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Serological Prevalence of Hepatitis B Virus in Peshawar, Khyber Pakhtunkhwa, Pakistan

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WS designed the study and wrote the article. TK performed entire experimental work. SZ helped in the collection of samples. MI and HB analyzed data.

Key words
Hepatitis B virus, Molecular prevalence, HBsAg, Polymerase chain reaction, Pakistan.

ABSTRACT

The present study aims to investigate an up-to-date prevalence of Hepatitis B Virus (HBV) infection among a mix population of Peshawar, Khyber Pakhtunkhwa, Pakistan. This study highlighted the relationship between HBV, demographic, clinical parameters and the risk factors in patients with HBV. A total of 4,758 blood samples from the mixed population were collected including 2382 male 2376 females. All the samples were subjected to hepatitis B surface antigen Immuno-chromatographic test (ICT), HBsAg enzyme-linked immunosorbent assay and by Reverse transcription polymerase chain reaction for the existence of antibodies against HBV. It was observed that 307 (12.8%) out of the 2382 individuals harbored antibodies in their blood against HBV. Among the different age groups, the highest number of incidences of HBV antibodies was found in the 16-25 age groups (7.01%). ICT positive samples were further screened by nested polymerase chain reaction (PCR) to determine the existence of active HBV-DNA. It was investigated that 5.7% (2382) of the total population (4758) tested was positive, among which the female 15.4% (367) possessed antibodies in their blood against HBV. Our results showed a higher male percentage than that of the female. The total HBV prevalence was recorded at 4.5% in all populations comprising both male and female. It was concluded that the highest prevalence of HBV was found in male and female in the age group 16-25, and then followed by the age group that lies between 26-55.

INTRODUCTION

Hepatitis is the foremost public health concern across the globe. It is estimated that 350-400 million people globally are carriers of the surface antigen (HBsAg) of the hepatitis B virus (HBV) (European Association for the Study of the Liver). There are five major viruses that affect the liver seriously, hepatitis A, B, C, D and E (HAV, HBV, HCV HDV, and HEV). All these vary in mode of transmission, pathology, and prognosis mean. Hepatitis B virus (HBV) is a major agent for silent contagious liver infection, may be acute or chronic. HBV leads to inhibition of the progression of liver fibrosis, normalization of biochemical indicators of liver damage, including hepatocellular carcinoma (HCC) and liver cirrhosis-related end-stage chronic liver diseases (CLD) (Hafeez-ud-Din *et al*., 2012; Flisiak *et al*., 2017). Hepatitis B was first identified in 1963 and till date according to a study, about 20 million people in Pakistan are suffering from hepatitis B and C, each year brings about 150 000 new cases (WHO, 2017).

Hepatitis not only causes great sufferings to patients and their families but also results in great economic losses. Hepatitis B virus share same mode of transmission with HCV, especially likewise transfusion of unscreened blood and blood products, use of non-disposable syringes/ needles, dental and general surgical methods and operation with unsterilized equipment or tools, shaving by local barbers with used or reused razors, ear and nose piercing and tattooing with unsterilized needles, use of

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non-sterilized scissors for circumcision, drug abuses and unsafe sexual practices (Rantala and Van de Laar, 2008; Kumar *et al*., 2017). Most of the people do not suffer from any visible symptoms in the course of the acute infection period. However, acute illness along with the common symptoms like general abdominal pain, extreme fatigue, yellowing of the skin and eyes (Jaundice), vomiting, nausea, and dark urine color is reported in some people. Whereas acute hepatitis may result in acute liver failure in a small percentage of the people that might lead to death (Chang, 2007; Rantala and Van de Laar, 2008).

Of note, the classification of age groups plays a key role in the study of geographical and demographic differences, as well as in molecular epidemiology, vaccine development, therapeutic decision-making, and the study of chronic infection (Gul *et al*., 2016; Kumar *et al*., 2017). Hence, it provides important clinical information for health improvement planning and policy making. The prevalence of hepatitis is slightly higher than in developed countries due to the limited availability of resources (Gul *et al*., 2016; Farooq *et al*., 2018). Even though the developing countries like Africa, Asia, and Pacific Island has a high prevalence of HBV in comparison with developed countries like Europe, America, Australia where there is the lowest prevalence. Pakistan is considered as a developing country globally, having viral hepatitis as a major public health issue (Hasnain, 1994). There is a need among the large populations of the developing world to study the relationship of HBV by age, gender, route of entry and the duration of infection.

The PCR based method for HBV provides an extra advantage over non-PCR immunoassays such as ELISA and ICT, as it is a quick and reliable tool to diagnose and quantify active HBV-DNA in the blood serum. However, to our knowledge, there is a lack of PCR based prevalence and relevant data availability studies with large sample numbers in the population of Pakistan. Peshawar city was decided upon as a good site to conduct the research due to the high population rate in almost all of its districts, the limited availability of health facilities, and the frequency of natural and human disasters (Kumar *et al*., 2017).

The main concern of the study was to attain an up-to-date HBV infection and distribution of HBV infection and its maximum and minimum viral load in Peshawar city, Khyber Pakhtunkhwa, Pakistan. We also analyze the relationship between demographic and clinical parameters, and to look at the risk factors present in patients with active or acute HBV. The present study will not only help to determine the seroprevalence of HBV and to compare it with previous investigations but will also attract the attention of local and foreign organizations towards this important issue.

MATERIALS AND METHODS

Study area and population size

Pakistan is capital territory consists of four provinces (Sindh, Panjab, Balochistan and Khyber Pukhtoonkhwa) along with the federally administered tribal areas. The Cross-sectional descriptive study was conducted in Peshawar city, the capital province of Khyber Pakhtunkhwa, Pakistan by the total population in 2014 counting 3,575,000, upon as a good site due to limited or poor availability of health care facilities. The main language is Pushto locally called (Pakhto). The terror activities, natural disasters and especially low literacy rate in females is the ground reality and the main reason for non-hygienic health care facilities in the last decade.

A total of 4,758 local residents of the area, most of whom came from different parts of the country, were a mixed population of Peshawar city, Pakistan. The majority of them are Pushtuns with other smaller ethnic groups such as the Hinkowans, Dards, Chitralis, Gujjars, and Afghan refugees participated in our prevalence exercises and were considered for the possible evaluation of the present study. All the individuals were assigned in eight different age categories 5-15, 16-25, 26-35, 36-45, 46-55, 56-65, 66-75, 76 to above age, respectively. Data of three years from 2013 to 2016 was recorded by using planed design questionnaire in a laboratory-based (Alfalah Medical Centre and Hospital, Dabgari Gardens, Peshawar, KPK, Pakistan) descriptive study. The population was selected on the basis of visiting the concern laboratory for diagnosis. This questionnaire included general information for example, marital status, socioeconomic conditions, the age of patient, gender, reference of doctor and diagnostic tests. All the confidential was used for research purposes only. The study was approved by the Ethical committee (Alfalah Medical Centre and Hospital, Dabgari Gardens, Peshawar, KPK, and minor collections center laboratories with the collaboration of Department of Allied Health Sciences, Iqra National University, Peshawar, Pakistan.

Inclusion and exclusion criteria

All subjects who gave full informed consent were included in the study. Subjects who had once been vaccinated with the required three doses and those who declined to offer consent were excluded from the study.

Initial screening by HBsAg ICT

Initially, all suspected samples of blood were examined by the immuno-chromatographic test for HBsAg. All the kits and reagents used in this screening were purchased from Abbot (global healthcare and research). Positive samples by ICT were further processed for the next round of examination.

ELISA test for HBV

ELISA test for HBV positive sera was investigated for the possible anti-bodies complementary to its antigen by commercially available kit following the manufacturer's instruction (BIOKIT, S.A, Barcelona-Spain). The test was performed in 96 well plates and the sample as showing positive results were read spectrophotometrically to calculate the cutoff value in response to its positive control. Samples showing ELISA positive were processed for DNA extraction and subsequent nested PCR.

DNA isolation and confirmation of HBV DNA

The DNA from blood samples was extracted using a DNA extraction kit (Invitrogen). 5ml of the blood samples were collected from patients in a disposable syringe from the radial vein and stored in sterilized gel tubes. Serum was collected by coagulation of blood and stored at -20°C for further analyses. The HBsAg positive ELISA samples were subsequently used for DNA extraction and nested PCR using commercially available kits by Sacace (Sacace, Biotechnology, Italy) as manufacturer's information inside the Cepheid smart cycler (Nasdaq: CPHD, California, US). For simple and nested PCR isolated DNA was dissolved in 50 µl of nuclease-free water. The initial round of PCR, specific primers were used as shown in Table I.

A total of 20 µL of the PCR mixture (1.5 µl of 25 mM $MgCl_2$ and 1 µl of 2.5 mM dNTPs) containing 1 µl each of 10 µM forward and reverse primers and Taq buffer, 3.3 µl nuclease-free water were added to 10µl of DNA for the first round in thermocycler machine. For the second round of PCR, the reaction mixture was same as of 1st except for 4 µl of DNA template and 9.3 µl of nuclease-free water. The parameters for PCR thermocycler were adjusted for denaturation (94°C) for 5 min, followed by annealing (52°C) for 40 sec and finally, extension (73°C) for 30 sec followed by 30 cycles to attain the final reaction mixture. The nested PCR was conducted under the same previous PCR conditions stated prior.

Table I.- Set of primer run in the nested PCR (*i.e.* first and second cycle of PCR) the primer pairs.

Cycle	Primers sequence
First	F: 5′-CATCCTGCTGCTATGCCTCATCT-3′,
	R: 5′-CGAACCACTGAACAAATGGCACT-3′
Second	F: 5′-GGTATGTTGCCCGTTTGTCCTCT-3′
	R: 5′-GGCACTAGTAAACTGAGCCA-3′

RESULTS

Gender and age-wise HBV distribution

In the present study, a total of 4,758 samples were collected, out of which 2382 were males and 2376 were females. All the tested samples were categorized into eight age groups that fall into different categories 5 to 75 years or above were initially screened for HBsAg by ICT. All the male samples were positive by HBsAg ICT (21.9%) and were further processed by ELISA. Our ELISA results revealed that out of the total number, 12.8% were positive for HBsAg (Table II). The nested PCR assay of the samples positive by either ICT or ELISA revealed that 136 (5.7%) male are with active HBV DNA in their blood (Table II). In order to calculate the age wise prevalence in male, our results revealed that all the age groups were affected while the prevalence deviates in different age groups. The highest incidence rate of 7.01% was observed in the age group of 16 to 25 years, followed by 6.4 % in the age group of 26-35 while a lower incidence of 2.08% was observed in the age group of 66-75 years, likewise no incidence was seen in the age group of 76 years or above (Table II).

Table II.- The prevalence of HBV among the different age group of males from Peshawar, KPK, Pakistan as revealed by immuno-chromatographic test (ICT), ELISA and PCR.

S. No.	Age categories (years)	No. of samples	HBsAg		Positive PCR	Prevalence (%)
			ICT	ELISA		
1	5-15	183	15	9	7	3.8
2	16-25	613	148	98	43	7.01
3	26-35	498	102	63	32	6.4
4	36-45	355	96	51	21	5.9
5	46-55	386	94	47	19	4.9
6	56-65	211	53	33	12	5.6
7	66-75	96	10	5	2	2.08
8	76-above	40	6	1	0	0
	Total	2382	524 (21.9%)	307 (12.8%)	136 (5.7%)	35.69%

Table III.- Prevalence of HBV among the different age group of female from Peshawar, KPK, Pakistan as revealed by immuno-chromatographic test (ICT), ELISA and PCR.

S. No.	Age group (years)	No. of sample	HBsAg		Positive PCR	Total prevalence
			ICT	ELISA		
1	5 to 15	487	21	10	7	1.4
2	16 to25	506	98	48	35	6.9
3	26 to 35	476	85	39	27	5.6
4	36 to 45	469	66	13	8	1.7
5	46 to 55	218	43	10	1	0.45
6	56 to 65	138	27	6	3	2.1
7	66 to 75	57	18	4	1	1.7
8	76 to 85	25	9	4	0	
	Total	2376	367(15.4%)	134(5.6%)	83(3.5%)	

Similarly, the age-wise prevalence in female was also evaluated (Table II). It was observed that all the age groups were affected while no PCR positive sample was detected in older age groups of 76 to above. The prevalence deviates in the different age group of the female gender. The highest incidence rate of 6.9% was observed in the age group of 16 to 25 years, followed by 5.6% in the age group of 26-35 while a lower incidence of 0.45 % was observed in the age group of 46-55 years (Table II). Interestingly, it was observed that the age groups, the 16-25 year the HBV prevalence was higher in the all young age group that is 16-25 year. The prevalence of HBV among the infected individuals showed a high incidence rate in males in comparison to females (Tables II, III). We investigated that 5.7% (136) of males showed positive PCR results for HBV and the prevalence in females was 3.5% (83) as shown in Table III.

Sex-wise distribution of HBsAg

During this study, out of 2382 male samples, 319 samples were found positive for HBs antigen and 136 were reported positive for HBV DNA as shown in Table IV. For females, 134 samples out of 2376 were reported positive for HBs antigen and for HBV DNA. 83 samples were found positive as shown in Table IV. To calculate the total prevalence in the whole selected population harboring HBs antigen 9.5%, while for HBV active DNA infection is 4.5%, typically gel photograph are shown in Figure 1.

Table IV.- Sex wise distribution of the anti-HBV antibodies and HBV DNA positive.

Sex	Total No. of samples	Anti-HBV +ve	HBV +ve
Male	2382	319	136
Female	2376	134	83
Total	4768	453(9.5%)	219 (4.5%)

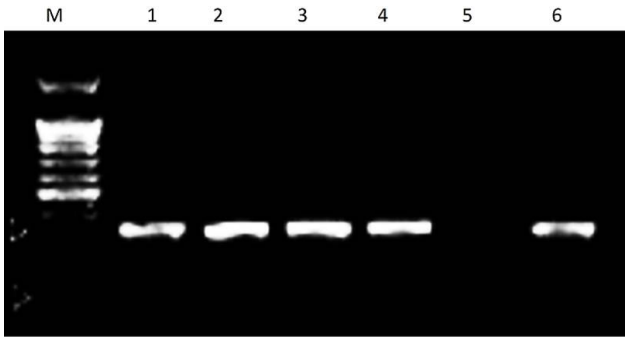

Fig. 1. Typical gel photograph of HBV amplified products. M, 1 Kb molecular marker; 1-4, positive samples for active HBV showing 180 bp band of hepatitis B virus; 5, negative control; 6, positive control.

Online data and its comparative study

Important findings reported, regarding the active HBV for Pakistan were reviewed from the World Wide Web (http://webfoundation.org/) and the data were examined manually. From all over the country the average prevalence of HBV in terms of the individual province as well as all four provinces was calculated as shown in Table V.

DISCUSSION

Emerging infectious diseases pose a serious threat to public health security; this is especially true in the underdeveloped world because of limited resources to combat them. These emerging pathogens are characterized by a novel mode of pathogenesis and in some cases, a broad host range (Khalil *et al.*, 2017). Hepatitis B infection is an international public health challenge which is alarmingly increasing with a prevalence ranging from 2 to 8% in different population groups and in Pakistan the

Table V.- Literature reviewed demonstrating the prevalence of hepatitis B across the different locations of Pakistan since 1996 up till now. Each technique has been mentioned in the following studies.

S. No.	Locations	Prevalence	Method	References	S. No.	Locations	Prevalence	Method	References
1	Karachi	2.28%	ICT	Kakepoto *et al*. (1996)	14	Karachi	4.7%	ICT	Mujeeb *et al*. (2006)
2	Bahawalpur	1.1%	ICT	Yosaf *et al*. (1998)	15	Liaquetpur	5.96%	ICT	Ayub *et al*. (2006)
3	Karachi	3.5%	ICT	Syed, (1998)	16	Northern areas	3.66%	ICT	Maqbool *et al*. (2007)
4	Faisalabad	2.06%	ICT	Hashmi *et al*. (1999)	17	Rawalpindi	2.45%	MEIA	Ishtiaq *et al*. (2007)
5	Abbottabad	1.55%	ICT	Faisal *et al*. (2000)	18	Balochistan	4.8%	ICT	Ahmad *et al*. (2007)
6	Rawalpindi	5.86%	ICT	Mumtaz *et al*. (2002)	19	Lahore	1.52%	ICT	Asad *et al*. (2007)
7	Rawalpindi	3.3%	ELISA	Khattak *et al*. (2002)	20	Rawalpindi	2.31%	ICT	Zeeshan *et al*. (2007)
8	Peshawar	1.9%	MEIA	Ahmad *et al*. (2004)	21	Peshawar	1.40%	ELISA	Alia *et al*. (2008)
9	Islamabad	2.51%	ICT	Asif *et al*. (2004)	22	Sindh	6.2%	ICT	Mujeeb *et al*. (2008)
10	Multan	3.37%	ICT	Mahmood *et al*. (2004)	23	Peshawar	1.16%	ICT	Ahmad (2016)
11	Karachi	2%	ICT	Akhter *et al*. (2005)	24	Kurram Agency	14.95%	ICT	Hussain *et al* (2016)
12	Punjab	4.93%	ICT	Waqas *et al*. (2005)	25	Mardan	20.79%	ICT	Khan *et al*. (2017)
13	Bahawalpur	2.69%	ICT/ELISA	Fayyaz *et al*. (2006)	26	Havelian City	6.39%	ICT	Ahmad *et al*. (2017)

situation is not different from that in the rest of the world (Ahmad *et al*., 2016). More specifically, in acute hepatitis patients who harbor the serum of HBs Ag are likely to be carriers or have a severe risk factor for causing chronic liver disease, in developing countries like India, Pakistan, and Bangladesh (Nadeem *et al*., 2011).

The causes of such disease outbreaks are complex and often not well understood.The experimental design of the current study aims to inquire about the prevalence of HBV in the Peshawar KPK, Pakistan,being the most important and central city to the north of Pakistan. Previous findings signify that most HBV positive subjects belong to the rural areas of low economic status (Akbar *et al*., 1997). The Peshawar city was taken as a model because its entire districts have a comparatively higher mix population. These districts are facing basic health prerequisites problems and suffer from natural disasters (*e.g.* floods, earthquake, *etc*). Previous findings were more concern about antibodies-based tests (Ahmad *et al*., 2017). In our recent findings advanced molecular-based techniques (RT-PCR) was used to draw the exact picture of active HBV prevalence and infection in between the different age groups comprising males and females. The data support the hypothesis of a homogeneous distribution of HBV infection between the male and female due to various outdoor factors.

It has already been notified that HBV infection is more likely to occur in individuals with low socioeconomic status (Akbar *et al*., 1997). The patients treated for hepatitis B and C in the past 2 years through Prime Minister Program for the Prevention and Control of Hepatitis Viral Infection were screened from 12 different regions. A success rate of 67% was declared for PCR reports of 1686 patients, which were available and about 33% were non-responders. About 3440 (45.4%) patients among 7572 patients, just 3440 completed 6 months of interferon therapy (Qureshi *et al*., 2013). In order to mitigate the risks associated with HBV, there is a greater need to understand the interactions of pathogen-host-environment, to monitor molecular evolution and genomic surveillance.

The present study confirms about 4.5% prevalence of HBV throughout Peshawar city. Prevalence frequency distribution of hepatitis B infection was reported lower in females (3.5%) in comparison with males where it is (5.4%). Our study suggests that the male's community is acquiring HBV infection more quickly and highly due to maximizing exposure to the outer environment in contrast to females who stay at their houses most of the time. Barber shaving, homosexuality (male to male mating) heterosexuality (male to female mating) and use of drugs are very usual in these areas which are in line for the arguments for such higher occurrence of HBV among males. Similarly, HBV higher incidence in males followed by females was observed when risk factors regarding hepatitis B and C infection were observed in liver, stomach in Karachi clinic (Khan *et al*., 2011; Shazi and Abbas, 2006).

Earlier studies also showed a high prevalence of HBV cases in males as compared to females in Pakistan (Alam *et al*., 2003; Mahtab *et al*., 2008). Similarly, the prevalence of HBV infection is high in Bangladesh, a study showed that the ration in males to female is almost 67.86% to 32.14% (Khan and Ahmad, 2014). The above-mentioned increase the prevalence of HBV in males reflects high risk factors in Asian males. These risk factors include a

high use of drugs, sharing of barber shaving razors and multisexual partners as compared to women. In Pakistan, a Disease Early Warning System (DEWS) was created in 2005 through the collaboration of WHO and the Federal Ministry of Health with the goal of early detection of infectious pathogens to reduce the morbidity and mortality rate; unfortunately, it was not implemented at the grassroots level (Tanveer and Shinwari, 2015). A recent survey conducted in Pakistan about the level of awareness among professional scientists raises alarms because it indicated that their awareness levels about biosafety and biosecurity issues are low (Janjua and Nizamy, 2004).

In this study, almost all age groups were found to be affected by HBV. However, a higher prevalence exists in young ages (both genders). The higher prevalence among younger age groups may be endorsed to the more frequent acquaintance to risk factors and persistent HBV infection. The barber risk factor is one of the major causes and the route of transmission for HBV infections in Pakistan. At a young age, peoples do not care and even do not complete the treatment. And second, due to low socioeconomic some child work as child labor and collecting raw and waste materials (used injection, *etc*.) from public spot and streets which may increase the rate of infection. The frequency of exposure to HBV is higher in male gender (32%) who routinely shaved with community barbers. Previous studies showed in Pakistan nearly 46% of the barbers reuse the blades, ultimately resulting in the increase transmission of HBV infection in the community blades (Mahtab, 2008; Haider, 2015). Other risk factors in this area may also be the contaminated dental instruments, large numbers of refugees from Afghanistan, an underdeveloped region that is at war, and internally displaced people (IDPs) for the reason of ongoing war against militants and terror represent another major obstacle to the health care of the people (Fox, 1996; Rauf *et al*., 2011).

Under-funded and ill-equipped healthcare facilities due to the ongoing war and infrastructure collapse of terrorist activities represent the biggest obstacles to health care for the people of Pakistan. The lack of emergency medical services (EMS) in Pakistan and initial help to victims are commonly provided by untrained but well-meaning people at the scene is another major cause of an abrupt increase in hepatitis (Bukhari *et al*., 1999). We also investigated that the majority of the patients associated with low economic status and visited by local medical practitioner's examinations. No proper sterilization methods and contaminated equipment reuse might be a possible reason for the high rate of prevalence in this site. Sharing personal materials like shaving razors, nail cutters, the needle used for ear and nose piercing, sewak (miswak), toothbrushes and close personal or sexual contact with an infected person has also been reported contributing as major risk factors for hepatitis B virus infection in the young age of these populations (Janjua, 2004). The dilemma is not only restricted to natural outbreaks but also bioterrorism attacks using engineered viruses by non-state agents cannot be ignored in the region. At present, Pakistan may not have the capacity to deal with these issues by itself, so efforts are required by the international community to reduce the existing threat of hepatitis diseases by educating communities, providing funds to facilitate healthcare personnel and by providing better treatment facilities to reduce the mortality rate. Risk factor study related to HBV infection in Pakistan would give important evidence to the health planners and the disease control department to overcome the spread of disease. Preventive measurements, public awareness and vaccination programme need to be more strengthen. The government and health authority need to re-enforce the management and control strategies. This was an initial study and case-control study should be conducted in the future.

CONCLUSION

The current studies highlighted the seroprevalence of HBV in between the various age groups along with the risk factors. Males have a higher prevalence in comparison with females. The infection was more proliferated between the younger group either male or female. Furthermore, Peshawar is still dominant in HBV infection, the capital province of KPK, Pakistan. Major awareness events should be launched by Government authorities and (NGOs) non-government organizations, ample use of vaccination and other preventive measures and cautions might be taken urgently to cease and prevent the outspread of such dreadful disease in Peshawar, Pakistan**.**

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Statement of conflict of interest

No conflict of interest is associated with this work.

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