Seronegative Occult Hepatitis C Virus Infection (OCI) in a Main Haemodialysis Centre In Pahang, Malaysia

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ABSTRACT

Introduction: Occult HCV infection has a predilection for specific populations such as haemodialysis (HD) patients. The exact natural course, epidemiology, pathogenesis and clinical importance of OCI are unknown. We investigated the existence of OCI among local patients undergoing routine HD at a referral hospital in Pahang, Malaysia. **Methods:** Serum and peripheral blood mononuclear cells (PMBCs) were collected from peripheral venous blood samples of seropositive (anti-HCV positive) and seronegative (anti-HCV negative) HD patients as well as healthy individuals (negative control group). Inclusion criteria for the seronegative patients included elevated liver enzymes. Both conventional PCR and strand-specific PCR were used to detect the viral RNA and to indicate active viral replication in PBMCs respectively. Direct DNA sequencing was done to confirm the viral HCV RNA and their genotypes. **Results:** In the majority (90-100%) of seropositive chronic hepatitis C patients, viral RNA was detected in both serum and PMBCs. Meanwhile, out of 22 seronegative patients, 6 (27%) showed active viral replication in PBMCs but no detectable viral RNA presence in the serum. None of the negative control group had detectable viral RNA. All seronegative patients with OCI were infected with HCV genotype 3 and two of them (2/6) had a slight elevation of their liver enzymes. **Conclusion:** Seronegative OCI does exist among local hemodialysis patients, with normal or persistently abnormal liver enzyme values. Further investigation is needed to study the mode of viral transmission and clinical significance of OCI in HD setting.

KEYWORDS: Occult Hepatitis C, Chronic Hepatitis C, Peripheral Blood Mononuclear Cells and Haemodialysis Unit.

INTRODUCTION

Hepatitis C virus (HCV) is one of the causative agents of chronic liver disease and liver carcinoma, causing 399,000 deaths worldwide. It has become a major public health concern worlwide with 75 million people are currently diagnosed with chronic hepatitis C infection (CHC), and in 2015, there were 1.75 million new HCV infection cases.[1] In Malaysia, 400,000 Malaysians are reported to be chronic HCV patients and liver cancer ranks the 8th leading cause of cancer in both genders.[2]

HCV is a member of the *Hepacivirus* genus which belongs to the *Flaviviridae* family. The virus can be spread among patients who have undergone haemodialysis (HD) treatment, received unscreened blood transfusion, shared needles among IVDA, had needlestick injury and less commonly sexual intercourse with an infected partner and vertical transmission from infected mother. Despite regular routine serologic and molecular testing, HCV infection in dialysis units remains a concern.[3] In 2016, the prevalence of HCV infection was 2% among the total of 102447 HD patients, thus approximately 2049 dialysis patients were chronically infected with hepatitis C. Since then, various infection control measures have been implemented to reduce the number of hepatitis C seroconversion.[4]

The gold standard for the diagnosis of HCV infection is the detection of specific anti-HCV antibodies and viral RNA in serum samples.[5] However, evidence of viral RNA presence especially in peripheral blood mononuclear cells (PBMCs) and/or hepatocyte, even in the absence of HCV RNA in serum, have been

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reported in numerous studies over the past few years.[6-10] This clinical status has been known as occult HCV infection (OCI), which can be categorized into two types. The first type is known as seronegative OCI, where patients are negative for both anti-HCV antibody and HCV RNA in their serum. Patients who are positive for anti-HCV antibody but negative for serum HCV RNA are considered as seropositive OCI or also called as secondary OCI [11]

Initially, detection of HCV RNA in hepatocytes from liver biopsy was considered as the gold standard method for diagnosing OCI. However, due to its invasiveness, study on PMBCs sample may be a reliable alternative method of diagnosis.[6,7,12,13] Other alternative methods such as combined HCV antigen-antibody assay or HCV-RNA detection in plasma or whole blood sample has been studied, however, they did not improve the diagnosis for individuals with OCI.[14] Thus, many previous studies have reported OCI among certain populations such as HD patients using PMBCs sample.[6,7,12,13,15]

Since its discovery, the existence of OCI in HD setting has been documented in several studies with prevalence ranging from 0 to 45%. A study done in Spain reported the highest prevalence of OCI cases with half of their HD patients were found to have the viral RNA in their PBMCs despite testing negative for both serum anti-HCV antibodies and viral RNA. They also found that the elevation of the liver enzymes was significantly associated with OCI.[7] Other than that, studies showing low prevalence rates of OCI concluded that the occurence of this disease is plausible and suggested further investigation.[6,12,13]

Until now, many aspects of OCI in HD patients has not been fully studied. Thus, we studied the existence of OCI in a main refferal hemodialysis centre in Kuantan, Pahang, the patients were further investigated for viral replication status in their PBMCs and viral genotypes. As far as we know, this is the first report of OCI in Malaysia.

Materials and Methods

Study design and sample size

This is an observational (cross sectional) and descriptive study. The sample size was calculated by using the following formula:

$$n = \frac{Z^2 P(1-P)}{d^2}$$

n = sample size; *P* = expected prevalence; *Z* = statistic corresponding to the level of confidence; d = precision

Since the nearest OCI study was done in Thailand in 2008, this calculation was based on the expected prevalence of 18% as reported by Thongsawat et al., (2008),[13] with the desired precision of 0.2 and confidence level of 95%, thus the minimum estimated sample size is 15 HD patients.

Study population and ethics

The study was conducted at the HD centre of Tengku Ampuan Afzan Hospital (HTAA), Kuantan, Pahang. This centre accomodated 60 routine HD patients. Twenty-two (22) HCV seronegative and 10 HCV seropositive patients were recruited in the study. The HCV seropositive patients were also chronically infected with hepatitis C and they were enrolled as positive control for this study. The inclusion criteria for test subjects include: 1) dialysis patient 2) persistently negative serologic and molecular markers of HCV (anti- HCV and HCV-RNA). The clinical data of each patient were obtained from patient's medical file record. Ten (10) healthy adults were also recruited for this study and their PBMC and serum samples were included in the assays as negative control. Ethical approvals were already obtained from IIUM Faculty of Medicine Ethics Committee,(IREC 631) and the Medical Research Ethics Committee (NMRR-16-1883-32319).

Sample processing

All sample processing was performed in Microbiology Laboratory, Basic Medical Sciences, Faculty of Medicine, IIUM. Blood samples (10 mL) were collected in sodium heparin tubes from each patient after receiving their written informed consent. PBMCs and serum samples were isolated from fresh venous blood by Ficoll gradient centrifugation method at 400xg for 35 minutes at 18°C. The PBMCs layer was collected and washed three times with 1× phosphate buffered saline (PBS) with a pH of 7.4 at 100xg for 10 minutes at 18°C. The cells were resuspended in culture medium [RPMI 1640 with 10% fetal bovine serum (FBS) and 1% Pen-Strep

antibiotics] and they were stored in freezing medium (80% FBS and 20% DMSO) at -80 ^{o}C until used.

Total RNA extraction from PBMCs and serum

Total RNA was extracted from both PBMCs and serum samples using SV Total RNA Isolation Sytem (Promega, Madison, WI) and High Pure Viral RNA Extraction kit (Roche, Swiss), respectively. The procedures were carried out according to the manufacturers' instructions. After precipitation, RNA pellets were dissolved in 30μL nuclease-free water and kept in -80 ^{o}C freezer until used.

Reverse transcription reaction

Total RNAs were reverse transcribed into complementary DNA (cDNA) at 42 ^{o}C for 1 hour in a final volume of 22μL containing 5μL of extracted RNA, 1μL of random primer, 4μL of 5X reaction buffer, 1μL of 10mM deoxynucleotides (dNTP) mixture, 1μL of Promega Reverse Transcriptase enzyme, 3μL of 25 mM magnesium chloride (MgCl$_2$), 1μL of RNasin®ribonuclease inhibitor and 6μL of nuclease-free water. The reaction mixture was denatured at 85 ^{o}C for 15 minutes to inactivate the reverse transcriptase enzyme.

Detection of genomic (positive strand) and anti-genomic (negative strand) of HCV RNA by PCR

PCR was performed using specific primers (Table 1) adopted from previous studies, targeting the 5'untranslated region (5'UTR) of both positive and negative strands. For the detection of genomic viral RNA, a set of primers (HCV-F and HCV-R) was used to generate a 212 bp amplicon. A set of different primers (HCV-negF and HCV-negR) was used to generate a 327 bp amplicon from anti-genomic viral RNA. Samples with negative amplification were tested in a second round of semi-nested PCR using HCV-nF and HCV-R primers for genomic viral RNA, and a second round of nested PCR using a set of inner primers (2NesF and 2NesR) for anti-genomic viral RNA.

PCR was conducted under standard conditions, using 2.5μL of cDNA from previous reaction, 4.0μL of Go Taq 5X Flexi reaction buffer (Promega, USA), 0.8μL of 25mM MgCl$_2$, 0.25μL of 10mM dNTP mixture, 0.25μL of Go *Taq* DNA polymerase (Promega, USA), 12.7μL of nuclease-free water, 1μl of each forward and reverse primer in a total volume of 22.5μL. The thermal profiles for the amplification of both strands were as follows: initial denaturation at 94 ^{o}C for 5 minutes; 35 cycles with denaturation at 95 ^{o}C for 30 seconds, annealing at 55 ^{o}C for 30 seconds and elongation at 72 ^{o}C for 1 minute. The reaction was terminated with a final extension at 72 ^{o}C for 7 minutes. For contamination control, nuclease-free water was used to replace cDNA template. For positive control, a standard hepatitis C virus (14/150) from National Insititute for Biological Standards and Control was used.[16]

DNA sequencing

PCR products were analyzed in 1.7% agarose gel electrophoresis and purified using MinElute gel extraction kit (Qiagen, Valencia, CA, USA). The procedure was carried out according to the manufacture's instructions. The purified PCR products were then sequenced using the same PCR primers. The concentration of the primers used was 10 pmole/μL and the minimum volume was 5μL for each reaction. The nucleotide sequences identity was then confirmed using BLAST from National Centre for Biotechnology Information (NCBI) homepage.

Table 1: List of primers used in this study. *UTR = untranslated region

Target gene	Primer's name	Purpose of the primer	Sequence (5'-3')	Amplicon's size (bp)	Reference
5'UTR*	HCV-F	Detection of genomic HCV RNA	AGT GTT GTG CAG CCT CCA G	212	(34)
	HCV-R		ACT GCC TGA TAG GGT GCT TG		
	HCV-nF		CGG TGA GTA CAC CGG AAT TG	153	
	HCV-negF	Detection of anti-genomic HCV RNA	CAT GGT GCA CGG TCT ACG AGA CC	327	(9)
	HCV-negR		GGC GAC ACT CCA CCA TGA ATC AC		
	2NesF		CTG TGA GGA ACT ACT GTC TT	266	
	2NesR		CTC GCA AGC ACC CTA TCA GG		

Statistical analysis

Data analysis was performed using SPSS (version 25 for Window) software. Results were expressed as mean±standard deviation (SD) or frequencies (%). Independent samples t-test was used for the comparison between different parameters in the studied groups. Meanwhile, Fisher's exact test was used to compare the categorical data. The differences in data results were considered statistically significant if the p value was < 0.05 and highly significant if the p value was < 0.01.

Results

The demographic and clinical data of all patients are summarized in Table 2. Of the total 42 recruited HD individuals, 21 (50%) were females and 21 (50%) were males. Duration of dialysis treatment in both seronegative group and seropositive control group ranged between 2 to 10 years (Mean: 3.94±1.664 SD). Statistical analysis showed highly significant differences (*p<0.01*) between the two groups regarding their duration of dialysis treatment (*p=0.008*). There were no statistical differences in age (*p=0.039*), gender (*p=1.00*) and creatinine values (*p=0.155*) between seronegative and seropositive groups. Besides that, there were statistical differences between both groups on the levels of alanine transaminase (ALT) (*p=0.015*) and aspartate transaminase (AST) (*p= 0.05*) as well as hemoglobin (*p=0.037*).

Out of 22 seronegative patients, 6 were identified as patients with OCI, where genomic HCV RNA was not detected in their serum samples but in their PBMCs (6/22; 27%). (Figure 1). Negative strands or anti-genomic HCV RNA were also found in their PBMCs (Figure 2, Table 3), indicating that there was detectable active viral replication within this extrahepatic site.

On the other hand, all the seropositive HD patients, yielded positive genomic viral RNAs in both PBMCs and serum samples (10/10; 100%). 9 out 10 of the patients showed positive result for anti-genomic viral RNA in their serum. While, all the negative control group yielded negative results (0/10; 0%) for both genomic and anti-genomic HCV RNAs in both types of samples.

Table 2: Demographic and clinical data of study patients including control groups. MD = missing data as it was not stated in their medical records. #NA = not applicable in which their samples were not subjected for liver function tests. *HD = hemodialysis

| Parameter		Seronegative HD* patients (n=22)	Seropositive HD* patients (n=10)	Healthy individuals (n = 10)	p-value
Age	Range	24.0 - 66.0	29.0 - 59.0	27.0 - 36.0	0.039
	Mean ± SD	40 ± 12.84	39 ± 8.88	30 ± 2.95	
Gender (F/M)		8/14 (36%/64%)	3/7 (30%/70%)	10/0 (100%/0%)	1.000
Duration of treatment (years)	Range	2.0 – 5.0	2.0 - 10.0	-	0.008
	Mean ± SD	3.5 ± 1.06	5 ± 2.33	-	
History of blood transfusion		MD	2 (20%)	-	-
Family history of hepatitis		-	1 (10%)	-	-
ALT (IU/L)	Range	7.0 – 56.0	14.0 – 31.0	NA#	0.015
	Mean ± SD	16.9 ± 13.2	18.6 ± 4.94		
AST (IU/L)	Range	9.0 – 40.0	15.0 – 29.0	NA	0.050
	Mean ± SD	17.0 ± 8.12	17.9 ± 4.58		
Hemoglobin (g/dL)	Range	7.0 -.12.4	7.9 – 12.0	NA	0.037
	Mean ± SD	9.34 ± 1.42	10.49 ± 1.51		
Creatinine (nmol/L)	Range	300.0 – 1156.0	345.0 – 968.0	NA	0.155
	Mean ± SD	576.1 ± 206.6	657.1 ± 203.4		
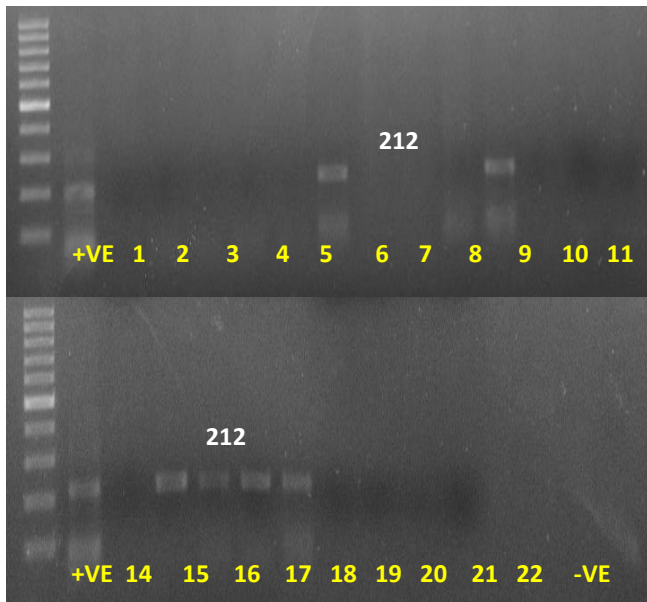
Figure 1: Gel electrophoresis of RT-PCR products from seronegative hemodialysis patients. Six (6) out of 22 patients have genomic viral RNAs in their PBMCs.
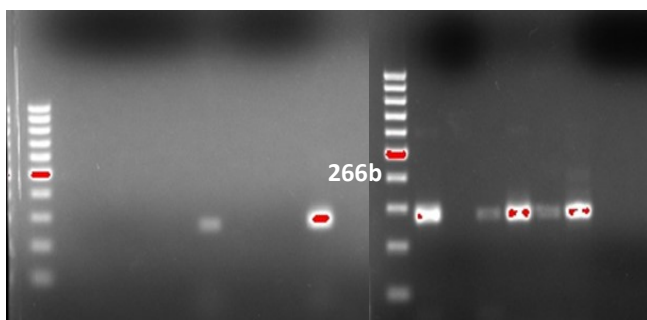
Figure 2: Gel electrophoresis of second round (nested) strand-specific RT-PCR products from seronegative hemodialysis patients. The first round strand-specific RT-PCR (not shown) gave very faint band of 327 bp. Six (6) out of 22 patients had anti-genomic viral RNAs in their PBMCs.

Nuclotide sequences of the amplified viral RNAs from all the OCI-positive samples were confirmed to belong to HCV as shown in the BLASTN result (Table 4). Their nucleotides composition were accurately matched with the reference sequences with 98-100% similarities.

Moreover, based on the phylogenetic analysis of the nucleotide sequences, all of them (6/6; 100%) were found to be infected with HCV genotype 3 subtype a (Figure 3).
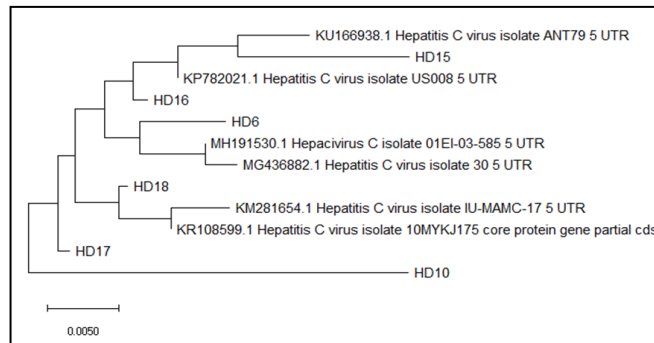
Figure 3. The evolutionary history of the 5'UTR was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.08698097 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Jukes-Cantor method and are in the units of the number of base substitutions per site. This analysis involved 12 nucleotide sequences. There were a total of 11371 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

DISCUSSION

Knowledge regarding the prevalence of OCI, its natural history and routes of transmission are limited. Hence, it is still controversial whether OCI is actually a new entity of HCV or just a new form of chronic hepatitis C infection.[17-18] Since the first description of OCI[8] many data supporting the presence of OCI in specific populations such as in HD and patients with cryptogenic liver cirrhosis.[12,19,20] Meanwhile, others have reported absence of OCI in patients with immunosuppressive conditions and HCV-associated diseases.[21-23] Nonetheless, the contribution of this extrahepatic reservoir could have clinical consequences in viral transmission and disease pathogenesis, thus, future studies are needed to add to our knowledge of the OCI clinical significance. In the present study, haemodialysis (HD) patients were chosen as the study group since information about OCI among local (HD) patients is limited or unknown and they are at an increased risk of HCV exposure due to vascular access, blood transfusions and the potential for nosocomial transmission. Therefore, our main objective was to investigate the existance of OCI in our local HD patients.

Table 3: Strand specific RT-PCR on two types of samples in three group of patients.

Sample's type	Seronegative HD patients (n=22)		Seropositive HD patients (n=10)		Healthy individuals (n=10)	
	Genomic HCV RNA (%)	Anti-genomic HCV RNA (%)	Genomic HCV RNA (%)	Anti-genomic HCV RNA (%)	Genomic HCV RNA (%)	Anti-genomic HCV RNA (%)
PBMCs	6/22 (27)	6/22 (27)	10/10 (100)	10/10 (100)	0/10 (0)	0/10 (0)
SERUM	0/22 (0)	0/22 (0)	10/10 (100)	9/10 (90)	0/10 (0)	0/10 (0)

Table 4: Direct DNA sequencing confirmed HCV-RNA amplification of PBMCs samples from seronegative patients. RT-PCR targeting the 5'UTR of viral genome. [a]Viral RNAs were amplified by nested strand specific RT-PCR (positive strand).

Id	Amplicon Size (BP)	Identities Score (%)	Genbank Accession Number	Genotype (Subtype)
HD 6	266[a]	234/234 (100)	MH191530.1 (Hepacivirus C isolate 01EI-03-585)	3(a)
HD10		222/226 (98)	KR108599.1 (Hepatitis C virus isolate 10MYKJ175)	3(a)
HD15	212	170/170 (100)	KU166938.1 (Hepatitis C virus isolate ANT79)	3(a)
HD16		170/170 (100)	KP782021.1 (Hepatitis C virus isolate US008)	3(a)
HD17	266[a]	235/238 (99)	MG436882.1 (Hepatitis C virus isolate 30)	3(a)
HD18		236/240 (98)	KM281654.1 (Hepatitis C virus isolate IU-MAMC-17)	3(a)

We have found 6 out of 22 serenegative patients from HTAA HD centre infected with OCI, indicating the strong existence of OCI among the local HD patients. The actual route of HCV transmission in these patients were unknown. In Malaysia, the transfusional safety regulations, the stringent nosocomial infection prevention practices and the high awareness among health-care professionals, have reduced HCV seroconversion in the last 10 years from 11% in 2007 to 2% in 2016.[4] However, whether similar awareness and practices might also contain OCI transmission is still in need to be explored.

HCV is known as a hepatotropic viral pathogen and hepatocytes are the reservoir for viral replication. Therefore, the existence of viral RNA in extrahepatic sites like peripheral blood mononuclear cells (PBMCs) raises important questions; whether the virus actively replicates within these cells? Or remain dormant within or just adherant to theses cells? The viral replication involves synthesis of a complementary RNA (anti-genomic RNA strand) that acts as a template for the production of a positive genomic RNA strand.[24,25] Presumably, the detection of negative strand HCV RNA indicates active synthesis of a new viral genomic strand. Demonstration of the viral RNA negative strand showed that HCV was actively replicating within PBMCs of all (6/6; 100%) of our OCI infected patients. This finding suggests that patients with OCI are potential HCV carriers and most likely contribute to the nosocomial transmission of HCV infection.

Previous studies have reported the presence of viral RNA and proteins in monocytes, B cells and T cells from OCI infected patients, indicating that PBMCs are indeed another reservoir for HCV beside hepatocytes.[26-28] The detection of HCV RNA in liver biopsy samples of an individual with undetectable viral RNA in serum was previously considered as the gold-standard for the identification of OCI. However, since it is an invasive procedure, carrying risk of complications and is rarely conducted according to the current National Haemodialyis Quality Standards 2018, other alternatives were investigated and several studies found that PBMCs sample is a significantly relevant and safe alternative to study OCI.[6,7,12,13,29,30]

Liver function tests are performed to show evidence of abnormality such as liver inflammation and cell damage by measuring the level of released liver enzymes. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are the central enzymes involved. The range of normal ALT and AST levels are 0 to 55 units and 5 to 34 units, respectively.[31] An elevated level of liver enzymes found in the bloodstream suggests that hepatocytes are undergoing injury and this can be inflicted by several causes besides hepatotropic viruses. For instance, medications that may potentially cause liver toxicity (e.g pain-relief medications), alcoholism and autoimmune diseases can all cause elevation in liver enzyme levels in blood.[32] In a situation where the liver enzymes are increased, these potential causes were studied but in some cases, the cause of the enzyme elevation remained unknown. Study done by Barril et al., (2008) showed existence of OCI among dialysis patients with negative test results for both serum anti-HCV antibodies and HCV RNA but positive result for HCV RNA in the liver and abnormal values of liver enzymes.[7] In our study, only two seronegative dialysis patients showed minor elevation in their liver enzymes (ALT: 56.0,55.0 IU/Ml; AST: 40.0,36.0 IU/mL) due to unknown causes (cryptogenic) and it was found that they harbor the virus in PBMCs. Based on the statistical analysis, ALT ($p=0.015$) and AST ($p=0.05$) values were significantly associated with

OCI, suggesting that OCI may be the cause for this unexplained minor elevation in their liver enzymes. However, OCI did not appear to have any impact on the serum creatinine level thus suggesting it does not probably have a recognizable deleterious effect on kidney function.

Besides that, all of the seronegative OCI patients in this study were found to be infected with HCV genotype 3. This finding agrees with the prior genotyping studies in which investigators had found genotype 3 as the most prevalent HCV genotype in Malaysia.[33-37]

This is also crucial as it is known that genetic heterogeneity of HCV may contribute to the differences in disease outcome and response to antiviral treatment observed in HCV infected patients.[38] Genotype 3 was reported to be the less aggresive strain with the rate of evolution to chronicity ranging between 33 to 50% as compared to genotype 1 (~92%).[39] In addition, genotype 3 is also associated with hepatic steatosis, a condition where excess fat is accumulated in the liver.[40]

Therefore, with these predictive variables that may occur later, it is best to acknowledge and manage OCI infected patients accordingly.

CONCLUSION

In conclusion, OCI does exist among hemodialysis patients in HTAA, with normal or mildly but persistently elevated liver enzymes. Although the present study has small sample size, it is the first to demonstrate the occurence of OCI among dialysis patients in Malaysia and to highlight the potential cause for ongoing HCV transmission within haemodialysis units. Further studies with larger sample size, multiple dialysis centres and extended parameters are recommended to explore the clinical significance of OCI.

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Conflict of interest

There is no conflict of interest to be declared

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