

Genotyping of Hepatitis C Virus Isolates from Iraqi Hemodialysis Patients by Reverse Transcription-PCR and One Step Nested RT-PCR

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Abstract

Background: Hepatitis C virus (HCV) infection is a major health problem among dialysis patients in developing countries. Geographical distribution of various genotypes of HCV is useful for understanding the epidemiological status, detection of mode and source of infection, designing the program of control, evaluating the response to treatment and development of diagnostic methods and vaccine production.

Objectives: To investigate the prevalence of HCV genotypes and subtypes (1a, 1b, 2a, 2b, 3a, 3b, 4, 5a & 6a) among hemodialysis patients.

Materials and Methods: The prevalence of anti-HCV antibody was determined by Enzyme Linked Immunosorbent assay (ELISA). Then, HCV specific RNA was detected in those anti-HCV seropositive and seronegative dialysis patients, utilizing reverse transcriptase-polymerase chain reaction technique (RT-PCR), Furthermore, Genotyping the HCV-RNA positive samples by one step nested RT-PCR technique.

Results: Genotyping analysis was performed in 29 HCV-RNA positive patients. Genotypes 1a, 1b, 3a and 4 were found in (34.48%), (13.79%), (3.45%) and (41.38%) patients, respectively. In addition, two patients (6.90%) had mixed infected with both 4 and 1b.

Conclusion: The genotype distribution in our study is comparable to that for non haemodialysis patients. Further analyze relatedness of HCV isolates by sequence analysis are required to trace the source of infection.

Key words: HCV Genotypes, Hemodialysis, RT-PCR.

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الخلاصة

تمهيد: تعتبر العدوى بالتهاب الكبد الوبائي (HCV) مشكلة صحية كبيرة بين مرضى غسيل الكلى في البلدان النامية. وأن التوزيع الجغرافي للأنماط الجينية المختلفة من فيروس التهاب الكبد نمط "ج" مفيد لفهم الحالة الوبائية، والكشف عن وضع ومصدر العدوى، وتصميم برنامج لمراقبة وتقييم الاستجابة للعلاج وتطوير طرق التشخيص وإنتاج اللقاحات

الأهداف: للتحقيق في انتشار الأنماط الجينية والأنواع الفرعية لفايروس التهاب الكبد نمط "ج" (1a، 1b، 2a، 2b، 3a، 3b، 4، 5a، 6a) بين مرضى الغسيل الكلوي.

الأشخاص وطرائق العمل: تم تحديد معدل انتشار الأجسام المضادة المضادة للفايروس عن طريق فحص الامتزاز المناعي المرتبط بالانزيم (ELISA)، وتم الكشف عن الرنا الفيروسي في مرضى غسيل الكلى، وذلك باستخدام تقنية سلسلة تفاعل البوليميراز العكسية (PCR-RT)، وعلاوة على ذلك تم إجراء التتميط الجيني للعينات الإيجابية للرنا الفيروسي باستخدام تقنية سلسلة تفاعل البوليميراز ذات الخطوة الواحدة المتداخلة.

النتائج: تم إجراء تحليل التتميط الجيني في 29 من المرضى الأيجابيين للرنا الفيروسي. وتم العثور على المورثات 1a، 3a، 2a، 1b، 4 في (34.48%)، (13.79%)، (3.45%) و (41.38%) من المرضى، على التوالي. وبالإضافة إلى ذلك، قد اثنتين من المرضى (6.90%) إصابة مختلطة مع كل من 4 و 1b.

الاستنتاج: أن توزيع الانماط الجيني في دراستنا مماثل للمرضى من غير مرضى غسيل الكلى، ويلزم إجراء المزيد من التحليل على العزلات عن طريق تحليل التسلسل الجيني لتتبع مصدر العدوى.

الكلمات المفتاحية: التهاب الكبد جيم التركيب الوراثي، الغسيل الكلوي، PCR-RT

Introduction

HCV infection is a major health problem among hemodialysis (HD) patients in developing countries. The prevalence rates reported in HD patients in Middle Eastern countries are 68% in Saudi Arabia with a range of 14.5% to 94.7%, 26% in Oman, and 80% in Egypt. The prevalence rates reported are 1%–29% from Western Europe, 8%–36% from North America, 5.9% in Australia, and 44%–60% in Far Eastern countries [1]. The seroprevalence of HCV in hemodialysis units in Jordan was 34.6% compares with 45% in Tunisia and 45% in Syria [2]. In Iran, the overall prevalence of HCV (HCV antibody and HCV-RNA) was 5.4%. Epidemiological studies about HCV infection among HD patients in Iraq have reported a prevalence of 7.1%–62% in different cities [3]. While the reported prevalence in the general population in Iraq is ranges from (0.2%) to (0.5%) [4] and is (8.18%) in blood bank [5].

On the basis of nucleotide variation, HCV is divided into six major genotypes and more than 80 subtypes [6,7]. The complex of genetic variants found within an individual isolate is termed the quasispecies

[8,9,10]. There is 30-50% variation among viral genotypes and 15-30% among different subtypes while there is 1-5% variation in nucleotide sequence from a single HCV infected patient [6,7]. Genotype 1a, initially described as an American variant, is distributed worldwide. So is genotype 1b (Japanese variant), the most widespread worldwide, and so are types 2 and 3. Genotype 4 is mainly found in Africa and the Middle-East. Type 5 has mainly been isolated in South Africa and Central Africa. Type 6 strains are essentially distributed in the Far East [11,12]. Several studies have shown that infection with genotype 1, with a prevalence of 70–80% in most western countries, is associated with a poor response to IFN- α therapy [12,13].

The importance of HCV genotyping has considerably increased in the last few years. It has been used to study worldwide and local molecular epidemiology of HCV, and to trace sources of HCV infection in risk groups such as drug users and blood products. Typing has also been used to study relationships between type/subtype and the clinical status, pathogenesis and/or outcome of disease. The major area of clinical

application of HCV genotyping has been in the study of the significance of types/subtypes, in response to antiviral treatment of HCV infection with interferon and ribavirin, as well as the identification of patients with mixed infections. It has also been a useful application in vaccine research and development [7,14].

Materials and Methods

Subjects & Samples: A total of 236 hemodialysis patients, 150 (63.6%) males and 86 (36.4%) females, their age range from 15 to 78 (44.39 ± 15.06 S.D.) years were included in this study. They were attendant's three haemodialysis (HD) centers in Baghdad; Al-Yarmouk Teaching Hospital, Al-Kadhimiya Teaching Hospital and Al-Karama Hospital. Sampling lasted from May to October 2010. The mean duration of HD treatment was (33.91 ± 25.75) months. All patients were dialyzed 2 or 3 times per week and each HD treatment took three to four hours. The clinical diagnosis was obtained from patient records and interview and ethical approval for use of all specimens was obtained. Our exclusion criteria were patient's on peritoneal dialysis or history receiving antiviral and/or interferon therapy for HCV (+) subjects. 10 ml of blood was obtained by vein puncture using disposable latex gloves and syringes immediately before HD sessions. Sera were separated from whole blood under optimal conditions for RNA extraction. For this purpose, The blood samples were allowed to clot in the room temperature for 20 minutes and then centrifuged at 2,000 rpm for 10 minutes (-4°C). All samples were divided into three aliquots then immediately frozen and stored at (-20°C) and (-80°C), for serological and molecular assays respectively to minimize degradation of viral nucleic acid, prevent cross contamination and unnecessary thawing and freezing

Methods: For anti-HCV antibodies detection, two commercial kits were utilized. The initial screening for anti-HCV IgG antibody was determined by Enzyme-Linked Immunoassay (ELISA) (Bioelisa HCV 4.0 ELISA, Biokit, Spain). All ELISA positive samples were subjected to confirmatory test using immunoblot assay (EIBA) (Bioblot HCV, Biokit Spain). The procedure was done according to manufacturers' instructions. For HCV RNA detection by RT-PCR, all seropositive samples were tested individually for the presence of HCV RNA by qualitative RT-PCR (Sacace Biotechnologies, REF V-1-100R, Italy). To permit the molecular analysis of the large number of seronegative samples, a pooling strategy was developed, similar to the method described by [15]. This involved the pooling of four seronegative serum samples and the analysis of the mixture for the presence of HCV RNA. Twenty-five μl of each of the four samples were mixed together, and then 100 μl pool was used for the assay. The RT-PCR procedure based on four major processes: isolation of HCV RNA from specimens using RNA/DNA extraction kit (Ribo-Sorb, Sacace Biotechnologies, REF K-2-1, Italy), reverse transcription of the RNA using reverse primer and M-MLV reverse transcriptase provided with the kit and incubated in thermal cycler at 37°C for 30 minutes, and then the cDNA was amplified by PCR with primers specific for the 5' untranslated region of the viral genome. The amplification were carried as follows: 95°C for 5 min, then 42 cycles of 95°C for 30 sec, 67°C for 30 sec, and 72°C for 30 sec, followed by a final extension at 72°C for 1 min. After that detection of the amplified products was done on agarose gel. The kit contains the internal control which may be used in the isolation procedure and serves as an amplification control for each individually processed specimen and to

identify possible reaction inhibition. Negative and positive controls were extracted, reverse transcribed, and amplified in each batch of samples tested by PCR. The serum samples proved to contain HCV RNA by RT-PCR were further subjected for HCV genotype/subtype analysis by Re-extraction of viral RNA from HCV RNA-positive clinical samples utilizing RNA/DNA Extraction Kit, Ribo Virus (HCV Real-TM Genotype) (Sacace Biotechnologies, REF K-2/C, Italy), which is designed for the rapid preparation of highly pure viral nucleic acids. The PCR method used for this part of the study utilizing one step reverse transcriptase nested PCR using HCV Genotyping Kit (Genekam Biotechnology, Germany, Ref.MK757). This ready to use PCR kit contains primers, buffers, PCR master mix, positive control, negative control, molecular marker and loading dye. The procedure was done according to manufacturers' instructions.

Results analysis & interpretation: The PCR products (10µl) were subjected to electrophoresis in agarose (2%) in the presence of ethidium bromide and visualized under UV transilluminator. The band size was assessed by direct comparison with a 100-bp DNA marker. The sample is considered to be positive for HCV RNA if the band of 240 bp is observed on agarose gel. The presence of a 440 bp fragment indicated positive result for internal control (IC) specific amplified DNA fragments. The negative control sample (NCS) and DNA-buffer values for the run are important for the run validity. For genotyping, the expected size for genotype conducted with tube C were: 234 bp-genotype 1b, 139 bp or 190 bp-genotype 2a, 337 bp-genotype 2b, and 176 bp-genotype 3b. While, the expected size for genotype conducted with tube H were: 208 bp-genotype 1a, 232 bp-genotype 3a, 99 bp-

genotype 4, 320 bp-genotype 5a, and 336 bp-genotype 6a.

Statistical analysis: Descriptive analysis was done using the statistical package for social studies (SPSS) program for windows software package release 15.

Results

HCV RNA Prevalence in Haemodialysis

Patients: HCV-RNA was detected in 29/236 (12.29%) of hemodialysis patients sera. In 92 of 100 (92%) confirmed anti-HCV antibody by bioblot; 24 of these sera were HCV RNA-positive. Five sera gave indeterminate results on immunoblotting; two were HCV RNA-positive and three were negative on immunoblotting; one was HCV RNA-positive), as shown in table (1). To permit the molecular analysis of the large number of seronegative samples, a pooling strategy was developed. This involved the pooling of four serum samples and the analysis of the mixture for the presence of HCV RNA. All 136 anti-HCV antibody-negative sera were tested by RT-PCR divided among 34 pools. Among the 34 pools of seronegative samples, 2 yielded positive signals for HCV RNA. Then these two positive pools were retested individually by RT-PCR and resulted in two of 136 (1.47%) seronegative sera were confirmed to be HCV-RNA positive.

HCV Genotyping in Haemodialysis

Patients: HCV genotypic distribution among Al-Yarmouk Teaching Hospital, Al-Kadhimiya Teaching Hospital, and Al-Karama hospital were summarized in table (2). The predominant HCV genotype was 4, detected in 12/29 (41.38%) of HCV RNA positive patients, whilst genotypes 1a and 1b were found in 10/29 (34.48%) and 4/29 (13.79%) of HCV RNA positive patients, respectively. One of 29 (3.45%) had genotype 3a and two of 29 (6.90%) had mixed infection with 1b & 4. There was no

genotype 2, 5, 6 or subtype 3b detectable in our study subjects. Our results demonstrated that HCV genotype 4 was the predominant

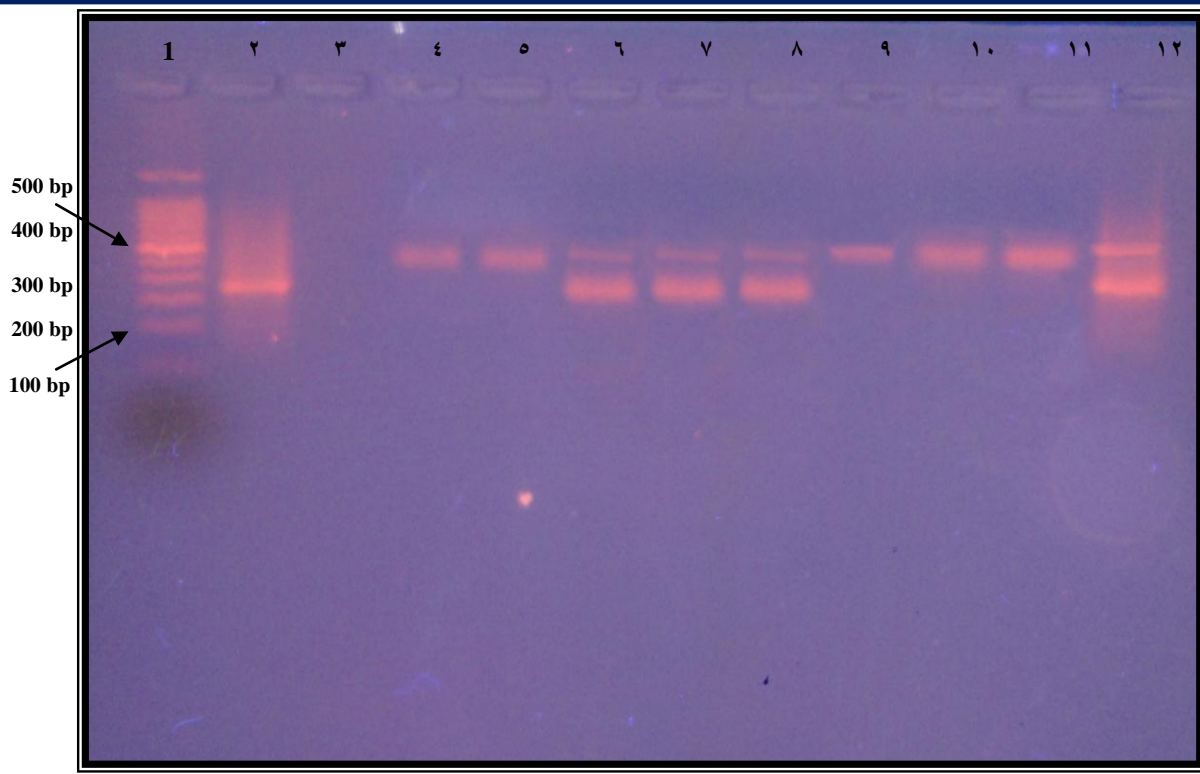
among haemodialysis patients whether as single or as co-infection with HCV 1b.

Table (1): Data of anti-HCV positive & negative with HCV RNA detection.

Test results		No. of patients (%)			
EIA	Bioblot	Total	%	PCR positive	%
Positive	Positive	92/100	92%	24/92	26.09%
Positive	Indeterminate	5/100	5%	2/5	40%
Positive	Negative	3/100	3%	1/3	33.33%
Negative		136/236	57.63%	2/136	1.47%
Total		236	100%	29/236	12.29%

Table (2): HCV genotype & subtype distribution of 29 HCV-RNA positive hemodialysis patients.

Haemodialysis units	HCV genotypes (% of RNA-positive)					
	1a	1b	3a	4	4&1b	Total
1- Al-Yarmouk Teaching Hospital	2	1	0	4	1	8
2- Al-Kadhimiya Teaching Hospital	7	2	0	6	1	16
3- Al-Karama hospital	1	1	1	2	0	5
Total	10 (34.48)	4 (13.79)	1 (3.45)	12 (41.38)	2 (6.90)	29 (100)



Figure(1):PCR products from hemodialysis patients. Lane 1, DNA marker (100-bp ladder).The 500bp band was present at triple the intensity of the other fragments and serves as a reference indicator, while all other fragments appear with equal intensity on the gel; lane 2, HCV cDNA (C+) serves as positive control for amplification; lane 3, DNA-buffer (C-) serves as negative control for amplification; lane 4, HCV IC Rec Fag serves as internal control; lane 5, Negative control sample (NCS) serves as negative control for RNA isolation; lane 6 to 8, DNA from hemodialysis patient positive for HCV antibodies; lane 9 to 11, DNA from hemodialysis patients negative for HCV antibodies; lane 12, HCVC+ Rec Fag serves as positive control for RNA isolation.

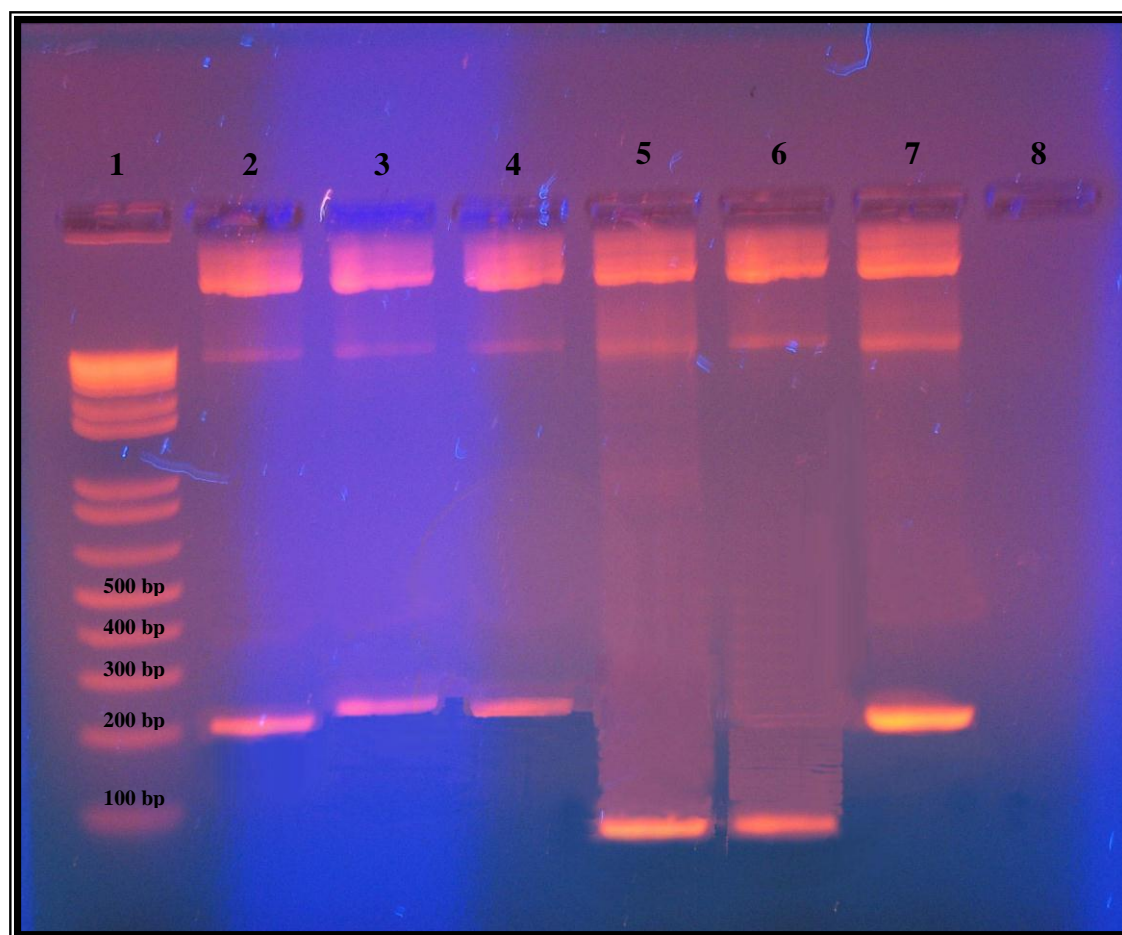


Figure (2): Electrophoresis of different HCV genotype PCR products (pattern of reaction in the second-round nested PCR). Lane 1, molecular weight DNA marker (100-bp ladder); lane 2, genotype 1a (208 bp, the test conducted with tube H); lane 3, genotype 1b (234 bp, the test conducted with tube C); lane 4, genotype 3a (232 bp, the test conducted with tube H); lane 5 & 6, genotype 4 (99 bp, the test conducted with tube H); lane 7, positive control; and lane 8, blank control.

Discussion

In the present study RT-PCR was used to screen for the presence of HCV RNA in all 236 serum samples. HCV RNA was detected in 29 (12.29%) samples, table (1). Genotype 4 was detected in (41.38%) in the tested HCV-RNA positive hemodialysis patients (Table: 2). Not only that but also genotypes 1a and 1b, were found in (34.48%) and (13.79%), respectively. The pattern of our genotypes is similar to those reported from other Middle East countries such as Saudi Arabia and Lebanon, where genotype 4 is the most prevalent [16,17]. A recently published

article in Al-Najaf governorate from hemodialysis units in Al-Hakeem Hospital and Al-Sadder Teaching Hospital, patients who were HCV-RNA positive, show genotype 4 was predominant (100%) of the patients[5]. However, this should not be generalized to all HCV cases prevalent in Iraq. Analysis should be done with a larger population, including hemodialysis patients and blood donors, to determine the prevalent HCV genotype [16].

HCV genotype 1a is the most prevalent genotype in Jordanian patients. This genotype is also predominant in Jordanian

blood donors and in haemodialysis patients of some Middle Eastern countries including Lebanon, Turkey, Cyprus and Syria. In contrast, HCV genotype 4 is the most prevalent genotype in other Middle Eastern countries including Saudi Arabia, Egypt, Yemen and Bahrain [2]. The present study agrees with study mentioned that genotype 1a (34.48%) is more likely to disseminate in the hemodialysis environment or could be more adapted to the immunosuppression of these patients[18]. Similarly, this genotype is also predominant among hemodialysis patients of other countries such as Jordan and the United States [2,19]. In contrast, other mentioned that genotype 1a or 1b predominates in the non-Arab countries of the Middle East[20]). Dominant HCV genotypes in our neighboring countries were found to be: 1b and 3a in Turkey [21] ; and 4 in Middle East countries [22], Saudi Arabia [23] , Kuwait [24] , and Egypt [25,26]. In Syria a study found that the HCV genotypes in hemodialysis patients from Syria are equally distributed between HCV genotype 1 and HCV genotype 4[27].

In our study, only one of 29 (3.45%) HCV-RNA positive samples had genotype 3a. This pattern is different when compared to reports from developed countries, where the molecular epidemiology of HCV seems to be influenced by lifestyles among young adults as reported from USA and Southeast Asia in their young drug addicts [17]. In our results, two samples had a mixed pattern of subtypes 1b/4 (6.89%). Mutations in the viral genome or co-infection may explain these findings [13]. Grouping of anti-HCV positive patients in dialysis units might thus increase their risk acquiring multiple HCV strains. Infection with two or more different HCV genotypes has been observed in HD patients and 13% of patients referred for renal transplantation [28] . In contrast, some studies have described super-infection in intravenous drug

users and hemodialysis patients were uncommon event [29].

We did not detect genotypes 2, 5 and 6 in any of the samples. There is no conclusive proof these genotypes are not present in Iraq, especially considering the fact that genotype 2b and 6a were reported in thalassemic patients [5]. Our result similar to findings of other studies [30,31] on the genotype distribution in Iran where none of these patients were found to be infected with genotype 2. This finding is in contrast with global HCV genotypes distribution [32] and the high frequency of genotype 2 in patients on hemodialysis and non-uremic patients living in Lebanon [33], Saudi Arabia [34], and Argentina [35]. Most patients infected with HCV subtype 4 were receiving treatment at the three hemodialysis units in Baghdad. The spread of viral strains with the same genotypes, could confirm eventually the possibility of the HCV dissemination in the hemodialysis setting. The homogeneity of the genotypes reflects a transmission nosocomial [36].

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