Prevalence and Risk Factors of Occult Hepatitis C Virus infection in One Tertiary Egyptian Centre

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Abstract

Background: Occult hepatitis C infection (OCI)is a new form of hepatitis C virus (HCV) in which HCV-RNA is present in hepatocytes or peripheral blood mononuclear cells(PBMCs)with undetectable plasma HCV-RNA , regardless of hepatic transaminase elevation. **Aim:** To find out prevalence of OCI infection in patients with anti HCV antibodies positive regardless to their transaminases and to study possible risk factors for OCI **Patients and Methods:** The current study included 100 patients in which HCV antibodies were detected by ELISA and HVC-RNA were examined in serum and PBMCs by real time polymerase chain reaction (PCR). **Results:** OCI was detected in 14 % of enrolled patients. Having BMI ≥35.5kg/m^2 demonstrating 5-times higher odds of exhibiting OCI in females while male sex had 10-times higher odds. **Conclusions and Recommendations :**Prevalence of OCI was 14% in anti HCV positive patients regardless hepatic transaminases level among them, obesity and male sex considered the main risk factors in this study, more studies on bigger number of patients are required.

Key words: Occult, Hepatitis C, Prevalence, Risk factors.

Introduction:

Hepatitis C virus (HCV) affects approximately 170 million people worldwide and accounts for an estimated 350,000 deaths annually. Detection of serum HCV-RNA and HCV antibodies are the basis of HCV diagnosis (1-4). However, a separate entity of this infection is occult HCV infection (OCI), characterized by detectable hepatocyte and peripheral blood mononuclear cell (PBMC) HCV-RNA in association with immeasurable

serum HCV-RNA (5, 6). The two currently recognized types of OCI are seronegative OCI characterized by negativity of both anti-HCV antibodies as well as serum HCV-RNA, and seropositive occult HCV infection, or secondary OCI, distinguished by positive anti-HCV antibodies but negative serum HCV- RNA (7, 8).

Occult HCV infection (OCI) has been detected in patients with cryptogenic liver disease at different rates e.g. about 10% as evidenced by some studies (2, 9) or about 74% as evidenced another (10). It also can be detected in the general population, as demonstrated in a study in which 9 out of 279 subjects (3.3%) with normal enzymes and negative HCV antibodies and HCV- RNA in serum, were shown to be peripheral blood mononuclear cell (PBMC) HCV-RNA positive. A Chinese similarly demonstrated that 2.2% of blood donors were diagnosed as OCI (11).

In 2018, some researchers reported that 3.4 % among blood donors have OCI (12).

Furthermore, OCI has been reported in special patient groups including autoimmune hepatitis (13), lymphoproliferative disease (14,15), glomerular nephropathies (16), antiphospholipid syndrome (17), Type II mixed cryoglobulinemia (18-20), hemophilia (21), hepatitis B infection (22), alcoholic liver disease (23) and HIV infection (24).These findings need more studies to clarify prevalence and risk factors for OCI

While liver biopsy is the primary diagnostic tool for OCI by identification of hepatocyte HCV-RNA, the risks associated with this procedure, such as bleeding and accidental organ puncture, call for testing of PBMCs in most studies in order to establish the diagnosis of OCI. However, PBMC testing for HCV-RNA is inferior to hepatocyte testing with regards to true estimation of OCI prevalence, as PBMCs are positive in only about 70% of patients when compared to hepatocytes. Nevertheless, this rate of detection may increase with serial PBMC testing in cases of high OCI suspicion (5, 25, 26), as evidenced in a recent study by Wang et al showing that detection rate of OCI was comparable between PBMC testing (92%) and liver biopsy (27).

During the era when pegylated-interferon plus ribavirin was the standard therapy for patients with chronic hepatitis C, Pardo et al conducted a study to evaluate the effect of this drug regimen on patients with OCI (28). Ten OCI patients with elevated ALT levels, positive PBMCs for HCV-RNA, and liver biopsy findings of necroinflammation were given treatment for 24 weeks then followed up for a further 24 weeks. By the end of the

therapy period, 80% of patients demonstrated normalization of ALT levels and undetectable HCV-RNA in PBMCs. However, a sustained response could be achieved in only 30% of patients after 24 weeks of post-treatment follow-up. Quantification of PBMC HCV-RNA was not recorded at this time, although this would have been beneficial to further assess the effect of the administered therapy.

After the follow up period, five patients opted to undergo repeat biopsy, showing persistence of HCV-RNA. However, post-treatment biopsy in three cases demonstrated lower viral load, decreased number infected hepatocyte, and diminished necroinflammation and fibrosis. These results on OCI patients were in accordance with those typically observed in patients with chronic HCV infection at the time.

Recent introduction of the more efficient direct-acting treatment regimens for chronic hepatitis C revealed an increased association with persistent occult HCV infection following cessation of therapy for overt infection, with percentage of OCI following achievement of sustained virologic response (SVR) estimated to be 11.3% even reaching up to 55% in patients re-infected with HCV following liver transplantation (8, 29).

Therefore we aimed to determine the prevalence of OCI in Egyptian patients who have positive anti-HCV antibodies but negative serum HCV-RNA by PCR, and to study the possible risk factors.

Patients and methods

Type of study is: Prevalence study

It was conducted on patients recruited from the outpatient Hepatology clinics of Mansoura University Hospital in Egypt during the period from March 2016 to March 2018. Patients included in this study were those with positive antibodies for hepatitis C virus (anti-HCV antibodies) detected by third generation immunoassay method and negative hepatitis C virus RNA (HCV-RNA) by PCR in three consecutive plasma samples taken 6 months apart. Criteria for patients excluded from this study were those co-infected with hepatitis B virus, patients with positive HCV-RNA, and patients with other identifiable causes of liver affection, such autoimmune or alcohol hepatic diseases.

Design for this study was approved by the Mansoura Ethical Committee and written informed consent was obtained from each patient before being subjected to full medical history taking, complete clinical examination, and radiological evaluation by abdominal ultrasound.

Blood sampling and DNA testing

Samples of 10ml blood were taken from each patient and divided into three aliquots, one without anticoagulant (for serum separation) and two aliquots with EDTA, one for plasma separation and the other for peripheral blood mononuclear cells (PBMCs) separation for determination of HCV-RNA.

Serum samples were used for determination of liver functions including aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin and albumin by automated system Dialab 480. The measurement of alpha fetoprotein was done by enzyme linked immunoassay (ELISA).

HCV-RNA Extraction from Plasma

HCV RNA extraction was performed using Qiagen extraction kit for RNA (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions.

RNA Extraction from PBMCs

Peripheral blood mononuclear cells (PBMCs) were prepared by density gradient method by use of Ficoll Hypaque density according to the manufacturer's instructions (Lymphoflot; Biotest, Derelict, Germany). The obtained mononuclear cells were washed with phosphate buffer solution and re- suspended after adjustment of the cell count to 1×10^{6}cells/ml in PBS. Lysate of mononuclear cells was prepared by using Trizol reagent and shaking well to mix the cells, after which 100µm chloroform was added to each eppendorff, shaken adequately, and centrifuged at 13,200rpm for 15 minutes. The obtained supernatant was transferred to a sterile eppendorff to which 500µm of 100% isopropanol was added then re-centrifuged at 13,200 rpm for 15 min.

The supernatant was then discarded and 500µm of 70% ethanol was added and centrifuged for 10min, after which the ethanol was poured out. After the eppendorff was left to dry for 10 mins, a 20µm of hydration solution were added then frozen at -80°C until amplification.

Hepatitis C virus amplification by real-time PCR

HCV-RNA was detected by using a kit supplied from Qiagen (HCV RT- PCR Kit lot No., Qiagen,courtaboeuf, France). Amplification was performed by use of Applied Biosystem. The quantitative real-time PCR kit of HCV is based on RNA reverse transcription process with consequent cDNA fragment amplification by PCR. The amplification process lies in repeated cycles

of thermal DNA denaturing, primer annealing with complementary sequences, and completion of further polynucleotide chains by Taq-polymerase. An internal control (IC) sample corresponding to a stabilized RNA fragment was added to the sample being examined at the stage of nucleic acid isolation and intended for estimation of the efficacy of all the examination stages. The HCV quantitative real-time PCR kit and DNA probes, each of which contains a fluorescence label and fluorescence quencher, were included in the PCR mix. In case of specific cDNA product formation, a probe becomes destroyed leading to a fluorescence level growth registered by special appliances. DNA probes used for sought nucleic acid and IC PCR product detection are labeled with FAM and HEX fluorescence probes accordingly, allowing separate registration of HCV cDNA and IC sample PCR results.

Statistical analysis:

Data were entered and analyzed using IBM-SPSS software (2017, Version 25, Armonk, NY, IBM Corp.). Categorical data were expressed as N (%) and compared using ChiSquare (or Fisher's exact) test. Quantitative data were initially tested for normality using Shapiro-Wilk test; data were considered normally distributed if p value > 0.050. Presence of outliers was tested for by inspecting the boxplots. Quantitative data were expressed as mean ± SD (compared between two groups using Independent-Samples t-test) if normally distributed with no significant outliers or as median [25^{th} percentile – 75^{th} percentile] (compared between two groups using Mann-Whitney U test) if not normally distributed and / or having significant outliers. The receiver operating characteristic (ROC) curve was plotted between the "sensitivity" (true positive rate) and "1- specificity" (false positive rate) across a series of cut-off points to find the optimal cut-off point that discriminates diseased and non-diseased subjects. Predicting the likelihood of a dichotomous variable was performed using logistic regression analysis.

Results:

Our main results are shown in fig . (1). HCV PBM level in Occult HCV was 8.1 ± 1.4 IU/ml, ranging from 5.5 to 10.5 IU/ml. Table 1 shows that BMI (particularly BMI $\geq$35.5kg/m^2) and moderate to morbid obesity were significantly higher in patients with occult HCV infection when compared to those without. Table 2 shows that higher BMI was a significant predictor of occult HCV infection (OCI), with patients having

BMI ≥35.5kg/m^2 demonstrating 5-times higher odds of exhibiting OCI.

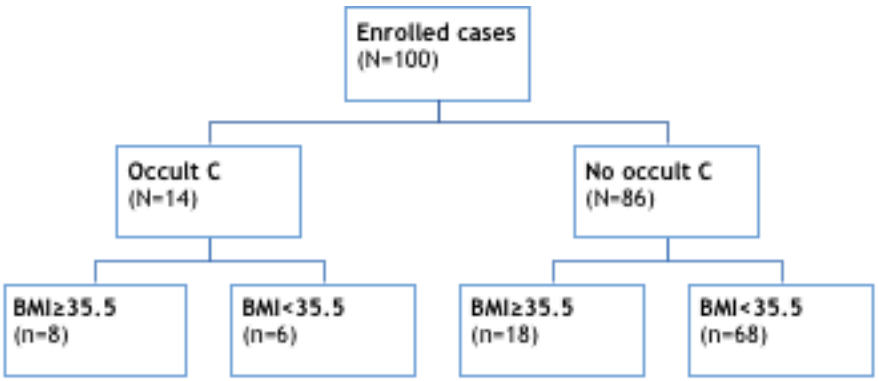

Fig. 1: Flow chart

Table (1): Comparison of clinical and laboratory data

Variable	Group		Test of significance	
	No occult C (N=86)	Occult C (N=14)	χ^2 or Z value	P value
Age (years)	55 (45.8-6)	50.5 (46.3-60.3)	Z=-0.866	0.386
Sex Male	60 (69.8%)	8 (57.1%)	χ^2=0.882	0.384
Sex Female	26 (30.2%)	6 (42.9%)		
Current smoking*	20 (23.3%)	5 (35.7%)	χ^2=0.997	0.318
Previous surgery*	36 (41.9%)	6 (42.9%)	χ^2=0.005	0.944
Presence of diabetes(DM)*	13 (15.1%)	4 (28.6%)	χ^2=1.545	0.214
BMI(kg/m2)	30.5 (28-34.9)	35.6 (29.9-36)	Z=-2.092	0.036
BMI ≥35.5	18 (20.9%)	8 (57.1%)	χ^2=8.206	0.004
Grade II or III obesity*	21 (24.4%)	8 (57.1%)	χ^2=6.262	0.012
Schistosomiasis*	16 (18.6%)	2 (14.3%)	χ^2=0.152	0.696
HB (Hemoglobin)	12.8 (11.2-13.55)	12.05(11.4313.93)	Z=-0.219	0.827
TLC (Total leucocytic count)	5.4 (4.75-6.35)	6.3(4.73-7)	Z=-1.11	0.267
PLT(Pletelet count)	203 (178-266)	199(157.75245.75)	Z=-0.517	0.605
ALB (Albumin)	4.2 (3.9-4.5)	4.15(3.9-4.55)	Z=-0.050	0.960
BIL (Bilirubin)	0.6 (0.58-0.7)	0.6(0.5-0.7)	Z=-0.795	0.427
AST(Aspartate transaminase)	23(18.8-32.8)	40(19.8-45)	Z=-1.46	0.143
ALT (Alanine transaminase)	29(19.8-40.5)	37(23-46.3)	Z=-1.59	0.113
RBG(Random blood glucose)	106(95.8-121.3)	113.5(97.5-188.3)	Z=-1.27	0.205
Creatinine	0.8(7-9)	0.9(0.7-1.1)	Z=-0.787	0.431
INR	1.05(1.0-1.2)	1.03(1-1.18)	Z=-0.5	0.960
FIB4	1.18(1.02-1.4)	1.5(1.05-2.06)	Z=-1.197	0.231

Median (IQR) and compared by Mann-Whitney U test.

Table (2): Univariate analysis for prediction of likelihood of OCI

Predictor		Crude odds ratio [COR] (95% CI for COR)	P value
Age (years)		0.430(0.912-1.040)	0.974
Sex	Female Male	R 1.731 (0.546-5.489)	0.352
BMI cutoff value	<35.5 ≥35.5	R 5.037 (1.549-16.378)	**0.007**
Smoking	Non-or ex-smoker Current smoker	R 1.833 (0.551-6.101)	0.323
Diabetic state	Non-diabetic Diabetic	R 2.246 (0.612-8.250)	0.223

P value by simple binary logistic regression analysis

Table (3): Independent predictors of the likelihood of occult HCV infection

Predictor		B	SE	Wald	P value	OR	95% C.I for OR	
							Lower	Upper
Age(years)		-0.067	0.043	2.388	0.122	0.936	0.860	1.018
Sex	Female Male	2.338	0.775	9.109	**0.003**	R 10.358	2.270	47.270
BMI cutoff value	<35.5 ≥35.5	1.668	0.847	3.882	**0.049**	R 5.301	1.009	27.856
Smoking	Non- or ex-smoker Current smoker	0.213	0.791	0.073	0.787	R 1.238	0.263	5.831
Diabetic state	Non-diabetic Diabetic	1.021	0.704	2.103	0.147	R 2.777	0.698	11.041

P value by simple binary logistic regression analysis; B=binary logistic regression coefficient; SE=standard error; OR=Odds ratio

Multivariate analysis was carried out to predict the likelihood of occult HCV infection being present. A binomial logistic regression was performed to ascertain the effects of age (in years), male sex, high BMI (≥35.5kg/m^2), current smoking status and presence of DM on the likelihood that participants had OCI. Four studentized residuals with values of 2.006, 2.559, 2.466 and 2.314 standard deviations were found but kept in the analysis. The logistic regression model was statistically significant,$\chi 2(5)=14.657$, p =0.012. The model explained 24.6% (Nagelkerke R^2) of the variance in occult HCV infection and correctly classified 86% of cases. Sensitivity was 71%, specificity was 98.8%, positive predictive value was 50% and negative predictive value was 86.7%. This signifies a specific rather than a sensitive model.Of the 5 predictor variables, high BMI and male sex were statistically significant independent predictors, as seen in Table 3. Patients with BMI ≥35.5kg/m^2 had 5 times

higher odds of exhibiting OCI whereas male sex had10 times higher odds.

Discussion:

Hepatitis C infection is a global health problem, the prevalence of it varies throughout the world, with the highest prevalence in Egypt (30).

Anew class of HVC infections, occult HCV, is defined as two different types either cryptogenic type in which patients have no anti HCV antibodies, or secondary type in which patients have anti HCV antibodies and had cleared HCV infection either spontaneously or after therapy, in both types detection of HCV-RNA in hepatocytes is the gold standard and most accurate alternatively detect HCV- RNA in PBMCs which is less invasive and easy to do.

Different studies have reported the prevalence of OCI in different populations; up till now, its associated risk factors have not been established yet (12).

Of the 100 patients included in the current study who spontaneously cleared HCV proved by positive test for anti-HCV antibodies and negative for serum HCV-RNA, 14 patients (14%) exhibited genomic HCV-RNA detectable in PBMCs. While findings from this study are consistent with different prevalence rates, with those reported by numerous other studies (27, 31, 32, 33, 34, 35, 36), they contradict some accounts reporting no occult HCV infection (37, 38). This may possibly be due to use of less accurate and less sensitive commercial assays in these studies. However, all these documented studies on OCI comprised a small number of patients, typically ranging from 5 patients to a maximum of 27.

A study done recently (27) evaluated the prevalence of OCI in hepatocytes and PBMCs of three groups of patients, either naturally resolved HCV infection or achieved SVR at 24 weeks (SVR24) following therapy with DAAs or pegylated\ribavirin (PR), finding HCV-RNA (by RNA scope assay of liver biopsies and PMBCs) in about 11% of all patients groups,15% in DAA group, 10% in PR group and 6.7% in spontaneously resolved group. This finding suggest that complete ‘cure’ is an unlikely occurrence as evidenced by the higher prevalence of HCV in patients treated with DAA and the relapse at 48 weeks post-treatment of one patient treated by PR. Peripheral blood mononuclear cells (PBMCs) were positive for HCV-RNA in almost all patients with detectable HCV-RNA in hepatocytes (12/13; 92.3%).

This finding is of particular interest in that it suggests that monitoring of PMBCs may be used for detection of occult HCV infection in

post-treatment longitudinal follow-up, reserving liver biopsy for cases demonstrating negative PBMC HCV-RNA. However, previous studies had demonstrated a lower detection rate of HCV RNA at about 70% by PBMC monitoring (5, 26).

Researchers found that the prevalence of OCI in blood donors population (seronegative to HCV antibodies) was 3.4%by testing HCV RNA in both hepatocyte and PMBCs (12) .On the other hand some other researchers test for HCV RNA in PMBCs in patients who are spontaneously or therapeutically clear the virus, and found complete clearance of HCV from PMBCs (39).

The current study examined PBMC only for genomic RNA, avoiding liver biopsy in an attempt to avert risk of complications. Studies with much larger sample size are needed to adequately determine whether PBMC viral load correlates with that of hepatocytes.

In the recent study (27), it was proved that prolonged hepatic inflammation and higher fibrosis score were more likely seen in patients with occult HCV infection whereas regression of fibrosis was characteristic of patients without OCI.

Continuous viral replication in hepatocytes following clearance of HCV leads to ongoing hepatic inflammation with deterioration of clinical condition. These histopathological findings seen in occult HCV infection are particularly concerning in cases of unchecked post -treatment patients because OCI does not necessarily manifest with abnormalities of transaminases and is unrelated to baseline viral loads. Therefore, it is essential to determine diagnostic and predictive biomarkers for occult HCV infection to be used particularly in absence of liver biopsy (27).

To predict the likelihood of presence of occult HCV infection being present, several independent predictor variables including age in years, male sex, high BMI $\geq 35.5 kg/m^2$, current smoking status and presence of DM were evaluated in this study. Of these, high BMI and male sex were found to be statistically significant, with patients having BMI $\geq 35.5 kg/m^2$ demonstrating 5- times higher odds of exhibiting OCI that come in concordance with results reported by researchers who found that overweight is a significant clinical risk factor (40). While male sex had 10 times higher odds this is compatible with the study which found that double numbers of males than females affected as OCI (12).

Conclusion and recommendations:

We concluded that prevalence of OCI was 14% after spontaneous clearance of the virus. BMI at cut off value ≥35.5kg/m^2 and male sex can be risk factors for OCI.

Further studies with larger patient groups are necessary to fully determine the risk factors of occult HCV infection thus enabling treatment options without entailing liver biopsy. To date, it remains unknown what duration is required for adequate monitoring of OCI patients and whether further treatment would benefit these patients.

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