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PREVALENCE OF HEPATITIS B VIRUS IN SOUTHERN PUNJAB, PAKISTAN USING REAL TIME PCR

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Abstract
Hepatitis B virus (HBV) can lead to the early onset of severe and prolonged liver infections that result in liver cirrhosis and hepatocellular carcinoma in infected individuals. These infections are a global concern, especially for developing countries like Pakistan. This study targeted the local population of the densely populated cities across Southern Punjab, who were found seropositive for hepatic infections, viz., hepatitis B (HBV). Accordingly, 176 seropositive samples (124 male and 52 female) were confirmed via real-time PCR. The real-time PCR detected and confirmed viral DNA in 81.8% (144) samples, while the remaining were negative. In case of male population, 105 individuals were positive, with a high viral load in 4/105, a moderate viral load in 78/105 and a low viral load in the remaining 23 individuals. In the female population, among 39 positive cases, 3 females were found to have a high viral load, 31 had a moderate viral load, and 5 had a low viral load. A high viral load could be related to the emergence of serious liver disease in both genders. The prevalence of HBV was significantly higher in the <25 years age-group than 26-50 years and 51-75 years age groups ($P<0.05$). The Southern Punjab population of Pakistan has a high prevalence rate of HBV due to a lower literacy rate, a lack of effective blood screening facilities, poverty, and ignorance regarding HBV risk factors. The high frequency of HBV infection in the studied area is of concern and highlights the need for adopting proper preventative measures. It is proposed that more initiatives should be taken to educate the general population and train health care workers regarding the adverse outcomes of HBV and how it transmits.

Keywords: Hepatocellular Carcinoma, Real Time PCR, Southern Punjab, Viral Load, Hepatitis B virus

Abbreviations: Hepatitis B Virus (HBV), World Health Organization (WHO), Hepatocellular Carcinoma (HCC), Cobas AmpliPrep-Cobas TaqMan (CAP-CTM), ethylenediaminetetraacetic acid (EDTA), Deoxyribonucleic acid (DNA), Polymerase Chain Reaction (PCR), Basic Health Units (BHUs), Hepatitis B surface antigen (HBsAg), Hepatitis B e antigen (HBeAg), Standard Operating Procedures (SOPs)

Introduction

The Hepatitis B virus infection is a major global health problem (Eke et al., 2011). It is a silent destroyer of the liver, and a lot of carriers do not realize that they have the HBV virus. The disease can result in acute or chronic liver damage, thereby leading to cirrhosis and other life-threatening consequences, such as hepatocellular cancer (Trépo et al., 2014). In Pakistan, chronic HBV infections are currently regarded as one of the most serious health complications, leading to an increased number of deaths every year (Bukhari and Zafar, 2013). According to the World Health Organization (WHO), 240 million people are frequently infected with HBV and an estimated 887,000 persons died from HBV disease complications in 2015 (Kitandwe et al., 2021; Zauli et al., 2016).

The infection is typically asymptomatic and/or subclinical, but persistent infections can cause the liver to scar and eventually develop cirrhosis, which can result in liver failure, hepatocellular cancer, or potentially fatal gastric and esophageal varices (Asad et al., 2013). Approximately 30% of infected people develop hepatocellular carcinoma (HCC) and liver cirrhosis (Zauli et al., 2016). Every year, over a half million deaths occur due to hepatocellular carcinoma and liver cirrhosis as a consequence of chronic HBV infection (Caliendo et al., 2011).

HBV viral transmission occurs mostly through blood, body fluids, unsterile needles, discarded syringes, during birth, and by sexual contact (Hebo et al., 2019; Mazhar et al., 2021). The symptoms included fever, fatigue, upset stomach, loss of appetite, urine color change, pale eyes and complexion, and grey stool (Pallavi et al., 2017). Blood tests, liver biopsy, or ultrasound are used to identify liver damage. Acute hepatitis does not have a specific treatment; however, it is curable with the intake of fluids, a healthy and balanced diet, and proper rest. In some circumstances, hospitalization is required. Specific treatment options involve using interferon, antiviral medicines, and/or liver transplants (Campos-Valdez et al., 2021).

Molecular techniques are reliable and accurate in confirming different infections. Moreover, these are quick at delivering authenticated results. There have been very few studies regarding HBV prevalence in Southern Punjab, Pakistan, focusing on age wise distribution in both genders. In this study, seropositive cases of HBV were confirmed using a fully automated system, i.e. COBAS AmpliPrep and COBAS TaqMan Hepatitis B (CAP-CTM; Roche Molecular Systems, Inc., Branchburg, NJ), that combines two integrated and highly sensitive platforms (S. Hochberger et al., 2006; Weiss et al., 2004). The distribution of the virus in different age groups, genders, and married/unmarried along with the viral load, was also accessed.

Material and Methods

Sample Collection and its Preparation

A total of 176 blood samples were collected from HBV seropositive patients identified in the districts located in Southern Punjab, Pakistan. These districts include Bahawalpur, Multan, Lodhran, Khanewal, Vehari, Bahawalnagar, Rahim Yar Khan (RYK), Dera Ghazi Khan, Muzaffargarh, Layyah, Rajanpur, Jampur, Kot Addu, and Taunsa Sharif.

The samples were collected in blood vacutainers and placed at room temperature for 10 minutes. In the next step, samples were centrifuged at 3000 rpm for 15 minutes for serum separation and all heavy molecules settled down. Then 500µL of EDTA plasma or serum was analyzed in the Molecular PCR Lab for qualification as well as for quantification of DNA HBV.

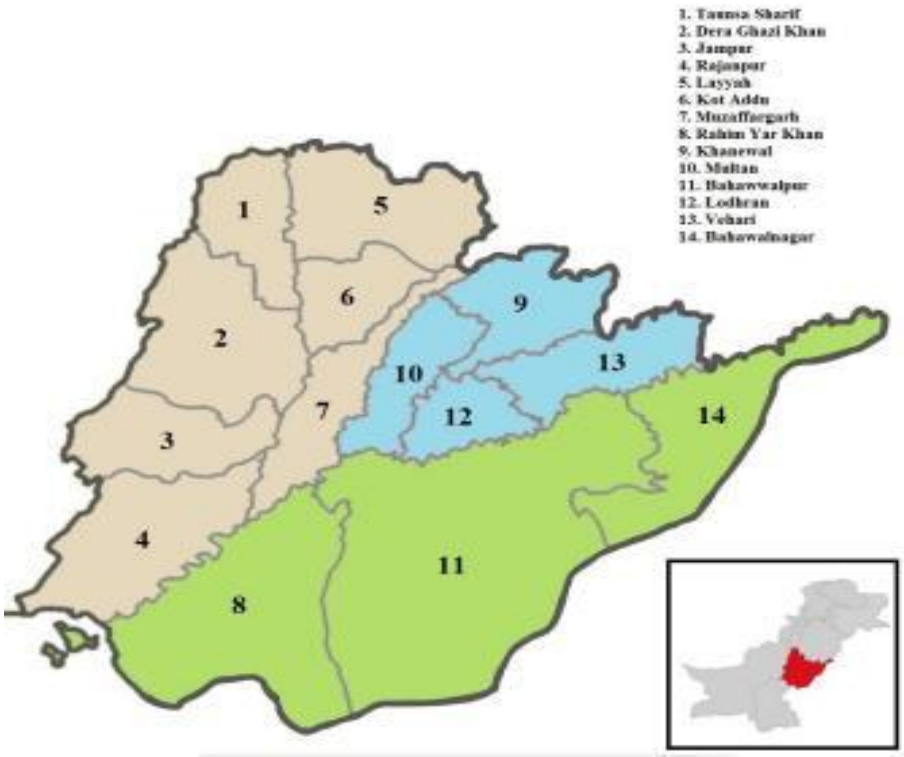

Figure 1: Southern Punjab Districts of Pakistan

Processing of HBV Seropositive Samples

The processed samples (n = 176) were subjected to the COBAS® AmpliPrep/COBAS TaqMan HBV Test, v2.0 for Nucleic Acid Extraction and its detection and measurement of the viral load in samples (Gullett and Nolte, 2015). For qualitative and quantitative analysis, the HBV virus particles, if present, are lysed by incubating at high temperature with protease and chaotropic lysis/binding buffer that release nucleic acids and protect the release of HBV DNA from DNases in serum or EDTA plasma. The processed specimen, containing the released HBV DNA and HBV IC DNA, is added to the amplification mixture and transferred to the CTM Analyzer.

Target Amplification and its Detection

The thermal cycler in the CAP/CTM 48 Analyzer heats the reaction mixture to denature the DNA and expose the specific primer target sequences. The Master Mix reagent along with specific primers are used in CAP/CTM HBV Test v2. It consists of primer pairs and probes specific to both HBV DNA and HBV Quantitative Standard (QS) DNA. Equivalent quantitation of all genotypes of Hepatitis B is ensured by the Master Mix reagent (S Hochberger et al., 2006). The CAP/CTM 48 analyzer amplifies the amount of DNA according to the number of cycles preprogrammed into the CAP/CTM 48 analyzer. Amplification occurs only in the region of the HBV genome between the primers; the entire HBV genome is not amplified. The use of dual-labeled fluorescent probes allows for real-time detection of PCR product accumulation by monitoring the emission intensity of fluorescent reporter dyes released during the amplification process.

Statistical Analysis

The secondary data of patients was also collected and assessed for completeness, intelligibility, and consistency. The information was recorded and a descriptive analysis was done to examine the prevalence of HBV in the studied population. Differences in prevalence were determined using the x2 test. An estimation of a p value ≤ 0.05 was considered significant. The Chi-square analysis was used to access statistically significant results and for comparing variables. The level of significance was set at $p \leq 0.05$.

Results

HBV Prevalence

One hundred and seventy-six seropositive samples were tested for HBV Qualitative DNA analyses by automated real time PCR. Among them, 144 (81.8%) samples were confirmed as HBV positive and 32 (18.2%) samples did not have HBV DNA.

District-wise prevalence of HBV infection w.r.t. literacy rate

The recorded literacy rate among individuals diagnosed with Hepatitis B virus (HBV) stood at 35.4%, as highlighted by the data analysis conducted on the studied participants. The distribution of literacy rates across different districts is visually represented in Figure 2, offering a comprehensive view of the educational landscape in relation to HBV prevalence. Most illiterate people were more susceptible to HBV (64.6%). Notably, the majority of HBV-positive subjects in the investigated region primarily hail from rural areas. The rural population faces significant challenges as essential health services, such as basic health units (BHUs) or hospitals, are either situated at a considerable distance or maintain suboptimal hygienic standards.

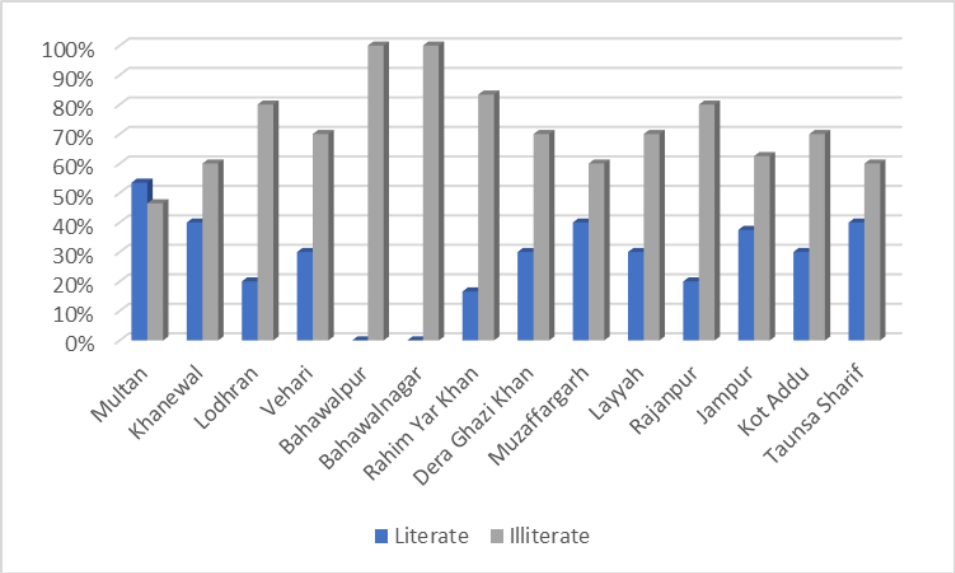

Figure 1: Prevalence of HBV w.r.t. literacy rate

While certain regions boast well-equipped private hospitals, their benefits remain largely inaccessible to the common populace due to financial constraints. The prevalence of HBV in the studied areas can be attributed not only to the lack of medical infrastructure but also to a broader issue encompassing insufficient knowledge about disease risk factors and its prevention.

Gender-wise prevalence of HBV

The real-time PCR confirmed 105 male and 39 female patients suffering from hepatitis B infection (Table 1). The initial screening as well as the confirmatory approach adopted have pointed out the high prevalence of HBV in the male population as compared to the female population. This gender-wise association of positive cases in the population, however, has a non-significant association ($P = 0.129$).

Prevalence of HBV infection in different Age groups (<25 years; 26-50 years; 51-75 years)

The molecular data confirmed 50 (92.6%) positive cases in the less than 25 years age group. Among 26-50 yrs age group, the number of positive cases were 69 (75.8%) while 51-75 years age group had 25 (80.6%) HBV positive individuals (Figure 4). The prevalence of HBV was significantly higher in the <25 years age group than in the other two age groups ($P<0.05$) (Table 1). The

incidence of HBV declines with increasing age, as shown by the age group (51 to 75 years). The younger age group had a higher HBV-infection percentage than the lower and older age groups.

HBV prevalence w.r.t. marital status of the patients

Out of 176 seropositive subjects, 40 were single patients and 136 were married. 34 (85%) patients having single status were having HBV. In the case of married individuals, 110 (80.9%) patients were HBV positive and 26 (19.1%) were HBV negative (Table 1). The prevalence of HBV in married people (80.9%) has a non-significant correlation with single ones.

Table 1: Prevalence of HBV w.r.t Gender, Age, and Marital Status

Factor	Patients	Total subjects	HBV DNA Detected	
Gender	**Male**	124	105	**P=0.129**
	Female	52	39	
Age Wise	**<25 years**	54	50	**P=0.04**
	26 – 50 years	91	69	
	51 – 75 years	31	25	
Marital Status	**Single**	40	34	**P=0.55**
	Married	136	110	

Prevalence of HBV in both genders w.r.t. viral load (high viral load >170000000µl and low viral load <20µl)

Out of 39 HBV positive female patients, 3 (7.7%) had a high viral load, 31 (79.5%) patients had a moderate viral load and 5 (12.8%) had low viral load. In 105 male subjects, 4 (3.8%) had high viral load, 78 (74.3%) had moderate viral load and 23 (21.9%) had low viral load (Table 2). The prevalence of low, moderate, and high viral loads in males was not significantly different from that in females (P= 0.334). A high viral load could be related to the emergence of severe liver cirrhosis and HCC.

Table 2: Prevalence of HBV in both Genders w.r.t Viral Load

Gender	HBV positive patients	High viral load	Low viral load	Moderate viral load
Male	105	4	23	78
Female	39	3	5	31

Risk factors linked to the transmission of HBV infection

The HBV-positive cases were more exposed to different kinds of risk factors, which we have tried to identify on the basis of the information collected from them. Age has been found to be a major determinant in predisposing an individual to a particular risk factor. In individuals under the age of 25, risk factors for hepatitis B primarily revolve around lifestyle choices and behaviors associated with increased exposure to the virus. High-risk activities such as unprotected sexual intercourse, frequent visits to barbers, dental treatments, ear and nose piercing, skin tattooing, sharing needles during drug use, or engaging in activities that involve close contact with infected blood or body fluids elevate the chances of contracting Hepatitis B in this age group. For individuals aged 26 to 50, risk factors broaden to include not only high-risk behaviors but also occupational exposure and healthcare-related risks. Healthcare workers, emergency responders, and individuals in close-contact professions are at increased risk due to potential exposure to infected blood or body fluids. The behavioral pattern associated with exposure to viruses in this age group was found to be less than that of the younger (<25 yrs) age group. In individuals aged 51 to 75 years, several risk factors contribute to the susceptibility and severity of hepatitis B infection. One significant factor is the cumulative impact of aging on the immune system, leading to a decreased ability to mount a robust response against the virus. Chronic conditions such as diabetes, which may become more prevalent in this age group, can further compromise the immune system and increase the risk of chronic hepatitis B infection. Additionally, lifestyle factors such as a history of high-risk behaviors in earlier years, including unprotected sexual activity or intravenous drug use, may persist as latent risks for Hepatitis B transmission. Furthermore, the potential for healthcare-associated exposures, including

medical procedures, surgeries, and interventions, become more pertinent in this age group, emphasizing the importance of vaccination and regular screening to mitigate the risks associated with Hepatitis B. Household contact may be a big reason to get infected by HBV, as aged people have more involvement in household chores and activities. The information gathered from patients and the correlation of associated factors has been shown in the table below.

Table 3: Risk Factors associated with transmission of HBV

Risk Factors	HBV %age
Barber Risk	16%
Dental Treatment	11%
Skin tattooing	2%
Household contact	20%
Sharing personal stuff	29%
Sexual contact	7%
Surgery	9%
Ear and Nose Piercing	6%

Discussion

HBV infection is a worldwide health issue and leads to hepatocellular carcinoma (HCC) and cirrhosis (Idrees et al., 2010). There are few treatment choices for patients with advanced HCC, which is the fifth most frequent malignancy globally and the third leading cause of cancer-related deaths (Rasheed et al., 2017). Over the past years, many serological surveys for HBV and its markers have been done throughout Pakistan. In our study, patients of both genders and different marital statuses detected and screened on the basis of serology were followed and confirmed for the presence of virus via real-time PCR.

Gender-wise distribution showed males infected with HBV in a higher number as compared to females. Awan et al., also found that males (70.43%) had a higher prevalence of HBV than females (29.57%) (Awan et al., 2012). A similar higher incidence of the hepatitis B virus was reported in the male population in studies conducted indigenously (Khan, Shams, et al., 2011; Zubair et al., 2010). This study also coincided with the findings of Riccardo Nevola et al., who showed that males have a higher incidence of HBsAg (1.8%) than females (1.2%; P=0.373) (Nevola et al., 2022). Fusco et al. found more HBsAg-positive in males as compared to females (OR = 0.6) (Fusco et al., 2008). Males are more likely to be infected with HBV due to their frequent contact with multiple HBV transmission factors, such as the use of drugs, blood transfusions, and frequent visits to barber shops, since barbers lack enough awareness about HBV infection and maintain an unsanitary business environment. In countries like Pakistan, where the male population is mostly responsible to do jobs and businesses, they are therefore more prone to be infected with HBV. Males frequently remain outside their home for business, and other activities, but females generally remain inside a house and are thus less prone to HBV infection to some extent since they have a low probability of interaction with HBV risk factors (Loomba and Liang, 2017; Watashi et al., 2014). Additionally, females have more intense innate, cellular, and humoral immune responses to combat viral infections than male patients. The sex hormones of both genders regulate immunogenicity to viruses in various ways due to their particular binding to specific hormone receptors present on immune cells. Generally, estrogens improve the immune system, whereas androgens suppress it (Montella et al., 2015; Ruggieri et al., 2018). But females are also susceptible to HBV infection, as they can get it via sexual contact, visiting beauty parlors and saloons and, to a minor extent drug usage as well. One of the risk factors for HBV might be that some females share their jewelry items also.

The younger age-group (<25 years) was found to have a higher rate of HBV infection than the other two categories. The higher prevalence among young individuals might be due to their more frequent exposure to HBV risk factors or prolonged disease (Khan, Akbar, et al., 2011a). Younger people are more likely to be involved in sexual activities, sharing stuff with friends and partners and this may be because of their frequent visits to unhygienic and unsafe barbershops or beauty salons (Samo et al., 2020). According to the age-wise distribution, the prevalence of HBsAg in age groups: <30

years was 7% (n = 10/138), 31-40 years was 5% (n = 10/195), 41-50 years was 13% (n = 15/116), and 51-60 years was 13% (n = 10/75), as investigated by Ayaz Ali Samo et al. in 2020 (Samo et al., 2020). Fawad Khan et al. (2011) found that the age group (46-60 years) had the greatest incidence rate of 29.13%, while the age group (<15 years) had the lowest incidence rate of 11.97% (Khan, Akbar, et al., 2011b).

This study reported a higher incidence of HBV in singles than married ones. A similar higher pattern was observed in previous findings as well. Considering that unmarried (single) people frequently engage in unprotected sex or have multiple sexual partners, this may imply that marital status is indeed a risk factor for HBV infection. Additionally, the presence of HBsAg in the married individuals in this study may be a sign that they acquired the virus through unprotected heterosexual sex or close contact with their spouse (Onu et al., 2023). A study in 2007 reported that HBV infection was higher in singles as compared to married patients (OR: 2.13; 95% Cl: 1.29-3.5) (Gholamreza et al., 2007). In another study, HBV prevalence w.r.t. marital status was highest among singles (26.6%) as compared to married (7.4%), divorced (8.5%) and widows (0%) (Onu et al., 2023). Unlike this study, it was discovered that married people had a higher HBV infection (P = 0.001) than singles or divorced people in Kogi state, Nigeria (Sule et al., 2011). There was a study that found married participants had a higher prevalence than single people (Motayo et al., 2015). Sujan Banik et al., 2022 discovered that married people were substantially more likely to have hepatitis B than unmarried or single people (OR 2.16, 95% CI 1.51, 3.10, P 0.0001) (Banik et al., 2022).

Intermediate levels of viral load were found in most patients, irrespective of gender. The variations in the viral load in both males and females are more likely influenced by factors such as the individual's immune response, coexisting medical conditions, genetics, and the specific stage of the infection rather than being predominantly determined by gender. It is essential that both males and females receive adequate therapy and regular monitoring under the supervision of healthcare specialists to properly control HBV infection. Studies have found that having a high amount of HBV in the blood can lead to severe liver problems, as it has been known that the viral load of HBV can remain constant over time until HBeAg seroconversion. Two pivotal studies revealed HBV viral load is associated directly with serious liver conditions—HCC and cirrhosis. These large-scale investigations in seven Taiwanese townships focused on chronic hepatitis B. The research established a significant correlation between HBV viral load and the development of cirrhosis and HCC (Sung et al., 2009; Wu et al., 2008). This study assessed the correlation between the risk of HCC and the serum HBV viral load. The HBV viral load was found to be an autonomous susceptibility for HCC, with a link between rising viral load and occurrence of HCC, even after controlling for other risk variables such as age, cirrhosis, and HbeAg status. Over a follow-up time period of up to 13 years, the cumulative incidence of developing an HCC varied from 1.3% in patients with a viral load of less than 300 copies/mL to 14.9% in patients with a viral load of at least 10^6 copies/mL (C J Chen et al., 2006). The second study by Iloeje et al., involving 3,582 untreated patients over an 11-year follow-up, revealed a clear association between higher HBV viral load at the beginning of the study and an increased incidence of liver cirrhosis, independent of serum ALT levels and HBeAg status. The occurrence of cirrhosis ranged from 4.5% in patients with a viral load less than 300 copies/mL to 36.2% in those with a viral load exceeding 10^6 copies/mL (Iloeje et al., 2006). From these studies, it is clear that a higher HBV-viral load is linked to a worse prognosis for liver disease caused by HBV if it remains untreated and is also associated with high mortality rates (G Chen et al., 2006; Iloeje et al., 2007; Yeo et al., 2007).

People are unaware of the risk factors exposing them to HBV in areas of southern Punjab, Pakistan. The major causes of this unawareness are the public’s unfamiliarity with the symptoms and transmission risk factors of these transmissible diseases and a lack of routine checkups. The previous published studies reported that Pakistan is highly endemic to HBV, with over 12 million infected people. And the areas of southern Punjab are also unlucky, because the prevalence is high

(Mirza et al., 2007). It is because of the greater number of patients and the huge population and area, and a lot of patients came to hospitals for their routine checkups (Janahi, 2014).
Dental treatments involving unsterilized equipment most of the time by non-professional diploma holders, and the reuse of razor, blades, utensils, etc. by barbers, which may not be considered dangerous due to ignorance and poverty, are also major risk factors for these diseases, particularly in rural areas. In addition, according to the WHO, an average person in Southeast Asia receives four infusions each day, a large portion of which is unnecessary and up to 75% is harmful or reused. Unnecessary infusions are frequently given in Pakistan due to the inevitable public belief that implanted treatments are more effective than oral drugs (Shah et al., 2013).
To conclude, our research presented information on the epidemiology of HBV infection among residents of southern Punjab, Pakistan. HBV infection is still a major health issue in this region, requiring public health initiatives. According to this study, the population of southern Punjab is infected with HBV due to a lack of adherence to proper general instructions and SOPs. The Ministry of Health must initiate a vaccination program in these areas' government and private hospitals. A national strategy for hepatitis B elimination is essential; however, it is not found in a third-world country like Pakistan.

Conclusion

Males have a higher incidence of HBV when compared to females in the studied population. This could be because men have more societal authority and participate in various activities than women. The HBV prevalence in single patients was higher than in married persons because of a lack of sex education. A higher viral load of HBV is related to a prognosis for severe liver disease caused by HBV if it is untreated and is also related to high mortality rates. The Southern Punjab population of Pakistan has a high prevalence rate of HBV due to a lack of education, a lack of effective blood screening facilities, poverty, and ignorance of HBV risk factors. The high frequency of HBV-infection in the studied area is of concern, highlights the need for taking preventative measures. It is proposed that more initiatives should be taken to educate the general population and untrained health workers about the adverse outcomes of HBV and how it transmits. A significant number of preventative measures should be employed during operational procedures and thorough monitoring during blood transfusion. The low, high, and moderate viral loads were not significantly different in both males and females. All the hospitals should implement proper infection control strategies. Furthermore, all clinically diagnosed patients should be tested for HBV serology to prevent mortality and morbidity.

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