INTRODUCTION

Hepatitis C virus is endemic in Pakistan; anti-HCV frequency is 4-9% in general population and 1.8-7.5% in blood donors. HCV screening of blood for transfusion is mandatory in Pakistan. All anti-body screening methods suffer limitation that anti-HCV is undetectable during the immunological window period. It is expected that in future, blood transfusion services in the country will face HCV as main threat if sensitive methods to screen blood and methods to eliminate the immunological window period are not adopted in time by Pardo et al.1 From the time of HCV entry in the body it takes about 60-90 days or longer for serum alanine aminotransferase (ALT) to rise, and about 44-82 days to show seropositivity depending upon the sensitivity of method used. There are exceptions to this normal response as well; in a few, ALT is not raised at all, and few become sero-positive by as late as 180 days.2,3 Based on these observations it can be inferred that more is required to be done before declaring blood transfusion safe. Current guidelines of World Health Organization (WHO) and American Association of Blood Banks (AABB) recommend serological screening of HCV or nucleic acid testing (NAT) irrespective of ALT status of blood donor. In Pakistan NAT screening of blood for transfusion is not performed in routine.

The initial test used to diagnose HCV was an enzyme immunoassay (EIA) for anti-HCV immunoglobulin G (IgG).4 Since then 3 generations of EIAs has been developed in the search of increase in sensitivity. Same is true for recombinant immunoblot assay (RIBA) Schroter et al.5 Both of the methods are dependant on the development of detectable levels of anti-HCV that takes many weeks.6 Globally sensitive methods that can detect HCV before seroconversion are being adopted. Assays that detect core antigen were devised, first such assay developed was a qualitative assay that significantly reduced the length of window period. A second test was subsequently developed for the detection and quantification of total HCV core antigen.7 This assay incorporating an immune complex dissociation step was designed for blood screening that reduced window period up to 21-30 days but lacks sensitivity as compared to HCV RNA screening. The methods of HCV RNA detection were explored that showed promising results.8 The molecular detection also passed through evolution, methods able to pick

ABSTRACT

Objective: To determine the frequency of HCV RNA in an anti-HCV non-reactive blood donor population with normal ALT, and its cost effectiveness.

Study Design: An observational study.

Place and Duration of Study: Baqai Institute of Haematology, Baqai Medical University, Karachi, and Combined Military Hospital, Malir Cantt, Karachi, from May 2006 to April 2008.

Methodology: After initial interview and mini-medical examination, demographic data of blood donors was recorded, and anti-HCV, HBsAg and HIV were screened by third generation ELISA. Those reactive to anti-HCV, HbsAg and/or HIV were excluded. Four hundred consecutive donors with ALT within the reference range of 15-41 units/L were included in study. HCV RNA RT-PCR was performed on 5 sample mini-pools using Bio-Rad Real time PCR equipment.

Results: All 400 donors were male, with mean age 27 years SD ± 6.2. ALT of blood donors varied between 15-41 U/L with mean of 31.5±6.4 U/L, HCV RNA was detected in 2/400 (0.5%) blood donors. Screening one blood bag for HCV RNA costs Rs 4,000.00 equivalent to 50 US dollars, while screening through 5 sample mini-pools was Rs. 800.00 equivalent to approximately 10 US dollars.

Conclusion: HCV RNA frequency was 0.5% (2/400) in the studied anti-HCV non-reactive normal ALT blood donors. Screening through mini-pools is more cost-effective.

Key words: HCV RNA. Hepatitis C virus. Blood donors. Anti HCV-negative.
5-10 copies/ml has been devised that can detect HCV RNA as early as 1-3 weeks post-exposure. HCV RNA testing by a sensitive and reliable method is expected to possibly interdict and virtually prevent most if not all transfusion associated HCV.\(^9\)

The aim of this study is to find out the frequency of HCV RNA in our anti-HCV non-reactive blood donor population with normal ALT, and the cost effectiveness of the method.

### METHODOLOGY

It was an observational study carried out on a cross-sectional sample of voluntary blood donors conducted at Baqai Institute of Haematology, Baqai Medical University, Karachi in collaboration with Combined Military Hospital, Malir Cantt, Karachi, from May 2006 to April 2008 after approval of research ethic committee of the institute. Blood donors volunteering for blood donation were included. The approximate sample size required for study was calculated assuming a power of 80% and a significance level 0.05. A written consent was obtained for blood donation and permission to perform relevant tests on blood. After initial interview and mini-medical examination, demographic data was recorded and donors were accepted for blood donation. Blood samples for blood grouping, cross-match, anti-body screening, ALT, syphilis serology, malaria slide examination, anti-HCV, HBsAg, and HIV were taken. Anti-HCV, HBsAg and HIV were screened by third generation ELISA. Those reactive to anti-HCV, HBsAg, HIV, syphilis serology or malaria on slide examination were excluded. Four hundred consecutive donors with ALT within the reference range of 15-41 U/L established by our laboratory as per recommendation of manufacturer were included in study. Blood donors, non-reactive to anti-HCV but positive for HCV RNA were retested for anti-HCV and HCV RNA after 3 months. All equivocal and borderline samples were retested to conclude the results.

HCV RNA Real time PCR was performed on 5 sample mini-pools using Bio-Rad Real time PCR instrument. Kits used for RNA extraction and reverse transcription were of “RoboGene® Hepatitis C virus Quantification Kit” manufactured by AJ Roboscreen GmbH, Leipzig Germany. A synthetic internal control RNA (IC RNA) is stabilized within the nucleic acid extraction tubes to be co-purified with the HCV target nucleic acid.\(^10\)

For RNA purification 450 µl lysis solution was added in extraction tube, 150 ml serum of sample to be analyzed was added to it, incubated at room temperature for 15 minutes, 600 µl binding solution was added to the ‘lysed’ sample. Lysed mix was placed in a 2 ml spin filter located receiver tube, centrifuged at 1000 x g for 1 minute. After discarding the receiver tube spin filter was placed in new receiving tube, and 500 µl of washing solution was added to spin filter, centrifuged at 1000 x g for 1 minute. Spin filter was opened and 650 µl washing solution was added, and it was centrifuged at 1000 x g for 1 minute. Spin filter was placed in new 2.0 ml receiving tube, centrifuged again for 3 minute to remove all traces of washing solution, and receiving tube was discarded. The spin filter was placed into a 1.5 ml elution tube, cap of spin filter was opened and 60 µl RNase-free water was added. Mix was incubated at room temperature for 2 min, and was centrifuged at 6,000 x g for 1 minute. The elution tube containing purified RNA was now immediately placed on ice while waiting for amplification process that was ensured to start within 20 minutes.

For RNA quantification 40 µl PCR grade water was added to amber cap vial containing lyophilized HCV/IC reagent mix, incubated at 37°C for 20 minutes vortexed and centrifuged briefly. Control strip from HCV/IC control RNA strips was taken, and loaded on reaction base for 0.2 ml tubes. Master mix was made using calculated quantity of PCR grade water, dye solution, buffer, Mg sulfate, primers/probe and RT-PCR Enzyme mix. 20 µl of master mix aliquots were mixed to each well of control and sample strips. Five µl aliquots of PCR grade water was added to non-template control (NTC) tubes, and 5 µl purified RNA sample was added to sample tubes. For annealing and amplification strips were placed in Real Time PCR instrument MiniOpticon\(^\text{™}\) System BIO-RAD. Wells were selected on screen and assigned respective number and text that identified standards, NTC, and samples. Ramping rate 2.5°C per sec was set, and thermal cycles were setup as per manufactures instruction. The process of annealing and amplification was completed in 2-3 hours.

The data was analyzed using the Statistical Package for Social Sciences (SPSS) version 15.0. Descriptive statistics of sociodemographic variables and other characteristics of the sampled population were computed. Mean values and standard deviations (SD) were calculated for quantitative variables and proportions for categorical variables. The cost effects of missing one case and sample pooling were calculated.

### RESULTS

Four hundred voluntary blood donors with normal ALT were included in study. All donors were male; with age between 18 and 54 years (mean 27 years). Ninety five present donors were donating blood first time while 5% were repeat donors. Mean weight of donors was 68 kg, while mean haemoglobin was 14.7 g/dl (Table I). Serum ALT varied between 15-41 U/L, mean 31.5+6.4 U/L, ALT profile has been shown in Figure 1.

HCV RNA was detected in 2 blood donors out of 400 (0.5%). In first donor ALT was 28 U/L. HCV RNA 7.414 million copies/ml were detected. Anti-HCV non-reactivity
consecutive anti-HCV-positive patients with persistently normal ALT were followed for 3.6 ± 2.3 years; serum HCV RNA was detectable by PCR in 94 patients (69%). During follow-up, all patients without detectable serum HCV RNA remained HCV RNA-negative and maintained normal serum ALT; all patients with detectable serum HCV RNA remained HCV RNA-positive, 20 (21%) had a slight fluctuation of serum ALT above the upper limit of normal.

In a study conducted at Canadian blood transfusion services by O’Brien et al. the incidences of transfusion transmitted infections (TTI) dropped rapidly after the introduction of HBsAg, anti-HCV and HIV screening, and a further drop was noted after introduction of screening by nucleic acid amplification testing. Although incidences of TTI are decreasing in Pakistan, still a high prevalence of HCV is reported from major parts of the country. In study of Idrees et al. anti-HCV seroprevalence of 14.63% was detected in healthy population which is alarming. In HCV endemic community a considerable proportion of blood donors is expected in immunological window period. It is assumed that HCV RNA screening of blood will eliminate most if not all of the potentially transmissible HCV blood units. It is likely to reduce window period from 66 (38-94) days to 19 (10-29) days. With the development of more sensitive methods of HCV RNA amplification detection up to 10 copies/ml is possible, that means a window period as short as 7-10 days and lesser risk of transmitting HCV then screening by ELISA. In a review by Espy et al. it was concluded that a Real-time PCR testing platform provides equivalent sensitivity and specificity as conventional PCR combined with Southern blot analysis. In blood banking since blood samples are pooled in 5, 10, 100 sera mini-pools for economy purpose, a sensitive system is required.

In this study, 2/400 blood donors were HCV RNA positive which is an alarming risk of transfusion associated viral transmission. Unfortunately little data regarding HCV RNA prevalence in anti-HCV non-reactive blood donors in Pakistan is available. Ahmed et al. performed a study in Faisalabad, Pakistan to find out the prevalence of HCV in general population, and to evaluate the importance of RT-PCR over HCV anti-body test in early diagnosis of HCV infection. They collected samples from HCV screening camps and blood donors. Blood samples from 300 subjects (77% males and 23% females) with a mean age of 32 ± 20 years were randomly collected, and analyzed. The results indicated that 48 (16%) cases were positive for anti-HCV antibody and 14 (4.7%) were on the borderline. By nested PCR, 84 (28%) samples were positive for HCV-RNA. Seroprevalence of anti-HCV in Faisalabad region and adjoining regions is 14-16% which is higher (1.3-7.5%) than the rest of the country. In addition all subjects in this study were blood donors included after vigilant

**Table I:** Descriptive statistics of blood donors N=400.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>N</th>
<th>Minimum</th>
<th>Maximum</th>
<th>Mean</th>
<th>Std. deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age in years</td>
<td>400</td>
<td>18.00</td>
<td>46.00</td>
<td>27.3225</td>
<td>6.10506</td>
</tr>
<tr>
<td>Haemoglobin g/dl</td>
<td>400</td>
<td>13.60</td>
<td>16.60</td>
<td>14.6695</td>
<td>0.52086</td>
</tr>
<tr>
<td>Serum ALT</td>
<td>1400</td>
<td>15.00</td>
<td>41.00</td>
<td>31.4250</td>
<td>6.41930</td>
</tr>
<tr>
<td>Weight in kg</td>
<td>1400</td>
<td>50.00</td>
<td>97.00</td>
<td>68.3025</td>
<td>8.71555</td>
</tr>
</tbody>
</table>

ALT: Alanine aminotransferase; Kg: Kilograms; g/dl: Grams per deciliter.

**Table II:** Cost incurred on HCV RNA screening of blood bags.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Cost in rupees</th>
<th>Cost in US Dollars**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Screening one blood bag for HCV RNA</td>
<td>4000/00*</td>
<td>50/00</td>
</tr>
<tr>
<td>Screening mini pool of 5 samples</td>
<td>800/00</td>
<td>10/00</td>
</tr>
<tr>
<td>Screening mini pools of 10 samples</td>
<td>400/00</td>
<td>05/00</td>
</tr>
<tr>
<td>Treatment of one missed case</td>
<td>200,000/00</td>
<td>2500/00</td>
</tr>
</tbody>
</table>

* Cost differs from lab to lab; this cost is average cost of three leading laboratories of city. ** Approximate cost in US dollars, may vary with exchange rates.

**DISCUSSION**

HCV RNA was detected in 2 blood donors out of 400 with normal ALT (0.5%), is quite alarming. While a raised ALT is taken with suspicion a normal ALT may not exclude the possibility of viral transmission. ALT remains normal during the initial 6-8 weeks of immunological window period, in addition a significant proportion of patients with detectable anti-HCV have normal serum ALT levels. In the study by Martinot-Peignoux et al. 135

and presence of HCV RNA were reconfirmed. The donor was called after 90 days for follow-up, his repeat ALT was 38 U/L, a repeat anti-HCV by third generation ELISA was carried out that was still non-reactive, and repeat HCV RNA performed revealed 0.27 million copies/ml of viral RNA. In second HCV RNA positive donor serum ALT was 35 U/L. HCV RNA 23,200 copies/ml were detected. Both of the blood donors were asymptomatic, and history of recent surgery, IV injections, tattooing, and high risk sexual behaviour was denied. Screening one blood bag HCV RNA cost Rs. 4,000.00 equivalent to 50 US dollars, while screening through 5 sample mini-pools was Rs. 800.00 equivalent to approximately 10 US dollars. As compared to the expenses of treatment an option of screening through mini-pools is cost effective (Table II).

![Figure 1](image-url) ALT profile of blood donors (n=400).
donor interview and after exclusion of high risk group, while the samples collected in Faisalabad study were from general population as well as from blood donors resulting higher prevalence of HCV RNA.

The sera were pooled in mini-pools of 5 samples, pooling sera enables screening of large number of samples in short time in economical way, without much losing the sensitivity. It has been used in blood banking by many investigators. In study of Seme et al. the usefulness of 24 mini-pool hepatitis C virus RNA screening was evaluated on 6432 consecutive anti-HCV negative specimens, 268 mini-pools were tested using an automated commercial PCR assay for qualitative detection of HCV RNA, with a lower limit of detection of 100 copies/ml. Eighteen (0.28%) anti-HCV negative/HCV RNA positive serum samples were detected. Bamaga et al. performed study on 3288 plasma mini-pool samples collected from blood donors. The samples were tested by RT-PCR, out of 3288 samples 1% were RT PCR positive. Palomäki et al. used mini-pools of 96 donations, 2423 mini-pools (232,600 donations) were screened, nine HCV RNA-positive mini-pool samples were detected. Forcic et al. performed study on anti-HCV negative plasma pools obtained from various Croatian transfusion centres, 2647 anti-HCV negative plasma pools were tested by NAT and 12 (0.45%) HCV RNA positive pools were detected. Koppelman et al. used mini-pools of 8 donors to screen out 2912 test pools, 11 confirmed positive. The specificity after initial testing and percentage of invalid results were 99.83 and 0.48%, respectively.

Use of HCV RNA screening of blood is cost effective. Commercially available HCV RNA differ in cost from lab to lab, on average it costs Rs 4,000 per test (50 US dollars), testing one donation in a mini-pool of 5 samples will cost Rs 800 (10 US Dollars) each if 1 in 200 samples is positive for HCV RNA. As compared to the cost of treatment of chronic HCV infection after transmission of virus (Rs 200,000 or 2500 US dollars) the additional cost of Rs 800 per bag will not be a burden. Screening through 5 sample mini-pools will save Rs 3,20,000 (4000 US dollars) per 100 tests. A risk of HCV transmission in 1 per 200 transfusions is alarming, that too in low seroprevalence population. The risk increases 3 times if blood bags are used to prepare fresh frozen plasma and platelet concentrates as well.

CONCLUSION

HCV RNA prevalence is 0.5% in this anti-HCV non-reactive blood donors with normal ALT. Screening by mini-pools is more cost-effective than expenses of treatment.

Declaration: This manuscript is based on thesis of research conducted for the attainment of PhD degree during 2008.

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