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Occult HCV in Egyptian volunteer blood donors

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Abstract:

Objective: The study aims to investigate the risk of post-transfusion transmission of hepatitis c virus (HCV) in the circumstances of occult HCV when anti-HCV is undetectable by ELISA and HCV-RNA is detected by RT-PCR in the plasma and or in peripheral blood mononuclear cells (PBMCs) of donor blood and the recipients are immunocompromised. **Patients & methods:** The study covered 18 chronic renal failure patients (CRF) [12 males (66.7%) their age ranged from 28 to 65 years and 6 females (33.3 %) their age ranged from 15 to 55 years] undergoing hemodialysis in Nile Hospital as part of their therapy have to receive blood transfusions (275 blood units) for the first time. Commercial ELISA kits for anti-HCV and nested-RT-PCR (N-RT-PCR) kits were used. **Results**: Anti-HCV was positive in one serum from the eighteen (5.5%) poly transfused CRF patients at the end of the study while the seventeen sera were negative. This serum was also positive for HCV RNA by N-RT-PCR. Out of the 20 transfused blood units, one blood unit (three components) were tested by blood banking anti-HCV negative by ELISA, were positive for HCV RNA by N-RT-PCR. The collective markers of this blood unit represent an occult HCV. The risk of acquiring post-transfusion HCV infection from an occult HCV blood unit is 5%. Real time PCR showed variation in the viral load of the serum of the infected CRF patient, the plasma of blood unit, the PBMCs of this blood unit whether activated by PHA-M or not.

Key words: Occult HCV, Egyptian, blood donors.

Introduction

The existence of hepatitis C (originally "non-A non-B hepatitis") was postulated in the 1970s and proven in 1989 (**1**).

HCV is a small, enveloped, single-stranded, positive-sense RNA virus (**2**)**.** It is a member of the hepacivirus genus in the family Flaviviridae (**3)**.

HCV is a human infection affecting primarily the liver. HCV infection is often asymptomatic, making it very difficult to detect it at an early stage and this is the major reason why early treatment is difficult. It is often referred to as "silent disease" (**4)**.

HCV is spread primarily by blood transfusion, intravenous drug abuse, poorly sterilized medical equipments and vertically from an infected mother. An estimated 130–170 million people worldwide are infected with hepatitis C. In some countries, e.g., Egypt, the prevalence is as high as 22% (**5)**.

There are seven major genotypes of HCV, which are indicated numerically from one to seven **(6).** The prevalent genotype in Egypt is type 4 (73%) followed by genotype 1 (26%) whereas mixed HCV genotypes infection was found in 15.7% **(7)**.

Although HCV is mainly hepatotropic, its presence in extrahepatic sites has been widely demonstrated (**8)**. Several investigators have demonstrated that HCV infected peripheral blood mononuclear cells (PBMC) are the major extrahepatic site of viral replication (**9)**.

Generally, most studies of HCV prevalence use nonpaid blood donors to represent the community infection frequency. Screening is usually done by anti-HCV antibodies. Using blood donors as a prevalence source may underestimate the real prevalence of the virus because donors are generally a highly selected population (**10)**. Also Blood banks do not report follow-up HCV testing of recipients.

Material and Methods

Study Samples:-

Eighteen CRF patients [12 males (66.7%) whose age ranged from 28 to 65 years and 6 females (33.3 %) whose age ranged from 15 to 55 years] undergoing hemodialysis in Nile Hospital who received blood transfusion (275 blood units) for the first time. For follow up study a 5 ml blood was collected from these CRF patients before dialysis every three months for a period from November 2011 to October 2012 for time-lapse detection of acute HCV infection by screening for anti-HCV in their sera by ELISA (Axiom kits, Germany).

Detection of anti-HCV by ELISA during follow up of transfusion-recipient CRF patients:-

Plasma samples of the transfused blood units (275 units) were screened for anti-HCV by ELISA using a commercial kit (Axiom, Germany). Peripheral blood mononuclear cells

(PBMCs) of blood units were isolated on Ficoll-Hypaque density gradient solution (**11)**; PBMCs of each blood unit were divided into 2 aliquots for invitro culture for 72 hrs. One PBMC aliquot to be treated with phytohemagglutinin-M (PHA-M) mitogen for T lymphocyte activation at a concentration of 1 million cells per 1 ml RPMI 1640 medium and the second PBMC aliquot without PHA-M mitogen.

The serum of a CRF seroconverted patient at the end of the study was tested for HCV-RNA by N-RT-PCR.

Twenty blood units transfused to the CRF patient who seroconverted from negative anti-HCV to positive antibody were tested for HCV RNA by N-RT-PCR.

The serum of the post-transfusion anti-HCV seroconverted CRF hemodialysis patient and the three samples of the transfused blood unit (Plasma, PBMCs with PHA-M activated 72 hrs invitro culture and PBMCs culture without PHA-M) were tested for quantitative HCV viral RNA load of by N-RT-PCR.

Detection of HCV RNA by Nested Reverse Transcriptase Polymerase Chain Reaction (N-RT-PCR):-

The serum of a CRF patient who was seroconverted at the end of the study and samples from the 20 blood units transfused into this CRF patient were tested for HCV RNA by N-RT-PCR.

HCV RNA extraction and reverse transcription:-

The commercial Viral RNA Extraction Kit "QIAGEN" was used for extraction of HCV RNA according to the manufacture's instructions. Also QIAGEN OneStep RT-PCR Kit was used for both reverse transcription and amplification PCR

Nested-PCR primers:-

N-PCR primers for detection of Hepatitis C Virus RNA in serum, plasma and lymphocytes extracts described by **El Awady et al. (12**) were used. The two pairs of outer and inner primers are:-

A- Outer primers:-

- Primer –1 (P1): is 21 nt sense primer sequence.

5'AACTACTGTCTTCACGCAGAA 3'

- Primer – 2 (P2): is 22 nt anti-sense primer sequence.

5'GGTGCACGGTCTACGAGACCTC 3'

B- Inner primers:-

- Primer –3 (P3): is 18 nt sense primer sequence.

5' GTGCAGCCTCCAGGACCC 3'

- Primer –4 (P4): is 20 nt anti-sense primer sequence.

5' ACTCGGCTAGCAGTCTCGCG 3'

An amplicon of 171 bp length was identified in positive HCV- RNA samples **(12).**

First step of simultaneous RT and PCR amplification of targeted HCV-viral RNA:-

The mixture of N-PCR for first amplification consisted of 10 µl template RNA extract, 0.5 µl sense primer (P1), 0.5 µl anti- sense primer (P2), 10 µl 5x Qiagen one step RT-PCR buffer, 2 µl dNTPs, 2 µl Qiagen one step RT-PCR enzyme mix (containing reverse transcriptase and taq DNA polymerase) and 25 µl DEPC treated water (RNase free) to complete the final volume to 50 µl **(11).**

The tubes were placed in the thermocycler for DNA amplification using the following protocol:-the reaction was incubated at 42ºC for 60 min. and denaturated at 98ºC for 10 min. followed by 30 cycles of amplification (denaturation at 94 ºC for 1 min, annealing at 55 ºC for 1 min then extension at 72 ºC for 1 min) then a final step of extension at 72 ºC for 7 min. for one cycle.

Second Nested PCR amplification:-

The mixture for nested PCR amplification consisted of 5 µl of first step of N-PCR product, 0.5 µl sense primer 3 (**P3)**, 0.5 µl anti- sense primer 4 (**P4)**, 25 µl master mix (fermentas) and 19 µl DEPEC treated water to complete the final volume to 50 µl **(12).**

The tubes were placed in the thermocycler for DNA amplification using the following protocol: - one cycle of DNA denaturation at 95 ºC for 5 min. followed by 30 cycles of amplification (denaturation at 94 ºC for 1 min, annealing at 55 ºC for 1 min then extension at 72 ºC for 1 min) then a final step of extension at 72 ºC for 7 min. for one cycle.

The amplified product was detected by 2 % agarose gel at 171bp.

Detection of HCV RNA by Quantitative Real time PCR:-

The serum sample of CRF hemodialysis patient and the three samples of the blood unit (Plasma, PBMCs with PHA-M activated 72 hours and PBMCs without PHA-M) were detected for quantitative HCV RNA viral load by using ABI 7300 prism ® (Applied Biosystem, Foster City, USA).

Results

Detection of anti-HCV in Hemodialysis patients sera by ELISA:-

Anti-HCV was positive in one serum from the eighteen (5.5%) poly transfused CRF patients at the end of the study while the other CRF patients sera remained negative. -Normal blood picture according to the sex and age.

Detection of HCV RNA by N-RT-PCR:-

The serum of the seroconverted CRF patient was positive for HCV RNA by N-RT-PCR. The **s**ixty samples (20 plasma samples, 20 PBMCs with PHA-M activated 72 hours and 20 PBMCs without PHA-M) from the 20 blood units transfused into this patient when tested for HCV RNA by RT-PCR showed one transfused blood unit out of twenty units which had no antibodies against HCV by ELISA, but was positive for HCV RNA by N-RT-PCR.

The markers of this blood unit represent occult HCV. The risk of acquiring post-transfusion HCV infection from an occult HCV infected blood unit is (1/20 x 100) = 5% in the blood banking service provided at the Nile hospital where the study was done.

Discussion

Hepatitis C virus (HCV) is a serious worldwide problem which has a great impact on health status in the field of liver diseases with an estimation of 170 million infected people worldwide **(5)**. Egypt has a very high prevalence of HCV antibody reaching up to 24% prevalence rate in some age groups and population sectors **(7,13**). More than 90% of HCV infection in Egypt is genotype 4 **(14)**.

Volunteer blood donors are considered to represent community HCV infection status that **El Gohary et al. (15)** found 14.5% HCV seroprevalence in blood donors at Ismalya, confirming the 14.4% seroprevalence in blood donors from Cairo **(16)** and of 19.4% in Egyptian blood donors tested in Saudi Arabia (**17**).

In the present work, we investigated post-transfusion transmission of HCV in the circumstances of occult HCV infected blood units. Occult HCV is considered as undetectable plasma anti-HCV by ELISA; detection of HCV-RNA by RT-PCR in the serum and PBMC. Also we investigated the effect of PHA-M mitogen activation of blood unit PBMC for improving HCV RNA detection in occult HCV.

We found in our study one blood unit out of 20 blood units 1/20 (5%) which had no HCV antibodies by ELISA, while its (three samples: Plasma, extracts of a 72 hrs invitro culture of PBMCs activated with mitogen or PBMC without mitogen) were positive for HCV RNA by Nested-RT-PCR. The markers of this blood unit represent occult HCV.

There was no association between the presence or/absence of anti-HCV in the serum and the presence of HCV RNA by PCR in this blood unit. Unfortunately this occult HCV infected blood unit caused the transmission of HCV infection into a CFR patient. Thus the risk of post-transfusion HCV infection was 5%, which is dangerous and requires immediate corrective action in Egyptian small blood banking utilities. Implementation of NAT screening should be enforced there.

The HCV RNA viral load of the posttransfusion HCV infected CRF patient serum (2150 IU/ml) was greater than the viral load of HCV in the donor plasma (998 IU/ml).This may be due the low immunity of CRF patient that allowed replication of the virus to take place heavily than in the case of the blood donor. The viral load of HCV in PBMCs of blood unit (2.153.000 IU/ml) was greater than the viral load of HCV in plasma and this affirms that the PBMCs are an extrahepatic site suitable for replication of HCV. The HCV viral RNA load of in extracts of PBMC invitro 72hrs culture activated with mitogen (PHA-M) (2.678.000 IU/ml) was greater than the HCV viral RNA in extracts of PBMCs without mitogen (2.153.000 IU/ml). This showed the ability of PHA-M to enhance the expression and replication of the HCV viral genome invitro cultures for 72 hrs.

The fact that mitogen stimulation can enhance productive virus replication raises the question if for example; a coincidental non specific or specific activation of lymphocytes may play a role in occult HCV infection disease course and augment hepato-pathogenicity of an ongoing chronic infection. In the case of HCV, virus reactivation in PBMCs has been reported following liver transplantation **(18).**

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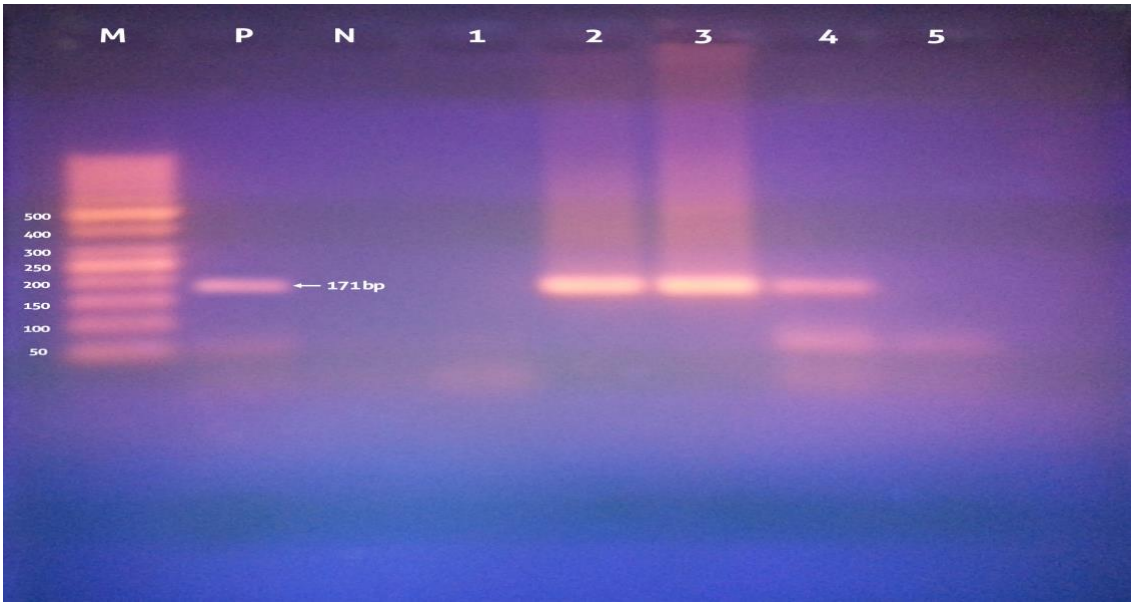

Figure (1): Nested-PCR showing HCV-RNA amplicon at 171 bp detected in occult HCV infected transfused blood unit: Results of two PBMCs extracts and one plasma sample.

Real Time Polymerase Chain Reaction for Hepatitis C Virus RNA load in serum, plasma and PBMCs:-

HCV-RNA load in the serum of post-transfusion HCV infected CRF patient as well as the occult HCV infected blood unit, plasma as well as PBMCs extracts (See methods) were tested by real time PCR showed that HCV replicated in the recipient and the blood unit PBMC load was higher than plasma.

Sample	**HCV- RNA by Real-time PCR (IU/ml)**
Serum of HCV infected CRF patient *	2150
Transfused blood unit Plasma	998
Extract of transfused blood unit PBMCs **	2.678.000
Extract of transfused blood unit PBMCs ***	2.153.000

Table (1): HCV-RNA load results of Real time PCR
* Acquired post-transfusion HCV infection.
** Extracts of an invitro 72 hrs PBMCs culture activated with PHA-M
*** Extracts of an invitro 72 hrs PBMCs culture without PHA-M