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PREVALENCE OF HEPATITIS B IN THE PATIENT COMING TO PATHOLOGY LABORATORY AT COMBINED MILITARY HOSPITAL (CMH) LAHORE FROM PUNJAB

[1]Sada e Batool, [2]Dr. Shakeel Mazhar, [2]Dr. Affaf Ahmad, [2]Dr. Muhammad Bilal
[1]GCU Lahore
[2]Services Hospital Lahore

Abstract:
Introduction; Hepatitis is a swelling or infection of the liver that makes it to stop work properly. Most of the times, viruses are the major cause of hepatitis but drug or alcohol use can also lead to hepatitis. In some cases, our body mistakenly attacks its own body tissues and leads to disease. Hepatitis B is a major health problem in Pakistan, India and all over the world.
Objective: To estimate the prevalence rate of hepatitis B infection among the patients of Punjab.
Result: ELISA and PCR techniques were used for the analysis of blood samples of the patients to determine the prevalence of Anti-HBV antibodies and HBV-DNA respectively. During the 5 month duration of my work 8000 blood samples were taken to find out the prevalence of hepatitis among the patients coming to Pathology Laboratory (CMH) Lahore. The analysis of the data showed that, total 8,000 blood samples taken from the people; 200 were HBV positive, 148 (74%) blood samples were detected to be positive for HBV in males and 52 (26%) in females by ELISA method.
Keywords: *ELISA and PCR techniques, Hepatitis B, Anti-HBV antibodies.*

Corresponding author:
Sada e Batool,
GCU Lahore

QR code

INTRODUCTION:

Hepatitis is a swelling or infection of the liver that makes it to stop work properly. Most of the times, viruses are the major cause of hepatitis but drug or alcohol use can also lead to hepatitis. In some cases, our body mistakenly attacks its own body tissues and leads to disease. Hepatitis B is a major health problem in Pakistan, India and all over the world. Hepatitis B is a potentially life threatening liver infection caused by the hepatitis B virus. Hepatitis B infection can be spread through having contact with the blood, semen, vaginal fluids, and other body fluids of someone who already has a hepatitis B infection. It has been found that the hepatitis B virus can be passed to an infant during childbirth if the mother is infected (Dienstag, 2008). HBV belongs to the family hepadnaviruses. The HBV genome is a relaxed circular, partially double stranded DNA of approximately 3,200 base pairs (Seegar and Mason, 1988). Viral proteins include the envelope protein, hepatitis B surface antigen (HBsAg); a structural nucleocapsid core protein, hepatitis B core antigen (HBcAg); and a soluble nucleocapsid protein, hepatitis B e antigen (HBeAg). Serum HBsAg is a marker of HBV infection, and antibodies against HBsAg signify recovery (Dienstag, 2008).

Early chronic HBV infection is associated with the presence of hepatitis B e antigen (HBeAg) and high serum HBV DNA levels. A National Institutes of Health (NIH) workshop defined three stages in the natural history of chronic hepatitis B infection (Lok, 2000). The first stage is characterized by a period of immune tolerance, lasting 2–4 week in healthy adults but often several decades in those infected neonatally or in early childhood (Lee, 1997). During this phase there is active viral replication, evidenced by secretion of HBeAg and high levels of serum HBV DNA, but no symptoms and no significant increase in serum alanine aminotransferase (ALT). An immunologic response may then develop during which HBeAg is still secreted, but serum HBV DNA levels decline as the number of infected cells decreases. This stage lasts a few weeks in patients with acute, symptomatic infection, but may persist for 10 or more years in those with chronic disease, potentially leading to cirrhosis and its complications. In second phase, this immunoactive phase is characterized by increase in serum ALT, with more frequent flares being associated with greater likelihood of progressive disease (Brunetto *et al*., 2002). In the third phase, the host immune response halts active viral replication; patients become HBeAg-negative and anti-HBeAg antibodies become

detectable (seroconversion). This phase is usually preceded by a marked reduction in serum HBV DNA to levels undetectable by hybridization techniques, followed by normalization of serum ALT and resolution of liver necroinflammation (Fattovich, 2003). Around 50% of patients clear HBeAg within 5 yr of diagnosis, 70% within 10 yr (Fattovich, 2003).

Immune system efforts to clear the infection result in elevated alanine aminotransferase (ALT) levels, as HBV-infected hepatocytes are destroyed during the inflammatory response to HBV infection. Persistent necroinflammatory liver injury can lead to the principal complications of chronic HBV infection; decompensate cirrhosis and related liver failure. In addition, HCC can develop during HBV infection (Rustgi *et al*., 2010).

Prenatal infection of infants from infected mothers and horizontal infection early in childhood from exposure to HBsAg-positive family members are the main routes of HBV transmission in highly endemic areas, such as Southeast Asia, Africa, the Pacific Islands, and the Arctic. In regions of low endemicity, such as Western countries, hepatitis B is primarily a disease of adolescents and adults as a result of high-risk sexual behavior and injection drug use. HBV infection is a dynamic process with replicative and nonreplicative (or low replicative) phases based on virus-host interaction. The presence of circulating HBsAg, hepatitis B e antigen (HBeAg), and high levels of serum HBV DNA characterizes the immunotolerant phase. This first phase is seen in patients with perinatal infection and often lasts for decades. During this phase patients have no symptoms, normal or slightly increased serum alanine aminotransferase (ALT) levels, and minimal histological activities, which imply that there is a lack of or a very weak immune response against the infected hepatocytes (Sharma *et al*., 2005).

Acute Hepatitis B

The incubation period of HBV ranges from 2 weeks to 4 months. Initially, patients complain of fatigue, malaise, anorexia, right upper quadrant discomfort, or flu-like symptoms (coryza, photophobia, headache, and myalgia); then jaundice becomes apparent, usually within 10 days of the onset of symptoms. Low-grade fever, jaundice, and mildly tender hepatomegaly are the most common signs. In the acute phase, ALT and AST levels rise. Although the peak ALT level reflects the hepatocellular injury, it has no prognostic value. With recovery, ALT levels normalize in 1 to 4 months (Schiodt *et al*., 1999).

Chronic Hepatitis B

Chronic hepatitis B is usually diagnosed as a result of a workup for abnormal liver function tests or as a result of screening patients at risk for HBV infection. Many patients with chronic hepatitis B have no symptoms or have nonspecific symptoms such as fatigue or right upper quadrant discomfort. Acute exacerbations due to HBV e antigen seroreversion (in which e antigen reappears) occasionally occur in patients with chronic hepatitis B. Most of these exacerbations are asymptomatic, but occasionally an acute hepatitis-like clinical picture with detectable IgM antibody against the core antigen occurs, leading to misdiagnosis of acute HBV infection in patients not previously known to have chronic HBV infection (Chu *et al*., 1989). In late cases, signs of cirrhosis such as jaundice, ascites, splenomegaly, pedal edema, encephalopathy, or variceal bleeding can be present. Hepatocellular carcinoma should be suspected in cirrhotic patients with new-onset right upper quadrant pain, rapidly developing ascites, a palpable liver mass or hepatic encephalopathy. Other nonspecific features of hepatocellular carcinoma include watery diarrhea, hypoglycemia, and certain cutaneous manifestations such as acanthosis nigricans. In chronic hepatitis B, liver enzyme levels can be normal, even in patients with well compensated cirrhosis. ALT levels may range from normal to five times higher than normal. The most characteristic histologic feature of chronic HBV infection is the "ground-glass hepatocyte," which is due to intracellular accumulation of HBV surface antigen (Gerber *et al*., 1974).

The hepatitis B virus is passed from person to person as a result of:

•   Blood to blood contact. For example, drug users sharing needles or other equipment which may be contaminated with infected blood. (Blood used for transfusion is now screened for hepatitis B virus.)

•   Having unprotected sex with an infected person.

•   An infected mother passing it to her baby.

•   A human bite from an infected person. This is very rare.

Although the overall incidence of hepatitis B virus (HBV) has declined since the introduction of universal vaccine guidelines, the incidence remains elevated in high risk groups. Recent guidelines from the Centers for Disease Control (CDC) have underscored the importance of vaccination against HBV in high risk individuals. However, the incidence of HBV in this group remains elevated, suggesting under use of vaccinations by healthcare providers (Foster *et al.,* 2011).

Eight genotypes of HBV (labeled A though H) have been identified. All eight have been found in the United States but genotype A accounts for 35% of cases, genotype B for 22%, and genotype C for 31% (Chu *et al*., 2003). Pakistan is highly endemic with HBV with nine million people infected with HBV and its infection rate is on a steady rise. The reason may be the lack of proper health facilities, poor economical status and less public awareness about the transmission of major communicable diseases including HBV, HCV and HIV. The clinical significance of HBV genotypes is not as clear as that of hepatitis C virus genotypes. Although recent data have suggested that different HBV genotypes may be associated with different rates of progression of liver disease and different rates of response to interferon therapy (Norder, 2004); these data were not enough to recommend routine testing for HBV genotypes in clinical practice (Lok, 2007). In HBV infection, the virus itself does not injure liver cells. Rather, the damage of hepatitis is immune-mediated and begins to appear as the host's immune system attempts to clear the virus.

The risk of HBV transmission from needle stick is 1% to 6%; if the blood is positive for HBV surface antigen but negative for HBV e antigen, and 22% to 40% if positive for both antigens (Mast, 1993, Werner 1982 and Gerberdind, 1995). Saliva, nasopharyngeal fluid, breast milk, semen, urine, and cervical secretions can also harbor HBV (Kidd, 2006).

Most cases of hepatitis B virus (HBV) infection acquired in adulthood resolve spontaneously within 6 months. In contrast, most infections acquired at birth or in early childhood persist and become chronic. HBV produces several proteins (antigens) that can be detected in the blood and that disappear as the body produces antibodies against them. The patterns of these and other markers provide clues to the phase of infection. Surface antigen and HBV DNA are often the first detectable markers of acute infection, appearing before the onset of symptoms or elevation of alanine aminotransferase (ALT).By definition, HBV infection is chronic if surface antigen persists longer than 6 months. HBV e antigen, derived from HBV pre-core protein, is considered a marker of HBV replication and infectivity. In chronic infection, e antigen can persist for years or decades. HBV core antigen cannot be detected in the serum, but antibodies against it can: first immunoglobulin M (IgM) and later immunoglobulin G (IgG) (Chu *et al*., 1989).

Our purpose of research is the data analysis of hepatitis B among patients' of different age groups belonging to Punjab. A person who is infected with hepatitis B virus can have up to a billion viral copies per drop of blood. To cure a patient, a drug needs to reduce those levels to zero. While existing medications are very powerful, they cannot quite deliver the knockout punch to hepatitis B. The drugs approved to treat the virus can reduce its numbers, make symptoms disappear for years and push it to the brink of extinction. But for most people, the medications can't kill the virus completely. And, as long as any virus remains, it can multiply and grow strong again (John, 2013).

Aims and objectives:
The present study, prevalence of hepatitis B in the patient coming to pathology laboratory at Combined Military Hospital (CMH) Lahore from Punjab, deals majorly with risk factors of hepatitis B. The major objectives of the study were as follows:

To estimate the prevalence rate of hepatitis B infection among the patients of Punjab.

To determine the ratio of male and females, that are positive for hepatitis B virus infection coming to pathology laboratory in (CMH) Lahore.

To evaluate the percentage prevalence of Hepatitis B among patients of different age groups.

LITERATURE REVIEW
Hepatitis B virus (HBV) infection is a wide and major global health problem especially in Asia, Africa, southern Europe and Latin America. About 2 billion people are infected with HBV worldwide and 400 million among them are suffering from chronic HBV infection. Pakistan is highly endemic with HBV with nine million people infected with HBV. The infection rate is on a steady rise. The reason may be the lack of poor economical status, less proper health facilities and   less public awareness about the transmission of HBV, HCV and HIV (Ali *et al*., 2011).
Kaur *et al*. (2000) study showed that in the United States (US), there are an estimated 1-1.25 million chronically infected persons. But HBV infections are most often associated with groups at high risk of intravenous drug users, sexually transmitted diseases, prisoners and other intimate contacts (Alter and Shapiro, 1998). The infections HIV, HBV and HCV were the most important that could be acquired through blood. Many rapid testing kits for these types of infections were available in the market, based on immunofilteration and immunochromatographic

techniques. Though ELISA was recommended and preffered screening technique in many blood banks and laboratories. In the laboratory for emergency services rapid detection kit for HIV, HBsAg and HCV are used.
Bernard *et al*. (2011) reported that in Ghana, HBV infections occurrence among blood donors in a rural areas was determined by using serological methods and the results were analyzed to assess the trends in three consecutive years, 2006, 2007 and 2008. The prevalence of viral carrier rates in the blood donors appears to be different to that of urban blood donors. Simultaneous increase of HBV and HCV infections might have been caused by sexual transmission through unprotected sex and other practices. The decreasing rate of positivity to HBV suggests that the transmission is horizontal rather than vertical. In areas of low endemicity, there is an age effect on the prevalence of HBV infections. Horizontal transmission of HBV has been related to age, socioeconomic conditions, socio professional status and risky behaviors such as sharing of bath towels,

partially eaten candies, chewing gum or dental cleaning materials, as well as biting fingernails in conjunction with scratching the backs of carriers. It has been shown that the improvement of socioeconomic conditions may lead to a decreased exposure to HBV infections thus an increased risk of HBV infections might be related to an increased exposure to risk factors in conjunction with poor sanitary and socioeconomic conditions. The decreasing trend of HBV and HCV infections in our study population might be due to decreased exposure to risk factors in conjunction with improving sanitary and socioeconomic conditions.

Zeinab *et al*. 2013 conducted a study in Egypt to determine the prevalence of anti-hepatitis B core antibody (anti-HBC) positivity in blood donors, highlight the residual risk of transmitting HBV in blood banks through blood transfusion and determine whether routine anti-hepatitis B core antigen screening of blood donations provides any concrete benefits with regard to HBV transmission risk reduction. The study was undertaken on 3167 blood donors negative for anti-hepatitis C virus (HCV), anti-human immunodeficiency virus (HIV) and HBsAg: 16.6% of blood units were positive for total anti-core, of these 64% were anti-HBsAg positive. Results showed that: anti-core screening would possibly eliminate the risk of unsafe blood donation; nucleic acid amplification should be considered as the primary screening method for high risk recipients.

In Turkey the prevalence of hepatitis B surface antigen (HBsAg) is less than 10% that shows that our country is within the moderately endemic region (Doganci *et al*., 1992). The horizontal transmission is very common in highly populated or moderate endemic countries and lead to acute form of hepatitis B (Erol *et al*., 2003).

According to the study that was carried out at Division of Infectious Diseases and Molecular Diagnostics, Centre for Applied Molecular Biology, Ministry of Science and Technology, Lahore, Pakistan from May 2002 to February 2004. HBV genotypes were determined in 112 HBV DNA positive sera by using simple and precise molecular genotyping system based on PCR using type-specific primers for the determination of genotypes of HBV A through H. Four genotypes (A, B, C and D) out of total eight reported genotypes so far were identified. Genotypes A, B and C were predominant. HBV genotype C was the most predominant in this collection, appearing in 46 samples (41.07%). However, the genotypes of a total of 5 (4.46%) samples could not be determined with the present genotyping system. Mixed genotypes were seen in 8 (7.14%) HBV isolates. Five of these were infected with genotypes A/D whereas two were with genotypes C/D. One patient was infected with 4 genotypes (A/B/C/D). Genotype A (68%) was predominant in Sindh; genotype C (68.96%) was most predominant in Khyber Pukhtoonkhwa (68.96); whereas genotypes C and B were dominant in Punjab that is 39.65% and 25.86% respectively. All the four common genotypes of HBV found worldwide (A, B, C and D) were isolated (Idrees *et al*., 2004).

HCC (Yuen *et al*., 2001 reported that the two established agents for the treatment of chronic hepatitis B are interferon (IFN)-α and lamivudine. Adefovir dipivoxil has also been licensed recently in the USA and in Europe. Their main modes of action are immunomodulation and direct suppression of viral replication. IFN-α induces HBeAg seroconversion in ~20–30% of patients. The proportion achieving HBeAg seroconversion is lower in Asian patients, who mostly acquire the infection at birth or early in life and consequently have a long period of immunotolerance. More importantly, IFN-α treatment in Asian patients does not prevent the occurrence of cirrhosis related complications and). The substantial side effects during treatment and the possibility of causing hepatic decompensation in patients with pre existing cirrhosis also limit the use of IFN-α.

HBV resistance is the main limitation of long-term therapy with oral HBV polymerase inhibitors, which represent the most common approach in the treatment of chronic hepatitis B. The complete suppression of HBV replication minimizes the risk of resistance and therefore close monitoring with sensitive HBV DNA determinations at least every 6 months is required. Lamivudine monotherapy has the highest risk of selecting resistant mutations compared with other anti-HBV agents and is not currently considered as an optimal first-line treatment. Adefovir has a similar profile but less potency than the other nucleotide analog, tenofovir, whereas telbivudine selects for lamivudine resistance mutants and therefore its place is currently unclear. Entecavir and possibly tenofovir are the two most potent anti-HBV agents with the best resistance profile in nucleo(s) tide-naive patients, while tenofovir represents the optimal treatment for patients with lamivudine resistance. Combination of two agents without cross-resistance should be used in any patient with HBV resistance (Papatheodoridis and Deutsch, 2008).

HBV is a small circular DNA genome that is partially double-stranded, HBV contains numerous antigenic components, including HBsAg, hepatitis B core antigen (HBcAg), and hepatitis B e antigen (HBeAg). Humans are the only known host for HBV, in some cases nonhuman primates have been infected in laboratory conditions. Hepatitis B virus exists in eight different genotypes (A-H) and its prevalence differs with differs by geography and ethnicity. Ten different studies conducted at different regions of Pakistan showed that the most prevalent HBV genotype in Pakistan is genotype D with overall prevalence rate of 63.71% followed by genotype A (10.036%), genotype C (7.55%) and genotype B (5.335%) while mixed genotypes were 2.377% and 9.931%, respectively (1drees *et al*., 2011).

Awan et al. 2011 conducted most detailed and recent study showed that the most emerging and common genotype in Pakistani population is genotype C with the prevalence rate of 27.66%, which is a bad news as it is more common in cirrhotic patients and is associated with more severe form of liver diseases. Moreover, Previous studies also shows that genotype D have more severe disease, and less responsive to interferon therapy as compared to genotype A and B and have higher HBV DNA levels. This genotype also has specific viral sequence patterns that may predict long term response to lamivudine treatment. However, further studies are needed to characterize prevalence of different genotypes, their relative severity and treatment response rates in Pakistani population (Ali *et al*., 2004).

Most of the laboratories in Pakistan have all the necessary facilities for conducting research on

hepatitis. These are comparable to the laboratories in the developed countries. Pakistan uses reagents and kits in the Public Health Pathology laboratories that are imported and we have not been able to produce the immunological and biochemical reagents locally. The preparation of Immunological reagents is a time consuming process. Locally produced anti-sera by our own purified strains would be more specific and would produce 'more sensitive reagents for diagnostic purposes. Imported kits nowadays are very expensive; for example an ELlSA kit for the detection of HBsAg from ABBOTT laboratories at present costs Rs. 18000/- for 100 tests. The cost/test comes to at least Rs. 250/-. In comparison our own produced standard ELlSA would cost about 1/10 of the price of the imported kit. This was one of the objectives that kept in mind to develop ELlSA for detection of HBsAg. The reagents produced were compiled in the form of kit with all the necessary buffers needed for the test (Kazmi, 1997).

MATERIALS AND METHODS:

Specimen collection

Pathology laboratory technicians at CMH helped to draw the blood for sampling from patients. Blood was collected under strict biosafety conditions. Blood was drawn from the vein by using disposable syringes (5 ml), usually from inside of the elbow. Most syringes come pre-packaged and contain a small amount of heparin, to prevent coagulation. Either serum or plasma can be used for test. Whole blood specimens should be separated from red blood cell as soon as possible in order to avoid hemolysis. Also clot must be removed.

After collecting the blood samples from patients, the next step was the separation of plasma or serum from the blood samples as soon as possible to avoid hemolysis. For this purpose blood was centrifuge in centrifuge machine at 3000-4000 rpm for about 5-10 minutes. After centrifugation, the light straw colored serum was separated and then transferred into the disposable serum cup with the help of sterilized micropipettes as shown in the Fig: 1

Fig: 1. Specimen collection and serum preparation after centrifugation

Storage

• Stored all components at 2-8 °C. Do not freeze. Avoid strong light.

• Placed unused wells in the zip-locked bag with desiccant provided, then seal zip-lock bag in the aluminum foiled pouch with a plate lid and return to 2-8 °C, under which conditions the wells will remain stable for 2 months, or until the labeled expiry date, whichever is earlier.

• Sealed and returned all the other unused reagents to 2-8 °C, under which conditions the

stability will be retained for 2 months, or until the labeled expiry date, whichever is earlier.

Immunoassay

HBsAg ELISA

Principle

This assay is based upon the one-step sandwich method. Sample, Anti-HBs coated microplate and enzyme-labeled Anti-HBs are combined. During the incubation, HBsAg present in the sample is allowed to react simultaneously with the two antibodies, resulting in the HBsAg being sandwich between the solid phase and the enzyme-linked antibodies. After

washing, a complex is generated between the solid phase, the HBsAg within the sample and the antibody in enzyme conjugate by immunological reactions. Substrate A and B are then added and then catalyzed by this complex, resulting in a chromogenic reaction. The resulting chromogenic reaction is measured as absorbance. The color intensity is proportional to the amount of HBsAg in the sample.

Components and reagents of the kit

1.   Coated wells

1 plate of 96 wells pre-coated with mouse monoclonal Anti-HBs.

2.   Enzyme conjugate

1 vial containing 7.5 ml of HRP (horseradish peroxidase) labeled sheep polyclonal Anti-HBs in a buffer containing BSA (bovine serum albumin).Contains 0.1 % Proclin 300 preservative.

3.   Negative control

1 vial containing 1 ml of phosphate buffered solution containing proteins of bovine origin. Contains 0.1 % Proclin 300 preservative.

4.   Positive control

1 vial containing 1 ml of phosphate buffered solution containing heat- inactivated human plasma positive for HBsAg and protein of bovine origin. Contains 0.1 % Proclin 300 preservative.

5.   Stop solution

1 vial containing 7.5 ml of 0.62mol/l sulfuric acid.

6.   Substrate A

1 vial containing 7.5 ml of hydrogen peroxide.

7.   Substrate B

1 vial containing 7.5 ml of TMB (3, 3', 5, 5'-tetramethylbenzidine) in a buffer solution.

8.   Wash solution concentrate

1 vial containing 30 ml of 20 times working strength PBS-Tween wash buffer.

9.   1 copy of instruction for use
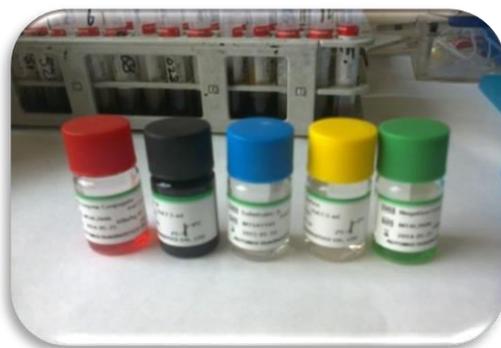
10.   1 piece of plate lid

11.   1 Zip-lock bag

Fig: 2 ELISA kit reagents for the detection of HBsAg

Materials

- Absorbent paper and paper towel
- Automated microplate strip washer
- Distilled water
- Disposable reagent troughs
- Incubator
- Magnetic stirrer
- Micropipettes and multichannel of appropriate of appropriate volumes
- Microplate
- Plate shaker

Reagent preparation

1.   Brought all reagents to room temperature (18-25°C) prior to use for at least 30 minutes. Mix all reagents through gently inverting before use.

2.   Adjusted the incubator to 37°C.

3.   Added 1 volume of wash solution concentrate to 19 volumes of distilled water to give the required volume, and mix well with a magnetic stirrer. The solution is stable at room temperature for 2 months.

Safety precautions:

1.   The positive control is made of HBsAg positive sera and has been heated at 60°C for 10 hours. This is generally a accepted method to inactivate the hepatitis agent. However, for safety precautions, it must be treated as potentially infectious material. Both positive and negative controls have been tested and found negative for HIV-1-2 and hepatitis C virus. However, they must be treated as potentially infectious agents.

2.   Do not smoke or eat where specimen or reagents of the kit are handled.

3.   Do not pipette by mouth. Wear PVC gloves when handling reagents kits or specimens and wash hands thoroughly afterwards. Non acid containing spill should be wiped up thoroughly with 5% sodium hypochlorite solution.

4.   All waste material should be properly disinfected before disposal. Both liquid and solid waste material can be autoclaved for 1 hour at 121°C. Solid waste material can also be incinerated. Non-acid waste material can be treated with sodium hypochlorite (bleach solution) diluted to a final concentration of 1.0%. Acid liquid waste material requires neutralization before similar treatments and should stand for 30 minutes to effective disinfections.

Procedure

1.   Used only the number of the wells required and format the microplates' wells for each control and sample to be assayed. Leaved well A1 as the blank well. To each plate added 50 µl of the negative controls to wells B1, C1, and D1 and 50 µl of positive controls to wells E1, F1. Added 50 µl of sample to each of the rest of the wells. (Do not blank well A1 when you plan to read the absorbance using a reference wavelength).

2.   Added 50 µl of enzyme conjugate to each well except the blank well.

3.   Shook a plate shaker for 30 seconds to completely mix the liquid within the wells.

4.   Covered the plate with a lid and incubate at 37°C for 30 minutes.

5.   Added 350 µl of wash solution, decant (tap and blot) or aspirate. Repeated 5 additional times for a total of 6 washes. An automated microplate strip washer can be used. At the end of washing, inverted the plate and taped out any residual wash solution onto absorbent paper.

6.   Added 50 µl of substrate A, then 50 µl of substrate B to each well, including the blank well.

7.   Gently mixed for 15 seconds and incubated at 37°C in the dark for 10 minutes without shaking.

8.   Added 50 µl of stop solution to each well, including the blank well and mix gently.

9.   Took the absorbance within 20 minutes at 450 nm (using a reference wavelength of 620-630 nm to minimize well imperfection) in a microplate reader. Alternatively, the actual absorbance of each well at 450 nm with the absorbance of the blank well at 450 nm. All steps are described in Fig: 3.
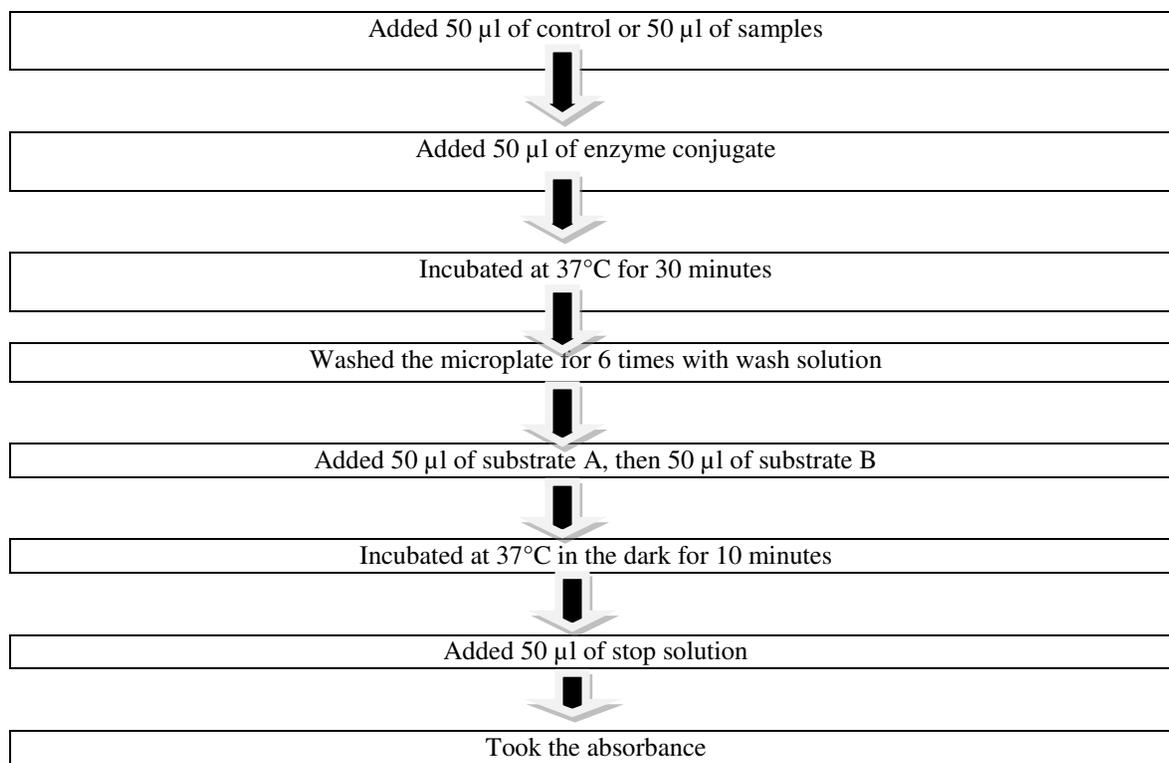
Procedural flow chart

Added 50 µl of control or 50 µl of samples

↓

Added 50 µl of enzyme conjugate

↓

Incubated at 37°C for 30 minutes

↓

Washed the microplate for 6 times with wash solution

↓

Added 50 µl of substrate A, then 50 µl of substrate B

↓

Incubated at 37°C in the dark for 10 minutes

↓

Added 50 µl of stop solution

↓

Took the absorbance

Fig: 3. Steps involved in performing the ELISA test

Measurement results

Each plate was considered separately when calculating and interpreting result of the assay.

Negative control

Calculated the mean absorbance of the replicates of the negative control

Cut-off value

The cut-off value is 2.1 times the mean of the negative control replicates (in case the mean absorbance of negative control replicates < 0.05, use 0.05 instead of the actual mean).

Example

Negative control absorbance:

Well 1 = 0.011, well 2 = 0.009, well 3 = 0.013

Mean negative control = (0.011+ 0.009+ 0.013) /3 = 0.011

Cut-off value   = 2.1 X0.05 = 0.105

Control procedure

The recommended control requirement for this assay is using positive and negative control to verify assay performance. The result is valid if the following criteria for the controls are both met:

Negative control

Mean absorbance of negative control is lower than 0.1.

Positive control

Mean absorbance of negative control is equal to or higher than 0.6.

Interpretation of results

Nonreactive

Samples giving an absorbance less than the cut-off value are considered nonreactive.

Reactive

Samples giving an absorbance equal to or greater than that of cut-off value are considered reactive. Such samples should be retested in duplicate using the original sample source. Samples that are reactive in at least one of the re-tests are presumed to contain HBsAg

and should be confirmed by using confirmatory kit and tests for other HBV markers. Samples that are non-reactive in both wells on retest should be considered non-reactive.

Quality control checks

Measurement precision

This assay is designed to have a within-run precision of <10%. 1 bovine serum panel member was assayed, using 1 batch of reagents, in replicates of 96. Data from this study are summarized in the following Table: 1a

Table: 1a precision values

Panel member	Batch	N	Mean	SD	% CV
1	1	96	1.117	0.068	5.58

This assay is designed to have a between-run precision of <15%. 1 human plasma based panel member was assayed, using 1 batch of reagents, in replicates of 8, across 12 separate runs. Data from this study are summarized in the following Table: 1b

Table: 1b precision values

Panel member	Batch	N	Mean	SD	% CV
1	1	96	1.150	0.084	7.35

Sensitivity

The diagnostic sensitivity of the product was determined by testing a panel of 412 positive samples, all samples resulted positive; therefore the diagnostic sensitive was 100%.

Table: 2 Sensitivity of the samples

Samples	Tested samples	Positive sample
HBsAg	412	412

Specificity
The diagnostic specificity was determined by testing a panel of 10040 negative samples. 30 samples were positive when first tested, and still those 30 samples were positive when retest. Then result obtained show a diagnostic specificity higher than 99.5%.

PCR (polymerase chain reaction)
Principle
Polymerase chain reaction is a highly sensitive technique for the detection of hepatitis B virus-DNA and hepatitis C virus-RNA in serum, liver tissue, and peripheral mononuclear blood cells. In chronic hepatitis B, it is particularly useful for identification of infectious subjects who are hepatitis B surface antigen positive and anti-hepatitis B e antigen antibody-positive, and for follow up of hepatitis B virus infections in liver transplantation programmes.

PCR reagents

- Acids & Bases
- Agarose
- Buffers
- Detergents
- dNTPs
- General Reagents
- PCR Enzymes

PCR reaction steps
There are three major steps in a PCR, which are repeated for 30 or 40 cycles. This is done on an automated cycler, which can heat and cool the tubes with the reaction mixture in a very short time.

Denaturation at 94°C: During the denaturation, the double strand melts open to single stranded DNA, all enzymatic reactions stop.

Annealing at 54°C: The primers are jiggling around, caused by the Brownian motion. Ionic bonds are constantly formed and broken between the single stranded primer and the single stranded template. The more stable bonds last a little bit longer (primers that fit exactly) and on that little piece of double stranded DNA (template and primer), the polymerase can attach and starts copying the template. Once there are a few bases built in, the ionic bond is so strong between the template and the primer, that it does not break anymore.

Extension at 72°C: This is the ideal working temperature for the polymerase. The primers, where

there are a few bases built in, already have a stronger ionic attraction to the template than the forces breaking these attractions. The bases (complementary to the template) are coupled to the primer on the 3' side (the polymerase adds dNTP's from 5' to 3', reading the template from 3' to 5' side, bases are added complementary to the template).

Samples
Fifty hepatitis B surface antigen (HBsAg) - positive samples were collected from patients. Stored at -70°C until DNA extraction will be done.

DNA extraction
Phenol-chloroform extraction procedure: a total of 200µl of each sample was added to 200µl of lysis buffer (25mM EDTA, 200mM Tris-HCl pH 7.5, and 250mM NaCl, 1% SDS) and was included at room temperature for 60 min.

400µl equilibrated phenol pH 7.8 was added, decanted and incubated for 10 min at room temperature. Tubes were centrifuged at 12000g for 5 min. Then mixed with an adequate volume of chloroform: isoamylalcohol (24:1). Then was precipitated with 3M sodium acetate pH 5.2 and 96% ethanol and incubated at -20°C for overnight. After centrifugation for 15 min at 4°C at 16000g, the pellet was washed with 70% cold ethanol and centrifuged at 16000g at 4°C. The pellet was air-dried and resuspended in 20µl double-distilled H_2O (dd H_2O) and then stored at -20°C until using.

Nested PCR Amplification
Principle
Nested polymerase chain reaction is a modification of polymerase chain reaction intended to reduce non-specific binding in products due to the amplification of unexpected primer binding sites, in that two pairs (instead of one pair) of PCR primers are used to amplify a fragment.

The first pair of PCR primers amplifies a fragment similar to a standard PCR. However, a second pair of primers called nested primers bind inside the first PCR product fragment to allow amplification of a second PCR product which is shorter than the first one.

The advantage of nested PCR is that if the wrong PCR fragment was amplified, the probability is quite low that the region would be amplified a second time by the second set of primers. Thus, Nested PCR is a very specific PCR amplification.

Nested PCR reaction

Nested PCR requires two sets of primers which are used to amplify a specific DNA fragment using two separate runs of PCR. The second pair of primers functions to amplify a smaller specific DNA fragment located within the first PCR product.

For evaluation of DNA extraction methods that are presented in this study, nested PCR was done using specific primers for HBV gene sequences. Nested PCR, in first- and

second-round PCR, was performed for 3 min at 94°C, following of denaturation at 94°C for 45 sec, annealing for 60 sec at 55°C and extension at 72°C for 90 sec. Final extension was done at 72°C for 6 min. PCR solution contained 2.5µl of extracted DNA, 0.5µl dNTP mix, 2.5µl 10x Taq polymerase buffer, 0.75µl MgCl2, 0.2µl Taq polymerase and 1µl of each primer (10 pmol), PrsS3 and S1R in first-round PCR and YS1 and YS3 in second-round PCR. PCR products were detected in 1% ethidium bromide stained agarose gel as shown in Fig: 4
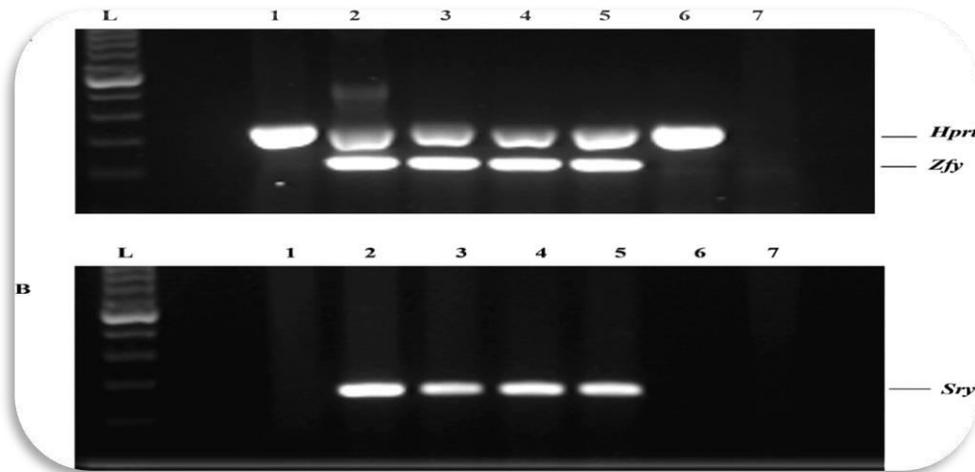

Fig: 4 Verification of the Nested PCR product on gel.

RESULTS AND DISCUSSION:

Results

ELISA and PCR techniques were used for the analysis of blood samples of the patients to determine the prevalence of Anti-HBV antibodies and HBV-DNA respectively. During the 5 month duration of my work 8000 blood samples were taken to find out

the prevalence of hepatitis among the patients coming to Pathology Laboratory (CMH) Lahore. The analysis of the data showed that, total 8,000 blood samples taken from the people; 200 were HBV positive, 148 (74%) blood samples were detected to be positive for HBV in males and 52 (26%) in females by ELISA method as shown in Fig: 5
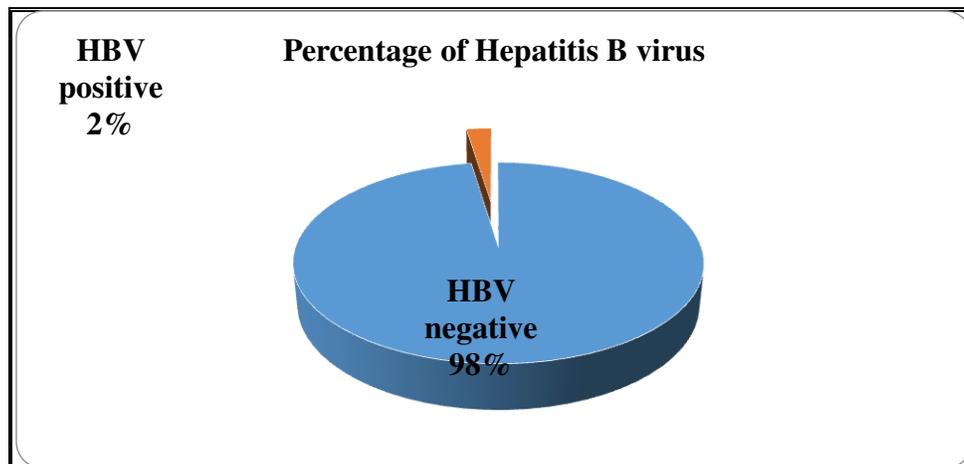

Fig: 5. Percentage of Hepatitis B surface antigen (HBsAg)

On the basis of this data analysis it is cleared that 98% population are HBsAg negative and 2% are positive for HBsAg as shown in (Fig: 5).

Table: 3. Percentages of sex wise distribution of Hepatitis B surface antigen (HBsAg) positive cases
Percentage of HBsAg positive male (74%) and females (26%)

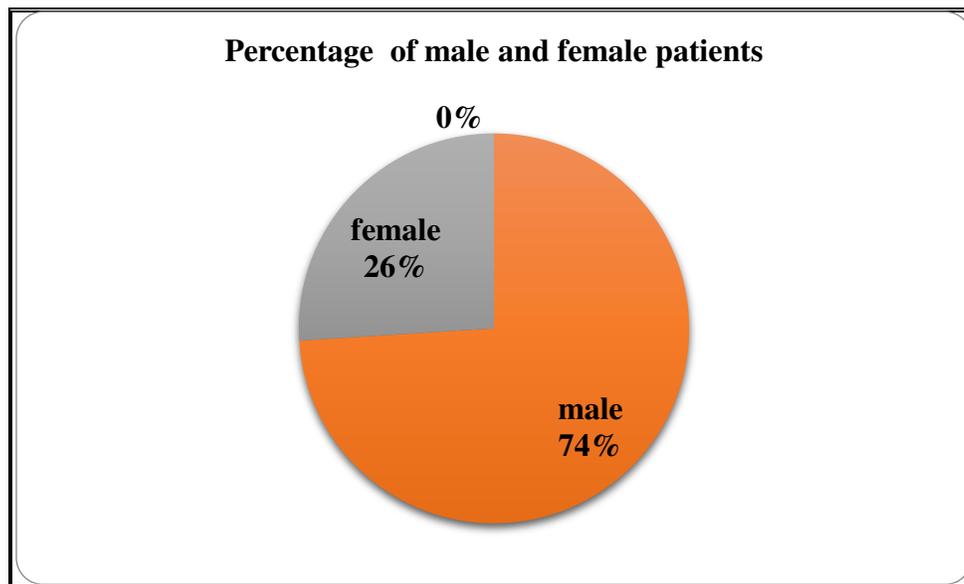| Subjects		Hepatitis B surface antigen positive cases
Sex	Total No.	%age
Male	148	74%
Female	52	26%

Fig: 6 Hepatitis B seropositive male and females

On the basis of analysis out of 200 samples 148 males and 52 females are positive for HBV as shown in (Fig: 6)

Table: 4 Percentage and number of HBV patients

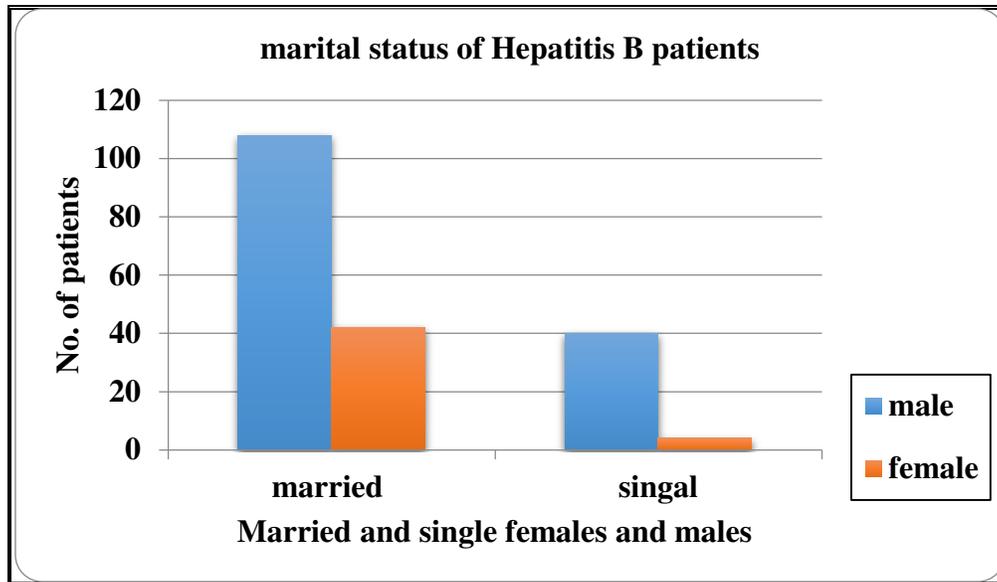| Sr. no.	Marital status	Total no of patients	%age
1	Married males	108	54
2	Married females	42	21
3	Single males	40	20
4	Single females	4	2

Fig: 7 Percentage of seropositivity of HBV with respect to marital status

Data related to marital status was analyzed and showed that married male were the highest 108 (54%) having hepatitis B while single male were only 40 (20%) with hepatitis B. Similarly the prevalence of HBV infection was higher 42 (21%) among married females as compared to the single females 4 (2%) as shown in Table.4 and Fig.7.

Percentage prevalence of patients Hepatitis B surface antigen

Percentage prevalence of patients Hepatitis B surface antigen (HBsAg) in =

$$\frac{\text{No. of positive patients}}{\text{Total no. of studied subjects}} \times 100$$

With the help of this formula percentage prevalence of total subjects along with their factors determined the ratio of infection by hepatitis B in different age group people.
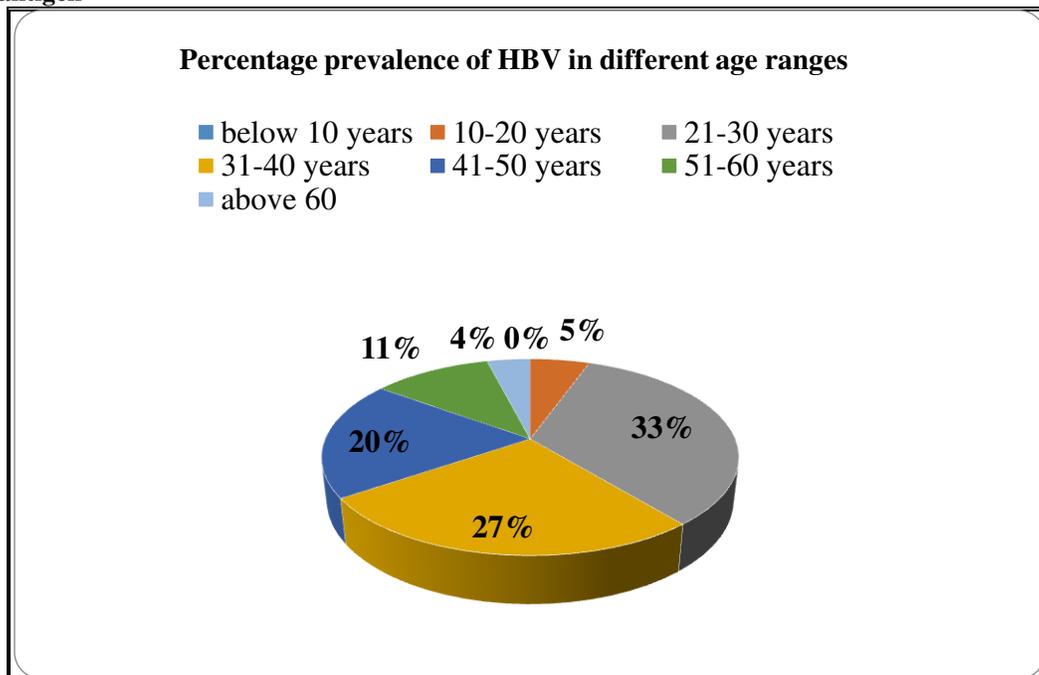

Fig: 8 Percentage prevalence of hepatitis B among patients of different age groups

On the basis of data collected with reference to age, patients were categorized into six age groups that are from 0-10 years, 10-20 years, 21-30 years, 31-40 years, 41-50 years, 51-60 and above 60 years. 0% belong to the age group of

'below 10 years', 5% belong to '10-20 years',33% belong to '21-30 years', 27% belong to ' 31-40 years', 20% belong to '41-50 years',11% belong to '51-60', years and 4% seropositive patients belong to the age group of 'above 60 years' (Fig: 8).

Discussion

In the present study Anti-HBV test was used to screen the blood samples at pathology laboratory of CMH Lahore. The work was performed to find out the prevalence of hepatitis B in the patients. Two hundred blood samples out of eight thousand that are HBV positive were taken, data collected and represented in graphical form on the basis of sex, marital status and different age groups regarding hepatitis B.

The prevalence of HBV infections determined by random sampling of male and female subjects and screening for HBsAg described in my study of general population. The samples found positive in the preliminary screening were further investigated for the presence of DNA HBV blood plasma.

The analysis of the data revealed that two hundred blood samples that were HBV positive were taken. Out of these 200 positive patients 148 (74%) were male while 52 (26%) were females (Fig: 2). These results showed that prevalence of HBV infection is almost 35% more in males as compared to females. The reason may be due to fact that men are more exposed to the risk factors as compared to women. Males are more commonly involved in the trends and practices leading to the transmission of infections like blood transfusions and visits to barber shop that make them more prone to get the infection at a much frequent rate as compared to females. The least incidence of HBV in females could be attributed to low exposure to HBV risk factors due to male dominating society of the area and also the estrogen hormone in females is considered to play a role in the spontaneous clearance of HBV infection and similar work is done by Yuen *et al*. (2001).

On basis of these factors the difference of frequency of infection among the gender category is well understood. My results have shown that the hepatitis caused by HBV is higher in male. The detailed histories of positive subjects have shown that the blood transfusion and surgical operation are the major risk factors. The study presented in this manuscript describes the status of viral hepatitis infection in the general population in an

area with environmental pollution and contamination in the food stuff which indicates an alarming condition of hepatitis in the general population and need for the country-wide screening of population to diagnose the disease at early stages and prevent the

threats to human life. The results show similarity with previous work of Rauf *et al*. (2010).

Demographic data showed that 0% belong to the age group of 'below 10 years', 5% belong to '10-20 years',33% belong to '21-30 years', 27% belong to ' 31-40 years', 20% belong to '41-50 years',11% belong to '51-60', years and 4% seropositive patients belong to the age group of 'above 60 years' (Fig: 4), this finding may be explained by a high risk of exposure to HBV over the years. The other reason may be the frequent use of injections in those age groups for different purposes. The results were in the favor of previously reported data in which majority of the hepatitis B seropositive patients belong to the age group of 20-40 years. For further confirmation of the results PCR was performed to check the viral load in the patients. Zou *et al*. (2001) found the similar results in which younger subjects had higher chance of infection.

A large proportion of Pakistani population is unaware about the epidemiology and risk factors of viral hepatitis. Although the screening and diagnostic recommendations advocate early detection of HBV and but most of the victims of viral infections are brought to the hospitals in Pakistan when they are at the end stages of liver damage. The late diagnosis increases the risk of hepatocellular carcinoma and decrease the effect of antiviral therapies. Risk of hepatitis B is increasing day by day. So it's an alarming situation in Pakistan. We need proper public awareness and pollution control programs to overcome this problem.

CONCLUSION:

This present study prevalence of HBV among different groups visited to CMH Lahore showed that, the male population is at higher risk of HBV viral attack because males are more commonly involved in the trends and practices leading to the transmission of infections like blood transfusions and visits to barber shop that make them more prone to get the infection at a much frequent rate as compared to females. Moreover, HBV more common at the age group of 20-40 years that is about 60%.

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