

Screening of Hepatitis C Virus Genotypes in Urticaria Patients in Saudi Arabia

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Objective: The main objective of this study was to estimate the prevalence of hepatitis C virus (HCV) infection and to determine the HCV genotypes in urticaria patients of Saudi Arabia. **Methods:** After thorough clinical examination by a consultant dermatologist, urticaria patients and individual healthy controls were enrolled. Venous blood collected from subjects was analyzed for LFT (aspartate transaminase [AST], alanine transaminase [ALT], albumin, and bilirubin), hepatitis B surface antigen (HBsAg), and HCV antibodies—HCV-RNA-PCR screening and genotyping. **Results:** Upon enzyme immunoassay (EIA) screening for HCV infection, 5/70 (7.1%) urticaria patients and none among the controls tested positive for the presence of anti-HCV antibodies ($p=0.005$). Genotyping analysis revealed that HCV belongs to types 3 and 4 with subtypes 3a, 4a, and 4c. No significant variations were seen in the mean serum levels of ALT, AST, albumin, and bilirubin between the patients based on their HCV sero-positivity status. **Conclusion:** This prospective study indicated that HCV infection plays a significant role in urticaria. However, larger studies in different ethnicities could ascertain the association between different HCV genetic variants and the urticaria.

Introduction

URTICARIA IS A COMMON DISORDER that affects as many as 20% of the population at some point during their life-time. Factors believed to cause or aggravate urticaria include drugs, foods, additives, connective tissue disorders, and infections (Hamid *et al.*, 1998; Siddique *et al.*, 2004). Urticaria has a spectrum of diverse clinical presentations and causes. Clinically, it is classified into ordinary urticaria, physical urticarias, contact urticaria, urticarial vasculitis, and angioedema (Grattan *et al.*, 2002). Although urticarial vasculitis and urticaria as part of mixed cryoglobulinemia are well known to occur, urticarial vasculitis is a form of small vessel vasculitis that may present with both urticaria and arthritis. Infections are well recognized as one of the exacerbating factors for urticaria. Infectious agents, such as bacteria, viruses, parasites, and fungi, were earlier reported to have association with urticaria. The temporal relationship between infections and urticaria onset, as well as its remission or improvement after treatment of a coincident infection, supports this link (Wedi *et al.*, 2004).

Hepatitis C virus (HCV) is a single-stranded RNA flavivirus that replicates in hepatocytes and peripheral blood mononuclear cells. Hepatitis C infection leads to liver cirrhosis and hepatocellular carcinoma (Reichel, 1990). Reports have also shown that 55–70% of the HCV patients go into the chronic course of the disease affecting organ systems, including joints, muscles, neural and gastrointestinal tissues, and skin (Blackard *et al.*, 2008). Cutaneous manifestations are

often the first signs (observed in 20–40% of the patients with chronic HCV infection) (Galossi *et al.*, 2007). However, the prevalence of skin manifestations varies from one geographic area to another. Blood or blood products constitute the primary transmission routes of HCV (Schwartz and Birnkrant, 2008). Whether hepatitis C infection is involved in pathogenesis of urticaria or not is still debatable, as conflicting reports have been presented both in favor and against to this topic. HCV-RNA tests were also performed on sero-positive patients for HCV antibody (Kaşifoğlu *et al.*, 2007) and the skin findings for HCV-RNA-positive and -negative cases were compared. For example, a previous report from Saudi Arabia suggested the involvement of different HCV genotypes in pruritus (Halawani and Bakir, 2011), but none of the studies have yet been carried out in urticaria patients. Hence, the objective of this study was to examine the involvement of HCV infection and to assess the prevalence of specific HCV genotypes in urticaria patients from the Saudi population.

Materials and Methods

Enrollment of subjects

This prospective study enrolled 70 urticaria patients attending dermatology clinics at the King Khalid University Hospital (KKUH) and King Abdul Aziz University Hospital (KAUH), Riyadh, between March 2007 and March 2009. Using the standard questionnaire, both demographic (age, sex, nationality, and marital status) and clinical details of each

patient (symptoms, sign, past-medical history, and predisposing risk factors for HCV infection, Hepatitis B virus (HBV) infection, urticaria, and its duration) were collected after obtaining their informed consent. Examination for urticaria included the history of onset of the symptoms, presence of any food allergies, underlying infections, or drugs as exacerbating factors. The presence of evanescent urticated plaques was considered suggestive of urticaria. Patients with drug, food, or infection-induced urticaria were excluded from the study. Similarly, patients with physical urticaria or those with a previous history of connective tissue diseases were excluded. In addition, 70 healthy blood donors, consisting of eight (11.42%) women and 62 (88.57%) men with a mean age of 28.45 ± 7.40 (18–41) years, were also studied as controls. Each participant was subjected to the detailed physical and dermatological examinations performed by a consultant dermatologist.

Clinical examination did not reveal any skin manifestations and none of them reported past exposure to any of the HCV risk factors. None of the 50 blood donors tested positive for the presence of anti-HCV antibodies, hence negative for HCV-RNA. All of the healthy blood donors also tested negative for hepatitis B surface antigen (HBsAg) also.

Biochemical testing

Ten milliliters of whole blood was collected from healthy blood donors and urticaria patients in properly labeled plain, Red-Top 10 cc containers. Sera were separated by centrifugation at 2500 g for 15 min at 4°C and stored at –70°C until further investigations were carried out. Serum levels of alanine transaminase (ALT), aspartate transaminase (AST), albumin, and bilirubin were determined using an automated multi-channel clinical chemistry analyzer according to the standard protocols followed at KAUH biochemistry laboratory.

Enzyme immunoassay and line immunoassay for anti-HCV antibodies

Anti-HCV antibodies were detected using the Ortho® HCV 3.0 ELISA Test System (Ortho-Clinical Diagnostics, Inc.). Test samples were added to the antigen-coated wells and incubated at 37°C. Plates were washed and conjugate was added and incubated at 37°C, followed by substrate addition and incubation at 37°C; finally, the reaction was stopped by sulfuric acid addition. A yellow orange color was indicative of the presence of HCV antibodies. Specimens with absorbance greater than or equal to the cutoff value were considered initially positive and were re-tested in duplicate. Anti-HCV positive samples were confirmed by LIA (line immunoassay; Lia Tek HCV III; Organon Teknika). Positive sera were incubated at 20–25°C and washed; conjugate was added and incubated at 20–25°C. Strips were washed and substrate was added and incubated at 20–25°C. The reaction was stopped with sulfuric acid. Results were recorded as positive with a visible color.

Screening of anti-HCV-positive serum samples for HCV-RNA by polymerase chain reaction

Anti-HCV-positive sera were analyzed by using COBAS AMPLICOR HCV ASSAY (Version 2; Roche Diagnostic Systems). Serum was incubated with lysis buffer, and then RNA was precipitated with isopropanol. Each specimen or control

was amplified with biotinylated primers. The unbound material was washed away, and the biotinylated amplicon was detected with an avidin-horseradish peroxidase conjugate-tetramethylbenzidine- H_2O_2 colorimetric reaction. The absorbance of each sample was recorded at 660 nm and compared with a predefined cutoff value to determine positive or negative results for both HCV and internal controls.

HCV-RNA genotyping and subtyping of polymerase chain reaction-positive serum samples

After HCV-RNA screening, positive sera were analyzed for HCV genotypes using a commercially available Line Probe Assay (LiPA; Innogenetics). Amplicons derived from the HCV-RNA screening were used for genotyping assay. Genotyping strips, including negative and positive controls, were placed in the typing tray. Denaturing solution and 10 µL of amplified DNA product were layered over each other and then mixed. Pre-warmed (37°C) hybridization solution was added and then incubated in a shaking water bath at 37°C. After hybridization, the strip was washed and incubated in 2 mL of stringent wash solution in a water bath at 50°C. Conjugate was added and incubated at 25°C, it was washed, substrate was added, and the conjugate was incubated at room temperature. The reaction was stopped by washing the strips in distilled water. Finally, alkaline-phosphatase-labeled streptavidin was added and incubated. A purple/brown line appeared only when there was a perfect match between the probe and the biotinylated polymerase chain reaction (PCR) products.

Determination of HBsAg

Commercially available ELISA kits (Hepanostika HBsAg Uni-Form II Microelisa system; Biomerieux, Boseind 15) were used. Test sera, and negative and positive control sera were added to anti-HBsAg-coated microelisa strips, incubated at 37°C, washed, and blotted dry. Substrate was added and incubated at 18–25°C. The reaction was stopped and the absorbance was recorded at 450 nm. A test serum was considered reactive if the mean absorbance of the test sample was greater than or equal to cutoff value, and nonreactive when the mean absorbance was less than the cutoff value.

Statistical analysis

The data were analyzed and the summary statistic was carried out by a statistical package, SPSS version 11.5. All the biochemical variables were represented as mean and standard deviation (SD), whereas frequencies were mentioned in the form of percentages (%). Student's *t*-test (two-tailed) was used to examine the significance of difference between the two sets of data, and a *p*-value of <0.05 was considered significant.

Results

The patient group was comprised of 23 (32.85%) men and 47 (67.14%) women, whereas the control group consisted of 32 (45.71%) men and 38 (54.28%) women. The mean $\pm$ SD age of our patients was 42.9 ± 15.06 years, with women being slightly higher in age (43.50 ± 14.78 years) as compared with men (38.75 ± 16.71 years). A majority of urticaria patients (65; 92.85%) were Saudi and only five (7.14%) were of other nationalities. A majority of the patients (31.6%) were middle aged (40–50 years) and were married (58; 82.85%). These blood

donors consisted of 6 (12%) women and 44 (88%) men with a mean age of 28.45 ± 7.40 (18–41) years. A majority of them were Saudi (96.0%) and were married (76%). Their clinical examination did not reveal any skin manifestations and none of the controls reported past exposure to any of the HCV risk factors.

In urticaria patient group, 51.42% were exposed to one, whereas 10% were exposed to more than one HCV risk factor. Among these, history of intramuscular injection (21.43%) followed by receiving single or multiple blood transfusion(s) (14.28%) in the past, contact with jaundiced patients (14.28%), contact with HCV-infected family members (7.14%), and sexual contact with an HCV-infected partner (4.28%) and jaundice (2.85%) were the major risk factors to which patients were exposed. None of the control subjects reported exposure to the risk factors discussed previously.

Comparison of liver function marker enzymes between case versus control groups showed that serum levels of ALT were 28.20 ± 17.46 versus 33 ± 12.96 ($p=0.07$), AST were 19.24 ± 6.46 versus 20.96 ± 4.23 ($p=0.06$), albumin were 40.40 ± 9.33 versus 42.62 ± 7.29 ($p=0.11$), and bilirubin were 9.34 ± 6.63 versus 8.68 ± 7.01 ($p=0.56$), indicating that they were within normal reference ranges.

Upon enzyme immunoassay (EIA) screening for HCV infection, 5/70 (7.1%) urticaria patients and none among the controls tested positive for the presence of anti-HCV antibodies. This difference in sero-positivity is significant ($p=0.005$). HBsAg was positive in two patients (2.85%), both of whom were anti-HCV negative.

Noticeably, 19/65 (29.23%) anti-HCV-negative patients reported exposure to single or multiple risk factors, whereas all the anti-HCV-positive patients (5/5, i.e., 100%) reported exposure to more than one of the HCV risk factors (Table 1).

No significant variation of ALT ($p=0.68$), AST ($p=0.59$), albumin ($p=0.45$), and bilirubin ($p=0.68$) levels between anti-HCV-positive and -negative patients was seen (Table 2). Three out of five anti-HCV-positive patients were positive for the presence of HCV-RNA. Further analysis showed that those HCV genotypes were of types 3 and 4. Majority of these genotypes belonged to type 4, subtypes 4a (1) and 4c (1), whereas genotype 3 was of subtype 3a (1) (Table 3).

Discussion

Many extrahepatic diseases, dermatologic in particular, have been described during the course of acute and chronic HCV infection (Alfredo Rebora, 2010). Identification of the risk factors for HCV infection plays a vital role in determining the source and mode of viral transmission (Flamm *et al.*, 1998;

TABLE 2. BIOCHEMICAL VALUES OF LIVER FUNCTION ENZYMES BETWEEN ANTI-HEPATITIS C VIRUS-POSITIVE AND -NEGATIVE URTICARIA PATIENTS

Biochemical parameters and their reference ranges	Anti-HCV positive	Anti-HCV negative
ALT (20–65 U/L)	27.1 ± 9.46	29.3 ± 11.56
AST (20–65 U/L)	20.16 ± 5.67	18.32 ± 7.3
Albumin (30–50 g/L)	38.68 ± 8.66	42.19 ± 10
Bilirubin (3–1 μ M)	10.83 ± 6.73	9.68 ± 5.98

All the biochemical values are represented as mean and standard deviations.

ALT, alanine transaminase; AST, aspartate transaminase.

Hadziyannis *et al.*, 1998). In the present study, a majority of the urticaria patients reported a history of intramuscular injection, followed by blood transfusion(s) as a major risk factor for HCV infection. Although the incidence of transfusion-associated hepatitis C infection has declined significantly since the development of screening tests, and the introduction of disposable syringes and other medical devices, this change has had little impact on overall disease incidence. Whatever the route of transmission, acute hepatitis develops after a 4- to 12-week incubation and most of the cases remain asymptomatic and are diagnosed while being tested for unrelated purposes. In developed countries, prevalence rates of sero-positivity are generally <3% whereas they may be as high as 10–30% in certain endemic areas, for example, in Egypt (El Sayed *et al.*, 1996). In the present study, 5/70 (7.14%) of the patients and 1/70 (1.42%) of controls were sero-positive for HCV. Earlier studies in Saudi Arabia reported the seroprevalence of HCV among the healthy population ranging from 0.2% to 1.7% for adults and 0.01% to 0.1% for children (Fakeeh and Zaki, 1999; El-hazmi, 2004; Madani, 2007).

The relationship between chronic HCV infection and urticaria is still debatable, as there have been conflicting reports both in favor of and against an HCV role in causing urticaria. Our results are in similar line with Malik *et al.* (2008), Siddique *et al.* (2004), Ahmed *et al.* (2003), Kanazawa *et al.* (1996), and Reichel and Mauro (1990), who have mentioned the positive relationship between HCV and urticaria but in contradiction with Raslan *et al.* (2009), Cribier *et al.* (1999), Llanos *et al.* (1998), and Smith *et al.* (1997), who reported the weaker or lack of association between HCV infection and urticaria. Specific pathological details are unclear, but immunological mechanisms arising from the viral-dependent proliferation of

TABLE 1. PREVALENCE OF RISK FACTORS AMONG ANTI-HEPATITIS C VIRUS-POSITIVE AND -NEGATIVE PATIENTS

Risk factor	Anti-HCV positive	Anti-HCV negative
Jaundice	0	2 (3.07%)
Contact with jaundiced patient	1 (20%)	9 (13.84%)
Blood transfusion	3 (60%)	7 (10.76%)
History of intramuscular injection	5 (100%)	10 (15.38%)
Sexual contact with HCV patient	1 (20%)	1 (1.53%)
Contact with family member with HCV	0	5 (7.69%)

HCV, hepatitis C virus.

TABLE 3. HCV-RNA-PCR SCREENING AND GENOTYPING RESULTS OF ANTI-HEPATITIS C VIRUS-POSITIVE URTICARIA PATIENTS

Anti-HCV positive	5/70 (7.14%)
HCV-RNA-PCR screening	
HCV-RNA-PCR positive	3/5 (60%)
HCV-RNA-PCR negative	2/5 (40%)
HCV genotypes	
Genotype 4	2/3 (66.6%)
Subtype 4a	1/2 (50%)
Subtype 4c	1/2 (50%)
Genotype 3	1/3 (33.33%)
Subtype 3a	1/1 (100%)

PCR, polymerase chain reaction.

monoclonal and polyclonal lymphocytes and extra hepatic tissue invasion by replicating HCV are currently postulated in the etiopathogenesis of extrahepatic and cutaneous findings (Hadziyannis, 1998; Bonkovsky *et al.*, 2001).

Based on the nucleotide sequence, HCV is categorized into six major genotypes and many more subtypes. In the present study, three out of five anti-HCV-positive urticaria patients were positive for the genotypes 3 (subtype a) and 4 (subtypes a and c). Presence of HCV-RNA in urticaria patients indicates the active replication of the virus. Although the specific influence of different genotypes on the long-term prognosis of HCV infection is still unclear, it is believed that patients infected with genotypes 2 or 3 are much more likely to respond to interferon therapy, whereas those infected with genotype 1 (particularly 1b) or 4 respond poorly. Kanazawa *et al.* (1996) have reported that anti-HCV was detected in 19 (24%) of 79 patients with urticaria, with genotypes of HCV II/1b in 12 (71%), III/2a in 4 (24%), and IV/2b in 1 (6%) of them. Liver function test results in the HCV-positive and -negative patients and that of controls were within the reference ranges. In addition, no specific differences in liver function enzymes were noted between the different HCV genotypes of urticaria patients, indicating the normal liver function.

In conclusion, increased sero-positivity rate of HCV infection among urticaria patients indicates a significant association with disease pathology. In addition, no significant variation of LFT markers was seen between patient and control groups based on their HCV status. This is the first report from Saudi Arabia, which describes the genotypes of HCV in urticaria patients. "Since the present study is cross-sectional in nature, further longitudinal studies involving a larger population are warranted to find out the association between HCV genotypes and urticaria."

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Author Disclosure Statement

No competing financial interests exist.

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