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Assessment of Occurrence of Hepatitis B Virus Genotypes among Population in Provisional Capital and Federal Capital of Pakistan

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Authors' contributions

This work was carried out in collaboration between all authors. Author JI designed the study, supervised and analysed all the experiments, corrected the first draft of the manuscript, managed literature searches, performed the experiments and wrote the first draft of the manuscript. Authors AR, ID and JZKK managed the analyses of the study and literature searches. All authors read and approved the manuscript.

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ABSTRACT

Aim: This study was aimed to figure out the prevalence of HBV genotypes among population in Pakistan. The areas which were not explored previously were studied in this research with special emphasis on finding out the new strains and sub genotypes of HBV. The genotypes were further confirmed by DNA sequencing. This study will help future researcher to study some other aspects of HBV related strains in Pakistan and also about its medical therapies.
Study Design: The Hepatitis B surface antigen (HBsAg) and HBV DNA positive samples were collected in the first step and sent to Nuclear Medicine Oncology and Radiotherapy Institute [NORI], for further analysis.

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Place and Duration of Study: Polymerase Chain Reaction (PCR) lab, Nuclear Medicine, Oncology and Radiotherapy Institute [NORI], Islamabad, between September 2012 to February 2014.
Methodology: A total of 450 Hepatitis B surface antigen HBsAg and HBV DNA positive samples including 100 from southern Punjab, 150 from Khyber pakhtoonkhwa (KPK), 100 from Islamabad and 100 from Quetta region were collected during the period of September 2012 to February 2014 and genotyped by type specific nested PCR primer pair method for 8 HBV genotypes from A through H. From the total 450 HBsAg positive samples, 41 were excluded from the study as they were either found to be negative for HBV DNA on or they had less than 100 IU/ml of viral load. So, out of the total 409 selected samples, 283 were of male gender and 126 of female with mean age of 35 years, ranging from 12 to 65 years. The patients were randomly selected irrespective of their age and gender and a written consent (parental consent in case of less than 18 years of age) was obtained. The study was approved by ethical review committee.
Results: Our results showed Genotype D as the most prevalent and dominant genotype in all regions studied. Mix HBV genotype infections of genotype A with B & C, D with B and C constitute about 17 percent of all the samples. A mixture of genotype A+D was detected from the majority of the samples among mixed genotype group (62/69) which was followed by C+D (3/69). Genotype B and C are not common to be detected and were also found in smaller proportions. Positive PCR results were repeated twice for confirmation.
Conclusion: Genotype D was identified and come out to be the most dominant genotype in Pakistani community showing sub-genotypes of D1 & D3 which is about 70.0% of the total sample size in our study. Genotype A and D are present as co-infection with each other and contributed as the second prevailing genotypic group.

Keywords: Hepatocellular carcinoma; hepatitis B virus; open reading frame; polymerase chain reaction; real time detection PCR; restriction fragment length polymorphism.

1. INTRODUCTION

Hepatitis B virus [HBV] is one of those agents causing acute and chronic infection in human beings, relics a major global health issue throughout the world. More than 400 million people out of which 350 are chronically infected by this disease shows high risk of liver cirrhosis [1]. The HBV infection rate has decreased significantly in developed countries [2] like United States of America where the acute HBV infection rate has decreased by 78% approximately from 1992 to 2005 [3]. However, the decline in the infection rate of HBV has not been observed in Asian countries like Pakistan although the vaccines and antiviral drugs are used constantly during HBV therapy. Moreover, the low economic condition of Pakistan imposes a considerable burden on poor people purchasing costly antiviral drugs and vaccines beside its threat to health and life [4].

HBV genotype shows a prominent geographical distribution pattern in their occurrence. Nine well known HBV genotypes have been identified on the basis of HBV DNA divergence in the genome, named as genotype A, genotype B, genotype C, genotype D [5], genotype E [6-7], genotype G [8] and genotype H [9] with specific distribution along the globe [10] which are common in the world while genotype I [11-12] and genotype J [13] are also identified but their status and geographical distribution are not well known.

HBV infection epidemiology reflects a complicated network of interrelated factors leading to acute and chronic liver ailment. Susceptibility of HBV infection is attributed to some extend to variation and modifications within human genome. Several viral factors persuade the outcomes of infection included are viral load, viral mutations, HBeAg seroconversion and response to antiviral therapy, and HBV genotype [14-18]. In one study, they showed miR-15b, an important miRNA during HBV infection and hepatocellular carcinoma development, directly binds hepatocyte nuclear factor 1 (HNF1) mRNA, a negative regulator of HBV Enhancer I, to attenuate HNF1 expression and resulting in transactivation of HBV Enhancer I. This process leads to the enhancement of HBV replication and expression of HBV antigens, including HBx protein, finally leading to the down-regulated expression of miR-15b in both cell lines and mice in a long cascade of events [19].

Various studies have been conducted on HBV genotypic distribution in Pakistan but still there are many areas in northern and southern part of Pakistan, specifically the areas in Baluchistan and in Khyber Pakhtunkhwa (KPK) are

unexplored so far. Genotype D is reported to be the most prevailing and dominant in about all the areas studied so far. Genotype A is the second most abundant with genotype D combination. Genotype B and C are also reported from some areas along with a considerable rate of mixed HBV infections, especially those with genotype A and D [20-22]. We elected samples randomly from provisional cities (Lahore, Peshawar, and Quetta) and capital city (Islamabad) which is more developed and people from rural areas visit frequently for their treatment purpose, so it gives us a better chance to carry research on HBV genotyping.

In this study, an efficient, more precise and more elaborated Real Time PCR based method was used to find out HBV genotyping with differentiation through A to G but A, D are most common in Pakistani population. Various responses have been observed by patients during antiviral therapy to medication and most important role played by the type of genotype they have. So it becomes very important to explore them and to know the role of genotype in this remedy. Up till now, all studies carried out did not consider this fact so it was our basic aim to consider in our study.

2. MATERIALS AND METHODS

2.1 Sample Collection and Selection

The samples for genotyping purpose were collected from provisional capital and federal capital of Pakistan where most people from rural as well as urban cities come for better treatment. The provisional capital and federal capital hospitals are equipped with latest techniques and highly qualified medical staff. So it gives us a better opportunity to study different genotypes dogging in people of Pakistan coming from different localities. A total of 450 HBsAg positive samples, including 100 Islamabad regions, 150 from Peshawar city, 100 from Lahore region, and 100 from Quetta city were collected during the period of September 2012 to February 2014. The patients were randomly selected irrespective of their age and gender and a written consent (parental consent in case of less than 18 years of age) was obtained. The study was approved by the ethical review committee (PC-44000). The samples were brought to Nuclear Medicine, Oncology and Radiotherapy Institute [NORI], Islamabad and tested for positivity and quantification of HBV DNA. The samples having more than 100 IU/ml of viral load were only selected and subjected to HBV genotyping. From the total 450 HBsAg positive samples, 41 were excluded from the study as they were either found to be negative for HBV DNA on or they had less than 100 IU/ml of viral load. Out of the total 409 selected samples, 283 were of male gender and 126 of female with mean age of 35 years, ranging from 12 to 65 years. The sample selection and gender information are shown in Table 1.

2.2 HBV DNA Extraction and Quantification

DNA was extracted from 200 µl of plasma using AJ ROBOSCREEN INSTANT virus DNA kit (GmbH, Germany) according to the manufacturers' protocol. Viral load quantification was done by ROBOGENE HBV DNA Quantification kit (AJ Roboscreen, analytikajena Biosolutions, GmbH, Germany) using Rotor-Gene™3000, Corbett Research, Germany.

2.3 HBV Genotyping

The genotyping was performed using genotype specific PCR primer method described by Naito et al. 2001 [23]. The PCR reaction was divided into two rounds, a regular and a nested. Both PCR rounds were performed. In first round, 10 µl of GoTaq Green Master mix (Promega, USA) was used with 1 µl of each universal primer P1-A and S1-2, 3 µl of ddH_2O and 2 µl of extracted DNA. The cycling profile used was: First incubation (HOLD 1) at 95℃ for 10 minutes, then 40 cycles of 94℃ for 20 seconds, 55℃ for 20 seconds and 72℃ for 60 seconds then extension (HOLD 2) at 72℃ for 7 minutes and 4℃ (HOLD 3). For nested PCR round, two mixtures were prepared. In mixture 1, 1 µl of each primer for genotypes A, B and C was included while 1 µl of each primer for genotypes D, E and F was included in mixture 2 [23] along with 8 µl of GoTaq Green Master mix (Promega, USA), 8 µl of ddH_2O and 2 µl of amplified product from 1st round of PCR. The mixtures were then subjected to thermal cycling with profile: incubation (HOLD 1) at 95℃ for 10 minutes, then 40 cycles of 94℃ for 45 seconds, 63℃ for 20 seconds and 72℃ for 60 seconds and then extension (HOLD 3) at 72℃ for 7 minutes and at 4℃ for 1 minute (HOLD 3). (RotorGene Q, Qiagen-Germany). Products of nested PCR round were subjected to electrophoresis on ethidium bromide stained 2% w/v xTBE agarose gel along with 50 bp DNA ladder (GeneRuler^{TM}, Fermentas) and analyzed in gel documentation system (BIORAD Gel Doc-XR, USA). The 1st round PCR was used to amplify the HBV DNA

while in 2nd round PCR, the genotype was determined by specific primers. The 1st round PCR primers pairs (outer primers) and 2nd round PCR primers pairs (inner primers) were premeditated based on conserved nature of nucleotide sequences in the pre-SI region through S genes. The primers combination for 2nd round PCR was designed based on the differences in product size. The genotypes were identified according to their product sizes.

3. RESULTS

From the total 450 HBsAg positive samples, 41 samples were excluded from the study as they were either found negative for HBV DNA or they had less than 100 IU/ml of viral load. Out of the total 409 selected samples, 283 were of male gender and 126 of female with mean age of 35 years, ranging from 12 to 65 years. The sample selection and gender information are shown in Table 1.

Table 2 shows the percentage distribution of different genotypes in different regions of Pakistan. Over all, HBV-D genotype was found to be the dominant genotype which was detected from 285 out of 409 cases (70.0%). It was the most pre dominant genotype among the prevailing one. Second most prevalent group was of mixed genotype infections (A+D) which were detected from 62 samples (15.15%). A mixture of genotype A+D was detected from the majority of the samples among mixed genotype group (62/69) which was followed by C+D (3/69) then C+D (2/69) and A+B (2/69). Genotype A was found to be 46 from out of 409 samples (11.2%), genotype C come 4 (1.0%) and genotype B from 2 (0.48%) samples overall from the study area (Table 2, Fig. 2).

Genotype B and C are not common to be detected and identified in our population. Overall, genotype B was detected from 6 (1.44%) samples and C from 7 (1.73%) either alone or mixed with other genotypes, largely with D. Except for two and four samples which were detected as genotype B, and C respectively. Genotype B was not found from Islamabad, Peshawar and Lahore region whereas C genotype had not come from Quetta city. Moreover, the mixed infection with genotype A and D was not much higher in any area other than Peshawar and Lahore where it constitute about 39.99% of all the samples.

Among total 128 selected positive samples from Peshawar, about 3 (2.34%) were not genotyped successfully and we call them as un-typeable genotypes. All other samples were detected with some genotype or a mixture of two. None of the samples was found to be having more than two genotypes. Genotype D was found from 71.09% of the total 128 samples; genotype A form 7.03% whiles the mixture of A+D form 16.40% of the samples. None of the samples from this region was detected as genotype C (Table 2).

Table 1. Region based samples selection and gender description

	Islamabad			Peshawar			Lahore			Quetta			Total		
	M	F	T	M	F	T	M	F	T	M	F	T	M	F	T
HBsAg Pos.	67	33	100	93	57	150	83	17	100	61	39	100	304	146	450
Neg.for DNA	2	0	2	12	7	19	3	8	11	4	2	6	21	17	38
<100IU/ml	0	0	0	0	3	3	0	0	0	0	0	0	00	03	03
Genotyped	65	33	98	81	47	128	80	9	89	57	37	94	283	126	409

**M=Male, F=Female, T=Total, Neg=Negative, Pos=Positive*

Table 2. Region wise distribution of HBV genotypes

Genotype	Islamabad	Peshawar	Lahore	Quetta	Total
A	17(17.34)*	9(7.03)	9(10.11)	11 (11.70)	46 (11.2)
B	0	0(2.0)	0	2 (2.12)	2 (0.48)
C	2(2.04)	1(0.78)	1(1.12)	0	4 (1.0)
D	63(64.28)	91(71.09)	55(61.79)	76 (80.85)	285 (70.0)
A+B	0	2(2.0)	0	0	2(0.48)
A+D	16(16.32)	21(16.40)	21(23.59)	4 (4.25)	62(15.15)
B+D	0	1(0.78)	0	1 (1.06)	2(0.48)
C+D	0	0	3(3.37)	0	3(0.73)
UT	0	3 (2.34)	0	0	3(0.73)
Total	98	128	89	94	409

***** *Parentheses show percentages*

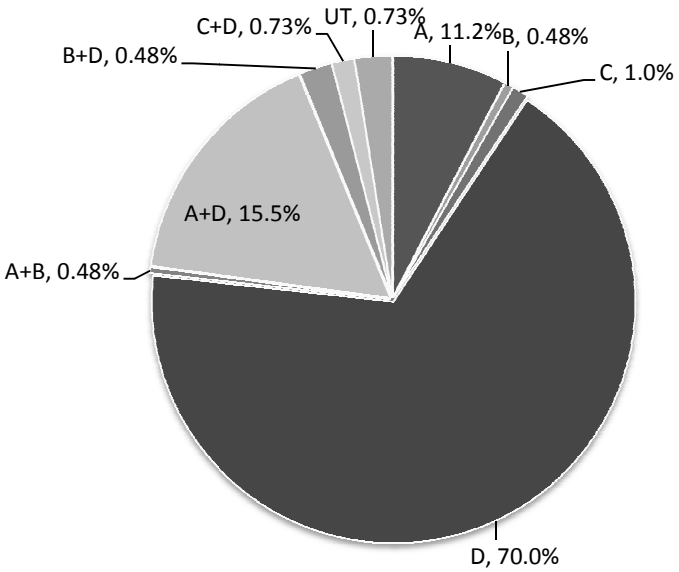

Fig. 1. Pie chart showing percentage prevalence of different HBV genotypes in overall studied population

A sample size of 98 from Islamabad region was found to be positive for HBV DNA by PCR having a viral load of >100 IU/ml. Out of these, genotype D was found from 63 samples, A from 17, B from none and C from 2 of the samples. Different combinations of mixed genotypes were found from 16 samples. Mixture of A+D was present in 16 samples; B+D, C+D and A+B was present in none of the samples (Table 2).

From Lahore region, we selected 100 samples (Table 1). This is congested populated city where most people found suffering from various diseases. Out of total 100 samples studied, 55 (61.79%) were typed as genotype D which was followed by a mixture of genotype A and D detected from 21 samples, mixture of genotypes C+D from 3, genotype A from 9 and C from 1 sample (Table 2).

Total 94 samples from Quetta region were genotyped. Genotype D was again the dominant genotype with prevalence of 80.85% (76/94). Genotype A was identified from 11, genotype B from 2, mix genotype A+D from 4 and mix genotype B+D was found from 1 sample (Table 2).

In a nut shell, genotype D was identified and come out to be the most dominant genotype in Pakistani community showing sub-genotypes of D1 & D3 and come from 285 samples which is about 70.0% of the total sample size in our study (Figs. 2 and 3). Genotype A and D are present as co-infection with each other and contributed as the second prevailing genotypic group from 62 samples (15.15%). Mixed genotypes C+D, A+B, and B+D are not much identified in Pakistan. In our sample size, only 3 samples from Peshawar were typed as un-typeable (Table 2).

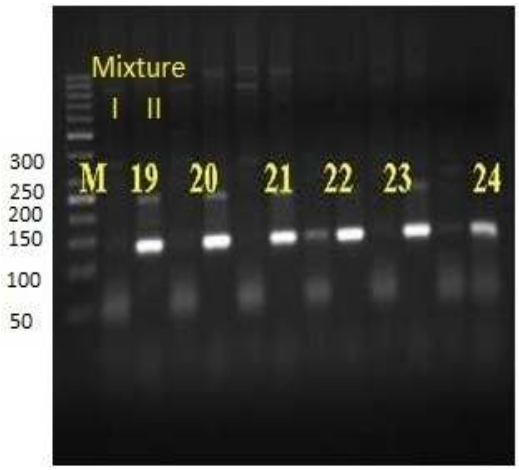

Fig. 2. Nested PCR products (second round PCR)

Lane 1: GeneRulerTM 50bp DNA ladder (Fermentas, Life Sciences) run as marker. Mix I & 2 run side by side. Lane 2-12: PCR product of HBV positive samples with 119bp size fragment (Genotype D)

Fig. 3. The map shows area of study in Pakistan

3.1 Statistical Analysis

Descriptive statistics and percentage were calculated with Microsoft Excel. The results for genotype prevalence in Pakistan are shown in simple percentages. Graph prism software was used during statistical analysis and the level of significant was set to 0.001 (Fig. 1).

4. DISCUSSION

The aim of the study was to investigate the genotypes of HBV customary in the areas of four regions of Pakistan; Punjab and Islamabad, Khyber pakhtoonkhwa (KPK) and Quetta. A couple of research work on HBV genotypes have been carried out in Islamabad region and some districts of Khyber Pakhtoonkhwa (KPK) but there was no information available on the topic for most of the districts in northern part of Punjab and Baluchistan. Previous studies (4, 19-20) reported genotype D as the most prevalent genotype in Pakistan except one study (21) which shows a nearby equal proportion of A, B, C and D with C as the most prevalent. Moreover, most of the previous studies also reported a high percentage of mixed co-infections of A+D genotype. Our results are consistent with most of the studies which shows the dominance of D genotype and presence of a high percentage of mix infection. But it indicates that the mix infections with genotype A and D are not so common in region of Baluchistan. However it is found from a large percentage of the samples from the north Punjab, Islamabad and Khyber Pakhtoonkhwa (KPK) areas. Moreover, north Punjab and Islamabad region do not have HBV/B and HBV/C infections which are found from 5% of the total samples from two other regions, either in combination with D or alone.

The Khyber pakhtoonkhwa (KPK) and Baluchistan have different pattern of genotype than in as they have a reasonable infections with genotype C and D. This could be correlated with the residents in these areas. The residents of Khyber pakhtoonkhwa (KPK) and Baluchistan have a very close relationship to Afghanistan as they have a lot of common traditions and common language. Besides, they frequent visits across the border for business and trade. An important factor is also the immigrants from Afghanistan who are living in this area for several decades. Similarly, these people have very close links to southern China, although the nature of this link is different and mostly business based. So, the introduction of genotype B and C in both these areas may be attributed to their relationships across the border. We did not found any sample with genotype C from Quetta region. However, 3 out of 94 samples had genotype B; mix with D in one sample and alone in two. The percentage is not so much high to be considered for discussion due to the effect of some specific factors like the relationship or link of people across the border. However, the people of all regions also have a very strong link with other countries of the world as about 28% of the total population is either living or working in Europe, America and Middle East and this relationship may exist as a reason for introduction of genotype C, B infection and co-infections.

5. CONCLUSION

Genotype D was identified and come out to be the most dominant genotype in Pakistani community showing sub-genotypes of D1 & D3 which is about 70.0% of the total sample size in our study. Genotype A and D are present as co-infection (A+D) with each other and contributed as the second prevailing genotypic group and come from those regions which were not explored earlier in any study. Moreover, the mixed infection with genotype A and D (A+D) has a very high prevalence of about 15.15% in all Pakistan with low contribution from Quetta city. Genotype A was the third one having 11.2% infection rates. Mixed genotype C+D is also present in our community but with very low contribution (Table 2).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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