VIROLOGY

Hepatitis C virus infection in Jordanian haemodialysis units: serological diagnosis and genotyping

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The seroprevalence and genotypes of hepatitis C virus (HCV) were studied in 283 patients attending six haemodialysis units in Jordan. In all, 98 (34.6%) patients were anti-HCV-positive by EIA, 92 (93.9%) of whom were also reactive in an immunoblot assay. The prevalence of anti-HCV was correlated with a history of blood transfusion before the introduction of blood donor screening for HCV and with duration of haemodialysis. HCV RNA was detected in 30 (30.6%) of 98 anti-HCV-positive sera. HCV viraemia was not associated with a particular antibody for the six HCV antigens studied by the immunoblot assay, although reactivity to the core antigens was greater in the HCV RNA-positive sera than in negative sera. Two HCV genotypes (1 and 4) were identified for the first time in Jordan by restriction fragment length polymorphism analysis of HCV 5'-NCR. The predominant genotype was HCV 1a (12 of 30). Genotypes 1b and 4 were detected in 10 and 8 patients, respectively. The antibody response to HCV antigens was genotype-dependent, with a wider range of antibody specificities detected in the immunoblot assay in the 12 patients with genotype 1a infection than in the 8 patients with genotype 4. However, there was no significant difference in the prevalence of antibodies to HCV antigens among patients infected with either genotype 1a or 1b. In conclusion, the prevalence of anti-HCV, blood transfusion, duration of dialysis and HCV genotypes suggest possible nosocomial HCV transmission among patients which needs confirmation by phylogenetic analysis of subgenomic HCV regions.

Introduction

Haemodialysis patients have a high risk of acquiring hepatitis C virus (HCV) infection. Transfusion of unscreened blood [1], duration of dialysis [2] and nosocomial transmission within haemodialysis units [3, 4] are implicated as the main transmission routes of HCV in haemodialysis patients. In these patients, HCV infection is usually asymptomatic [5] and can be diagnosed by serological methods and by amplification of HCV RNA by reverse transcriptase-PCR (RT-PCR) [6], which distinguishes between viraemic and non-viraemic HCV patients and also is used for HCV genotyping [7]. HCV isolates have been classified into six major genotypes, many of which contain a number of closely related subtypes [8]. New HCV variants from Vietnam, Jakarta and Indonesia have been

described as genotypes 7, 8, 9, 10 and 11 [9–10]. The distribution of HCV genotypes varies in different countries as documented in blood donors, and haemodialysis and chronic hepatitis patients [11–13]. There has been no report on HCV genotypes in Jordan. Therefore, this study used both serological and molecular methods to assess (i) the prevalence of anti-HCV and HCV RNA among haemodialysis patients in different dialysis units in Jordan, (ii) the influence of blood transfusion and dialytic age on HCV infection, (iii) the HCV genotypes and (iv) the correlation between HCV genotypes, viraemia and the antibody response to various HCV antigens.

Materials and methods

Patients

In all, 283 patients from haemodialysis units at six government hospitals were studied, with informed, written consent. Plasma samples were separated from

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the whole blood within 1–3 h of vein puncture, divided into small volumes and stored at -70°C .

Information on the age of patients, duration of haemodialysis, number of blood transfusions and renal diagnosis was obtained from patient records and interviews. The renal diagnoses were: chronic renal failure and hypertension ($n = 81$), chronic glomerulonephritis (46), diabetic nephropathy (42), small kidney (33), failed kidney transplant (12), polycystic kidney (6) and others (63).

Biochemical tests

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured by spectrophotometry with kits from Menagent (Italy) according to the manufacturer's instructions.

Serology

All plasma samples were tested by the Innostest HCV Ab IV (Innogenetics, Belgium) enzyme immunoassay (EIA) for the presence of antibodies against proteins derived from four regions of the HCV genome: core, NS3, NS4 and NS5. EIA-positive samples were re-tested by the Inno-EIA HCV Ab III immunoblot system (Innogenetics), according to the manufacturer's instructions. Each strip incorporates HCV antigens derived from the core (C1, C2), E2, NS3, NS4A, NS4B and NS5 regions.

Reverse transcriptase nested PCR (RT-nested PCR)

RNA was extracted from 200–400 μl of all seropositive plasma samples with the commercially available high pure RNA Kit (Boehringer Mannheim, Germany). To detect contamination, an HCV-negative plasma was extracted with each batch of five RNA extractions.

Extracted RNA was reverse transcribed and amplified with nested primers matching the conserved sequences in the 5' non-coding region (5'-NCR) of HCV RNA [14]. A negative PCR blank with distilled water instead of RNA was included with each set of five reactions. The PCR products (5 μl) were subjected to electrophoresis in agarose (Promega, USA) 3% and the band size was assessed by direct comparison with a 100-bp DNA marker (Promega).

Genotyping analysis

HCV genotypes were determined by restriction fragment length polymorphism (RFLP) analysis of the 5'-NCR. The positive RT-nested PCR products were digested separately by two pairs of restriction endonucleases (*HaeIII/RsaI* and *MvaI/HinfI*; Boehringer Mannheim) for differentiation into genotypes 1–6 [15]. Subtypes 1a/c and 1b were further differentiated by digestion with the restriction endonuclease *BstUI* (New England Biolabs, USA). Band size was assessed by direct comparison with a 20-bp DNA marker (BioRad, USA).

Statistical analysis

The χ^2 test was used for statistical analysis, with Fisher's exact test where any one value was <5 ; p values <0.05 were considered significant. Correlation coefficients were determined with an Excel programme.

Results

In all, 174 male and 109 female patients aged 9–80 years were enrolled into the study (Table 1). Sera from 98 patients (34.6%) were anti-HCV positive. Seropositivity rates did not differ between male (36.8%) and female (31.2%) patients. The duration of haemodialysis treatment was grouped into <12 , 12–48 and >48 months. Rates of anti-HCV seropositivity were corre-

Table 1. Characteristics of haemodialysis patients tested by EIA

Parameter	Number (%) of patients		
	Total ($n = 283$)	Anti-HCV-positive ($n = 98$)	Anti-HCV-negative ($n = 185$)
Sex			
Male	174	64 (36.8)	110 (63.2)
Female	109	34 (31.2)	75 (68.8)
Duration of haemodialysis (months)			
<12	45	3 (6.7)	42 (93.3)
12–48	135	27 (20)	108 (80)
>48	103	68 (66)	35 (34)*
Previous blood transfusion			
before 1993	191	62 (32.5)	129 (67.5)
after 1993	16	11 (68.7)	5 (31.3)
	175	51 (29.2)	124 (70.8)*
Elevated			
ALT	7	6 (85.7)	1 (14.3)
AST	7	5 (71.4)	2 (28.8)

* $p < 0.001$ for <12 months dialysis versus >48 months, and for receipt of blood transfusion before and after 1993.

lated with the duration of haemodialysis ($r^2 = 0.988$). The prevalence of anti-HCV in those who had undergone haemodialysis for >48 months was significantly higher than in those who had undergone dialysis for <48 months ($p < 0.001$). In Jordan, anti-HCV screening was introduced to blood banks in 1993. The prevalence of anti-HCV was significantly higher in patients who received blood transfusion before 1993 (68.7%) than those who received blood transfusion after 1993 (29.2%, $p < 0.001$). Only six (6.1%) and five (5.1%) of anti-HCV positive patients had elevated ALT and AST levels, respectively.

Variation in the prevalence of anti-HCV was observed between the haemodialysis units (range 13.6–47.1%) (Table 2).

HCV RNA was detected in 30 (30.6%) of the 98 anti-HCV-positive patients, with the prevalence of HCV RNA varying from 0% to 41.6% in the six dialysis units.

In 92 of 98 anti-HCV EIA-positive sera, the result was confirmed by immunoblot; 29 of these sera were HCV RNA-positive. Four sera gave indeterminate results on immunoblotting (one was HCV RNA-positive) and two were negative (one was HCV RNA-positive).

There were no significant differences in the prevalence of antibodies to different HCV antigens between HCV RNA-positive and HCV RNA-negative sera. However, HCV RNA-positive sera were more likely to have high intensity antibody bands (3+ or 4+) to core antigens (C1 20/30 *versus* 10/68, C2 17/30 *versus* 9/68; $p < 0.001$) than HCV RNA-negative sera.

The predominant HCV genotype was 1a, detected in 40% of HCV RNA-positive patients, whilst genotypes 1b and 4 were found in 33.3% and 26.6% of patients, respectively. All isolates from patients in Unit 3 were genotype 1 (Table 2).

Patients infected with genotype 1a were more likely than those infected with type 4 to have detectable antibodies in the immunoblot assay to core 1 (12/12 *versus* 5/8, $p = 0.049$), E2 (10/12 *versus* 3/8, $p = 0.06$), NS4 (11/12 *versus* 2/8, $p = 0.004$) and

NS5 (6/12 *versus* 0/8, $p = 0.04$) antigens. There were no other significant differences in antibody distribution between patients with genotypes 1a, 1b or 4.

Discussion

This is the first study to report on the seroprevalence and genotyping of HCV in haemodialysis patients in Jordan. The seroprevalence of 34.6% compares with 41.9% in Saudi Arabia [16], 45% in Tunisia [17] and 45% in Syria [18]. HCV RNA was detected in only 30.6% of anti-HCV positive patients (Table 2). Possible explanations for this low percentage of viraemic patients include (i) false positive serology, (ii) intermittent viraemia, (iii) unusually high rates of clearance of infection in these dialysis patients or (iv) levels of viraemia in these patients below the lower limit of detection of the genome detection assay. The low rates of strong positive (3+ or 4+) antibody bands in the immunoblot assay in the HCV RNA-negative sera support the first hypothesis. However, it has also been reported that 7–68% of haemodialysis patients have intermittent viraemia with periods of undetectable HCV RNA for up to 4 weeks [7, 19–21]. The viral load is relatively low in this group of patients and long-term maintenance haemodialysis decreases the HCV RNA level but does not produce clearance of viraemia [22–24]. It is not possible to discern for certain which of the four suggested hypotheses pertains in this group of patients.

HCV viraemia was not associated with a particular antibody specificity, as also reported elsewhere [25, 26]. In contrast, others have reported that antibodies to capsid antigen (C22) [27] or NS3 region [28] are more likely to be correlated with viraemia. However, in the present study, HCV RNA was associated with stronger reactivity (3+, 4+) to HCV core (C1, C2) antigens in the immunoblot assay, as compared with the HCV RNA-negative sera. The low reactivity (1+) to C1 and C2 in HCV RNA-negative patients suggests resolved infection with loss of antibodies. Such a profile has been reported by other investigators [29].

The ALT and AST values in haemodialysis patients are reported to be lower than in healthy individuals [30–

Table 2. Prevalence of HCV RNA and HCV genotypes in anti-HCV-positive haemodialysis patients in Jordan

Haemodialysis units	Number (%) of patients					
	Total	Anti-HCV-positive	HCV RNA-positive	HCV genotypes (% of RNA-positive)		
				1a	1b	4
1. Princess Basma Hospital	65	23 (35.4)	7 (30.4)	2 (28.5)	1 (14.3)	4 (57)
2. Jarash Government Hospital	22	3 (13.6)	0 (0)	0 (0)	0 (0)	0 (0)
3. Al-Salt Government Hospital	38	12 (31.6)	5 (41.6)	3 (60)	2 (40)	0 (0)
4. Al-Basheer Hospital	70	22 (31.4)	7 (31.8)	4 (57)	2 (28.5)	1 (14.2)
5. Al-Nadeem Hospital	21	6 (28.6)	0 (0)	0 (0)	0 (0)	0 (0)
6. Zarqa Government Hospital	68	32 (47.1)	11 (34.4)	3 (27.3)	5 (45.4)	3 (27.3)
Total	283	98 (34.6)	30 (30.6)	12 (40)	10 (33.3)	8 (26.6)

32]. In the present study, most anti-HCV-positive patients had ALT and AST values within the normal range, indicating that transaminases are not sensitive markers for ongoing HCV replication in haemodialysis patients. Similar results have been reported by others [5]. The elevated level of transaminases observed in some anti-HCV-negative patients (Table 1) could be due to other factors including drugs or other viral agents.

The prevalence of anti-HCV in Jordanian haemodialysis patients was correlated with history of blood transfusion before the introduction of anti-HCV screening for Jordanian blood banks in 1993, suggesting this as an important route of infection. The seropositivity to HCV also increased significantly with increase in the duration of haemodialysis (Table 1), suggesting possible nosocomial transmission between patients. Numerous studies in recent years have provided molecular evidence for the nosocomial transmission of HCV within haemodialysis units [3, 4]. The greatest risk of transmission of HCV within dialysis units is *via* dialysis next to an HCV-positive patient [33]. In the present study, the route by which the haemodialysis patients acquired HCV was not determined. All Jordanian haemodialysis units use hypex and formalin to disinfect the haemodialysis machines at the end of each session. Filters and tubes are discarded after each use. Nurses do not regularly wear gloves when dealing with patients. The hands of dialysis personnel have been implicated as a potential mode of facilitating transmission of HCV between haemodialysis patients [34]. Furthermore, variations in the level of hygienic standards may explain the differences in prevalence of anti-HCV positivity among the dialysis units (Table 2). To limit the spread of HCV infection in haemodialysis units, precautionary aseptic measures should be improved. Anti-HCV-positive patients should be considered potentially infectious and should be isolated from the anti-HCV-negative patients – dialysing HCV-positive patients in a separate unit or in a defined sector of a dialysis unit significantly reduces nosocomial HCV infection [33]. Furthermore, education programmes for staff on the risk of transmission of blood-borne viruses should be considered.

HCV genotype 1a is the most prevalent genotype in Jordanian patients (Table 2). This genotype is also predominant in Jordanian blood donors (unpublished data) and in haemodialysis patients of some Middle Eastern countries including Lebanon, Turkey, Cyprus [11] and Syria [18]. In contrast, HCV genotype 4 is the most prevalent genotype in other Middle Eastern countries including Saudi Arabia, Egypt, Yemen and Bahrain [11, 15, 36, 35].

The prevalence of antibodies to different HCV antigens in patients infected with genotype 4 was significantly lower than in patients infected with genotype 1. The difference in this serological response may be due to differences in virus replication capacities or in the

immune response to viral antigens, or to the presence of different epitopes in the two genotypes. Genotype dependence of antibody to HCV core, NS3 and NS4 antigens has been reported by others [36, 37].

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