Egyptian Journal of Medical Microbiology Volume 25 / No.3 / July 2016 1-8

ORIGINAL ARTICLE

Prevalence of Occult Hepatitis C Virus in Blood Donors in Zagazig City Blood Banks

Omar M. Eldaly, Eman M. Elbehedy, Ahmed Elsadek Fakhr, Alshimaa Lotfy*
Medical Microbiology and Immunology Department, Faculty of Medicine, Zagazig University, Zagazig, Egypt

Key words:

Occult HCV, Zagazig city blood banks, blood donors

ABSTRACT

Background: *Egypt is reported as the country of highest incidence of HCV worldwide. The high rate of new cases reported require investigating uncommon sources of infection.* ***Objectives:*** *The study aimed to investigate the prevalence of occult hepatitis C infection (OCI) among blood donors from Zagazig city blood banks as a probable hidden source of transmission; and to evaluate HCV core Ag detection as a method for diagnosis of occult hepatitis C infection.* ***Methodology:*** *One hundred and forty blood donors from Zagazig university blood bank were incorporated into this study. Reverse transcription nested polymerase chain reaction from Peripheral Blood Monocyte (PBMC) and plasma obtained from subjects was done using HCV specific primers and HCV core antigen was detected using commercial ELISA kit.* ***Results & Conclusion:*** *HCV RNA was detected in PBMC extracted from 10 subjects (7.1 %). Two of them were also positive in plasma (1.4%). The overall incidence of OCI was 8/140 (5.7%). Out of them only one was also identified by ELISA HCV core antigen detection kit. The Comparison between ELISA technique for detection of HCV core antigen and nested PCR revealed sensitivity 30%, specificity 99.2% in PBMC and sensitivity 100%, specificity 98.6% in Plasma.*

INTRODUCTION

The hepatitis C virus (HCV), an enveloped single-stranded RNA virus, classified as a separate genus (Hepacivirus) within the Flaviviridae family[1]. The virus replicates by the synthesis of the so-called negative or antigenomic complementary RNA strand[2]. So far, six major genotypes (HCV-1 to HCV-6) have been defined, each encompassing multiple subtypes with substantial differences in their global distribution and prevalence [3].

The disease is affecting about 170 million people worldwide with a high incidence of chronic liver illness including cirrhosis and hepatocellular carcinoma [4, 5].

An estimated 8-10 million among Egyptian population have been exposed to the virus and 5-7 million have active infections [6] with up to 100,000 new infections occur each year in the country.[7]

Originally, the diagnosis of HCV infection was made by the detection of antibodies against the viral antigens and/or by detecting the presence of its RNA in plasma or serum[8]. However, a new entity of HCV infection was first designated in 2004 in some patients with persistently elevated liver function tests and who were anti-HCV and serum HCV RNA negative [9].

Corresponding Author:
Alshimaa Lotfy
Medical Microbiology and Immunology Department, Faculty of Medicine, Zagazig University, Egypt
Email: drshimo82@yahoo.com; Tel.:01009816616

Fifty seven % of these patients were found to have HCV RNA in the liver in the absence of conventional HCV markers and this clinical situation was termed "occult HCV infection". It was also verified that the antigenomic HCV RNA strand could be detected in the liver cells of many patients with occult HCV infection indicating active viral replication [10].

Occult HCV infection (OCI) has also been described in two other different clinical situations. One of these is positive for anti-HCV, serum HCV-RNA negative with persistent normal values of liver enzymes, of whom approximately 90% have detectable viral RNA in their liver cells and in peripheral blood mononuclear cells (PBMCs)[11,12]. The second one is in individuals who resolved HCV infection after antiviral treatment or spontaneously. In these patients, viral RNA remains detectable in hepatocytes and in PBMCs years after apparent recovery from the disease (normalization of liver enzyme values and loss of serum HCV RNA). This occult infection could be related to the continuity of necro-inflammatory process in the liver of the sustained responders[13]. The main risk is that general population who are apparently diseases free could have occult HCV infection. The viral replicative ability in PBMCs could carry the potential to spread HCV through transfusion or haemodialysis, and probably predispose to liver neoplasia in occult HCV infected subjects [14].

Being the highest prevalence of HCV worldwide with high incidence of new infections, facts require the determination of the actual rate of (OCI) among the Egyptian population especially groups like blood donors and hemodialysis patients. New methods of diagnosis rather than the routine detection of HCV Ab in serum need also to be evaluated.

Detection of hepatitis C virus core antigen (HCV core Ag) in serum or plasma is a promising test which could be an alternative to the molecular techniques, and is particularly important during the window period of HCV infection occurring after HCV infection and before the appearance of antibodies[15]**.** It can be used as a screening assay for blood or plasma donations in different countries [16]**.**

METHODOLOGY

1. Study population:

One hundred and forty blood samples from donors attending Zagazig university blood bank were incorporated into this study (10 females and 130 males) with age. All of them were apparently free from liver diseases and negative for HCV IgG by ELISA.

Peripheral Blood Monocytes (PBMCs) were obtained by the standard density gradient technique using (Lymphoflot; Biotest, Dreieich, Germany) following the manufacturer instructions. The PBMC pellet was directly used for RNA extraction.

Plasma was obtained by centrifugation of Heparinized blood for 15 minutes at 2,000 x g to remove cells and platelets then the supernatant was divided into aliquots of 0.5 ml and stored at -20°C for further use.

RNA extraction from PBMCs and plasma was performed using Viral Gene-spin™Kit **(iNtRON Biotechnology, Korea)** and TIANamp Virus RNA Kit **(TIANGEN, China)** respectively. RNA was kept at -20˚C till use.

2. Nested PCR:

RNA from both plasma and PBMC was subjected to Nested PCR reactions using Maxime RT-PCR PreMix Kit **(iNtRON Biotechnology, Korea)** in the first round PCR and PCR Master mix Solution **(i-Taq TM) (iNtRON Biotechnology, Korea**) in the second round PCR using primers (Table 1) previously described by Ray *et al*. (2000) targeting the 5' Non coding region (NCR) of the HCV genome [17]**.**

Table 1: PCR primers

Primer	***Sequence (5`-3`)***	***Extension from 5` base of the HCV genome***
Outer forward primer (P1)	5`-GTGAGGAACTACTGTCTTCACGCAG-3`	(47-71)
Outer reverse primer(P2)	5`- TGCTCATGGTGCACGGTCTACGAGA -3	(324-348)
Inner forward primer (P3)	5`-TTCACGCAGAAAGCGTCTAG-3`,	(63-82)
Inner reverse primer (P4)	5`-CTATCAGGCAGTACCACAAGG-3`,	(279-299).

All PCR reactions were carried out on thermal cycler **(Biometra, Cambridge U.K)**. 20 picomoles of each outer primer (P1 and P2) were added to 10ul purified RNA and 25 ul of master mix. A total volume reaction of 50 ul was reached by adding nuclease free water. The purified RNA samples were subjected to reverse transcription at 45° c for 30 min followed by inactivation of RT enzyme at 94° c for 5min. then amplification was done for 35 cycles of denaturation at 95˚C for 15 sec, annealing at 55˚C for 15 sec and extension at 72˚C for 1 min. A final cycle of 72°C for 10 minutes was done.

Nested PCR was performed by the transfer of 10 µl of the first PCR product to 40 µl of the second master mix containing 1X Taq buffer, 0.2 mM dNTP's, 20 pmole of each nested primer P3 and P4. The second step (Nested) PCR was done using the same cycling conditions of the first step omitting initial RT and RTase inactivation.

Amplicons were analyzed on 1.5% agarose gel stained with ethidium bromide and visualized by ultraviolet transillumination. The presence of a 236 bp amplification fragment indicated HCV-RNA positivity in the sample.

3. Detection of HCV core antigen:

It was detected by qualititative measurement using ELISA kit **(MyBioSource, USA)** according to the manufacture instructions.

RESULTS

Socio demographic characteristics of studied donors included revealed that most donors were middle age group males from rural areas (Table2). Five donors gave past history of surgery and dental procedures whereas 4 had received at least one setting of blood transfusion. However, none of the previous data showed statistical significance in correlation with HCV PCR positive results (Table 3).

Among the 140 studied donors, 10(7.1%) were HCV RNA positive from their extracted PBMC. Two of them (1.4%) HCV RNA was also detected in their

plasma. According to the OCI definition, results of nested PCR for plasma and PBMC demonstrated that occult cases were 8 out of 140 donors (5.7%). (Table 4).

The results of ELISA showed that only 4 out of 140 donors (2.9%) were positive for core antigen detection. In relation to results of nested PCR for RNA extracted from PBMC, ELISA technique for detection of HCV core antigen showed 30% sensitivity, 99.2% specificity with 75 % and 94.9% positive and negative predictive value, respectively. (Table 5). However; in relation to results of plasma samples nested PCR the sensitivity was 100%, specificity 98.6% and 100% and 100%% positive and negative predictive value, respectively. Only one case (12.5%) among those confirmed as OCI was also positive for HCV core antigen using ELISA technique.

Table 2: **Association between socio demographic characteristics and results of nested PCR of HCV cases of studied donors (N= 140)**

Socio demographic data	*PCR (+ve) No.=10*		*PCR (-ve) No.=130*		*X2*	*P. value*
	No.	**%**	**No.**	**%**		
Age (years)						
▪ 18- (53)	2	20.0	51	39.2		
▪ 28- (59)	3	30.0	56	43.1	6.13	0.047*
▪ 38-49 (28)	5	50.0	23	17.7		
Sex						
▪ Female (10)	1	10.0	9	6.9	0.13	0.716
▪ Male (130)	9	90.0	121	93.1		
Geographic distribution						
▪ Rural (78)	7	70.0	71	54.6	0.89	0.345
▪ Urban (62)	3	30.0	59	45.4		

Table 3: Association between past history and results of nested PCR of HCV cases of studied donors (N= 140)

Past history	*Total No.*	*+ve HCV*		*-ve HCV*		*P.value*
		No.	**%**	**No.**	**%**	
▪ **Jaundice**	0	0	0.0	0	0.0	---------
▪ **Drug intake**	0	0	0.0	0	0.0	----------
▪ **Surgery and dental procedure**	5(3.6)	3	37.5	2	1.5	0.00**
▪ **Previous blood transfusion**	4(2.9)	2	25.0	2	1.5	0.01**

-----=invalid **= highly significant

Table 4: Correlation between results of nested PCR for plasma and PBMC

	PCR(PBMC)		
	(+ve) cases	**(-ve) cases**	**Total**
PCR(plasma)			
(+ve) cases	2	0	2
(-ve) cases	8	130	138
Total	**10**	**130**	**140**

Table 5: Comparison between ELISA technique for detection of HCV core antigen and nested PCR (PBMC) and plasma in detection of HCV.

	PCR (PBMC)		*PCR(Plasma)*		*Total*
	(+ve) cases	**(-ve)cases**	**(+ve) cases**	**(-ve)cases**	
ELISA					
(+ve) Cases	3	1	2	2	4
(-ve) Cases	7	129	0	136	136
Total	**10**	**130**	**2**	**138**	**140**

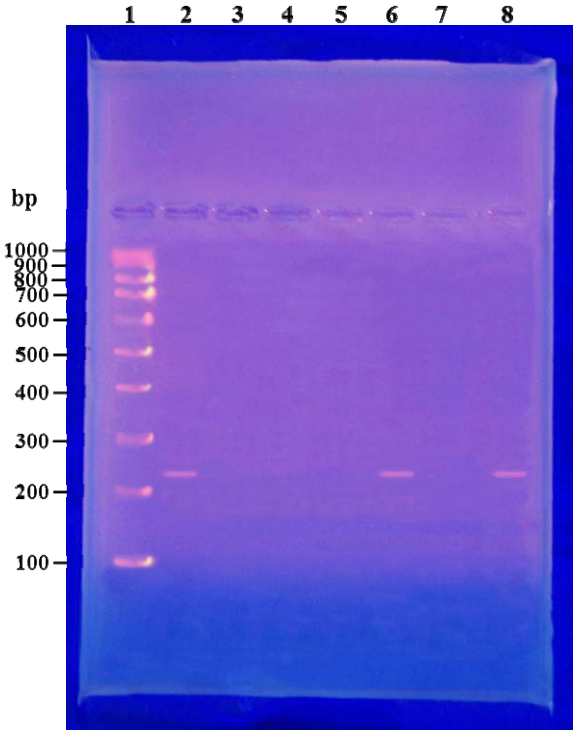

Fig. 1: Results of PCR amplification of HCV RNA in PBMC. Lane (1) is 100Bp Mwt marker, lane (2) is positive control , lane (3) is negative control , lanes(6,8) are positive PCR products of amplification of HCV RNA from PBMC of blood donors and lanes (4,5,7) are negative.

DISCUSSION

Occult HCV infection (OCI) is a different pathological entity from typical HCV infection. Authors have long struggled to prove the existence of occult hepatitis C infection (OCI). In the last years, it has been documented in haemodialysis patients, in general populations and in chronic liver disease patients of unknown etiology [18, 19, 20, and 21.]

HCV infection is routinely diagnosed and monitored by the detection of HCV antibodies and/or HCVRNA in plasma or serum. Subjects affected by occult HCV infection test negative for HCV-RNA in serum, but they are HCV-RNA positive in liver biopsies and may display abnormal values of liver enzymes[22]. Also identified in peripheral blood mononuclear cells (PBMCs) which represent alternative extra hepatic site of HCV replication [9].

In a country like Egypt, which has the highest prevalence of hepatitis C virus (HCV) in the world, ranging from 6% to 28% with an average of approximately 15% in the general population [23,24,25] and genotype 4 represents over 90% of Egyptian cases[26], with no sufficient data about occult HCV. Therefore, this study tried to investigate the prevalence of occult HCV in healthy blood donors in Zagazig University blood bank. A percent of (7.1%) was found which is near to the results of another study performed by Mostafa *et al*[27] who studied prevalence of OCI in Egyptian volunteer blood donors and was (5.5%). However, less percentage was detected by De Marco *et al* [18] who found that (3.3%) of blood donors in Italy have OCI. This could be interpreted by the lower prevalence of HCV there in comparison to Egypt.

The figures were higher when observing patients with risk factor, cryptogenic or overt liver disease. Keyvani and his colleagues [28] identified OCI in (8.9%) from liver transplant candidates with cryptogenic cirrhosis. Also, In Kasr El-Eini Hospital Samir *et al* [30] diagnosed OCI in (20%) among the studied patients with chronic lympho-proliferative disorder in comparison to (4%) of the control healthy group of their study.

The unexpected result of this study raises the issue of occult HCV infection frequency in the general population and its potential implications on the safety of medical procedures particularly blood transfusion which may never be completely risk-free.

The study revealed also a ratio of (1.4%) prevalence of hepatitis C virus by nested PCR from plasma among studied donors which is incompatible with the criteria of chosen donors who must be negative for HCV antibodies by ELISA, the test routinely performed for donated blood at Zagazig University blood bank. This may be attributed to false negative ELISA result due to technical error or possibly those

donors were early during incubation period before the seroconversion. This also raises the need to introduce new confirmatory methods in addition to ELISA like Nucleic acid testing (NAT) or core antigen detection methods for decreasing such false negative cases in our blood banks.

This means that the actual prevalence of OCI in this study was eight out of 140 (5.7%). This was slightly near to other study of Shazly *et al* who reported that the prevalence of OCI was 4% among healthy Egyptian sexual partners of patients with chronic HCV genotype 4 infections. Also Abdelrahim.S found a 3.7% prevalence of OCI among 81 hemodialysis patients at Minia Governorate, Egypt. [31, 32]

Furthermore, in this study, the association between socio-demographic characteristics and results of nested PCR of HCV cases of studied donors was investigated.

First, as regard age, 50% of HCV positive cases fall in the eldest age group (38-49 years) which was statistically significant (P. value=0.047). The age group was in agreement with the study performed by Castillo *et al* [29] who noticed the existence of positive HCV RNA in PBMC in age group (40- 52 y) and P value was 0.6 , Castillo *et al* [33] who compared the clinical, virologic, and histologic characteristics of patients with occult dual infection (HBV and HCV) to those of patients with single occult HBV or HCV infection by real-time PCR and found that occult HCV cases concentrated around 46 years and also L´opez-Alcorocho *et al* [34] who hypothised that ultracentrifugation of serum samples allows detection of hepatitis C virus RNA in Patients with occult hepatitis C by strand-specific real-time PCR and found that positive cases concentrated around 42 years and p value was 0.1.

However, association with older age was not found in all related studies. Samir *et al* [28] detected OCI in a younger age group (18 years) among chronic lympho proliferative disorder patients, while Saad *et al* [35] found that age was statistically insignificant (P. value 0.646).

Second, regarding sex of the donors, no statistical significant difference was demonstrated. The P. value was (0.716). The same finding was raised by Saad *et al*.[35] who found P. value was (1.0). However, the low number of females in the study makes it difficult to evaluate a solid relationship regarding this point. More numbers are needed to assess any possible association between the sex and OCI.

Finally, the different geographic distribution weather rural or urban was found to be statistically insignificant P. value was (0.345) and this also agrees with Saad *et al* who found that geographic distribution was statistically insignificant. Further studies are required to exclude or confirm any association between OCI and geographic distribution of the subjects.

Possible predisposing factors to OCI were also investigated by studying association with previous blood transfusion, dental procedures, surgery, drug intake, jaundice and fever.

Fortunately, there was high statistical significant difference between these factors and this result was co matched with Keyvani *et al*. who found that blood transfusion and history of travel to endemic areas is statistical significant P-value (0.02) and (0.001) respectively mean while, dental procedures and surgery were insignificant P-value (0.601) and (0.938) respectively.

On contrary, Samir *et al* found that blood transfusion as a possible risk factor was insignificant. Further studies are required to outline the possible risk factors.

HCV RNA detection by PCR is the gold standard for diagnosis of HCV infection; however HCV core antigen (Ag) tests have been introduced to supplement anti-HCV tests or HCV Real-time quantitative reverse transcription-PCR (qRT-PCR) analyses over the last decade [37]. These quantitative HCV Ag assays could be used for the monitoring of antiviral therapy as well as for diagnosis of HCV infection [38].

The test is a simple and reliable direct method for detection of HCV infection. Since this assay is based on ELISA technology, it can be easily performed in most laboratories and is cost-effective. Further it does not need any specially trained personnel, special equipment or infrastructure except that which is required for ELISA tests. It also reduces the problems of possible cross contamination and false positives as in a nested PCR protocol. Hence it could be a suitable alternative to HCV RNA detection. HCVcAg is also more stable as compared to RNA, the latter more likely to be degraded by the RNAase in the blood sample [40, 41].

Further, it was made mandatory for European Blood banks in January (2002) to test every blood and plasma donation for either HCV core Ag or HCV RNA in order to reduce the residual risk of HCV transmission through blood banks [36].

To estimate this test in correlation with nested PCR for diagnosis of OCI, It was investigated among the studied donors.

HCV core antigen by ELISA technique was detected in (2.9%) out of whole subjects. A result which is slightly higher than that found by Alzahrani and Obeid[15] who evaluate the presence of hepatitis C virus core antigen (HCV core Ag) in HCV antibodies negative blood donors at King Fahd Hospital of the University and was (2%). However, Gaudy *et al*[39] performed HCV core Ag screening among seronegatives cases with elevated liver enzymes and only (0.7%) out of them were found to harbor the antigen in their sera. Different locations of the studies and difference in kits or reagents could interpret this variation.

The results of comparing core Ag detection against nested PCR from PBMC was different when compared with that from plasma. Out of 10 who were positive by nested PCR from PBMC, only HCV core Ag was detected in sera of 3 of them, while only one sample of

those which were negative by nested PCR was positive for HCV core Ag, with 30% sensitivity and 99.2% specificity.

Nevertheless, results were better when comparing results of HCV core Ag against nested PCR from plasma samples. Both samples, which were positive by nested PCR, the core Ag was also detected and 2 samples of those which were negative by nested PCR were positive for HCV core Ag (100% sensitivity and 98.6% specificity).

However; this correlation between ELISA technique for detection HCV core antigen and nested PCR from plasma were different from that reached by Daniel and his colleagues [36] who tried to evaluate the role of core antigen as a marker of active HCV infection in comparison to HCV RNA. They found that sensitivity (85.3%) and specificity (95.8%). Also, Gaudy et al [39] who tried to detect usefulness of the hepatitis C virus core antigen assay for screening of a population undergoing routine medical checkup and found that sensitivity of the test in relation to HCV RNA detection (85.3%), specificity (99.3%).

In this study, only one case of those diagnosed as OCI, was also positive by HCV core Ag which means that the test has a very low sensitivity (12.5 %) in the diagnosis of OCI cases. These matches with the results reached by Juan and his colleagues[42] who evaluated a combined hepatitis C virus (HCV) antigen-antibody assay for 115 seronegative individuals with occult HCV infection and only 4 were positive. They concluded that the HCV core Ag assay does not improve the routine serological diagnosis of occult HCV infection.. The low sensitivity when comparing results against OCI may be due to very low count of the virus.

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