THE HEPATITIS C UNTYPE-ABLE GENOTYPES, AN EMERGENCE OF QUASI-SPECIES IN HCV INFECTED PATIENTS

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ABSTRACT

Objective: To determine the frequency of Type-able and untype-able genotypes in hepatitis C infected patients, and to observe their association with gender, age, Alanine Aminotranferease and viral load.
Study Design: Cross-sectional analytical study.
Place and Duration of Study: Department of Microbiology, Combined Military Hospital, and Lahore, Pakistan from Sep 2017 to Mar 2018.
Methodology: Six hundred forty seven anti HCV antibodies positive serum samples by Enzyme Linked Immuno Sorbant Assay were received from a total of 6791 serum samples. The positive sera were subjected to qualitative PCR and quantitative real time (RT) PCR to determine pre-treatment viral load. Quantitative PCR positive sera with viral load >500IU/ml were further subjected to molecular genotyping by using Ohno *et al* method.
Result: Out of 647 positive serum samples, type-specific PCR fragments were seen in 424 sera, while 13 (3.1%) of serum samples were of untype-able genotype. In all age groups genotype 3a had emerged as a predominant genotype 397 (93.6%), followed by 1b 8 (1.9%), 3b 4 (0.9%), 1a 2 (0.5%), while no sample detected to have 2a, 2b, 5a, 6a and mixed genotypes. The highest prevalence of untype-ables were seen in 61-70 age group.
Conclusion: Need of the hour is proper sequencing of untype-able genotypes via upgrading existing methodologies. It will not only help the clinicians in achieving sustained virological response but also help in identifying new genotypes/subtypes.

Keywords: Hepatitis C-virus, Genotypes, Untype-able variants.

INTRODUCTION

Hepatitis C virus (HCV), was discovered in 1989. For long it was thought to be a non-A, non-B virus acquired after blood transfusions. Quickly this discovery led to the development of serological and molecular methods for HCV detection. Its major value was observed in transfusion set ups where Hep C screening dropped the incident of transfusion acquired infection to nil, and add another test in the panel of investigations for clinical hepatitis[1].

One observes varied differences in the HCV nucleotide sequences. These variations has led to the formulation of genotypic classification of HCV which accommodates variants from all over the world and classify them into six main genotypes. Genotype 7, a rare genotype has also been reported. These genotypes are further divided into subtypes. A 30% sequence divergence was observed in various groups of these genotypes resulting in modifications of their antigenic properties and biological behaviour i.e. (type of treatment and response)[2].

A Worldwide distribution has been observed in HCV genotypes like 1a, 2a and 2b, while in South Africa and South East Asia genotype 5a and 6a are common[3]. In USA and countries of Western Europe frequent culprits causing chronic hepatitis are 1a, 1b, 2a, 2b, and 3a genotypes. Moreover, their frequencies may vary geographically as in south and Eastern Europe 1b is frequently diagnosed as infection causing genotype while Genotype 1a and 3a are more commonly seen in drug users. In Pakistan most prevalent Genotype is 3 with 3a and 3b subtypes. The 3a and 3b subtypes are circulating with equal frequency among males and females[3-6]. Furthermore, prevalence of Genotype 4 is high in North Africa and Middle East[3]. Mainstay of proper treatment of HCV in-

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fected patients rely on accurate detection of HCV genotype[7].

Current statistics suggest that HCV is responsible for >200 million infections worldwide[8], and over 10 million confirmed infections in Pakistan[9]. It has listed itself as one of the leading cause of cirrhosis 27%, Hepatocellular carcinoma (HCC) 25%, chronic hepatitis 70%-85% and liver transplantation[10-12]. According to World Health Observatory, the leading cause of deaths Worldwide in year 2013, responsible for 1.46 million deaths was hepatitis C viral infection and in years 2015–2030, 7.2 million deaths were expected[13].

For the prevention and control of viral hepatitis World Health Organization (WHO) for the year 2016–2021, has announced its first Global Health Sector Strategy (GHSS). Through varied steps GHSS would not only try to achieve the chalked out health targets of 2030 Agenda but also try to combat the expected 7.2 million HCV associated deaths during years 2015–2030[14].

High mutation rate of approximately 10^{-3} has been observed in HCV genome. This mutation happens at each replication of a nucleotide[15] which leads to formation of higher number of quasi species (untype-able) in the infected population. This error prone nature of HCV genome resulting in circulating large proportion of HCV variants in affected population is disturbing for clinicians and treating physicians as to what type and duration of therapy be selected for infected patients. Moreover, this fact is gaining popularity that these untype-able variants which we encounter during routine genotype diagnostic test are resistant to treatment[16].

This study was done to determine the frequency of type-able and untype-able HCV genotypes in patients suffering from chronic hepatitis C and to observe the biological behaviour of untype-able genotype in terms of gender, age, Alanine Aminotranferease and viral load.

METHODOLOGY

The study was done in the department of Microbiology, Combined Military Hospital, Lahore, Pakistan from September 2017 to March 2018. CMH Lahore serves as Primary Referral Centre for central and southern Punjab and adjacent areas of Sindh and Baluchistan. We included a total of 424 HCV positive sera in our study which were collected from patients, who were registered in Army Health Management System (HMS) as per their consent and ethical approval of the Institutional committee from September 2017 to March 2018. Patients were selected on the basis of having chronic HCV infection and were seropositive for anti-HCV antibodies by third-generation ELISA (Adaltis, Milano, Italy).

Selection of serum samples was done on non probability consecutive basis for the study after taking proper consent in written form from each patient. Duplicate samples were excluded from the study. The questionnaire included age, sex, rank, name, number, duration of having HCV infection, Liver Function Tests, Ultra sound abdomen report, quantitative estimation of HCV RNA to conclude association along with complete address and phone numbers.

Sera which were positive for HCV RNA were quantified as per manufacturer's instructions by using Real Time PCR System with real time specific software using HCV-RNA quantification kits (Robogene, AJ Roboscreen GmbH, Germany). The Real Time System undergoes amplification, identification and quantification at the same instant using fluorescent probes (HCV RNA: FAM, IC RNA: VIC/JOE) and results could be visualised on computer screen. It has a very low detection limit of 70 IU/mL. Only those sera having a viral load of >500 IU/ml were further selected for molecular genotyping by using Ohno *et al* method[17].

All the 424 HCV positive sera (qualitatively and quantitatively) were genotyped by the Ohno et al method, as described previously[17]. In Ohno *et al*, method for genotyping, two different primer mixtures were used. Mix-A had a set of primers for the detection of genotypes 2a, 1b, 2b and 3b genotypes and mix-B had a primer set to detect genotypes 1a, 3a, 4, 5a and 6a. Amplification was

carried out in two rounds. The amplified product of first round of PCR was then subjected to second round nested PCRs for each sample. An 08 µl of the second-round PCR product was electrophoresed on a 2% agarose gel, stained with Ethidium bromide, evaluated under UV light and compared with 1000 bp ladder. Samples with known genotypes were run with each round of nested PCR as controls.

The expected sizes of the genotype-specific bands amplified by PCR typing were as follows:

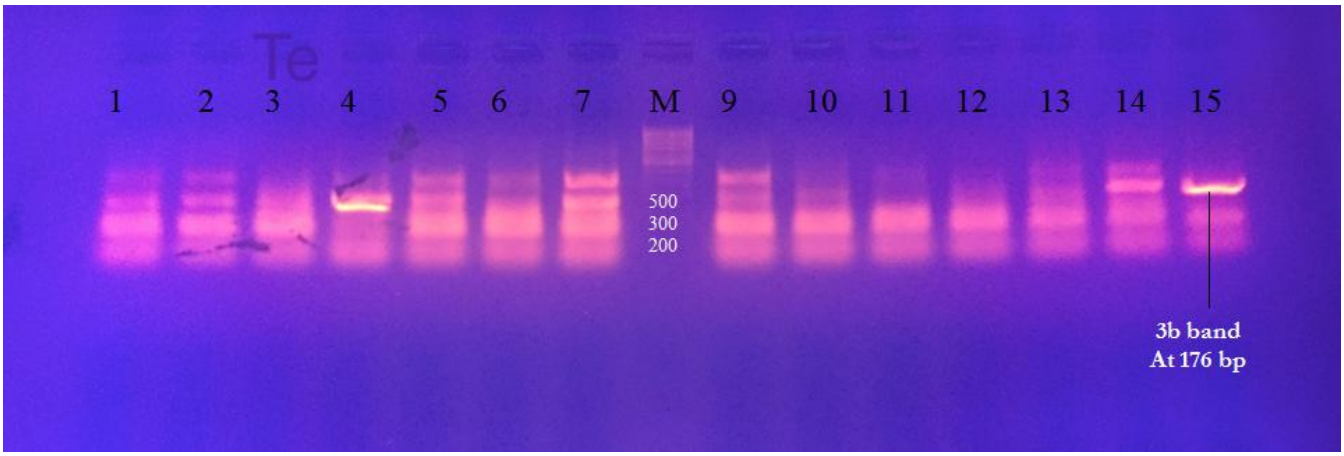

Figure-1: Agarose Gel electrophoresis (2%) showing patterns of PCR products from different HCV specimens by multiplex PCR[17]. Lane 1 shows negative control, lanes 2,3,5,6,7,9,10,11,12,13,14, showing presence of untype-able genotypes by showing no specific bands. lanes 4 and 15 showing bands of genotype 3b. Lane M shows 50-bp DNA ladder marker.

genotype 1a, 208 bp in size; genotype 1b, 234 bp; genotype 2a, 139 bpand 190 bp. Expected sizes of the genotype 2b, 3a, 3b, 4, 5a and 6a specific bands were 337 bp, 232 bp, 176 bp, 99 bp, 320 bp; and 336 bp.

SPSS version 21.0 for Windows was used for the analysis of data and statistics. The results for all variables were entered in the form of rates (%). T-test and chi-square tests were applied to find out associations used. Log- Likelihood Ratio test (LR) and Cramer's V statistics were applied to find out the significance of association in terms of *p*-value amongst untype-ables and the categorical variables. The *p*-value of <0.05 was considered significant.

RESULTS

From a total of 6791 serum samples, 647 samples were positive while 6144 were found negative for anti-HCV antibodies by using 3rd generation ELISA. After confirmation through qualitative PCR when these 647 serum samples were subjected to quantitative PCR, only 424 samples showed viral titres of >500 IU/ml whereas, 223 samples either showed a titre of <5001 U/ml or no titre at all (detection limit 70 IU/mL). They were excluded from the study and rest of the samples were further tested for specific genotype bands. Out of these 424 serum samples specific genotype bands were seen in 411 serum samples whereas, 13 samples (3.1%) were found to have untype-able genotypes as a specific genotype band was absent in these samples (fig-1). Furthermore, in these 411 sera genotype 3a emerged as a predominant genotype (93.6%), followed by 1b

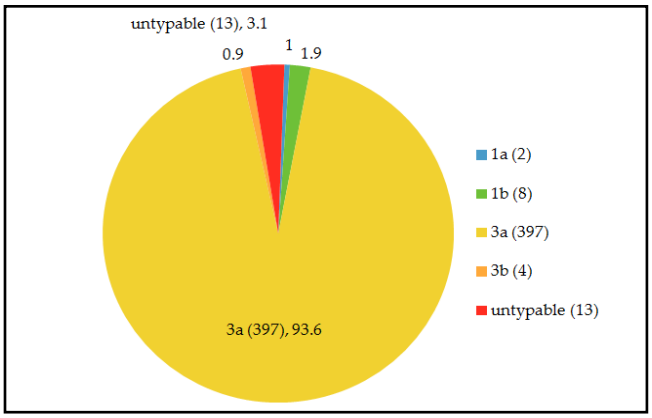

Figure-2: Frequency of various HCV genotypes n=424.

(1.9%), 3b (0.9%), 1a (0.5%) respectively, while no sample was detected to have 2a, 2b, 6a and mixed genotype. Genotype 7 was out of scope of available PCR method (fig-2).

Table-I: Percentages of HCV genotypes in different age groups of HCV patients (n=424).

Age Group		Genotypes				
		1a	1b	3a	3b	Un-type
21-30	count	1	-	33	1	1
%age within Age group		2.8	-	91.7	2.8	2.8
%age within Genotype		50	-	83	25	7.7
31-40	count	-	-	53	-	3
%age within Age group		-	-	94.6	-	5.4
%age within Genotype		-	-	13.4	-	23.1
41-50	count	-	-	83	-	-
% age within Age group		-	-	100	-	-
% age within Genotype		-	-	20.9	-	-
51-60	count	-	6	127	1	3
% age within Age group		-	4.4	92.7	0.7	2.2
% age within Genotype		-	75	32	25	23.1
61-70	count	1	2	69	1	5
% age within Age group		1.3	2.6	88.5	1.3	6.4
% age within Genotype		50	25	17.4	25	38.5
71-80	count	-	-	25	1	1
% age within Age group		-	-	92.6	3.7	3.7
% age within Genotype		-	-	6.3	25	7.7
81-90	count	-	-	7	-	-
% age within Age group		-	-	100	-	-
% age within Genotype		-	-	1.8	-	-
91-100	count	2	8	397	4	13
% age within Age group		0.5	19	94	0.9	3.1
% age within Genotype		100	100	10	100	100

Table-I shows cross tabulation of HCV genotypes among studied patients with gender. Out of 424 HCV patients, there were 217 (51.2%) females and 207 (48.8%) were males. Mean age with standard error of mean was (51.64 ± 0.675), and age range was 20-92 years. Distribution of genotypes was equal between both the sexes. Moreover, no significant association had been seen of any genotype with gender, as all the *p*-values were greater than 0.05.

Table-II: Gender wise distribution of HCV genotypes among the studied patients n=424.

		Genotypes					
		1a	1b	3a	3b	Un-type	Total
Gender							
Female	count	1	3	203	2	8	217
% age with Gender		0.5	1.4	93.5	0.9	3.7	100
% age with Genotype		50	37.5	51.1	50	61.5	51.2
Male	count	1	5	194	2	5	207
% age with Gender		0.5	2.4	93.7	1	2.4	100
% with Genotype		50	62.5	48.9	50	38.5	48.8
Total	count	2	8	397	4	13	424
% age Prevalence		0.5	1.9	93.6	0.9	3.1	100
p-value		0.973	0.435	0.942	0.962	0.448	100

Table-III: Summary of statistical tests / strength of association among all genotypes vs. gender, ALT, viral titres and age group in n=424 HCV suspected patients.

	Method			
	Likelihood Ratio		Strength of Association	
	L-R statistics	*p*-value	Cramer's V	*p*-value
Genotype* Gender	11.175 (10)	0.344	0.355	*0.001
Genotype* Age group	44.106 (40)	0.302		*0.001
Genotype* ALT	159.922 (545)	NS	0.611	*0.001
Genotype* Quantitative PCR	302.272 (2040)	NS	0.989	0.195

**0.001 shows highly significant result, NS; Not Significant, Test statistics along with degree of freedom are mentioned in brackets.*

In this table, a comparison had been shown of percentages of a given genotype within an age group with that of another age group. For example, the percentages of the untype-able genotype within age-group for age categories 21-30, 31-40, 41-50, 51-60, 61-70, 71-80 and 80 were 2.8%, 5.4%, 0%, 2.2%, 6.4%, 3.7% and 0%. Highest per-centage of untype-able genotype and prevalence within genotypes 38.5% was seen in 61-70 age-group. Data had shown reduced number of untype-able genotypes in extreme of ages. Whereas, the highest prevalence of genotype 3a is seen in 41-50 age category (100%) with highest prevalence within genotypes was in age category 21-30.

Table-III shows an effort that had been done to calculate strength of association among all the genotypes vs. gender, ALT, viral titres through quantitative PCR and different age groups. Significant association was seen between age groups, genders and ALT and genotypes as *p*-value observed was <0.001. Same tests when applied on untype-ables with ALT, quantitative PCR, age groups and gender did not give any results due to small sample size.

So in order to observe biological behaviour of untype-ables we had classified Pretreatment viral titre into categories as high (>80, 00000 IU/ml) and low (<60, 0000 IU/ml) for untype-able genotypes. HCV viral titre was seen high (>80, 00000 IU/ml) in 8/13 cases while rest of the 5/13 cases had shown low viral titres between 1000-8000 IU/ml, so untypability could be attributed to both, low viral titres and high rate of mutations. Untypability was confirmed after running the HCV serum specimens in both the mixes. Serum specimens having Genotype 3b were used as positive control.

DISCUSSION

Many reports published in recent 4 years from within the country and abroad clearly indicates towards an upward trend of untype-able genotypes in HCV seropositive. Samples[18-24]. According to a recent review article published by Abdul-waheed *et al*, concluding results of 4534 HCV patient samples during the years 2008-2016 showed that 11.51% samples were untype-able stressing upon the fact that this issue should be resolved on priority basis[19].

Interestingly, our current study indicates a small chunk of untype-able HCV subtypes i.e,. (3.1%) as compared to Waheed *et al* where a considerable number (11.51%) of samples fall in this category suggesting not only a well controlled drug regulation system in Army but also raises the question as what future we are heading towards if we will not ensure compliance[19].

These untype-able subtypes of HCV genome pose complications both diagnostically and therapeutically[15,19,21].

Question arises as how these HCV variants produce in a population. According to literature every individual carries a slightly different variant of its own genotype in blood. As time passes these variants due to pressures from host immune system[3,11,15], persistent infection[4,12], treatment with Interferone, ribavarin in non-responders [3,7,15,16,21], develop greater genetic diversity. These variants/quasi species continue to evolve in an individual. Besides creating confusion for clinician as of what duration and type of treatment to be advised to these patients the brighter side of these quasi species is that their molecular analysis can be used to prove linkages of infections from mother to infant, in concordant sexual couples, to provide a clue for nosocomial transmission of infection between a health care provider and patient and between a needle stick injury recipient to the source of infection[15,16].

In developed countries, sequencing is performed in routine for the determination of underlying subtype in untype-able case[17,19]. However, in Army setups and in Pakistan, both the treatment and its duration are ascertained in term of viral titres of HCV (qualitative and quantitative detection) and type-able genotypes, which is a standard way of estimation of therapy duration. No sequence determination for underlying subtype in untype-able cases has yet been explored[3,7]. Furthermore, with emergence of these untype-able variants, requirement is generated for an

updated genotyping assay, based on data of indigenous viral sequences, which will have the capacity to accommodate these outliers or untypeables. These quasi species are beyond the scope of our genotypic methods and are contributing towards misdiagnosis and mistreatment resulting in non-responsiveness to the treatment regimen of interferon or direct acting antivirals (DAA)[16, 19-21].

Is this untypability is because of the sequencing method? as there are various methods in vogue for HCV genotyping e.g type-specific PCR, line probe hybridization assay (LiPA), genome sequencing, and restriction fragment length polymorphism (RFLP)[19,25]. In Pakistan two methods Ohno *et al*, method and Idrees *et al* method are generally practiced[2,17]. At our centre type specific PCR method devised by Ohno *et al* is used to detect six types and subtypes of HCV genotypes (1997)[17]. Method proposed by Idrees (2008) has more specificity as it is developed in the light of local sequences, excluding foreign sequences which could be one of the reason for untype-ables in our study. However various studies conducted in Pakistan, used Idrees *et al*, method had reported presence of untype-ables as well, further suggesting lack of complete sequencing ability of this method too[18,20,21].

In our study a correlation was tried to calculate between genotypes and gender. It was concluded that there were no variations in distribution of HCV genotypes amongst male 207 (48.8%) and females (217, 51.2%) and was equally distributed between both the sexes with no significant difference (p>0.05). This evidence is supported by other studies as well where there was no variation found in distribution[4,8,19].

The results of the present study indicate that highest % prevalence of untype-ables was seen between 61-70 age group with percentage prevalence of untype-able genotype of 38.5%. It has already been reported that HCV genotype distribution varies with age in both male and female patients[19,21,25]. This could be seen in our study as well with most of the untype-ables seen in older age group.

Further analysis regarding age groups, ALT and Gender with all genotypes, shows significant association (p<0.001) through Cramer's v-test (table-III) suggesting that HCV irrespective of genotype invariably causes hepatic damage, its % prevalence varies with age and has an equal distribution among both the sexes .

Mainstay of HCV standard treatment is to achieve sustained viral response. DAA/interferon therapy is very costly and has varied results against different genotypes[20]. Although, outcome of DAA therapy is very promising with very high SVR but the major hurdle is high cost[3,7,15,20]. In Pakistan, the allocated budget for health is already pretty low (just 2.7% of GDP)[3,22] and without knowledge of causative genotype, it is difficult for clinicians to start the therapy.

CONCLUSIONS

Untype-able genotypes do exist in our stream of patients and showed significant association with ALT levels (p-value 0.001). Hereby, stressing the fact that untype-able genotypes do have new genotype/subtypes and should be properly sequenced to help clinicians to attain sustained viral response. By exploring the phenomenon of untypability one would be able to tackle the forthcoming giant of HCV therapy resistance. This surge of untype-ables is an eye opener and promulgates the need of a well developed surveillance system.

CONFLICT OF INTEREST

This study has no conflict of interest to be declared by any author.

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