Original Article

Hepatitis C Genotype 4: Genotypic Diversity, Epidemiological Profile, and Clinical Relevance of Subtypes in Saudi Arabia

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ABSTRACT

Background/Aim: Hepatitis C virus genotypes 4 (HCV-4) is the most prevalent genotype in Saudi Arabia, although it's various subtypes, mode and route of transmission remains unknown. The aim of this study was to analyze (i) the variability of the HCV-4 subtypes, the route and source of HCV transmission and (ii) the influence of HCV-4 subtypes on their therapeutic response. **Patients and Methods**: Sixty-four HCV-4 patients were analyzed retrospectively for the prevalence of various sub-genotypes and the possible mode of transmission, and it was correlated with their treatment response to pegylated interferon (PEG-IFN) α-2a and ribavirin therapy. **Results:** Positive history of blood or blood products transfusion was noted in 22 patients (34%), hemodialysis in 10 patients (15.6%), surgery in 7 patients (11%), and unknown etiology in 25 patients (39%). Prevalence of HCV-4 subtypes was 4a = 48.4% (31/64), 4d = 39% (25/64), 4n = 6.25% (4/64), and remaining combined (4m, 4l, 4r, 4o) 6.25% (4/64). No significant correlation between subtypes and the source of transmission was recognized ($P = 0.62$). Sustained virological response in all HCV-4 patients was 64% (41/64), while in each subtypes separately it was 4a 77.4% (24/31), 4d 52% (13/25), and combined (4n, 4m, 4l, 4r, 4o) 62.5% (5/8) ($P = 0.046$). **Conclusion:** No obvious cause for the mode of HCV transmission was noted in majority of the patients. No significant correlation was observed between HCV-4 subtypes and the source of HCV infection. 4a and 4d subtypes were the most common in Saudi Arabia, and patients infected with 4a subtype responded significantly better to combination therapy than to 4d subtype.

Key Words: Hepatitis C virus, HCV genotype 4, subtypes of HCV-4

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More than 170 million people throughout the world are infected with Hepatitis C virus (HCV). HCV genotype 4 (HCV-4) is the most common genotype in the Middle East and Africa. It is responsible for > 80% of HCV infections, which has recently also spread to several European countries.[1] Previous studies in Saudi Arabia have indicated that the anti-HCV prevalence was 0.4-1.7% for adults and 0.1% for children.[2-5] The frequency of different genotypes in Saudi Arabia were as followed: HCV-4 genotype (69.2%), genotype 1a (12.8%), genotype 1b (11.4%), genotypes 2b (1.4%), genotype 5 (1.4%), and mixed infections with genotypes 4 and 5 (3.6%).[6] HCV-4 is a very heterogeneous genotype, showing significant genetic divergence compared with other HCV genotypes. Phylogenetic analysis of the NS5B region is commonly used for sub-typing. Until date, the number of sub-types has increased up to 20.[7,8] Of the 20 different subtypes identified, the most common ones are 4a and 4d, while others 4b, 4c, 4e, 4f, 4g, 4h, 4i, 4j, 4k, 4l, 4m, 4n, 4o, 4p, 4q, 4r, 4s, and 4t have been identified in different geographic regions of the world. The full clinical significance of HCV-4 subtypes is not known, because very few studies have correlated HCV-4 subtypes to the epidemiology, natural history, pathogenesis, disease severity, and outcomes of therapy. A recent study from Egypt reported a significant association between subtype 4o and hepatocellular carcinoma.[9] Sub-genotype 4d has been linked particularly in epidemiological studies to intravenous drug abusers in Poland and southern Europe.[10,11] Prevalence of HCV-4 subtype and their response to treatment are not

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known in Saudi Arabia, such knowledge is crucial for clinical and epidemiological analyses that will be required for the development of effective vaccines and antiviral therapies against HCV-4.

PATIENTS AND METHODS

Patients

Sixty-four consecutive adult Saudi patients with chronic HCV-4, who were referred to King Faisal Specialist Hospital and Research Center (KFSHRC) between 2006 and 2008, treated with PEG-IFN α-2a and ribavirin, and had completed 48 weeks of treatment, were included in this study. Pre-treatment demographic, clinical, biochemical, serological, and virological data were retrieved from our previous database that was established while assessing the sustained virological response (SVR) to combination therapy.[12] Serum samples collected for routine HCV quantification and genotyping in the molecular diagnostics laboratory were used for HCV-4 subtyping. All samples were stored at −70°C until assayed. The study was approved by the ethics committee of KFSHRC Research Advisory Council.

Treatment

All patients were treated with PEG-IFN α-2a 180 μg subcutaneously once weekly (Pegasys, F. Hoffmann-LaRoche, Basel, Switzerland) and ribavirin twice daily (Copegus, F. Hoffmann-La Roche, Basel, Switzerland) at a total daily dose of 1000 mg for patients weighing < 75 kg, and 1200 mg daily for those ≥ 75 kg. This standard combination therapy was administered for a total of 48 weeks and patient's follow-up was continued for another 24 weeks after completion of treatment to achieve SVR. RVR was defined as undetectable HCV RNA (<15 IU/ml) after 4 weeks of therapy, and EVR were defined as > 2 $\log_{10}$ reduction in serum HCV RNA at 12 weeks of treatment. End of treatment virological response (ETVR) and SVR were defined as viral load < 15 IU/ml at 48 weeks and 24 weeks after the end of treatment, respectively. Pretreatment phylogenetically analyzed subtypes were assessed by univariate analyses in relation with the EVR, ETVR, and SVR.

HCV-RNA quantification

The concentration of HCV-RNA was determined by reverse transcription PCR of plasma using CobasAmplicor HCV Monitor version 2.0 (Roche Diagnostics, Branchburg, NJ) following manufacturer's instructions. HCV-RNA quantification was performed at 4, 12, 48, and 72 weeks of therapy.

HCV Genotype

HCV genotype was performed using INNO-LiPA HCV II (Innogenetics NV, Ghent, Belgium).

Subgenotyping of HCV

Nucleic acids were extracted from sera using QIAmpMinElute Virus Spin Kit (QIAGEN, Santa Clarita, CA, USA) following manufacturer's instructions. Extracted RNA was converted to cDNA as described before.[13] Subgenotyping was performed according to the procedures described by Murphey *et al*.[14] PCR product were sequenced using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) following manufacturer's recommendations. DNA sequence was then analyzed using BLASTN 2.2.23+ software (http://www.ncbi.nlm.nih.gov/blast/) against all sequences in the database for subgenotyping of each sample. The sequence that showed the lowest value and maximum identity was taken as the subgenotype of the sample analyzed.

Statistical analysis

Data were collected initially in a specialized data collection form, introduced into a Microsoft Excel worksheet, and finally transferred to the statistical package for social sciences (*SPSS*) version 13.0 (SPSS, Chicago, IL, USA) for analysis. Results were reported as mean ± SD. HCV RNA levels were logarithmically transformed for analysis. Continuous variables were compared using the two-tailed student's *t*-test. Categorical data were compared using the two-tailed χ^2 test or Fisher's exact test. Factors associated with any specific subtype were analyzed by univariate analysis. *P* values ≤0.05 were considered statistically significant.

RESULTS

The average age of 64 (male:female = 41:23) Saudi patients was 38.7 ± 11.5 years. There was a positive history of transfusion of blood or blood products in 22 patients (34%) before 1991, while 25 patients (39%) did not give history of any significant illness in their life that could be attributed to the mode of HCV transmission. Another 10 patients (16%) were infected during the period of dialysis (they were HCV negative before the initiation of dialysis), and the remaining 7 (11%) had definite history of surgery (before 1991) but denied receiving blood transfusion [Figure 1]. None of the patient in our cohort had a history of IV drug abuse and only one patient had HIV that was transmitted by factor VIII (AHF) transfusion (before the discovery of HCV virus). No family history of HCV infection and no history of vertical or sexual transmission were reported.

The prevalence of HCV-4 sub-genotypes were 4a = 48.4% (31/64), 4d = 39% (25/64), 4n = 6.25% (4/64), and the remaining combined sub-genotypes were 12.4% (4n was 4/64 = 6.25%, while others 4o = 1 (1.5%), 4l = 1 (1.5%), 4m = 1 (1.5%), and 4r = 1 (1.5%). The most common HCV-4 subtype in our cohort was 4a (48%), followed by 4d (39%) and then 4n (6.25%) [Figure 2].

The details of the various modes of transmissions and the distribution of HCV-4 subtypes are shown in Table 1. There was no significant relationship between the various subtypes and the route of transmission, ($P = 0.6296$). There was no history of blood transfusion, minor or major surgery, hemodialysis, IV drug abuse, or any unusual sexual behavior in 25 patients (unknown etiology: 39%), with the majority belonging to subtype 4d. Out of the 64 patients, 22 (34%) reported a history of blood transfusion (subtype 4a 12/22 patients: 54%).

RVR, EVR, ETVR, and SVR were obtained in 18.8% (12/64), 81.0% (47/58), 81.0% (47/58), and 64.1% (41/64), respectively. Of the patients with RVR and EVR, 83% and 87.2% achieved SVR, respectively. The overall SVR in HCV-4 patients in our cohort was 64% (41/64), where 81.25% (52/64) were treatment naïve. When we analyzed the SVR in various sub-genotypes, 4a was achieved in 77% (24/31), 4d in 52% (13/25), and combined (4n,4m,4l,4r,4o) in 62.5% (5/8) the difference of response was statistically significant ($P = 0.046$) [Figure 3].

DISCUSSION

The importance of HCV genotyping has considerably increased in the past few years. Genotyping has also been used to study relationships between type/subtype to epidemiology, clinical status, pathogenesis, and the outcome of a disease.[15] Previous studies have confirmed the predominance of HCV genotype 4 (60-80%) in Saudi Arabia,[16-19] but there is no sufficient data available on HCV-4 subtype. This study was designed to analyze the prevalence of HCV-4 subtypes and their effect on the spread of HCV infection in Saudi Arabia. There is only one previous report published regarding the prevalence of HCV-4 subtypes in Saudi Arabia,[20] which while reporting the prevalence of all HCV genotypes, also reported on the prevalent subtypes: 4c/4d:33%, 4e:7%, 4h:14%, 4:20%, 1b:14%, 3a:5%, 5a:1%, and 6a:1%, but there was no specific reporting for HCV-4 subtype. Versant HCV genotype assay (LiPA) 1.0, used in a previous study, had an overall accuracy of 74% for the subtypes,[21] while the new assay outperforms the previous version of the line probe assay. The newer Versant HCV genotype assay (LiPA) 2.0 (used in the present study) yields an interpretable genotype result for 96% of the samples, and 99.4% of the interpretable results agreed with the reference method, rendering it an accurate and reliable assay for genotyping. Many studies have shown that the NS5B region is discriminative for determining HCV genotypes and their subtypes.[22,23] As such, in the present study, HCV subtype was determined by sequencing a fragment within NS5B region of the HCV genome.

Table 1: Prevalence of HCV-4 subtypes and their relationship with possible source of infection

HCV-4 subtypes	Blood transfusion or blood products 22 (34%)	Unknown etiology 25 (39%)	Dialysis 10 (16%)	Surgery 7 (11%)	*P* value
4a (31)	12	10	4	5	0.6296
4d (25)	6	13	4	2	
4n (4)	1	2	1	0	
4l (1)	1	0	0	0	
4o (1)	1	0	0	0	
4m (1)	1	0	0	0	
4r (1)	0	0	1	0	

In our cohort, predominant subtype among HCV-4 was 4a (48%) followed by 4d (39%), while in neighboring Egypt, the distribution of HCV-4 subtypes were completely diverse: 4a was detected in 80.6%, whereas 4g, 4l, 4n, 4o, 4f, and 4m were identified in 7.7%, 4.7%, 3.9%, 1.6%, 0.8%, and 0.8% of cases, respectively.[24] Surprisingly, 4d was rarely reported (<1%) from Egypt. However, the route of transmission of HCV in any region detects the incidence of genotype and subtypes for that particular region. The spread of 4a infection in Egypt coincided with the mass campaigns of intravenous antimony treatment for schistosomiasis.[25] In Saudi Arabia, 4a has been mainly imported from Egypt via blood donation from Egyptian volunteers in Saudi Arabia, and surgical procedures or IM/IV injections undertaken in rural Egypt.[26] The higher prevalence (48%) of 4a subtypes among Saudi patients is attributed to many factors, including the presence of workers from the Middle East, in addition to African and Asian nationalities with higher incidence of 4a. These individuals may have donated blood that had been used among Saudi patients before the era of HCV testing as observed in our cohort, where 39% of 4a patients had history of blood transfusion. Sexual transmission may also occur, although it is less frequently noted from this region.[27,28] Arif *et al.*,[29] clearly indicated that the intra-familial transmission of HCV was not the route

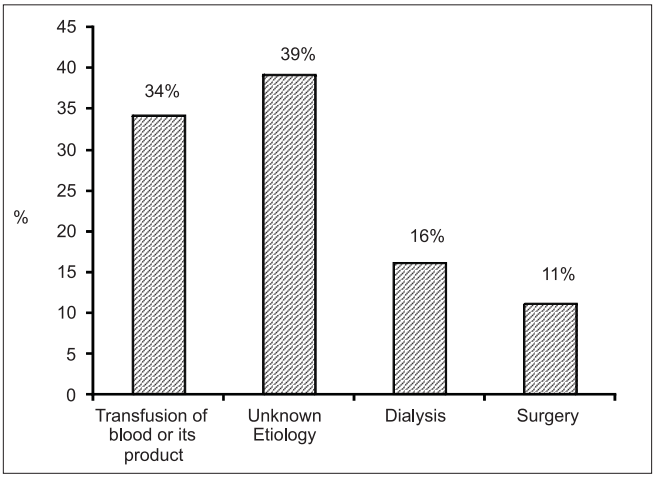

Figure 1: Possible mode of hepatitis C virus transmission

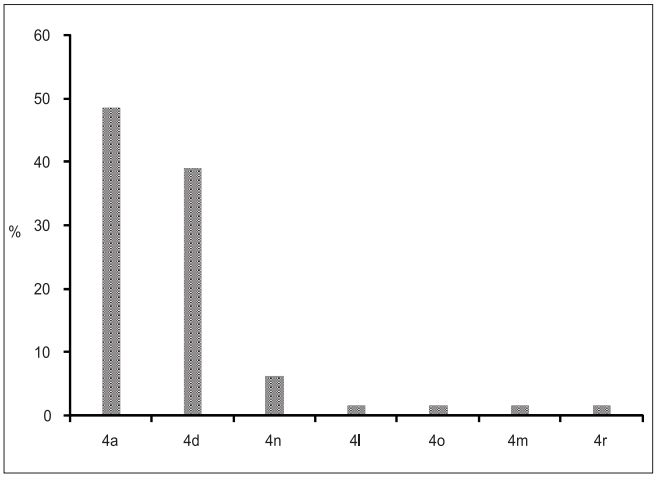

Figure 2: Occurrence of HCV-4 subtypes in Saudi Arabia

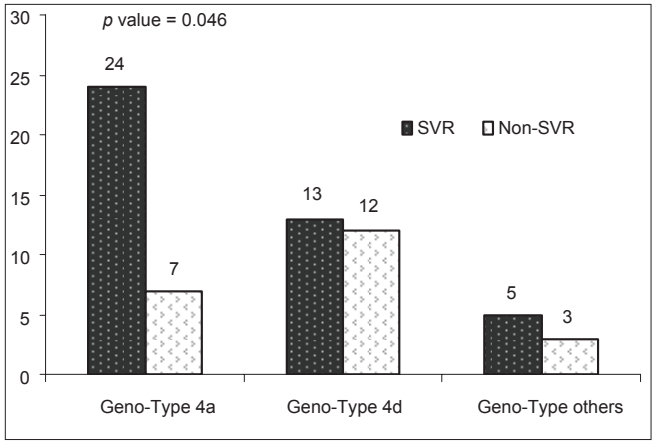

Figure 3: Sustained Viral Response in various HCV-4 subtypes

of transmission among Saudis and their results argued against sexual transmission of HCV despite a relatively long duration of marriage. This hypothesis is supported by our data as there was no history of HCV transmission reported among spouses. Another large epidemiological study in 2008 from Saudi Arabia[30] reported that HCV infection in children (0.012%) was much lower than that among adults (0.202%), suggesting that perinatal and childhood transmission was not a major mode of infection. In our cohort, none of the patients had a history of vertical transmission.

All European studies on HCV-4,[31-36] confirmed the presence of 4d in almost similar percentage (32-55%) as seen in Saudi Arabia, where 4a was mainly transmitted to Europe through IV drugs abusers from the Middle East and central African countries. In the present study, only a minority of 4d patients (24%, 6/25) had a history of blood transfusion. Mode of transmission was unknown in majority of 4d subtype (52%, 13/25), where no history of blood transfusion, surgery, dialysis, IV drug abuse, or abnormal sexual behavior was reported. In Saudi Arabia, the mode of transmission in 39% patients is unknown, but sporadic iatrogenic transmission is possible though not evident by previous studies from Saudi Arabia. Probably, in those group of patients, the infection might have occurred by needle-stick injuries among medical personnel, circumcision, cupping (Hijama), tattooing, ear and body piercing, razor sharing at barber shop, contaminated endoscopy, inadequately sterilized needles and syringes, tooth brush or syringe sharing, and unhygienic dental procedures. These varied probable causes could explain the presence of different subtypes among Saudi patients. There is a need to extend the molecular epidemiology for more prospective larger scale studies to other regions of the country to obtain a better evaluation of the HCV epidemic dynamics in Saudi Arabia.

Knowledge of HCV genotypes is essential not only for epidemiological reasons but also from a clinical standpoint. The overall SVR in HCV-4 patients in our cohort was 64% (41/64); this improved response to combination therapy was related to the inclusion of mostly naive patients (81.25%) who had strict compliance to 48 weeks of combination therapy. We also analyzed the SVR in various subtypes, 4a achieved 77% (24/31), 4d 52% (13/25), and combined subtype 62.5% (5/8), and the difference of response was statistically significant ($P = 0.046$). A previous retrospective study[37] showed similar response that was observed in French patients infected with HCV-4, where subtype 4a had significantly higher rate of SVR (58%) than subtype 4d (43%, $P = 0.035$). It was unclear from this study whether the difference in SVR was related to ethnicity, HIV infection, or IV drug use. Another study[38] from France has reported a poorer response to 4d group of 10 HIV-positive patients, who were acutely infected. None of the 10 patients treated early with antiviral therapy had SVR, suggesting that this subtype is less sensitive to interferon-based therapy. However, in another larger study[39] also from France, observed no significant difference in the virological responses of various HCV-4 subtypes. They reported SVR among 4a (51.3%), 4d (51.7%), and other subtypes (48%, $P = 0.16$), where 31.8% of the subjects were co-infected with HIV, 14% were cirrhotic, and 22% had received interferon therapy in the past; these factors did affect the response to therapy. Response to therapy in various other subtypes has not been reported earlier, except a very recent publication[40] that showed the influence of HCV-1 subtypes on the virus response to combination therapy, where patients infected with HCV subtype 1b had a higher probability of SVR than those infected with subtype 1a.

In summary, around 60% of our cohort had H/O surgery, blood transfusion, or hemodialysis, but the remaining 40% did not have any significant history attributed to HCV infection,

where the mode of transmission can only be speculated as unknown. No significant correlation was observed between HCV-4 subtypes and the source of HCV infection.

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