulshan Ravi, Lahore. Email: rabiyaghaffar@gmail.com

MATERIALS AND METHODS

Study Design: It was a cross sectional study conducted at Shaikh Zayed Medical Complex and Lab one Pvt. Limited. The study duration was October 2011 to April 2012. The sample size was 1000 and the donors were included through consecutive sampling after informed consent and considering following inclusion and exclusion criteria;

Inclusion Criteria: Blood donors with Anti-HCV negativity detected by ICT device (particle agglutination) method. Donors aged 17 – 60 years, voluntary non-paid donors having weight ≥ 50 Kg, Hb ≥ 13gm (males) or ≥ 12 gm (females) with minimum blood donation interval of 12 weeks and no history of cardiovascular, gastrointestinal, nervous system disease or jaundice in last 6 months were included.

Exclusion Criteria: HBsAg or HIV reactive patients or those not consenting for the study were excluded.

Sample Preparation: Blood was collected in a blood bag containing CPDA-1 (citrate phosphate dextrose adenine – one) as anticoagulant. In addition the aliquots were made for routine blood bank procedures. 10 ml additional blood was collected for ELISA and RT – PCR procedures. Serum from each sample was stored in 2 sterile plastic vials each containing 1.5 ml serum. One for anti-HCV testing by ELISA and the other for HCV – RNA testing by RT – PCR. Vials were labeled and kept frozen.

ELISA: All sera were reconfirmed for anti-HCV by 3rd generation ELISA. Detection of anti HCV was done by CobasElecsys Anti HCV assay Roche diagnostics.[10] Results were determined automatically by Elecsys software by comparing the electroluminescence signal obtained from the sample with the cut off value obtained by anti-HCV calibration. The analyzer automatically calculates the cutoff based on the measurement of positive and negative controls. The result is interpreted as negative and positive in the form of cutoff index;

- Negative: Samples with cutoff index < 0.9.
- Positive: Samples with cutoff index > 1.
- Equivocal: Samples with cut off ≥ 0.9 and ≤ 1.

Plasma Pooling Preparations: The plasma pool preparations were made for HCV – RNA detection by RT – PCR. Each plasma pool consists of 5 donors samples which were confirmed as anti-HCV negative by 3rd generation ELISA. Each plasma pool was made by mixing 200 microliters of each donors serum to make a final volume of 1ml and was stored in sterile storage vials. The vials were labelled and immediately frozen until RT – PCR was done. The original sample of donor was stored until the results of PCR was available.

RT – PCR: RT – PCR was performed by using Stratagene Mx3005 real time PCR instrument. The PCR kit used for qualitative detection of HCV RNA were of AmpliSens HCV – FRT Roche diagnostics.[11] Kits used for RNA extraction were of QIAamp® manufactured by Qiagen GmbH Roche Diagnostics. A synthetic internal control RNA (ICRNA) was stabilized within the nucleic acid extraction tubes to be co-purified with HCV target nucleic acid. In samples with negative or low positive result for HCV infection the IC RNA should always produce a positive result which will confirm that extraction has been performed correctly. All steps were carried out according to manufacturer's instructions. The results were interpreted by the real time PCR instrument software by the crossing or not crossing of the threshold line by the fluorescence curve. In case of HCV positive PCR result the infected donor was identified by retesting of 5 individual samples of the reactive pool.

RESULTS

After interviewing 1050 blood donors, fifty donors were excluded from the study who have past history of hepatitis or jaundice in last six months, any bleeding disorder, blood donation in last six months, close contact with a patient of hepatitis, or suboptimal physical or clinical status. Initial 1000 anti-HCV negative blood donors were included in the study. All these donors are healthy non-paid donors. The average age of donors was 29.0 ± 5.8 years and majority (85%) were below the age of 35 years, as shown in Figure 1. Among donors there were only two females, which was only 0.2% of the whole sample.

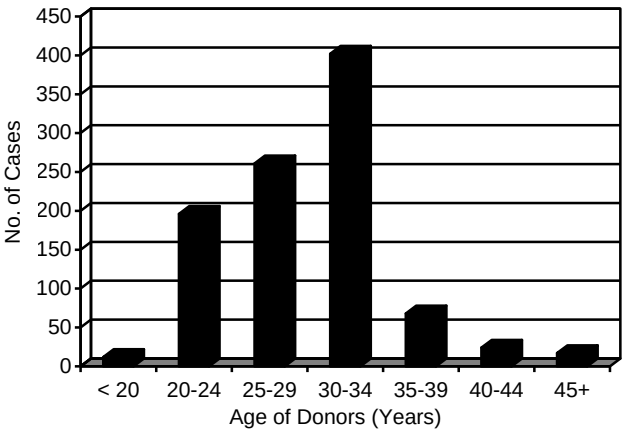

Fig. 1: *Age distribution of blood donors.*

When blood samples screened for anti-HCV by third generation ELISA it was noted that all samples were negative. On PCR testing initially four pools were found to be reactive for HCV, these pools were than segregated and the PCR testing of the individual sample of the reactive pools reveals two HCV positive samples in one pool and one HCV positive sample in each of the other 3 pools. Thus total 5 samples were found to be positive for HCV RNA on NAT testing.

When HCV positivity of the cases is related with age of blood donors, it was observed that all the positive cases were between 20 and 39 years among whom

4 were between 20 and 30 and 1 between 30 and 40 years. Statistically no significant difference found in the HCV positivity in different age groups of blood donors as shown in Table 1.

Table 1: *Results of NAT as per age of the donors.*

Age	*NAT result*		*Total*
	Positive	*Negative*	
<20	0	12	12
20 – 24	3	193	196
25 – 29	1	260	261
30 – 34	0	402	402
35 – 39	1	87	88
40 – 44	0	24	24
45+	0	17	17
Total	5	995	1000

p-value = 0.249

When we categorized the sample in two age groups ≤ 30 years and > 30 years there were 4 cases in group ≤ 30years, and one case in the age group of >30 thus the presence of Hepatitis C virus infection is not associated with age of the blood donor, no statistically significant difference found in two age groups (p value = 0.704).

However, all five positive cases were males. It was obviously due to the rarity of female gender in the study population.

DISCUSSION

There are serious concerns regarding the transmission of hepatitis C virus through blood transfusion because of the relatively longer 'window' between the infection and the appearance of detectable antibodies in the serum. In these cases the screening tests are negative yet the persons harbor the virus and are able to transmit the disease. Current study was conducted to assess the gravity of this problem in our set up, where the hepatitis C is endemic. Finding the frequency of HCV – RNA to be 0.5% in seronegative blood donors substantiated our fears regarding this critical issue. The recruitment of healthy blood donors from the low risk ones is the primary step in the establishment of safe blood transfusion.[12] In our study we excluded 50 (4%) of the blood donors on the basis of donor interview and clinical examination.

In general there are three types of blood donors i.e. voluntary unpaid donors, paid professional donors, and family / replacement blood donors who donate blood without any commercial incentive.[13] It is the goal of WHO that all the countries obtain their blood supplies from voluntary unpaid donors by the year 2020.[14] In Pakistan, out of 1.5 million blood transfusions annually 65% is from family / replacement blood donor. 25% from voluntary and 10% from paid professional blood donors.[1] In our study all the blood donations collected during the consecutive six months of the study period were from family/ replacement blood donors. Predominance of family/ replacement blood donors were also reported to be 99.9% and 98% and 90% in the studies conducted in Northern Pakistan,[15] Karachi[12] and Lahore[1] regions respectively.

In our study the mean age of blood donors was 29.5 ± 5.8 years. When donors were categorized into different age groups and HCV positivity was correlated, no significant difference is found in HCV positivity among different age groups (p value = 0.207). Comparable results have been obtained in a study on blood donors from Philippine[16] and in a study conducted in Baltistan[17] region of Pakistan.

In the present study female donors comprise only 0.2% of the study population and none of them was positive on NAT screening. All the 5 NAT positive cases were males. This is probably because of the reason that only a very small proportion of the study population was comprised of females. Most of the previous studies conducted on blood donors in Pakistan also showed the predominance of male blood donors as compared to females to variable extent. Male blood donor predominance was observed to be 100%, 99.8%, 98%, 95%, 67% respectively in the studies conducted in Karachi,[12] Khyber Pakhtunkhwa,[18] South Punjab[19] and Rawalpindi[20] regions. Similar results are reported in the studies from Japan,[21] Philippine[16] and Nigeria[22] where predominance of male blood donors was observed to be 95% and 88% respectively. However the data form most of the European countries[23] seems to show a different picture, with women playing a major role in blood donation.

In our study we screened the blood donors by the most sensitive 3rd generation EIA which has sensitivity of 97% in a high prevalence population, and has been used as a preferable method used in most of the recent studies conducted on NAT for HCV and other viral infections. It was observed in the studies conducted in Mexico[24], Saudi Arabia,[25] Germany,[26] Brazil,[27] France,[28] Iran[29] and also in Pakistan,[12] where EIA 3.0 has been used a preferable serological screening test for HCV and other viral infection. Our results are similar to a study conducted in Croatia where 2647 blood donors were screened by using NAT for HCV and 12 HCV RNA positive but antibody negative donors were found, thus giving a frequency of 0.4%.[30] Our results are also comparable with a Pakistani study conducted in Karachi in which 800 seronegative blood donors were screened for HCV – RNA by NAT and 0.375% blood donors were found who were NAT positive and anti-

body negative.[12] In contrast to our results the studies conducted in various Western countries show an extremely low or zero yield of NAT in blood donors. A Brazilian study in which 139,678 blood donations were tested but no NAT positive anti-HCV negative blood donation was found.[27] Similarly, in a German study no viremic but seronegative blood donor has been found after screening 331,783 donations.[31]

In order to analyze the enormous number of test procedures and to economize the high cost of NAT, mini pooling of sera has become a routine practice, although the number of samples in a pool varies greatly from place to place. The studies conducted on NAT from Saudi Arabia,[25] Pakistan,[12] Iran,[29] China,[32] Cyprus,[33] Germany[26] and Finland[34] the pool size varies from 2, 5, 8, 8, 8, 40 and 96 respectively. There are various views over the use of pooled NAT and individual NAT (ID – NAT). Pooled NAT has the advantage of being cost effective however sensitivity may be reduced due to dilutional effect especially in larger sized pools, while ID – NAT is very sensitive but cannot be implemented as a mandatory procedure due to its high cost. In many countries where NAT has been implemented as a mandatory procedure, MP – NAT is routinely practiced predominantly in pools of 96 donations.[35] In our study we selected a minipool of five individual samples. This is one of the smaller sized pool ever used. The system we used has the assay sensitivity of 10 IU/ml for HCV. Thus in a pool of five the sensitivity can be reduced to only 50 IU/ml which is quite higher as compared to other studies. In a German study 2,51,737 blood donations were tested by NAT using pools of 40 donations. For initial 81,456 donations HCV virus test (Roche Diagnostics) was used which has detection limit of 150 – 450 IU/m, thus in a pool of 40 the sensitivity was reduced to 6000 to 8000 IU/ml. For the remaining donations HCV CobasAmplicor was used which has detection limit of 49 IU/ml, this time sensitivity reduced to 2000 IU/ml.[26] In our study using 5 donation mini-pools, four pools were found initially reactive. When the pools were segregated and individual samples were tested by NAT, two positive samples were found in one pool and one positive samples in each of the other three pools. Thus retesting of the individual samples did not reveal any false positive result. Considering the international studies, we are confident that there is no impairment of sensitivity in the present study owing to a very small sized pool and the use of a sensitive system for NAT procedure.

CONCLUSIONS

Following conclusions may be drawn:

1) Seroprevalence of HCV – RNA in blood donors is found to be 0.5% in 1000 anti-HCV ELISA negative blood donors, which is quite alarming.
2) Pooling sera enables screening of large number of samples in an economical way without dropping the sensitivity.

ACKNOWLEDGEMENTS

Authors thank Mr. Yaseen Shad and Mr. Akram for their help in composing and preparing the manuscript and Mr. Asim Jaja for statistical analysis.

REFERENCES

1. Manzoor I, Hashmi N, Daud S, Ajmal S, Fatima H, Rasheed Z, Syed S. Seroprevalence of transfusion transmitted infections (TTIS) in blood donors. Biomedica, 2009; 25: 154-8.
2. Standards for blood banks and transfusion services ed. 18th American Association of Blood Banks, Bethesda MD, 1997; 41: k2.000.
3. Bhil F, Castelli D, Marincola F, Dodd RY, Brander C. Transfusion transmitted infections. J Transfusional Med 2007; 5-25.
4. Donhue JG, Munoz A, Ness PM, Brown OE, Yawn D, McAllistor HA. The declining risk of post transfusion hepatitis C virus infection. N Engl J Med. 1992; 327: 369-73.
5. Singh B, Verma M, Verma K. Markers for transfusion associated hepatitis in north Indian blood donors, Prevalence and Trends. J Infect Dis. 2007; 57: 49-51.
6. Leslie H, Tobler, Busch MP. History of post transfusion hepatitis. Clin Chemistry, 1997; 43: 1487-93.
7. Hans R, Marwaha N. Nucleic acid testing benefits and constraints. Asian J Transfus Sci. 2014; 8: 2-3.
8. Roth WK, Busch MP, Schuller A, Ismay S, Cheng A, Seed CR, et al. International survey on NAT testing of blood donations: Expanding implementation and yield from 1999 to 2009. Vox Sang. 2012; 102: 82-90.
9. Yaseen SG, Ahmed SA, Johan MF, Kiron R, Daher AM. Evaluation of serological transfusion – transmitted viral diseases and mutliplex nucleic acid testing in Malaysian blood donors. Transfusion Apher Sci. 2013; 49: 647-651.
10. Anti-HCVCobas, Antibody to hepatitis-C. December 22, 2014.
11. Guidelines to AmpliSens HCV - FRT, PCR kits. Available from URL: http://www.pcrdiagnostics.eu/files/ documents/ productspdf/20140310/HCV-FRT%20200612.pdf (Accessed December 22, 2014).
12. Ali N. Molecular screening of Hepatitis C virus in anti HCV negative blood donors. Pak Rese Repository [online] cited Dec 23, 2014] Available from: URL:http:/ www.cprints.hec.gov.pk/2744
13. Ramarkoto CE, Rakotomanana F, Ratsitorahina M, Vaomalala R, Razafindratsimanresy R, Randrimanana P. Seroprevalence of hepatitis C and associated risk factors in Urban areas of Antananrivo, Madagascar. BMC Infect Dis. 2007; 24: 13.
14. Blood safety and availability. Available from URL http://www.who.int/mediacentre/factsheets/fs279/en/index.html (Accessed December 19, 2014).
15. Khattak MF, Salamat N, Bhatti FA, Qureshi TZ. Seroprevalence of hepatitis B, C and HIV in blood donors in Northern Pakistan. JPMA, 2002; 52: 398.
16. Rodenas JG, Bacasen L, Que ER. The prevalence of HBsAg (+) and anti-HCV (+) among healthy blood donors at East Avenue Medical Centre, Quezon City. Phil J Gas-

troenterol. 2006; 2: 64-70.
17. Aziz MS. Prevalence of anti-hepatitis C antibodies and hepatitis B surface antigen in healthy blood donors in Baltistan. Pak Armed Forces Med J. 2006; 2: 2-5.
18. Safi SZ, Afzal MS, Waheed Y, Butt UJ, Fatima K, Pervoz Y, et al. Seroprevalence of hepatitis C and human. Immunodeficiency viruses in blood donors of north Western Pakistan. Asia Biomedicine, 2011; 5: 389-92.
19. Jahangir W, Ali F, Shahnawaz U, Iqbal T, Qureshi HJ. Prevalence of hepatitis B, C and HIV in blood donors of South Punjab. Esculapio, 2006; 2: 6-7
20. Gul-e-Atif, Nasir J, Hayat A. Seroprevalence of HBsAg and anti-HCV in Rawalpindi / Islamabad and analysis of risk factors. Ann Pak Inst Med Sci. 2009; 5: 242-4.
21. Myo K, San San O, Oo KM, Shimono K, Koide N, Okada S. Prevalence and factors associated with hepatitis C virus infection among Myanmar blood donors. Acta Med Okoyama, 2010; 64: 317-21.
22. Jaremiah ZA, Koate B, Buseri F, Emelike F. Prevalence of antibodies to hepatitis C virus in apparently healthy port Harcourt blood donors and association with blood groups and other risk factors. Blood Transfusion, 2008; 3: 150-55.
23. Bani M, Giussani. Gender differences in giving blood: a review of the literature. Blood Transfus. 2010; 8 (4): 278–287.
24. Chiquete E, Sanchez LV, Becerra G, Quintero A, Maldonado M, Panduro O. Performance of serological and molecular screening of blood donations for hepatitis B and hepatitis C viruses in a Mexican transfusion center. Annals of Hepatology, 2005; 4: 75-8.
25. Bamaga MS, Bukhari FF, Aboud MM, Malki MA, Alenzi F. Nucleic acid amplification technology screening for hepatitis C virus and human immunodeficiency virus for blood donations. Saudi Med J. 2006; 27: 446-53.
26. Hitzler WE. Runkel S. Routine HCV PCR screening of blood donations to identify early HCV infection in blood donors lacking antibodies to HCV. Transfusion, 2001; 41: 333-7.
27. Wendel S, Levi JE, Takaoka DT, Silva IC, Castro JP, Torezan – Filho MA et al. Primary screening of blood donors by NAT testing for HCV RNA: development of an "in-house" method and results. Rev Inst Med Trop Sao Paulo, 2007; 49: 177-85.
28. Leparche S. Blood safety and nucleic acid testing in Europe. Eurosorveillance, 2010; 10: 1-73.
29. Mehmoodian SM, Bahrami H, Shariti Z. Molecular study of HCV RNA among anti-HCV negative blood donors. Sci J Iran Blood Transfus Org. 2009; 6: 191-8.
30. Forcic D, Zgorelec R, Kosu-Gulija T et al. Screening of serologically negative plasma pools by nucleic acid amplification technology in Croatia 2001 – 2003. Transfusion Apher Sci. 2005; 33: 75-9.
31. Cardoso MS, Koerner K, Kubanek P. Minpooling screening by nucleic acid testing for hepatitis B virus, hepatitis C virus and HIV: Preliminary testing. Transfusion, 1998; 38: 905-8.
32. Nantachit N, Thaikruea L, Thongsawat S, Leetrakool N, Fongsatikul L, Sompan P. Evaluation of a multiplex human immunodeficiency virus-1, hepatitis C virus, and hepatitis B virus nucleic acid testing assay to detect viremic blood donors in northern Thailand. Transfusion, 2007 Oct; 47 (10): 1803-8.
33. Shang G, Yan Y, Yang B, Shao C, Wang F, Li Q et al. Two HBV DNA+ / HBsAg- blood donors identified by HBV NAT in Shenzhen, China. Transfus Apher Sci. 2009 Aug; 41 (1): 3-7.
34. Roth YK, Weber M, Buhr S, Drosten C, Weichert W, Sireis W et al. Yield of HCV and HIV-1 NAT after screening 3.6 million donations in Central Europe. Transfusion, 2002; 42: 862-8.
35. Allain JP. Genomic screening for blood borne viruses in transfusion settings. Clin Lab Haem. 2000; 22: 1-10.