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Association of genotypes with viral load and biochemical markers in HCV-infected Sindhi patients

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ABSTRACT

The presented study had two objectives. The first was to examine distributions of Hepatitis C Virus (HCV) genotypes in Sindh, Pakistan, where HCV is prevalent. The other was to explore clinically relevant relationships between the genotypes, viral load (measured by real-time polymerase chain reaction assays) and biochemical markers. For this, 1471 HCV-infected patients in six cities in Sindh were recruited and sampled. HCV genotype distributions varied among the cities, but genotype 3a was most prevalent, followed by 3b, 1a and 1b (detected in 51.5, 22.7. 9.25 and 3.2% of the cases, respectively). No type-specific sequences were detected in serum samples from 189 (12.8%) of the 1471 patients. Frequencies of low (<200,000 IU/mL serum), intermediate (200,000–600,000 IU/mL serum) and high (>600,000 IU/mL serum) viral loads were respectively 45.4, 16.5 and 38.1% for patients infected with genotype 3, and 16.9, 36.9 and 46.2%, respectively, for patients with other genotypes. Infection with genotype 1a was associated with significantly higher ($p < 0.005$) alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase titers than infection with genotype 3a. The results will help in the formulation of treatment strategies.

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Introduction

Infection by the hepatitis C virus (HVC) is a serious health issue,[1] with an estimated global of prevalence of 2.35% in 2010, increasing to 2.8% in 2013, and hence >185 million infections.[2–4] The mortality rate is also very high; more than 350,000 individuals die annually from life-threatening complications, which include cirrhosis.[5] Countries with the highest reported rates of HCV infection are in East Asia, Africa, North America and Thailand.[4,6,7] Pakistan has the second highest infection rate in the world, 4.5–8%.[8]

HCV is a single-stranded linear RNA virus with a high mutation rate; an estimated frequency of 10^{-2} mutations per nucleotide per year.[9] Its genome is approximately 9.6 kb

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long, and consists of a single open reading frame encoding a polypeptide chain of 3000 amino acids.[10] The number of recognized genotypes has recently increased from six to seven, and numbers of recognized subtypes has greatly expanded to 67.[11]

Genotypes 1, 2, and 3 are most prevalent globally,[12] while other genotypes are limited to specific regions. HCV genotyping provides information about variability in the viral genome, likely disease progression and possible treatment strategies.[13] The most common genotypes, collectively accounting for approximately 80% of the infections, in the Pakistani population are reportedly 3a, followed by 3b and 1a.[14]

Early diagnosis and treatment of HCV infection minimize risks of both long-term complications like fibrosis and cirrhosis developing, and transmission of infection in the community.[15] Further, various biochemical and molecular markers are now available that can be used in screening for hepatitis C infection, and for both diagnosis and monitoring chronic HCV infection. To determine viremia a number of methods are available, but quantitative polymerase chain reaction (PCR) and branched-chain DNA (bDNA) assays are most widely used. Determination of viral loads is useful for monitoring both treatment responses and relapse rates.[14] In chronically infected individuals, both viral load and HCV genotype may have clinical relevance, as virus loads at the time of diagnosis are correlated to required treatment durations.[9] The HCV mortality rate is growing in Pakistan due to deficiencies in both diagnosis and treatment.[8]

In HCV infection, the clinical findings, quantification of virus and genotype determination are strong predictors for the antiviral therapy. Different studies have been conducted exploring the clinical significance and relationship between viral load and genotype.[16] It is essential that the method used to quantitate viral load be unaffected by the variability of HCV.[9]

The present work has been conducted to document the prevalence of HCV genotypes and their association with viremia and biochemical markers in patients from different cities of Sindh province. Diagnosis, prognosis, interferon therapy, and clinical management of HCV are very poor due to the lack of health facilities in developing countries.[17] Different studies are available, where the relationship between HCV genotypes, viral load and different biochemical markers has been explored in Pakistani population.[8,18,19]

Diagnosis, prognosis, therapy, and clinical management of HCV are very poor due to the lack of health facilities in developing countries.[17] Accordingly, the HCV mortality rate is growing in Pakistan due to shortcomings in both diagnosis and treatment,[8] and it is important to improve the genotyping and quantification of viral loads, which are strong indicators of optimal therapies.[16] Clearly, the method used to quantify viral load must not be affected by the variability of HCV.[9] Further knowledge is also required of the distributions of genotypes in the country, and their relationships with viral loads and clinically relevant biochemical markers. Several studies have explored distributions of genotypes and viral loads in the country,[8,18,19] but higher-resolution data are required. Furthermore, as already mentioned numbers of recognized genotypes and subtypes has greatly increased recently, and more biomarkers are now available.

Thus, the study presented here had two objectives. The first was to document distributions of HCV genotypes in patients from six cities in Sindh (the second most heavily populated province of Pakistan). The other was to acquire more detailed knowledge of relationships between the genotypes and both viral loads and biochemical markers.

Materials and methods

Study area and subjects

The study was carried out between July 2010 and July 2013 at Citi Lab and Research Center, Lahore. HCV-infected patients who attended medical centers/sub-centers in six cities of Sindh province (Shikarpur, Nawabshah, Kashmore, Kandiaro, Dadu, Khairpur) were recruited, following international guidelines for human studies (The NuGO, 2007). Criteria for inclusion were: residence in these cities; HCV-positive results from an Enzyme Linked Immunosorbent Assay (ELISA, see below); detection of HCV RNA in serum by reverse-transcription polymerase chain reaction assays; and absence of detectable infection by other viruses, e.g. hepatitis B Virus (HBV), Hepatitis D Virus (HDV) and HIV. Patients were informed about the study and those recruited consented to participate. Their demographic characteristics, including age, marital status and complications, were documented. Serum samples were collected from all of the patients and stored at −80 °C until further analysis.

ELISA test for anti-HCV antibodies

A commercial kit (3rd Generation Micro ELISA HCV Ab, Amgenix, USA), which includes a 96-well antigen-coated plate, was used to detect anti-HCV antibodies. Following the manufacturer's instructions, serum samples were individually added to the wells, followed by horseradish peroxidase labeled with monoclonal antibody. After five washings with phosphate buffered saline (PBS) incubation titers were obtained by measuring absorbance at 450 nm using a RT-6000 microplate reader (Rayto, Germany).

Isolation of HCV RNA, cDNA synthesis and qualitative PCR analysis of HCV

HCV RNA was isolated from each patient's serum using Trizol following instructions of the manufacturer (Invitrogen, USA), except that the centrifugation time was reduced from 15 to 12 min. After adding TRIzol reagent RNA was extracted with chloroform and alcohol.

HCV RNA was then detected by nested reverse-transcription PCR using 5′ UTR primers, as previously described by Idrees et al.[8] Briefly, 10 μL portions of extracted RNA were reverse-transcribed into cDNA, using maloney murine leukemia virus reverse transcriptase (M-MLV RT, 100 units) and 1 μM of downstream primer (Outer anti-sense). The products generated from each sample were subjected to two separate PCR amplifications, one with primer set A and the other with primer mix B, in 20 μL reaction volumes containing 4.0 μL of 5× First Strand Buffer, 1.0 μL, dNTPs (10 mM), 3.0 μL

dH_2O and Taq polymerase (Fermentas Life Sciences, USA). The resulting specific HCV bands were visualized by ethidium bromide following electrophoretic separation on 2% agarose. Primer set A includes primers for genotypes 1a, 1b, 1c, 3a, 3c and 4, while set B includes primers for genotypes 2a, 3c, 3b, 5a, and 6a. The two sets are grouped according to sizes of sequences they amplify, to avoid generating products that would migrate to similar positions during electrophoresis.

Viral load and genotype determination

HCV RNA was quantified by real-time PCR (Bio-Rad Min-iOpticonTM, USA) using a RoboGene® Hepatitis C virus Quantification Kit (AJ Roboscreen GmbH, Leipzig Germany) according to the manufacturer's instructions.

The method described by Ohno et al. was used for the HCV genotyping. Briefly, cDNA was synthesized using 50 ng of HCV RNA and 100 U of M-MLV RTEs with incubation at 37 °C for 50 min.[20] In first-round PCR amplification a 470-bp region from the HCV 5′ UTR and core region was obtained using 2 μL portions of the synthesized DNA solutions. The products were then subjected to two second round PCR amplifications with the sets of primers (A and B) described above. Following amplification and electrophoretic separation, bands were detected by ethidium bromide staining, observed under a UV transilluminator, and HCV genotype-specific PCR bands were identified using a 100-bp DNA ladder (Fermentas, USA).

Biochemical markers

Clinical chemistry kits (Human, Germany) and an automatic biochemistry analyzer (Micro Lab 300, Merck Germany) were used to analyze biochemical markers such as aspartate aminotransferase (AST), alanine transaminase (ALT), γ-glutamyl transferase (γ-GT), alkaline phosphatase (ALP), bilirubin and total protein – in the serum samples.

Statistical analysis

The data were analyzed using the statistical package, SPSS version 18. All presented quantitative data are means ± standard deviations. Means of pairs of groups were compared by Independent Sample *t*-tests, and ANOVA was used to compare means of more than two groups. *p*-values < 0.005 are considered significant.

Results

We recruited 1471 HCV-infected patients (1043 males, 428 females: male to female ratio, 2.4) from six cities of Sindh province, the second most heavily populated province of Pakistan. As shown in Table 1, significant between-gender differences ($p < 0.005$) were detected in prevalence of all genotypes among the patients except 2a ($p = 0.257$), with male to female ratios ranging from 1.72 for genotype 3b to 5.48 for genotype 1a. Overall genotype 3a was most prevalent among the patients, followed by 3b, 1a, 1b and 2a (detected in 51.5, 22.7, 9.2, 3.2 and 0.48% of the samples, respectively). No genotypes were obtained for 12.8% of the patients (Table 1). Genotype 3a was most prevalent in patients from all of the cities (Table 2), but it was 12.9% more prevalent in patients from Dadu (59.7%) than in those from Shikarpur (46.8%), while genotype 3b was ca. 14% more prevalent among patients from Shikarpur (26%) than among patients from Kandiaro (12.2%). HCV genotype 1a was highly prevalent among patients from Kandiaro (19.8%), three times more frequent than among patients from Dadu (6.04%). Genotype 1b was most frequent in Shikarpur (detected in 5.09% of the patients). Genotype 2a was the least prevalent (<1%) in patients from all study areas, and it was not detected in any patients from Dadu and Khairpur. The patients from Dadu had the highest proportion with undetermined genotypes (16.1%). There were significant ($p < 0.005$) variations in prevalence of all genotypes among patients from all six cities according to ANOVA, except for genotype 2a ($p = 0.666$).

Analysis of associations between the patients' ages (in 10-year intervals) and HCV genotypes showed that HCV was most frequent among individuals aged 30–40 years followed by those aged 40–49 and 50–59 years (Table 3).

Genotype 3a was the most prevalent in all age groups. Infection rates of genotypes 3b and 1a were respectively highest among the 40–49 year-olds (35.6%) and 50–59 year-olds (12.3%). Interestingly, genotype distributions in the groups aged 10–19 and >70 years were very similar. Following Ali et al., we categorized viral loads of <200,000, 200,000–600,000 and >600,000 IU/mL as low, intermediate and high, respectively (Table 4), and found a non-significant association ($p = 0.031$) between the load and gender.[21] However, we detected significant between-genotype differences in viral loads, as genotype 3 was associated with low HCV viral loads while the others were associated with intermediate or high loads (Table 4).

Analysis of associations between HCV genotypes and biochemical markers showed that levels of ALT, γ-GT and ALP significantly differed ($p < 0.005$) among patients infected with 1a, 1b, 3a and 3b genotypes, but no significant between-genotype differences in levels of the other biomarkers were detected (Table 5). Genotypes 1a and 3b were respectively associated with the highest and lowest levels of ALT, γ-GT and ALP (Table 5).

Discussion

Sindh is the second most populated province of Pakistan, with a population of 35.2 million people. Distinctive distributions of HCV genotypes have been observed geographically and distribution changes even in different regions of the same community.[5] The HCV prevalence rate varies among the four provinces of Pakistan in the following order: Punjab having 6.7%, Sindh 5%, Baluchistan 1.5%, and 1.1% in Khyber Pakhtunkhwa (KPK).[22] It was reported that genotype 3 was the predominant strain in Pakistan.[7] In Punjab major prevalent genotype was 3a, followed by genotype 1a.[23] We investigated whether there are geographical differences concerning the HCV genotypes frequencies in different cities of Sindh and evaluated their association with viral load and serum biomarkers Sindh is the second most populated province of Pakistan, with a population of 35.2 million people and it has the second highest prevalence of HCV: ca. 5%, compared to 6.7,

Table 1 – Gender wise genotype distribution in HCV infected patients of Sindh ($n = 1471$).

Genotype	Male		Female		p-value	Total		Gender
Subtype	Frequency	Percentage	Frequency	Percentage		Frequency	Percentage	Ratio
Genotype 1	149	14.28	34	7.94	0.001	183	12.44	4.39
1a	115	11.02	21	4.9	0.001	136	9.25	5.48
1b	34	3.26	13	3.04	0.002	47	3.2	2.62
Genotype 2	5	0.48	2	0.47	0.257	7	0.48	2.5
2a	5	0.48	2	0.47	0.257	7	0.48	2.5
Genotype 3	750	71.91	342	79.9	0.001	1092	74.24	2.2
3a	539	51.67	219	51.16	0.001	758	51.53	2.47
3b	211	20.23	123	28.74	0.001	334	22.71	1.72
Undetermined	139	13.33	50	11.69	0.001	189	12.84	2.78
Total	1043		428			1471		

$p < 0.005$ was considered as significant.

Table 2 – Distribution of genotypes of HCV isolates in different cities of Sindh ($n = 1471$).

Genotypes/cities	1a	1b	2a	3a	3b	UN	Total
Shikarpur	38 (9.22)	21 (5.09)	1 (0.24)	193 (46.84)	107 (25.97)	52 (12.63)	412 (28)
Nawabshah	33 (8.29)	12 (3.01)	3 (0.75)	192 (48.24)	101 (25.38)	57 (14.32)	398 (27)
Kashmore	20 (8.10)	06 (2.43)	2.0 (0.80)	142 (57.49)	53 (21.46)	24 (9.71)	247 (16.8)
Kandiaro	26(19.84)	03 (2.29)	01 (0.76)	73 (55.72)	16 (12.21)	12 (9.15)	131 (8.9)
Dadu	9.0 (6.04)	01 (0.67)	0 (0)	89 (59.73)	26 (17.45)	24 (16.1)	149 (10.1)
Khairpur	10 (7.46)	04 (2.98)	0 (0)	69 (51.49)	31 (23.13)	20 (14.93)	134 (9.2)
p-value	0.001	0.001	0.666	0.001	0.001	0.001	

Note: Percentages (%) in parenthesis, UN, undetermined/untypable, $p < 0.005$ was considered as significant.

Table 3 – Age wise HCV genotype/subtype distribution in Sindh ($n = 1471$).

Age groups (years)	1a	1b	2a	3a	3b	UN	Total
10–19	2 (8.0)	0 (0)	0 (0)	17 (68.0)	05 (20.0)	01 (4.0)	25
20–29	11 (7.05)	01 (0.64)	03 (1.92)	76 (48.72)	39 (25)	26 (16.67)	156
30–39	39 (7.54)	28 (5.4)	01 (0.19)	263 (50.87)	122 (23.6)	64 (12.37)	517
40–49	34 (10.53)	11 (3.40)	0 (0)	119 (36.84)	115 (35.60)	44 (13.62)	323
50–59	39 (12.30)	3 (0.95)	02 (0.63)	206 (64.98)	28 (8.83)	39 (12.3)	317
60–69	10 (9.0)	4 (3.60)	01 (0.90)	62 (55.86)	22 (19.81)	12 (10.8)	111
>70	01 (0.93)	0 (0)	0 (0)	15 (68.18)	03 (13.64)	03 (10.02)	22

Note: Percentages (%) in parenthesis, UN, undetermined.

1.5 and 1.1% in Punjab, Baluchistan and Khyber Pakhtunkhwa, respectively.[22] Clear variations in geographical distributions of HCV genotypes have been observed, even within regions of the country.[5] However, genotype 3 is the predominant strain in Pakistan, according to Rasheed et al.,[7] and 3a is the most prevalent in Punjab, followed by 1a, according to Aziz et al.[23] To acquire higher-resolution data and explore associations between genotypes and clinically relevant biomarkers we investigated possible geographical variations in frequencies of HCV genotypes among cities in Sindh, then evaluated their associations with viral loads and a set of serum biomarkers.

We found that genotype 3 accounts for more than 70% of HCV infections in Sindh (at least amongst our patients), and that genotype 3a is most prevalent, followed by 3b, 1b and 2a (accounting for 51.5, 22.7, 9.25, 3.2 and 0.48% of the examined cases, respectively). These findings are consistent with previously reported patterns of HCV variants' distributions in Pakistan,[21,24] although genotype 4a is emerging in the country, according to Mahmood et al.[25] We did not detect genotype 5 or 6 in any of the analyzed samples.

Diverse distributions of HCV variants have been detected across the world, and we found significant variations in populations from the six cities of Sindh. Genotypes 3a and 3b were most frequent in patients from all of the cities, followed by 1a and 1b. Similar patterns have been widely observed in both Pakistan and neighboring countries,[9] including Bangladesh,[26] India,[27] Nepal[28] and Iran.[29] However, the incidence of every genotype varied significantly, although the infection rate of genotype 2a was consistently very low (accounting for <1% of cases). Genotype 3a was ca. 12.9% more frequent in patients from Dadu (59.7%) than in patients from Shikarpur (47%), who had a ca. 14% higher frequency of infection by genotype 3b than patients from Kandiaro (26% and 12.2%, respectively). The prevalence of genotype 1a was highest among patients from Kandiaro (19.84%).

The differences in distributions of HCV genotypes are likely to be due to several factors, including secular changes in HCV

Table 4 – HCV viral load categories and their distribution by gender and genotypes in different cities of Sindh.

	HCV viral load						
	<200,000		200,000–600,000		>600,000		
	IU/mL	(%)	IU/mL	(%)	IU/mL	(%)	*p*-value
Gender							
Males	387	37.1	215	20.61	441	42.28	
Females	173	40.42	105	24.53	150	35.04	0.031
Sindh							
Genotype3	496	45.42	180	16.48	416	38.1	
Others	64	16.89	140	36.94	175	46.17	0.001
Shikarpur							
Genotype3	103	34.33	34	11.33	163	54.33	
Others	16	14.29	43	38.39	53	47.32	0.001
Nawabshah							
Genotype3	157	53.58	47	16.04	89	30.38	
Others	18	17.14	37	35.24	50	47.62	0.001
Kashmore							
Genotype3	90	46.15	43	22.05	62	31.79	
Others	12	23.07	21	40.38	19	36.54	0.004
Kandiaro							
Genotype3	29	23.58	27	30.34	33	37.07	
Others	7	16.66	21	50	14	33.33	0.056
Dadu							
Genotype3	60	52.17	16	13.91	39	33.91	
Others	4	11.76	10	29.41	20	58.82	0.001
Khairpur							
Genotype3	57	57	13	13	30	30	
Others	7	20.59	8	23.53	19	55.88	0.001

Note: Percentages (%), $p < 0.005$ was considered as significant (others = all studied genotypes except 3).

distribution and associated risk factors. In Brazil, significant variations ($p = 0.001$) in frequencies of genotypes among areas have been found in two studies, but both found that genotype 1a was consistently predominant.[30,31] This pattern of distribution is similar to the present study. Age-related analysis of infections revealed that the most common genotype was 3a variants among all age groups as reported by Lazo et al.[12] In agreement with previous reports, the highest HCV prevalence was reported among individuals between 30 and 40 years.[23] Our results contradict with Nafees et al., who reported that older females (41–50 years) had higher HCV prevalence than males. Genotype 3b showed the highest infection rate (35.6%) in age group 40–49 years.[32] It has been reported from China that males have persistent elevated HCV RNA levels and are at higher risk as compared to females.[33] In contrast, we found that that the most common genotype was 3a, among all age groups of our patients from Sindh, as also recorded by Lazo et al.[12] In agreement with Aziz et al., we found that HCV was most prevalent among individuals between 30 and 40 years old, but our results conflict with findings by Nafees et al., that HCV was more prevalent among 41–50 year-old females than among males of this age.[23,32] The most common genotype in this age group (accounting for 35.6% of cases) was 3b. Our results also conflict with findings that infected males have persistently higher HCV RNA levels than infected females, and are at higher risk,[33] as we found no clear or

Table 5 – Association of HCV genotypes with biomarkers in Sindh Province.

Genotype	*n* (%)	ALT	AST	γ-GT	ALP	Bilirubin	T. Protein	Albumin
1a	136 (9.25)	121.3 ± 11.2	91.3 ± 8.6	97.3 ± 8.9	483.2 ± 11.2	2.26 ± 0.71	9.04 ± 0.51	3.26 ± 0.28
1b	47 (3.20)	90.4 ± 8.8	80.2 ± 8.0	89.4 ± 8.7	442.1 ± 10.5	1.98 ± 0.37	8.95 ± 0.61	3.71 ± 0.33
3a	758 (51.53)	69.2 ± 5.7	61.7 ± 4.8	62.1 ± 5.9	390.8 ± 8.5	1.78 ± 0.43	8.61 ± 0.31	3.64 ± 0.41
3b	33.4 (22.71)	87.1 ± 7.6	79.4 ± 6.4	84.3 ± 7.1	425.3 ± 10.6	1.94 ± 0.0.46	9.26 ± 0.0.53	3.12 ± 0.11
p-value	–	0.001	0.0264	0.001	0.001	0.418	0.321	0.145

Normal values: alanine amino transferase (ALT) < 40 U/L, aspartate amino transferase (AST) < 40 U/L, alkaline phosphatase (ALP) < 306 U/L, gamma-glutamyl transferase (γGT) < 40 U/L, Bilirubin < 1 mg/dL, T. Protein: 6–8.5 g/dL, Albumin: 3.4–4.8 g/dL, Significant $p < 0.005$, Insignificant $p > 0.005$.

significant ($p < 0.005$) differences in viral loads between both genders.

However, we detected significant differences in associated viral loads between genotype 3 and the others. The percentage of patients with a low load (<200,000 IU/mL) was highest (45.4%) among those infected with genotype 3, while 36.9% and 46.1% of patients with other genotypes had intermediate (>200,000 IU/mL and <600,000 IU/mL) and high (>600,000 IU/mL) viral loads, respectively. Similarly, Rong et al., reported that genotype 1 (included in other genotypes in this study) was associated with higher HCV RNA levels than genotype 3.[34]

Relationships between the genotypes and serum biomarkers are ambivalent, possibly due to the heterogeneity in HCV genetic variants, according to Al-Swaff.[35] However, Ijaz et al., detected relationships between HCV genotypes and serum levels of both ALT and ALP.[18] Extending these results, we found that genotype 1a was associated with consistently and significantly ($p < 0.005$) higher serum levels of ALT, ALP and γ-GT than genotype 3a.

In conclusion, the most prevalent HCV genotypes in Sindhi patients appear to be 3a followed by 3b, 1a and 1b. However, there are significant differences within the region, for example 3a is more prevalent in patients from Dadu than in those from the other cities. Genotype 3 is associated with the lowest viral loads, and infection by genotype 1a is associated with higher levels of the serum biomarkers. More importantly, the results reveal strong within-region variations in genotype frequencies, associated viral loads and biomarker levels. Hence, they highlight the need for high-resolution analyses of these variables to facilitate formulation of robust therapeutic strategies.

Conflicts of interest

The authors declare no conflicts of interest.

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