Evaluation of HBcAb and HBsAg Diagnostic Tests in Comparison with Nucleic Acid Testing for the Diagnosis of Hepatitis B Virus

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With the Name of ALLAH, the Most Merciful, and Beneficent

and Muhammad (PBUH) who is City of Education and

Hazrat Ali (R.A) the door of this city.

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FINAL APPROVAL

It is certified that we have read the thesis submitted by Ms. Sadaf Moneeba and it's our judgment that this project is of sufficient standard to warrant its acceptance by International Islamic University, Islamabad for the MS degree in Biotechnology.

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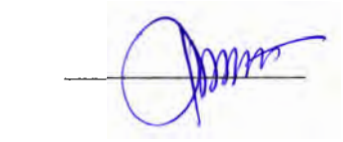

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DECLARATION

I, SADAF MONEEBA declare that the material contained in this thesis is my own, original work. It is being submitted for the degree of MS in Biotechnology. It has not been submitted before for any degree or examination elsewhere.

Sadaf Moneeba

October, 2016

To

My Parents Mr. and Mrs. Zia-ur-Rehman

For their Endless Love, Supports and Courage

TABLE OF CONTENTS

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LIST OF ABBREVIATIONS

Acronym	Abbreviation
HCC	Hepatocellular Carcinoma
HBeAg	Hepatitis B Envelope Antigen
RLUS	Relative Light Units
HBV	Hepatitis B Virus
HBsAg	Hepatitis Surface Antigen
Anti-HBs	Hepatitis B Surface Antibody
HBcAb	Hepatitis Core Antibody
DNA	Deoxyribonucleic Acid
EDTA	Ethylene Diamine Tertra Acetic Acid
IgG	Immunoglobulin G
IgA	Immunoglobulin A
IgM	Immunoglobulin M
NAT	Nucleic Acid Testing
NPV	Negative predicted values
PPV	Positive Predicted Values
RNA	Ribonucleic Acid
VCT	Voluntary Counseling and testing
WHO	World Health Organization
CMIA	Chemiluminescent Microparticle Immunoassay
PCR	The Polymerase Chain Reaction

HBIG	Hepatitis B immune globulin
CLIA	Chemiluminescence Immuno Assay
CHB	Chronic Hepatitis B Virus
SBTP	Safe Blood Transfusion Program
OBI	Occult HBV Infection
TTIs	Transfusion Transmissible Infections
ALT	Alanine Aminotransferase
EIA	Enzyme Immunoassay
HSCT	Haematopoietic Stem Cell Transplant
S/Co	Serum Cut off Value
Nm	Nanometer
µl	Microliter
°C	Degree centigrade
KDa	Kilo Dalton
UVI tec	UV Transilluminator
OLT	Orthotopic Liver Transplantation
SDS	Sodium Dodecyl Sulfate
EDTA	Ethylenediaminetetra Acetic Acid
HBIG	Hepatitis B Immunoglobulin
LAM	Lamivudine
HBIGþLAM	HBIG+LAM combination therapy
TTV	Transfusion Transmitted Virus
HBxAg	Hepatitis B X Antigen
MTC	Mother to Child

rpm	Revolutions per minute
RCF	Relative Centrifugal Force
PIMS	Pakistan Institute of Medical Sciences
PPV	Positive Predicted Values
NPV	Negative Predicted Values
DLR	Diagnostic likelihood Ratio
TN	True Negative
TP	True Positive
FN	False Positive
FP	False Negative
CI	Confidence Interval
MES	Morpholino EthaneSulfonic Acid
MOPSO	Morpholino Hydroxy-Propanesulfonic Acid
ACIP	Advisory Committee on Immunization Practices

List of Figures

List of Tables

Abstract

Safe blood transfusion plays important role in patients' health. For the sake of harmless and infectious free transfusion, WHO recommends quality-assure screening for TTIs namely HBV, HCV, HIV, malaria and syphilis. Hepatitis B is among the leading life threatening infections in the developing countries. Diagnosis of HBV infection depends on serological testing due to similarity in clinical symptoms of all types of hepatitis infection. Total 200 samples of blood donor were screened on HBsAg, Anti-HBc and PCR for the detection of HBV in blood donors and positivity ratios were 47.2% (93/197), 52.3% (105/197) and 47.7% (94/197) respectively. Three samples were used in testing so reaming 197 samples is considered as total number of sample. The sensitivity of HBsAg and HBcAb were 98.9% (95% CI: 94.2% - 99.9%) and 89.5% (95% CI: 82.0 % - 94.6%). Furthermore the specificity of the HBsAg and HBcAb were 98% (95% CI: 94.7- 99.9) and 99.5% (95% CI: 83.3% - 95.0%). The positive Predictive values (PPV) were 90.38% (95% CI: 94.2% - 99.9%) and 89.5% (95% CI: 82.0%-94.6%) for HBsAg and HBcAb respectively. The Negative Predictive value for the HBsAg and HBcAb were 99.0 % (95% CI: 94.7% - 99.9%) and 90.35% (95% CI: 83.3% - 95.0%). The Negative Diagnostic Likelihood Ratio (NDLR) and Positive Diagnostic Likelihood Ratios for HBcAb were 0.12% (95% CI: 0.07%-0.20%) and 9.28 (95% CI: 5.27%-16.33%) are respectively whereas Negative Diagnostic Likelihood Ratio (NDLR) and Positive Diagnostic Likelihood Ratios for HBsAg was observed as 0.12 (95% CI: 0.07%-0.20%) and 10.2 (95% CI: 14.63%-723.77%) correspondingly. The test agreement i.e. Kappa Agreement between the HBsAg and HBcAb assays was high both for HBsAg detection (κ = 0.96; 95% CI: 0.93%- 0.99) and for HBcAb detection (κ = 0.88; 95% CI: 0.85%- 0.93%).The false positive rate of HBsAg was observed as 0.5% and false for HBcAb was 5.5% as compare it with gold standard (PCR).

As core gives false negative results so it must be neglected in routine screening NAT is thought to be better in response to HBV infection but it will be expensive approach to developing country like Pakistan so only HBsAg screening could be considered as a best alternative and give satisfactory results at the window time of infection occurs HBsAg results for the reduce risk of HBV infection of transmission are beneficial.

Chapter 1

Introduction

Hepatitis B is among the leading life threatening infections in the developing countries. The virus affects liver cells causing malfunctioning ranging from liver failure to cancer depending on the stage (WHO, 2015). The liver performs important role in excretion of toxic waste products through bile and is involved in the proteins, carbohydrates, and lipids metabolism (Knolle and Thimme, 2014).

Williams 2006, Koruk and colleagues in 2015 reported that around 240 million people are infected with HBV and approximately 600,000 people die annually. HBV vaccine was introduced in 1982 and proven 95% efficacy but HBV is still transmitted. The WHO approved vaccine for hepatitis B which is HBIG (hepatitis B immune globulin) for newborns to prevent HBV transmission and increase the protection rate up to 90–98%.

HBV is consists of outer lipid envelope its outer envelope contains a high quantity of hepatitis b surface proteins. The envelope surrounds the inner nucleocapsid which is made up of 180 hepatitis B core proteins arranged in an icosahedral arrangement. These particles are not infectious and are composed of the lipid and protein that forms part of the surface of the virion, which is called the surface antigen (HBsAg), and is produced in excess during the life cycle of the virus as shown in fig 1.1 (Howard, 1986).

1.1 Classification:

The classification of HBV on the basis of genetic material and vertical or horizontal mode of transmission has now become a standard. HBV is divided in to 8 main types (A-H). In Asia and Alaska the F and C type of Hepatitis are associated with the hepatocellular carcinomas (HCC) in children and adults (PinkBook, 2015).

HBV can be chronic or acute depending upon short or long term illness (CDC, 2015).

1. Chronic Hepatitis B Virus (long term illness)
2. Acute/Occult Hepatitis B Virus (short term)

The main cause of chronic hepatitis B virus is basically ineffective response for reduction of viral load by the immune system of the host also and also the structural changes in host cells that are caused by viral invasion (non-cytopathic) and unique (hepatotrophic) characteristics of Hepatitis B Virus plays significant role in viral existence (Zheng *et al.*, 2015).

The natural development of chronic HBV infection involves four characteristic phases:

1. Immune active (HBeAg) positive.
2. Immune inactive phase.
3. Immune active phases (HBeAg negative).
4. Immune tolerant phase (Lok *et al.*, 2015).

The chronic infection depends on age of individuals as 90% children become chronically infected but 2-6% adults (CDC, 2015). According to European Association for the Study of the Liver (2009), the chronic hepatitis B virus (CHB) have 5 sequential phases and from them 5^{th} is the OBI (occult HBV infection).

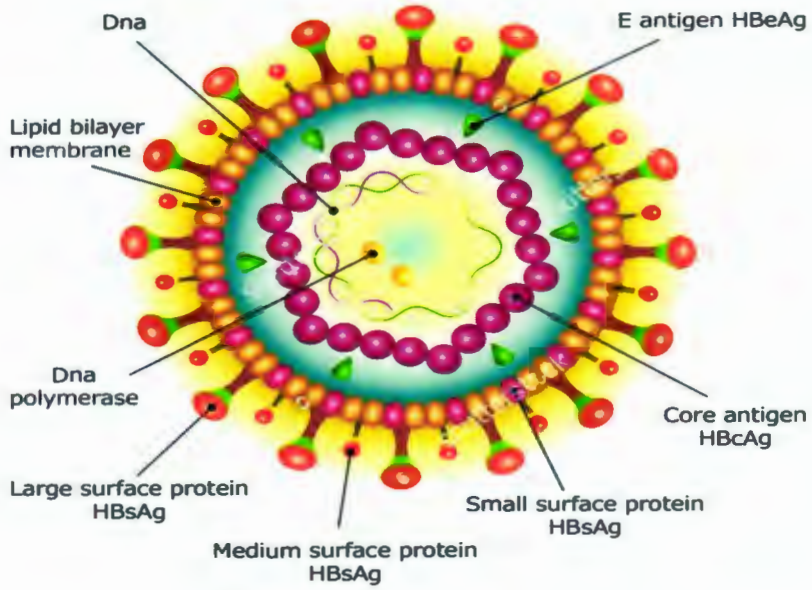
Allain (2004) gives peculiar definition of occult HB infection that the presence of Hepatitis B Virus DNA without Hepatitis B virus antigens and antibodies outside from acute phase in window time period.

Raimondo (2013) defined OBI (Occult HBV Infection) as enduring existence of HBV genomes in liver of individual donor is testing negative for HBsAg with help of detectable DNA of hepatitis B virus (serum).

Raimondo and group (2007) reported that the prevalence of hepatitis B surface antigen (HBsAg) and IgM antibody (immunoglobulin M antibody) to the (core antigen) are actually the characteristics of the acute hepatitis B Infection.

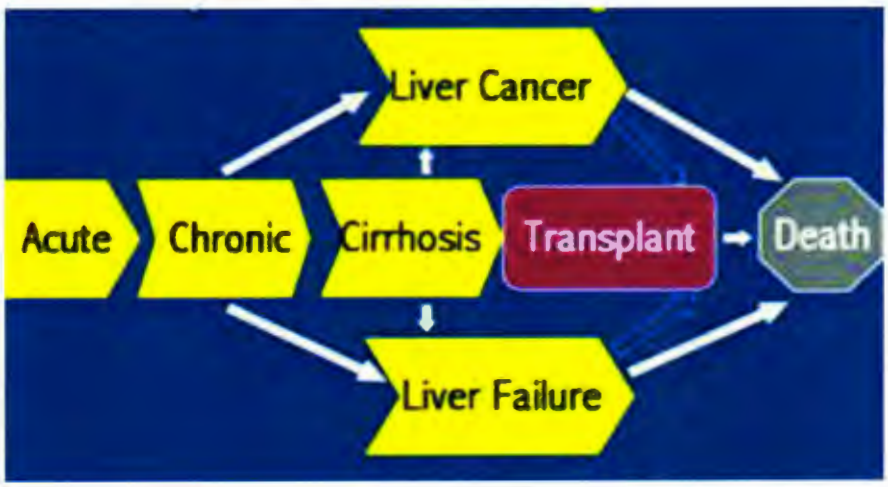
1.2 HBV Disease Progression

HBV is basically Liver carcinoma the acute infection is when a person is first infected with the hepatitis B virus some adults are unable to get rid of the virus after six months then it lead to chronic infection. Which cause death by completely damaging the liver. It may cause liver cirrhosis, liver failure, liver cancer and death of the individual if it is not treated properly Fig 1.2 (CDC, 2015).



Adapted from:" http://www.shutterstock.com/pic-173727872/stock-photo-diagram-of-hepatitis-b-virus-particle-structure.html"

Fig 1.1: *Structure of Hepatitis B Virus*



Available at:" http://www.hepb.org/expforum/media-player.aspx?speakerID=drTobias&topicID=treatment&language=English&mediaType=text&topicList=Questions"

Fig 1.2: *Hepatitis B Disease Progression*

1.3 Mode of Transmission:

HBV transmitted by individuals via occupational contacts with body fluid, blood and blood products (Kohn *et al.,* 2003). HBV occupational risk differs with reference to work place and time when an individual expose to infectious agent (Ciorlia and Zanetta, 2005). The blood which is used for the transfusions must be safe and free from any transfusion transmitted infections (CDC, 2015).

The modern practices of medicine added in controlling the spreading of disease in society. HBV spread due to carelessness in proper management of hospital waste as 10-20% wastes are dangerous to the health (Taneja and Biswal, 2006). HBV transmission is a big challenge for those who regularly come into contact via blood and patients and persons who are involved in transmitting blood between donor and patient (Health Workers) if carelessness showed. Regardless of health works common people can also be infected with the virus easily for instance;

1. Drug users or injectors
2. People who pierce or tattoo their bodies
3. Unprotected sex engaged in by adolescents

Hauri and friends in 2003 reported that 21 million people with HBV infections observed annually because of these reasons. HBV is also a social injustice and main cause of spreading HBV is wrong information about health cares and different Myths in some endemic countries for instance China (world largest chronically infected population) they are not permitted to work in food industries are suggested to go to routine test for HBV. These people can be expelled from work or school if HBV test come positive (CDC, 2006; Chireh and Lennarth 2011).

HBV transmission can occur through;

1. Sexual contact.
2. Sharing needles.
3. Re-use of contaminated Syringes.
4. Other drug-injection equipment.
5. Mother to child (MTCT) during childbirth.

6. By exposure to percutaneous injury or mucous membrane with secretions within family.
7. The Saliva which have HBV.
8. Blood injected accidently during dental and surgical procedures.

1.4 Pathogenesis of HBV:

Autophagy (Destruction of cells in the body) occurs in the life cycle of HBV the Dane particle of HBV get enter into the nucleus of host body and then Viral Protein of HBV is synthesized by RNA viral particle which is made after transcription process. The HBX (hepatitis B virus X) protein is synthesized and this HBX protein then activates the PI3K (phosphoinositide 3-kinase) pathway Beclin-1 synthesis (Role in the regulation of both autophagy and cell death) Via Autophagy replication of viral DNA occurred. (Fig 1.3).

1.5 Signs and Symptoms:

In case of acute infection of HBV signs and symptoms are;

1. Illness lasts for a few weeks
2. Loss of appetite
3. Nausea
4. Vomiting
5. Body aches
6. Mild fever
7. Dark urine
8. Development of jaundice.
9. Itchy skin(for all types of HBV)

1.6 Global Prevalence of Hepatitis B:

HBV is a main global health issue. Komatsu with colleagues (2015) stated that annually 240 million persons are exposed to chronic HBV and 600,000 infected people die. Hu in 2016 reported that 2 billion people have been infected worldwide

and 240 million are with chronic HBV; 800,000 annually deaths are observed due to hepatitis. In the understanding of viral infection its outcome and distribution, the epidemiology of HBV exemplifies milestone.

According to resolutions of the World Health Assembly (2010-2014) they indicated that HBV is on 15th position in all infections which cause the global mortality. Global prevalence of chronic HBV ~20% observed, ~15% to 40% are ratio of those chronic infected HBV patient which develop liver cancer and 15% to 25% die in 2014. In contrast with former published measurements (350 to 400 millions) this shows 31% decline in the HBsAg presence. This decrease is due to follow safe blood transfusion strategies and due proper to vaccination (Hu, 2016).

According to Lavanchy (2005) 1/3 of total global population are infected with HBV. Mother-to-child (MTC) transmission also main issue world widely and it is reported that 8 to 30% immunoprophylaxis strategies are useful in protecting MTC HBV transmission.in contrast with Western Europe or North America the Asian HBsAg positive Pregnant women are observed (Sellier *et al.*, 2015).

Chen (2000) stated that in the pacific region of Asia, the primary goal for the control of Hepatitis B virus control is to avert chronic infection, which is mostly the consequence of mostly from prompt exposure to HBV in the age 2-9 years. Because the Persistent infection is basically infection at early age that is typically asymptomatic.

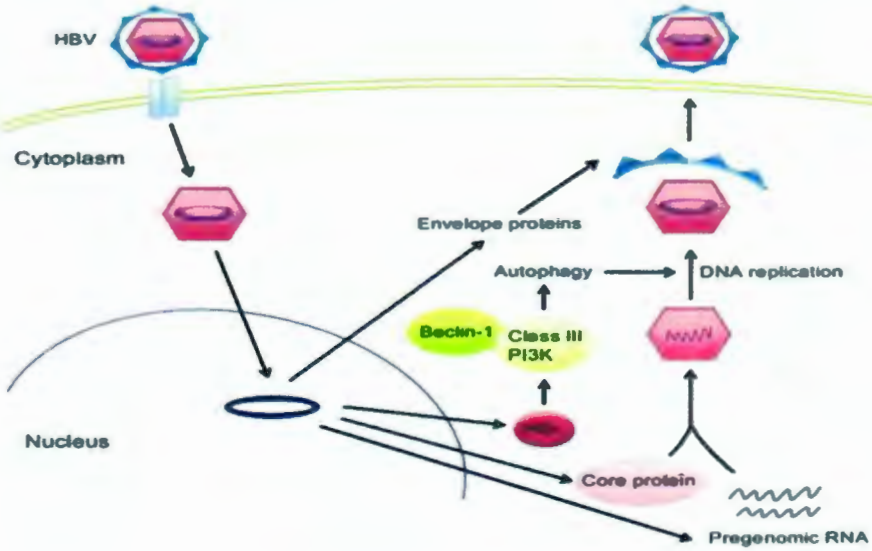
1.7 Pakistan Scenario:

HBV is major health issue in all over the world especially in developing countries including Pakistan. HBV is causative agent for most liver diseases in humans for instance liver cirrhosis, liver cancer (hepatocellular carcinoma) and liver fibrosis. Ullah and group (2016) described that prevalence of HBV is 2.5% in Pakistan and total 2200 doubted (suspected) people have visited for conformation of HBV in which 181 got positive results of diagnosis in these positive cases they observed 62.98% males and 37.01% females, in addition to age 50.27% are of 21 to 40 years, in contrast with marital HBV prevalence the married 61.32% and unmarried

ratio 38.67%. The ratio of HBV prevalence in persons who shared personal items is 35.91%.

WHO (2015) reported that in Pakistan approximately 4 million people are infected with HBV. Although HBV is controlled in Pakistan including in Punjab, Sindh and in few districts of Balochistan but still population is at high risk. General prevalence of HBsAg was observed as 2.5%, highest prevalence was noticed in Balochistan 4.3% in respect to Sindh that is 2.5% and 2.4% in Punjab which has significant high rate in correlation with Khyber Pakhtunkhwa (1.3%) (Qureshi *et al.*, 2010).

Immunoassays for the detection of total anti-HBc involve both IgM and IgG class antibody to the core protein and indicate current or past exposure to virus and viral replication. IgG anti-HBc appears shortly after HBsAg among persons with acute disease and generally persists for life therefore total anti-HBc is not a good marker for persons with acute disease. The detection of IgM anti-HBc is diagnostic of acute HBV infection. In persons with chronic HBV infection, HBsAg remains persistently detectable, generally for life. HBeAg is variably present, and IgM anti-HBc generally becomes undetectable 6 months after acute infection (Fig. 1.4).



Source: http://www.medscape.com/viewarticle/754992_7

Fig 1.3: *Pathogenesis of HBV*

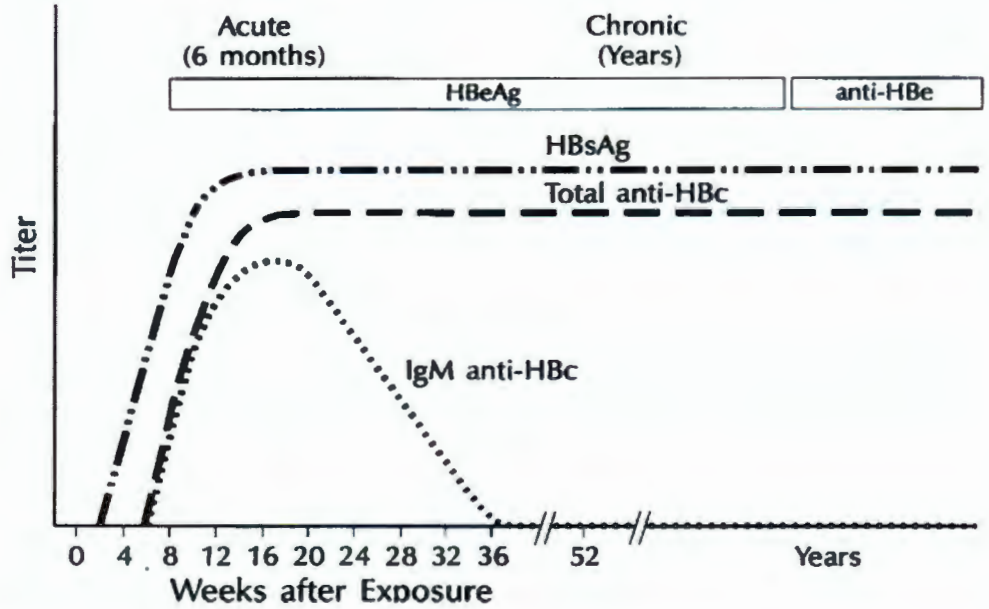


Fig 1.4: *Characteristics of progression to chronic HBV infection*

1.8 Diagnosis:

Diagnosis of HBV infection depends on serological testing due to similarity in clinical symptoms of all types of hepatitis infection. Table 1.1 shows the serological test results interpretation. Vaccinate according to current ACIP recommendations. If a person is only positive for anti-HBc while negative for HBsAg and anti-HBs, there are 4 possible explanations:

1. May be recovering from acute HBV infection (vaccination not indicated).

2. May be distantly immune and test not sensitive enough to detect very low level of anti-HBs in serum.

3. May be undetectable level of HBsAg present in the serum and the person is actually a carrier (acute or chronic).

4. May be susceptible with a false positive anti-HBc.

In the course of acute infection HBeAg is detectable. For the period of convalescence the anti-HBe , anti-HBs and anti-HBc developed, HBsAg and HBeAg are cleared (Mahoney, 1999).

1.9 Hepatitis B surface Antigen Test (HBsAg) through CLIA:

The CLIA is based on detection of luminescence which is emitted from labeled substrate during chemical reaction. Reagents are required for reactions that produce CL (Chemiluminescence) May be coupled to antibodies or antigens. The incubation time is variable depending upon the nature of sample and analysis. The results are achieved in 15 to 45min (Brown *et al.*, 2014).

Table 1.1 *Interpretation (analysis) of HBV the serological test result*

HBsAg	IgM anti-Hepatits B core	Total anti-Hepatits B core	Anti-HBs	Interpretation (analysis)
Positive	Negative	Negative	Negative	Indicates that early hepatitis B Virus infection before anti-HBc response.
Positive	Positive	Positive	Negative	Early HBV infection. Because IgM anti-HBc is positive, the onset is within 6 months. IgGantibody usually appears shortly after IgM; therefore, both are usually positive when IgM is positive.
Negative	Positive	Positive	Negative or Positive	Recent acute HBV infection (within 4–6 months) with resolution; i.e., HBsAg has already disappeared. Anti-HBs usually appears within a few weeks or months of HBsAg disappearance.
Positive	Negative	Positive	Negative	HBV infection, onset at least 6 months earlier because IgM anti-HBc has disappeared. Probable chronic HBV infection.
Negative	Negative	Negative	Positive	Response to hepatitis B vaccine. No evidence of infection
Negative	Negative	Positive	Positive	Past HBV infection, recovered.

1.10 Scope of Research Work:

The present risk of viral infections expected 1 in 153,000 for the HBV (liver infection), for each blood component received. 2.5% HBsAg positivity in general population of Pakistan (Idrees *et al.*, 2011). The improved system of blood transfusion is implemented in Pakistan through SBTP (Safe Blood Transfusion Program) including digital data entering management system. Currently the blood transfusion sector lack efficient tools for information and knowledge management and process automation (Waheed, 2015).

Blood remained a valuable source for diagnostic purpose since 1930; however, the rate of viral transmission is also increasing with the improvement in the storage capacity and number of blood banks due to poor quality measures (i.e. unscreened blood transfusion, reuse of unsterilized syringes and medical equipment). Other than the medical practitioners, barbers, ear piercing, tattooing, and circumcision are also major cause of Hepatitis B. For instance, WHO's statistics (2010) claimed that the seroprevalence rate of Hepatitis B under surface antigen is 2.5% for Pakistan but this could be high because people do not have adequate knowledge for early screening and documentation. Therefore, Government should launch a proper educational campaign educating general public regarding precautions and treatment of hepatitis B (Idrees *et al.*, 2007).

So the most successful way to stop transmission of Hepatitis B virus in the high endemic areas is to avert vertical transmission. In 1940s and 1940s syphilis and HBsAg tests introduced respectively for the blood donation screening (Stramer and Dodd, 2013).The first level of contacts (Health care person) should have enough awareness about HBV, hygienic measures and TTI (Othman *et al.*, 2014).

According to Blood Transfusion Services in Pakistan over 3.5 million units of blood are collected each year in which 65% source of blood is replacement (patient's relatives or friends) donors, study shows 10% are paid donors and 25% donations are volunteer non-remunerated blood donors (Bosan *et al.*, 2010).

Blood safety has been improved due to HBcAb along with HBsAg screening for blood donor but leads to excessive loss of blood units due to positivity rate of HBcAb which is around 10-20% (Laperche, 2005).

The best substitute for improving blood safety in our country is to add NAT testing to donated blood. It will detect most potentially infectious units of blood including window period donations and seronegative infections (Makroo *et al.*, 2012). Consequently, the applicability of anti-HBc testing in routine screening of donated blood is questionable.

According to Laperche (2015) HBV DNA screening would be more effective in countries with high or medium endemicity, and where anti-HBc testing should not routinely done.

Objective of study:

(1) To evaluate the performance and diagnostic effectiveness of two different HBV serological screening assays.

(2) To formulate an alternative cost-effective confirmatory screening approach for HBV that is suitable for use in Pakistan.

Chapter 2

Literature Review

Screening for Transfusion Transmissible Infections (TTIs) is a critical part of the process of ensuring that transfusion is as safe as possible. In Pakistan with an only incipient culture of voluntary donations and the lack of systematic screening, the infection risks are at the upper end (Zaheer and Waheed, 2014).

Around 5000 children are diagnosed with Thalassemia annually and large amount of blood is required by these patients regularly. Approximately 1.5 million donations of blood units are donated in Pakistan annu0ally. Almost 90% blood is collected from the relatives of patients and other 10% from voluntary donors (WHO, 2016).

In the blood bank of Pakistan HBV screening test HBsAg and HBcAb is compulsory for the detection of HBV. But around the world test for anti-HBc is still unanswered. In the field of Transfusion medicine the most sensitive issue is the safety of Blood and its components by which HBV can be transmitted if one shows carelessness (WHO, 2015).

HBV is enveloped DNA virus. The Time when infection is occurred hepatitis virus produces Australia antigen or surface antigen of HBV (HBsAg). It is found in the blood of infected person and responsible for the binding of virus to the liver cell it neutralized the antibodies (Neurath *et al.*, 1986). HBV is belongs to the Hepadnavirus family. The virus particle, called Dane particle HBV have several antigen antibody system which are shown in Table 2.1

2.1 Hepatitis B Antigen and Antibody System:

Presence of HBV DNA indicates that how severe is infection and the HBV presence. Hepatitis B has several Antigens and Antibodies system.

1. Hepatitis B Surface Antigen (HBsAg).
2. Hepatitis B Core Antigen (HBcAg).
3. Hepatitis B envelope Antigen (HBeAg).
4. Hepatitis X Antigen (HBxAg).

Table 2.1 *Hepatitis B and its virulence*

Type of virus	Genome	Family belongs to	Hepatitis B virion				Antigenic components	Existence of Virus (Remain Infectious)	Identified in serum	Incubation time
			Particle	Size	Composed of					
					Viral glycoproteins (outer shell)	Nucleocapsid (inner shell)				
Double shelled DNA virus	It has Circular DNA genome which is partially double-stranded	Hepadnaviridae viruses family	Dane particle	~ 42 nm in size	Consist of HBsAg (Hepatitis B surface antigen)	It made up of HBcAg (Hepatitis B core antigen) that carries enzymes and viral DNA for viral replication (DNA Polymrase)	1.HBsAg 2. HBcAg 3. HBeAg	7 days at room temperature	30-60 days after exposure to HBV	75 days but it could be 30-180 days

2.1.1 Hepatitis B Surface Antigen:

From the Blumberg's discovery of hepatitis B surface antigen, it is used in diagnosis of hepatitis B infection as hallmark (Blumberg BS with colleagues, 1986). Appears normally after 1-10 weeks of acute exposure and before commencement of symptom or by assessment of Serum Alanine Aminotransferase (ALT). It alone is not infectious but whole virus. HBsAg have 2 pair of d,y,w and r (mutually exclusive antigens) i.e. results from four main subtypes of adw, ayw, adr and ayr.

It helps virus in attachment to the cell membrane by binding with the liver cells (hepatocyte) plasma membrane it initiate mechanism of infection. Its persistence for 6 months leads to chronic infection. Huge amounts of Hepatitis B surface antigen (HBsAg) may paly role in inducing energy of T cell as a result of which decrease in antibody-mediated neutralization of HBV is observed and generalized hyporesponsiveness towards pathogens (Jayalakshmi *et al.*, 2013).

2.1.2 Hepatitis B core antigen (HBcAg):

Hepatitis B Core antigen is basically nucleocapsid protein and cannot be detected in the serum of a person but found in liver tissues by conventional techniques. In addition to HBeAg the HBcAg is 1000 times more immunogenic. The formation of hepatitis B core antibody is occurred when body is exposing to acute hepatitis it will firstly produce antibody.

During acute infection the HBcAg comprises of IgM and total anti-HBc present with chronic infection. Six months after exposure the antibodies made up of IgG anti HBc (Fig 2.1).When non-specific antibody (IgM) binds to core antigen of HBV as indicated in the diagram. In few cases antibodies will produced in the body which then cross react with hepatitis B core antigen and gives false positive serologic response (Fig 2.2) (Quaglio *et al.*, 2001).

Hepatitis B core antigen is necessary for replication of virus. It is multivalent protein antigen which functions as T cell independent and dependent antigen. It is consider as a marker for the viral replication (Lee *et al.*, 2009).

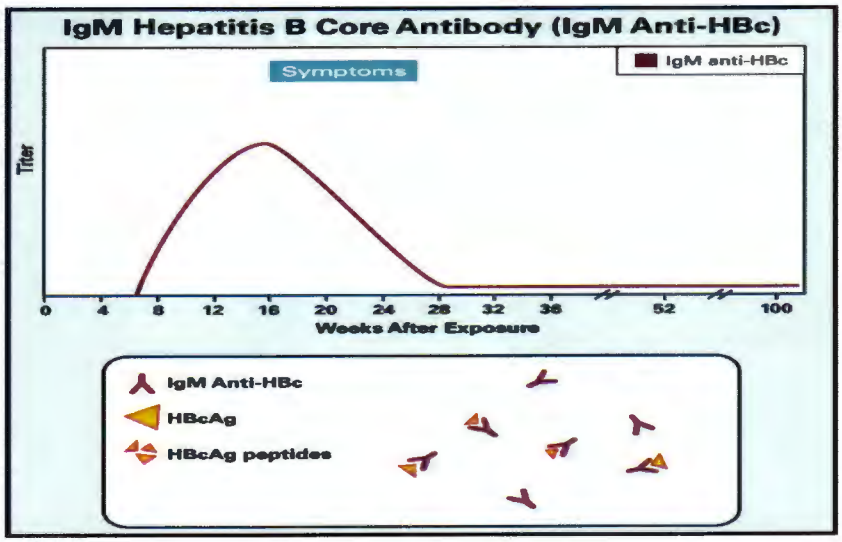


Fig2.1 *IgM Hepatitis B Core Antibody Response to Infection with HBV*

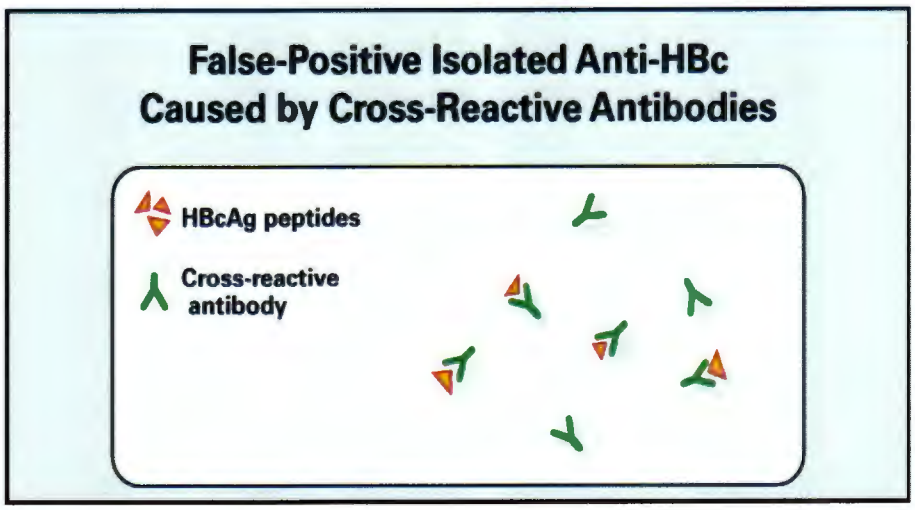


Fig2.2 *False-Positive Isolated Anti-HBc Caused by Cross-Reactive Antibodies*

2.1.3 Hepatitis B envelope antigen (HbeAg)

HBeAg is secretory protein which is discovered in 1972 by Magnius and Espmark. Envalope antigen of HBV (HBeAg) is found in plasma at concentrations of 10μg mL-1, identified by agarose gel immunodiffusion. HBeAg as a soluble protein is secreted into the blood from liver cells (hepatocytes) directly, and maintain persistence of infection. The occurrence of HBeAg in blood indicates high levels of virus.

It is important for causing infection but not necessary for viral Replication. Clinically used as index for: Viral replication, infectivity, severity of disease and response to treatment. On the period of perinatal transmission HBeAg function as spreading of viral infection. HBeAg have role in immunomodulatory function in antigen identification by CD4+ cells (Milich *et al.*, 1999).

2.1.4 Hepatitis B X antigen (HBxAg)

Hepatitis B X antigen (HBxAg) has molecular weight 17kDa. It is smaller than other antigens, nonstructural, multifunctional protein, composed of 154 conserved sequences of amino acids across all mammalian infecting Hepadnaviridae.

Causing hepatocellular carcinoma (liver cancer) related to HBV. Crucial to virus replication propagation and integration of viral genome into host. This promotes gene expression of virus. Accumulation of it can boosts replication of virus by changing biological functions containing peculiar expression of molecules involved in host cell signal transduction, transcription and proliferation, leading to viral persistence and hepatocarcinogenesis (Jayalakshmi *et al.*, 2013). Nomancalture of are Shown in Table 2.2.

Table 2.2 *Nomenclature of HBV*

Abbreviation	Term	Definition and comments
HBV	Hepatitis B	virus Etiologic agent of "serum" hepatitis; also known as Dane particle
HBsAg	Hepatitis B surface antigen	Surface antigen(s) of HBV detectable in large quantity in serum; several subtypes identified
HBeAg	Hepatitis B e antigen	Soluble antigen; correlates with HBV replication, high-titer HBV in serum, and infectivity of serum.
HBcAg	Hepatitis B core antigen	Core antigen of HBV; no commercial test available
Anti-HBs	Antibody to HBsAg	Indicates past infection with and immunity to HBV, passive antibody from HBIG, or immune response to hepatitis B vaccine
Anti-HBe	Antibody to HBeAg	Presence in serum of persons with chronic HBV infection indicates low titer of HBV
Anti-HBc	Antibody to HBcAg	Indicates prior infection at some undefined time in past.
Pre-S1, pre-S2	Envelope protein epitopes	Envelope proteins containing pre-SHBs epitopes
SHBs	Major surface antigen protein	The major protein that forms HBsAg particle is the smallest gene product (SHBs)
MHBs	Middle HBs protein	The middle HBsAg protein, containing pre-S2 gene product
LHBs	Large HBs protein	The large HBsAg protein, containing pre-S1 and pre-S2 gene products
Immune globulin HBIG	Hepatitis B immune globulin	Contains high titers of antibody to HBsAg

The central theme of research was to check prevalence of reactive anti-HBc (anti-HBc) and HBV DNA in the serum of healthy blood donors and who are negative to both assays in Iran population because it is not in routine practice. 2000 serum samples were taken from the healthy blood donors, those that negative to HBsAg tested for prevalence of HBcAb. All reactive samples to HBcAb were then scrutinized for the identification of anti-HBe antibody, anti-HBs titre, anti-HBc titre and HbeAg through EIA (enzyme immunoassay). All samples either positive or negative for both Assays or alone were then confirmed with PCR to determine the presence of HBV DNA. Results show that 6.5% samples were reactive to HBcAb.

From HBcAb reactive specimens 12.2% samples detected HBV DNA. They conclude that Anti-HBc must include in daily routine screening and reactive unites to HBcAb must be discarded nevertheless of HBsAg titre results and further more testing for HBV DNA must follow for the HBV infection (Behzad-Behbahani *et al.*, 2006).

Amini and colleagues (1993) studied epidemiologic features of HBV in the all-inclusive community of Hamadan area of Iran were examined they worked on an aggregate of 4930 subjects. 25.72% was found as Seropositivity of HBV marker out of these 2.49% were carriers of HBsAg, 18.09% were reactive to anti-HBs and 5.13% to anti-HBc only. They concluded that the 5.1% blood donors were anti-HBc reactive and totally nonreactive to HBsAg though did not find DNA of Hepatitis Virus.

The study of Chen and group (1999) focus on the relationship between S gene variant of HBV and HBcAb positive and HBsAg negative phenomenon. Out of 2700 replacement blood donors 300 were HBsAg positive and 336 were HBcAg, HBsAg and HBsAb positive. Total 3 blood donors had HBV DNA out of 29 donors who were positive on HBsAg and negative on HBcAb. They concluded that S gene variants of HBV are main cause for HBsAg negative and HBcAb in blood donors however the more commercial assay is required for the detection of S gene variants.

NAT is supposed to be very effective for the diagnosis of HBV in window time period specially in developing countries though it is cost affective (Hennig *et al.*, 2002).

Kuhns and group (2004) studied HBsAg and HBcAg by PCR and found that 200 results were HBsAg and HBcAg positive in which 90% were chronically HBV infected. 63% had high level of DNA and resting 72 samples were sensitive to PCR however 3% had 1.3copies/mL for reactive samples. They concluded that the DNA of HBV was very low in HBcAb and HBsAg positive blood donors. With HBV NAT detection 6% were negative, 3% undetected. These outcomes demonstrate the caution in any deliberation of dropping HBsAg screening.

Busch (2004) said that in some countries the HBsAg screening considered to be progressive and more sensitive assay for the prevention of transmission of HBV. NAT added for the screening of donor with minimum incremental output (yield) of HBsAg and anti-HBc-negative donations in few countries like Germany and Japan. For the plasma donor HBV NAT become a standard for donor screening with pressure to include HBV DNA detection as a required procedure for use of recovered plasma in manufacture of fractionated derivatives. In addition effect of usage of HBV NAT is needed for maintenance of HBsAg and anti-HBc screening. Finally it will address the cost effectiveness of incorporation of HBV DNA detection into HBV screening and NAT testing algorithms.

Laperche (2005) stated that in the high/medium endemic countries screening of DNA of HBV is more effective where anti-HBc not in routine.

Behzad-Behbahani and group (2006) establish study in Iran on 2000 healthy blood donors he determind that 6.55% were HBsAg non reactive while these are positive on HBcAb. The conclusion of the study is that the HBcAb must be applied on routine screening of volunteers blood donors and further testing for HBV DNA may be appriciatable for the detection of HBV infection in donors.

NAT is use to detect the non-reactive blood unites of both assays at the time of acute infection. Studies shows high yield of NAT in small area which are highly endemic for Hepatitis B Virus. They said that NAT might not be for the replacement for HBcAb screening because they have low level of DNA which is undetectable

associated with TTV (Transfusion Transmitted HBV) but HBsAg screening still required. The parallel testing needed to comparison two assays (HBsAg and HBcAb) and NAT in such a way that might it be sufficient for the detection of infectious donors.

They concluded that in the countries where HBcAb/HBsAb screening is not implicated they would be means of screening of donation from chronic infectious individuals only even single donor NAT were not detected. Studies shows that completely automated HBsAg screening may consolidate in critical affectability changes (sensitivity improvements). Every nation should build up its own blood screening strategy for HBV endemicity, out of infectious units which were identified by various serologic/NAT screening assays, and cost adequacy of test have ensuring blood safety (Kuhns, 2006).

The HBcAb test is implicated in United States from more than 20 years which is permit due to false positive results. In this study Researcher took 1324 donors which are tested on HBsAg, HBcAb and HBV DNA test. 37% donors were anti-HBc non-reactive while retesting this donor after 10 months and donor were negative to all viral infection of HBV. Viral DNA was fund in the serum of only those donors who were positive to HBsAg. This population considered as noninfectious and must back to the donor pool. Evidence from the study was ant-HBc gives false positive results it is not more specific and sensitive result (Katz *et al.*, 2008).

The study comprises of total 729 HBsAg non-reactive blood donor units included from which total 30.1% (220) donors were HBsAg negative while were reactive to anti-HBc from 220 only 66 (30%) were positive to HBV NAT. Predominant prevalence of HBV DNA is observed in HBsAg individuals confirm through NAT. They conclude that if alone HBsAg practice for screening then significant number of infected donors will be undiagnosed in contrast with HBcAb screening it will lead to elimination of a considerable units of blood and help in reducing HBV transmission including in immunocompromised individuals (Rajesh *et al.*, 2010).

They study on recipient of OLT (*Orthotopic liver transplantation*) received liver graft from antibody to HB core antigen reactive (HBcAb), HBV surface antigen non-reactive (HBsAg) donors who had at high risk of HB infection development. They also assessed mortality rate because of de novo HBV infection. The rate of occurrence of de novo HBV were 2.7% patient were receiving LAM prophylaxis versus, 3.6% patients receiving HBIGþLAM (combination therapy).

Due to incomplete information (in every treatment group) and limited cases of de novo HBV infection the risk of developing this infection could not be calculated. In summary on the basis of results it is concluded that Nucleoside analogue monotherapy could be applied when any HBV patient receive liver graft from HBcAb(þ) donors (Saab *et al.*, 2010).

The issue focused in this study is the current donor screening for the detection of Surface Antigen and core Antibody of the HBV. HBsAg is a marker of active viremia while HBcAb is a marker of exposure to HBV. Donors that have isolated HBcAb positivity may represent latently infected individuals or a false-positive result. The risk of transmission of HBV from an HBcAb-alone donor is about 10% to 12% for non-liver recipients Additional testing of these donors with HBc, IgG, and IgM as well as HBV, DNA, and NAT would further stratify the risk of transmission, with the highest risk in the IgM-positive, IgG-negative, and NAT-positive donor. Liver recipients of HBsAg-positive or HBcAb-negative donors should be monitored closely for the presence of active viral replication with expansion of therapy based on these results (Ison, 2010).

The cross sectional analysis is conducted in the Rasht in which they describe usage of sensitive screen assay is important before transfusion of the blood for the sake of prevention of blood borne diseases for instance HBV. They took 2041 non-reactive to HBsAg and these were tested for HBcAb reactive samples were further tested for identification of HBV DNA. The presence of HBcAb is 3.8% and DNA of HBV is detected in only 1 sample they concluded that HBcAb perhaps the source of transmission of HBV furthermore analysis of HBV DNA in reactive HBcAb is obligatory (Ali *et al.*, 2011).

This Study is conducted in an endemic area of Tzukuan Township to scrutinize achievement and disappearance of HBsAg. HBsAg and alanine transaminase (ALT) were checked in all selected people and DNA of HBV, against HBs, anti-HBc were examined in various groups. The annually record shoes that rate of clearance of HBsAg were 1.5% and no new HBsAg carrier observed. One out of 17 cases (HBsAg clearance) had positive DNA of HBV. Quantitative analysis of HBsAg shows that 78% had low level of titration, Anti-HBc alone 32% involved and dominant in end age. In this study very higher rate of anti-HBc and HBsAg seroclearance were observed (Tsai *et al.*, 2011).

Presence of anti-HBc antibody in the blood of donors considered as infected individual. The 10.2% donors were reactive to HBcAb and non-reactive to HBsAg and DNA HBV was 9.17% so the rate of occurring of anti-HBc is high so these non-infectious units must return into donor pool. This large unit should not be discarded as it will require fulfilling need of patients and NAT must be added (Makroo *et al.*, 2012).

During 1 year of time period two assays HBsAg and HBcAb analyzed through NAT. total donation was 6.5 million out of which 699 HBV infected of which 444(64%) were reactive to HBsAg, HBcAb and PCR. 697 positive to may be both or with one serological tests and analyzed that 477 (68%) positive to NAT. they said HBsAg shows no blood safety in comparison with anti-HBc (Stramer, 2012).

The study is conducted on Australian patients, they evaluated the sensitivity of alone HBsAg assay and in combination as well HBcAb. Blood samples were obtained to analyze the correlation between different serological marker and HBV DNA for the detection of HBV. From 331 samples 0.3% was negative to Anti-HBc II assay and positive for HBsAg in the presences of HBV DNA and positive in the absence of HBV DNA was 20%. Out of these 67 (20%) 18 had HBcAb negative for all other tests. No any DNA of HBV is found in negative serology. They concluded that the HBcAb Alone may miss an acute infection so the combination of two assays HBcAb and HBsAg is needed with nucleic acid (NAT) for reliable screening (Ba Alawi *et al.*, 2013).

Donor's samples were screened and analyses by molecular level on ELISA to check the presence of core antibody, surface antigen HBe for those who were negative HBsAg positive NAT. From 28,134 samples 25 had HBV DNA out of these 18 samples were selected for further work. By which 12 samples were positive to HBcAb and from them 3 were positive to both HBsAb and HBcAb. In brief among 28134 the 18 were positive to HBsAg and NAT. out of these 18 the 6 samples were in WP (Window Period) and 12 were with Occult HBV infection (OBI). They concluded that from every 3.6 NAT positive samples 1 were negative to serological markers (Doda and Arora 2014).

In this study Allogeneic HSCT (haematopoietic stem cell transplant) recipients from 2000- 2010 were evaluated, the risk of transferring of HBV in HBsAg-negative and HBcAb-positive in HSCT recipient is high. In which prior to HSCT 18% were HBsAg nonreactive but positive to HBcAb. Reactivation observed in 10%. They observed that reactivation hazard was reduced in HBV immune or exposed donors. The donor's immunity was independently and consistently associated with a decreased risk of HBV reactivation, while rituximab and cyclosporine treatments increased the probability (Mikulska *et al.*, 2014).

Summary of all the studies related to current study are given in Table 2.3

Table 2.2 Summary of Literature Review

Name of Author	Year of publication	Country	Assay used in Study	Results	Conclusions
Veena Doda and Satyam Arora	2014	India	ELISA HBcAb HBsAg NAT	HBcAb positive = 12 HBcAb and HBsAg positive = 3 out of 12	From every 3.6 NAT positive samples 1 were negative to serological markers
Ba Alawi et al	2013	Australia	HBcAb HBsAg NAT	Negative to Anti-HBc II = 0.3% and positive for HBsAg in the presences of HBV DNA	HBcAb Alone may miss an acute infection so the combination of two assays HBcAb and HBsAg is needed with nucleic acid (NAT) for reliable screening
Makroo *et al.,*	2012	India	Anti-HBc	Anti-HBc positive = 10.2% Reactive to HBcAb and negative to HBsAg = 9.17%	Anti-HBc positive units must not be discarded

Ali Khamesipour et al	2011	Northern Iran	HbcAb HbsAg NAT	HBcAb = 3.8% HBV DNA detected = 1 sample only	With HBcAb HBV DNA presences is obligatory
Tsai PS et al	2011	Kaohsiung, Taiwan	HBsAg Anti-HBc NAT	HBsAg = 78% Anti-HBc = 32%	higher rate of anti-HBc and HBsAg seroclearance were observed
Saab et al	2010	America	HBcAb HBsAg	2.7% patient were receiving LAM prophylaxis versus 3.6% pateints receiving HBIGþLAM	Nucleoside analogue monotherapy could be applied when any HBV patient receive liver graft from HBcAb(þ) donors.
Ison	2010	Philadelphia	HBsAg HBcAb NAT	Risk of transmission of HBV by an HBcAb positive donor is 10% -12% for non-liver transplant recipients.	Liver recipients of HBsAg Reactive or HBcAb non-reactive donors should be monitored closely for the prevalence of active viral replication with expansion of therapy.
Katz, Strong, Tegtmeier & Stramer	2008	Washington	HBsAg HBcAb HBV DNA test	37% donor were HBcAb positive while Reentry for anti-HBc gives non-reactive results.	HBcAb gives false positive results it is not more specific and sensitive test for the HBV screening for donors.

Kuhns, M. C., & Busch, M. P.	2006	California	HBsAg Anti-HBc NAT	High yield of NAT in small area which are highly endemic for Hepatitis B Virus.	The conclusion was the countries where HBcAb/HBsAb screening is not implicated they offer donation with chronic infection. Every nation should build up its own blood screening strategy for HBV which were identified by NAT screening assays.
Chen et al	1999	china	HBcAg, HBsAg and HBsAb	Out of 2700 Blood donors 300 were HBsAg positive and 336 were HBcAg, HBsAg and HBsAb positive. Total 3 blood donors had HBV DNA out of 29 donors who were positive on HBsAg and negative on HBcAb	S gene variant are involved in positivity and negativity of HBsAg and HBcAb. Commercially improvement is required for proper screening of donors.

Chapter 3

Material and Methods

This study is conducted in Department of Pathology & Blood Transfusion Services, Pakistan Institute of Medical Sciences, Islamabad, and CIRBs and at the Biotechnology laboratory, Department of Bioinformatics and Biotechnology, International Islamic University Islamabad from Nov 2015 till April 2016. Every donor who visit the Department of Blood Transfusion Services, have to fulfill the general criteria for blood donation as described in Standards and Guidelines for Blood Transfusion Services Ministry of National Health Services, Government of Pakistan. (Transfusion, 2010-2015)

Blood samples of 200 donors was collected from Blood Bank of PIMS; SZABMU. All the specimen were collected by the puncture of a vein with a needle to withdraw blood after pretest counseling.

The samples were tested for HBV surface Antigen by CMIA (Abbott, ARCHITECT system) and by using commercially available HBsAb kits (Abbott, ARCHITECT system; HBsAb Qualitative II), Anti-HBc II kits (Abbott, ARCHITECT system; anti-HBc II) and confirm by Nucleic Acid Testing. Both kits were stored according to manufacturer's instructions. The specificity, sensitivity and positive predictive values and negative predictive values were calculated using the NAT results as gold standard.

3.1 Blood sample collection:

Blood (3ml) samples of 200 donors were collected in Vacutainer tubes from Blood bank of PIMS; SZABMU. Blood collection Work was held in a quiet, clean and well-lit area provide soap and water or alcohol swabs (all hand hygiene materials), well-fitting non-sterile disposable gloves, single-use dispensable needles, and syringes or lancing devices in adequate numbers to pledge that every donor has a sterile needle and syringe or proportional for every blood testing; make accessible adequate tubes to forestall perilous practices (e.g. emptying blood to reuse tubes for sample collection). Clear information written and verbal provided to each donor who undergoes venipuncture technique.

Donor was put in comfortable position and a soft elastic tourniquet was connected on the upper arm around 3 inches above to the elbow. The Puncture side was cleaned with 70% liquor swabs for skin purification and open the dispensable syringe. The needle was conveyed parallel to the vein, skin was penetrated needle was progressed about ½ cm and after that vein

was pricked. Adequate amount 5ml of the blood was collected, tourniquet was removed. The needle was withdrawn and a cotton-wool ball was applied over puncture site. The blood was transferred to a plain sterile Vacutainer without anticoagulant and laboratory specimen was labeled with particular unique Donor ID and left Vacutainer at room temperature for at least two hrs (Fig 3.1).

3.2 Centrifugation of Blood:

To confirm consistency in results, samples were transferred to centrifuged tube, after collection of the blood; blood was allowed to clot the blood leaving it for 15 minutes or 1 hour at room temperature. The tube was centrifuged at >10,000 RCF (Relative Centrifugal Force) for 10 minutes or at 4,000 rpm for 5 minutes Serum were separated as shown in Fig 3.2 ,serum transferred to the eppendorf for tests. And samples were stored as frozen at -70 C so it is thawed before testing; the resulting supernatant is utilized for further serological testing. Donors history is available in Appendix IV.

3.3 Analytical Techniques:

3.3.1 Hepatitis B surface Antigen test (HBsAg) through CLIA:

The CLIA is based on detection of luminescence which is emitted from labeled substrate during chemical reaction. Reagents are required for reactions that produce CLIA May be coupled to Antibodies or Antigens. The incubation time is variable depending upon the nature of sample and analysis. The results are achieved in 15 to 45min (Brown, Waheed, Malik, Farooq, & Ansari, 2014). All 200 samples will be tested via CMIA technique on ARCHITECT system by Abbott Diagnostics according to manufacturer instructions.

HBsAg is the first serological marker that appears 1-10 weeks it is used to identify infected person (Having HBV) and to avoid transmission of the infection by blood and blood products and additionally to screen the status of infected people in combination with different hepatitis B serological markers.

Figure 3.1 *Blood Samples Collection*

Figure 3.2 *Centrifugation of Blood*

It is also used for the detection of acute and chronic HBV infections. All 200 samples were tested via CMIA technique on Archietc system by Abbott Diagnostics according to manufacturer instructions. The relative light strong and weak reaction are showed in fig 3.5

3.3.2.1 Principle

1. Firstly rHBcAg coated paramagnetic Microparticles, assay diluent; samples and specimen diluent were combined.
2. In the sample Anti-HBc present binds to rHBcAg (coated with Microparticles) reaction mixture is washed.
3. Then 2nd step is addition of conjugate (anti-human acridinium-labeled) another wash cycle followed, trigger and pre- trigger solution added to reaction mixture.
4. The resulting chemiluminescent reaction is measured as relative light units (RLUs).
5. A direct relationship exists between the amount of anti-HBc in the sample and the RLUs detected by the ARCHITECT i System optics.
6. Relative Light Measurements for Strong and Weak Reactions are observed by which positive and negative samples was analysis. (Appendix III)

3.3.2.2 Reagents

Reagents kit (ARCHITECT Anti-HBc qualitative ll reagent kit 2G21)

1. Microparticles: rHBcAg coated paramagnetic Microparticles

2. **Conjugate:** Anti-human acridinium-labeled

3. **Specimen Diluent:** containing reluctant in MOPSO buffer

Reagents kits for ARCHITECT Anti-HBc are shown in Fig 3.6

The relative light measurements for strong and weak reaction are shown in Fig 3.7

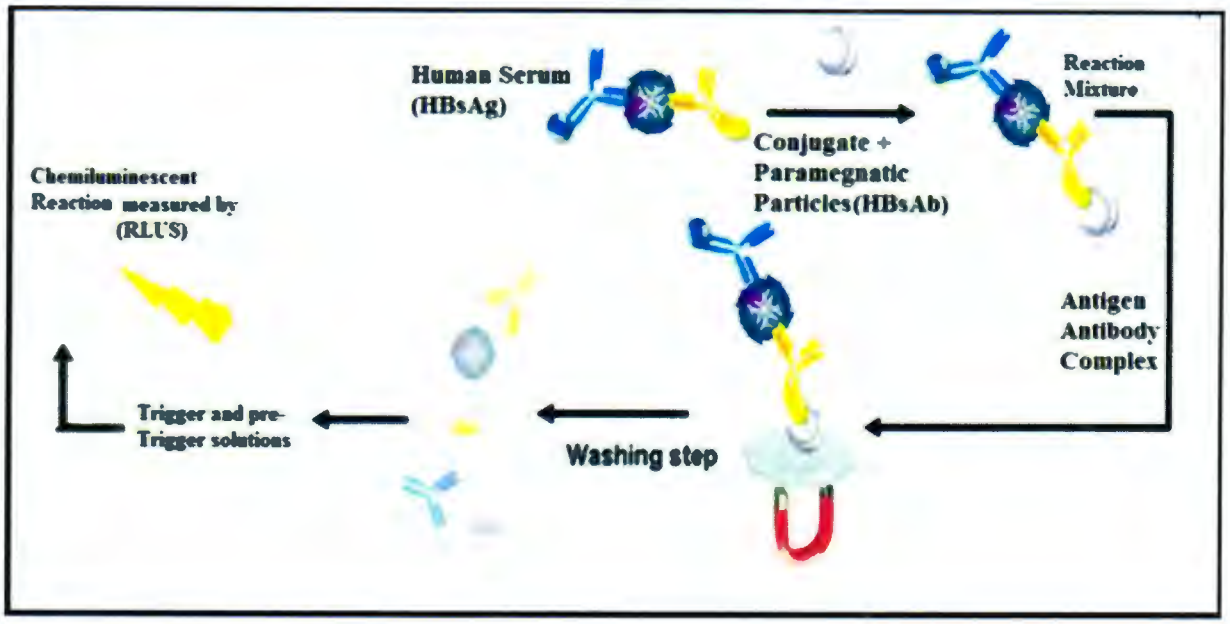


Fig 3.3 *CLIA principle flow sheet diagram of HBsAg:*

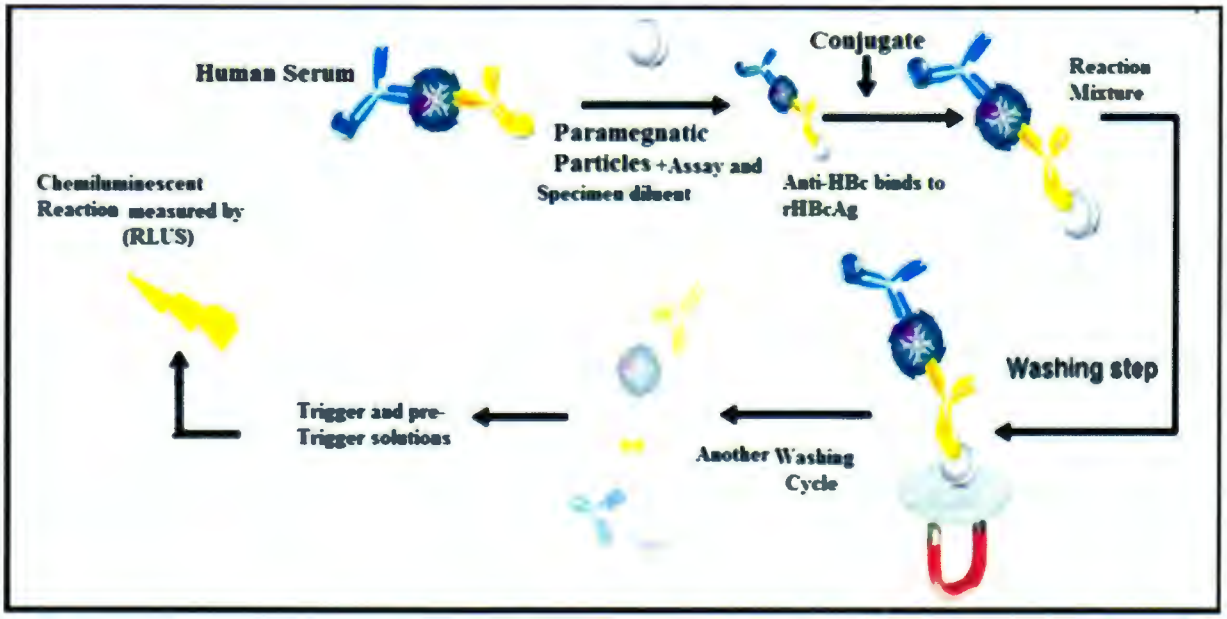


Fig 3.4 *CLIA principle flow sheet diagram of HBcAb*

3.3.3 DNA Extraction:

DNA isolation is basically a process of purification of DNA from sample using a combination of physical and chemical methods. Breaking the cells open commonly referred to as cell disruption or cell lysis, to expose the DNA within.

3.3.3.1 Principle:

Good quality DNA is a prerequisite for all experiments of DNA manipulation. The basic steps of disruption of the cell wall, cell membrane and nuclear membrane to release the DNA into solution followed by precipitation of DNA while ensuring removal of the contaminating biomolecules such as the proteins, polysaccharides, lipids, phenols and other secondary metabolites.

The samples with HBsAg and HBcAb positivity or negativity are followed by the DNA extraction procedure to further analysis on PCR.

3.3.3.2 DNA Extraction Protocol:

Standard protocol by Sambrook and Russel was followed for isolation of HBV DNA.

1. 50 µl serum of each Donors either positive or negative on three assays was taken in the eppendorf.
2. Then add 350 µl of TE buffer and maintain pH 8.0 was incubated at 56 °C for 2 h.
3. While incubation add 2 µl of 1 mg/ml of proteinase K and 50µl of 0.66 % SDS.
4. After 2h eppendorf were taken out from incubator and solution was brought cooled kept on room temperature.
5. Then subsequently extracted the solution with the equal amount of the PCI (phenol:chloroform:isoamyl alcohol::25:24:1).
6. The 5th step was repeated until the white precipitate at the interphase stopped appearing.
7. 0.3 M Sodium acetate salt solution was prepared by maintain pH 5.2
8. Then added Sodium acetate solution to the aqueous layer and protein was precipitated and form a pellet.
9. Then supernatant was extracted to another fresh autoclaved eppendorf.
10. Then add 2.5 µg of glycogen in supernatant, DNA was precipitated with 2.5 volumes of 95 % ethanol and then kept it at −20 °C for 4 h.

11. Then centrifuge it at 13,000 rpm for 15 min DNA was separated by centrifugation process.
12. Then rinsed it with 70 % ethanol (contains 70ml ethanol in 100ml water).
13. After wash at the end dried pellet was dissolved in 20 µl of TE buffer.
14. Now stored this DNA at −20 °C for PCR process (Fig 3.8). (Appendix I)

3.3.4 Polymerase Chain Reaction (PCR):

Polymerase Chain Reaction (PCR) is a powerful and sensitive technique for DNA amplification. The nucleic acid was extracted from 50-µl serum samples of blood donors by using *Sambrook* and *Russel* Organic protocol for DNA isolation. The resulting pellet was re-suspended in TE Buffer at -20°C and then subjected to nested PCR. Then amplification of HBV genome by nested PCR done as using the universal reported primers (Table 3.1) oligonucleotide primer selected from the highly conserved HBV surface gene, for the outer primers, followed by two different mixtures containing type-specific inner primers.

PCR was carried out in a tube containing 5µl of sample was added to 45 µl of reaction mixture made up of the following components:

3.3.4.1 Reaction Mixture:

- 50 ng of each outer primer
- 22.5 picomol/µl of each primer
- a 200 µM concentration of each of the four deoxynucleotides
- 1 U of AmpliTaq Gold DNA polymerase (Perkin-Elmer, Norwalk, Conn.)
- 2.5 unit Taq polymerase
- 1× PCR buffer containing 1.5 mM MgCl2
- 5 µl of reaction buffer (50 mM KCl, 10 mM Tris - HCl, pH = 8.3).

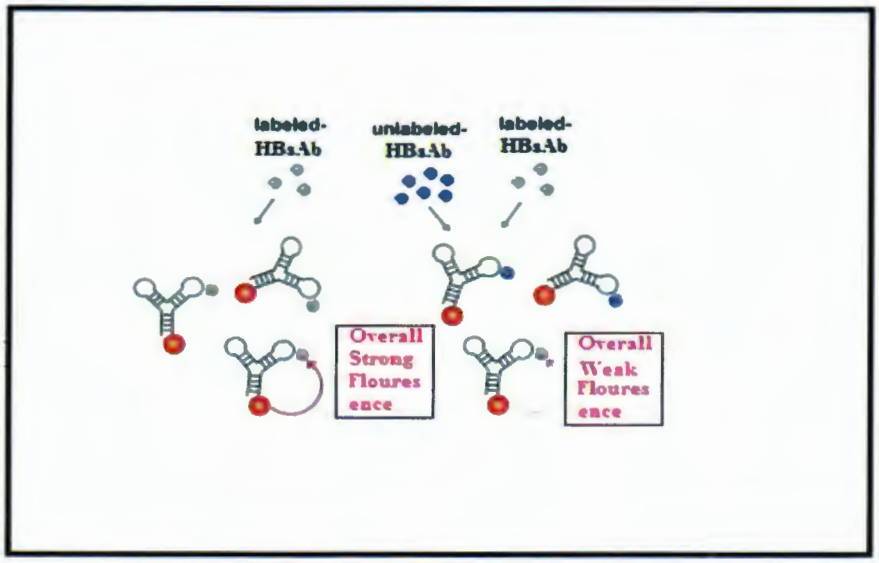


Fig 3.7 *Relative Light Measurements for Strong and Weak Reactions*

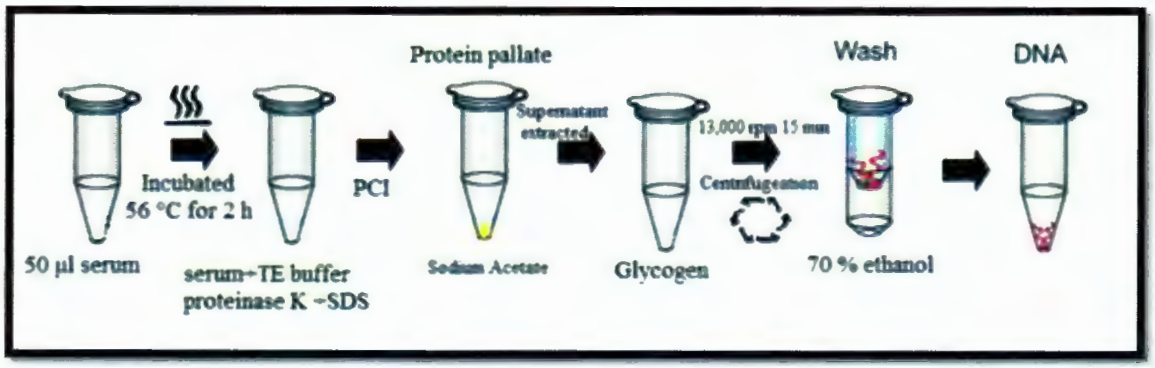


Fig 3.8 *DNA Extraction Protocol by Samrook and Russel* **:**

AmpliTaq Gold DNA polymerase was use to obtain an automatic hot-start reaction. Samples were denatured in an thermal cycler for 5 min at 94 °C and then subjected to 40 cycles of 1 min at 58 °C, 1 min at 72°C and 1 min 94°C and few samples were followed by 40 cycles consisting of 94°C for 20 s, 55°C for 20 s, and 72°C for 1 min in thermal cycler. The observed HBV (Hepatitis B virus) Genome is shown in Fig 3.9

3.3.5 Gel Electrophoresis:

Gel electrophoresis is a method for separation and analysis of macromolecules (DNA, RNA and proteins) and their fragments, based on their size and charge. It is used in clinical chemistry to separate proteins by charge and/or size (IEF agarose, essentially size independent) and in biochemistry and molecular biology to separate a mixed population of DNA and RNA fragments by length, to estimate the size of DNA and RNA fragments or to separate proteins by charge.

Ten µl of reaction product was electrophoresed in a 1.5 per cent agarose gel made in Tris-acetated-EDTA (TAE) buffer with pH=8 and visualized by UV illumination after ethidium bromide (10 µg/ml) staining. Positive and negative controls were also treated as samples. The intensity of expected PCR product band, were then compared with serial dilution of standard control after electrophoresis and ethidium bromide staining and evaluated under UV light. The sizes of PCR products were estimated according to the migration pattern of a 1kb DNA ladder (Naito H, 2001).

Table 3.1 *Primer sequences used for HBV genotyping by nested PCR*

	HBV Primers	Primer Sequences	GC %	Tm(c)
P1[b]	Forward primer(HBVF1)	5′-TCA CCA TAT TCT TGG GAA CAA GA-3′ (nt 2823–2845, universal, sense)	39.13	59.3
S1-2	Reverse Primer(HBVR1)	5′-CGA ACC ACT GAA CAA ATG GC-3′ (nt 685–704, universal, antisense)	50.0	58.4

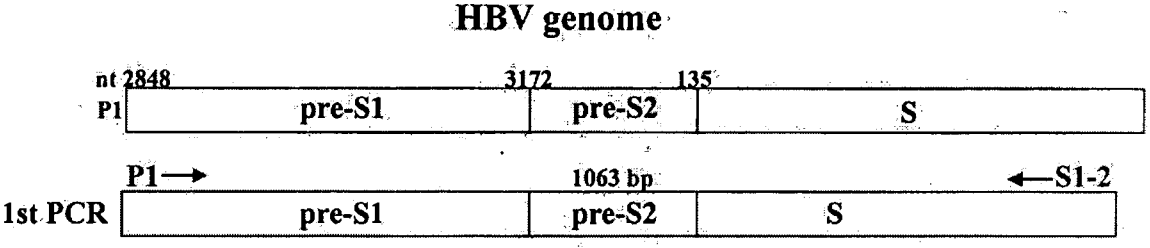


Fig 3.9 *Hepatitis B Virus Genome*

3.3.6 Statistical Analyses:

3.3.6.1 Sensitivity:

The sensitivity of a test is the probability that it will produce a true positive result when used on an infected population. The sensitivity of three assays which are employed for evaluation was determined by formula

$$\text{Sensitivity} = \frac{\text{Number of true positive specimens (TP)}}{\text{TP+ Number of false negative specimen (FN)}} \times 100$$

3.3.6.2 Specificity:

The specificity of a test is the probability that a test will produce a true negative result when used on a non-infected population. The specificity was determined by given formula:

$$\text{Specificity} = \frac{\text{Number of true negative specimens (TN)}}{\text{TN+ Number of false positive specimens (FP)}} \times 100$$

3.3.6.3 Positive and Negative Predictive Values (PPV and NPV)

The positive value of a test is the probability that a person has a proven infection when a positive test result is observed. The positive predicted value of three assays was determined by calculating:

$$\text{Positive predicted value} = \frac{\text{Number of true positive specimen (TP)}}{\text{TP+ Number of false Positive specimen (FP)}} \times 100$$

The negative predicted value of a test is the probability that a person is not infected when a negative test result is observed. The negative test result is observed. The negative predictive value was calculated as follow:

$$\text{Negative predictive value} = \frac{\text{Number of true negative specimen (TN)}}{\text{TN + Number of false negative specimen (FN)}} \times 100$$

3.3.6.4 Positive and negative Diagnostic Likelihood Ratio (DLR):

Diagnostic likelihood ratios (DLR) are valuable tool for comparing the accuracy of several tests to the gold slandered.

3.3.6.4.1 Positive Diagnostic Likelihood Ratio:

The "positive likelihood ratio" tells us how much to increase the probability of disease if the test is positive. The positive DLR represent the odds ratio that a positive test result will be observed in an infected population compare to the odds that the same result will be observed among a non-infected population. The positive DLR was determined by calculating:

$$\text{Positive Diagnostic Likelihood Ratio (DLR)} = \frac{\text{Sensitivity}}{\text{1- Specificity}}$$

3.3.6.4.2 Negative Diagnostic Likelihood Ratio:

The "negative likelihood ratio" tells us how much to decrease it if the test is negative. The negative DLR represents the odd ratio that a negative test results will be observed in an infected population compared to the odds that the same result will be observed among a non-infectious population. The negative DLR for a test was determined by given formula:

$$\text{Negative Diagnostic Likelihood Ratio (DLR)} = \frac{\text{1- sensitivity}}{\text{Specificity}}$$

3.3.7. Disease Prevalence

Hepatitis B virus prevalence was measured by giving formula:

$$\text{Disease Prevanlce} = \frac{a + b}{a+b+c+d}$$

3.3.8.Kappa Agreement:

Kappa tells us the extent to which one Assay and the other assay agree with each other beyond what anyone might expect to see based on chance alone. The kappa is determined by given formula:

$$\text{Kappa} = \frac{\text{Observed Agreement} - \text{Expected Agreement}}{1 - \text{Expected Agreement}}$$

Observed agreement was calculated by calculating the frequency with which the two measurements agreed this is determined by given Formula:

$$\text{Observed Agreement} = \frac{(a+d)}{N}$$

Expected Agreement was calculated by first calculating the expected values of the cells in the 2×2 table using the marginal frequencies, then using those cell numbers to calculate the frequency with which the two measurements are expected to agree this is determined by given formula:

$$\text{Expected Agreement} = \frac{\text{Expected (a)}}{N} + \frac{\text{Expected (d)}}{N}$$

Chapter 4

Results

4.1 Ethical consideration:

The study was approved by Ethical Comity board PIMS and study was carried out according to the standard ethical procedure. All HBV testing was conducted under the conditions of the "3 Cs": involved informed consent, be confidential, and include counseling. Informed written consent were taken from the Donors/patients participated in this study. Considerable efforts were taken to maintain to confidentiality of participants. This included non-disclosure of subject's identity and the use of unique ID number.

4.2. Demographic Characteristics of Donors

Total 200 registered donors were selected for evaluation studies each donor was subjected to blood donation procedure. Among these 200 majority of donors were male i.e. 176 (almost 90%) while females were in minority at 10% i.e. 34 out of 200 (Fig 4.1 A). Mean age of donors was calculated to be 40 years. Donors are distributed in 5 groups according to age: group-1, 2, 3, 4, 5 as shown in (Fig 4.1B). Group 1 had aging up to 18 years. There were 20 donors in the group having age 20-28 (group-2). Large number of donors observed i.e. 95 donors with age of 28-40 (group-3). Forty five subjects were aging from 40-50 (group-4) and the remaining 25 donors were up to 50 years (Fig 4.1 B).

4.3. Hepatitis B Surface Antigen Assay

HBsAg Qualitative II is Chemiluminescent Microparticle Immunoassay (CMIA) and is used for the qualitative detection of hepatitis B surface antigen (HBsAg) found in human serum.

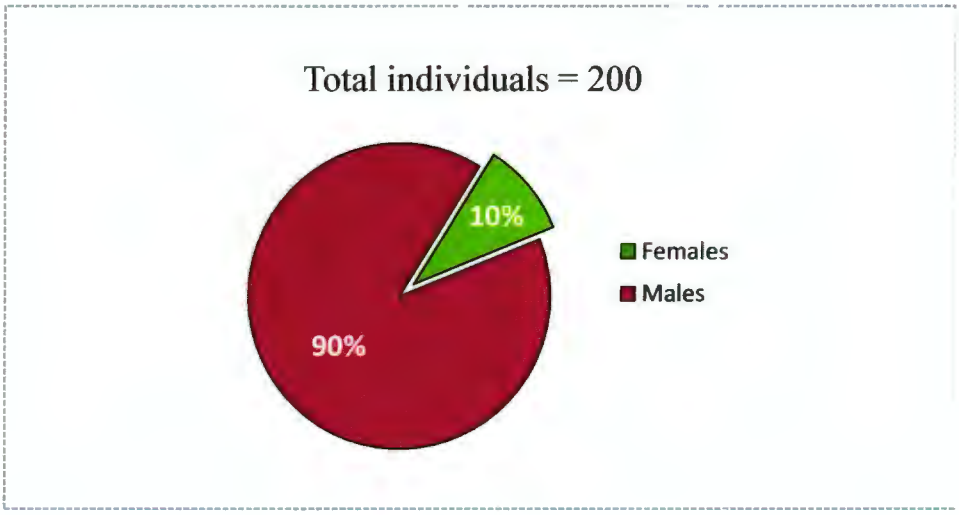


Figure 4.1(A) *Gender wise Distribution of Study Population*

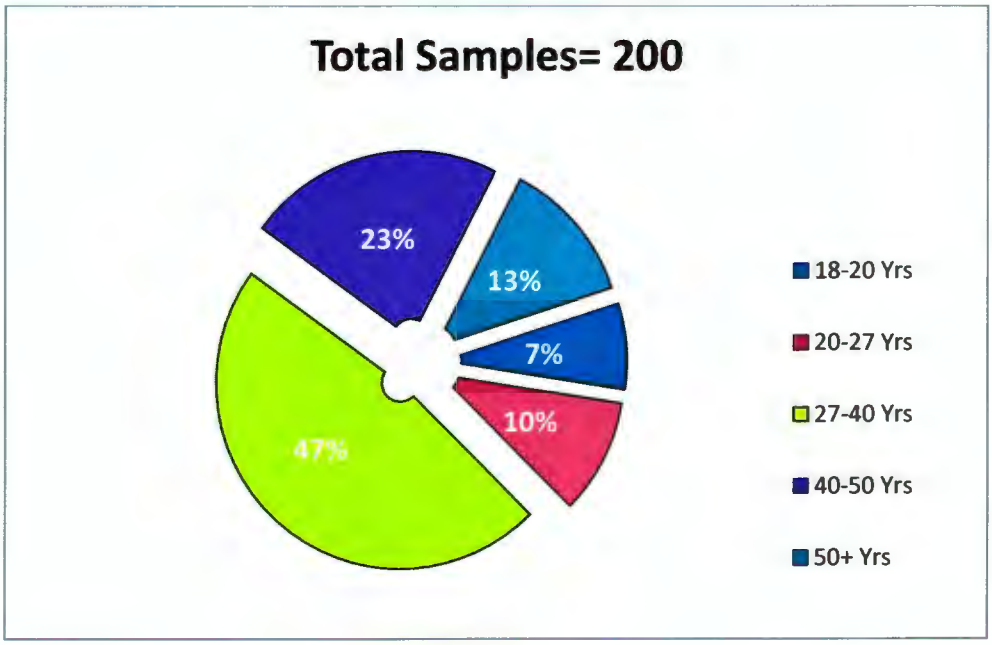


Figure 4.1 (B) *Age-Wise distribution of Study Population*

4.3.1 Detection of Hepatitis B surface Antigen

Diagnosis of HBV is very crucial for all the donors to prevent Transmission Transmitted Infections (TTIs) and for the transfusion of safe blood to the patients. Different techniques are used to diagnose HBV.

Total 7053 samples were screened and out of 5000 only 200 positive samples were taken for study. The results were achieved in 15 to 45min on HBsAg (Hepatitis B surface antigen test, 2000*i* ARCHITECT system by Abbott) diagnostics according to manufacturer instructions. The positivity and negativity of the samples were observed according to standard serum cutoff value (s/co) as shown in Table 4.1. The one step immunoassay HBsAg was run to all samples which yielded an overall 47.2% (n=93) positivity while remaining specimens 52.7% (n=104) were found as negative, results are shown in Fig. 4.2

4.4. Hepatitis B Core Antibody Assay

Anti-HBc II assay is also a CMIA which is used for the qualitative identification of Hepatitis B core antibody (anti-HBc) in human serum. The ARCHITECT Anti-HBc II assay is also used for screening purpose for blood and plasma to prevent transmission of HBV to the donors and blood components and also considered as an aid in the diagnosis of HBV infection.

4.4.1. Detection of Hepatitis B Core Antibody

Anti-HBc II is intended to detect previous exposure to HBV infection. Different techniques and methods are used to detect the core antibody of Hepatitis B. All 200 serum samples were again tested (Hepatitis B Core Antibody (Anti-HBc II)), 2000*i* ARCHITECT system by Abbott) for the detection of core antibody of HBV according to manufacturer instructions.

Results were achieved in 20 to 60 min. The positivity and negativity of the samples were observed according to standard serum cutoff value (s/co) as shown in Table 4.2. The two step immunoassay HBcAb was run to all samples which yielded an overall 53.2% (n=105) positivity while remaining samples 46.7% (n=92) were detected as non-reactive, results are shown in Fig.4.3. Results of two assays are shown in Table 4.3 and results with percentages are shown in Table 4.4

Table 4.1 Interpretation of Results

ARCHITECT HBsAg Qualitative II Initial Result

Initial Results(S/CO)	**Instrument Interpretation**	**Retest procedure**
<1.00	Non-Reactive	No retest required.
≥ 1.00	Reactive	Retest in duplicate.

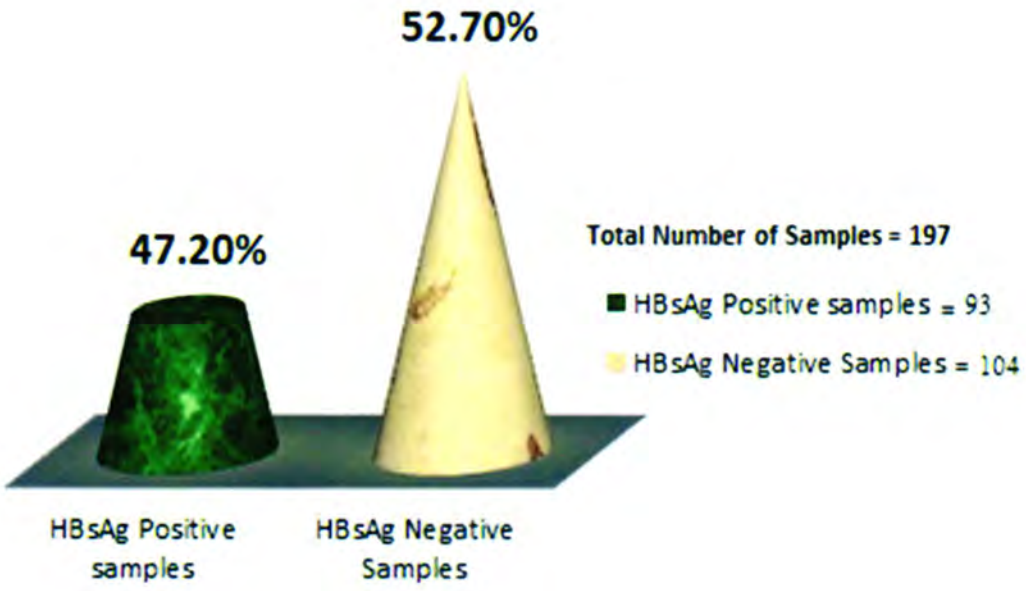


Figure 4.2 *Results of HBsAg Assay of study population*

Table 4.2. Interpretation of Results for HBcAb

ARCHITECT HBcAb Qualitative II Initial Result

Initial Results(S/CO)	**Instrument Interpretation**	**Retest procedure**
<1.00	Non-Reactive	No retest required.
≥ 1.00	Reactive	Retest in duplicate.

Figure 4.3. *Results of HBcAb Assay of study population*

Table 4.3. Test Results of HBcAb and HBsAg

Immunoassay	**Total Number of Samples**	**Number of positive outcome**	**Number of Negative outcomes**
Anti-HBc	197	105	92
HBsAg	197	93	104

Table 4.4 *Results of two assays with percentages*

Assays	***Reactive***	***Percentage***	***Non-Reactive***	***Percentage***
HBsAg	*93*	*47.20%*	*104*	*52.7%*
HBcAb	*105*	*53.20%*	*92*	*46.7%*

n=197

4.5. Polymerase Chain Reaction (PCR)

PCR was done to scrutinize that whether the detection of HBV DNA by PCR could be useful for comparing the two different diagnostic assays used for the detection of hepatitis B virus infection.

4.5.1. Detection of HBV DNA by Polymerase Chain Reaction (PCR)

PCR results were considered as the standard for the detection of HBV infection in particular area population. HBV DNA was identified by PCR with a PCR system (GeneAmp 5700, Applied Bio- systems). The extracted DNA samples of all donors were go through amplification process. The DNA product (amplified) was run on 1.5% percent agarose gel with 150 V and analyzed with a UV transilluminator (UVItec). In all the rounds of amplification the Positive and negative controls were run for HBV.

The results of two assays were noted and observed all the samples which were already diagnosed on HBsAg Qualitative II and Anti-HBc II. The results on PCR showed that out of 197 samples only 94 samples (47.7%) detect the HBV DNA and remaining 103 samples were negative to the HBV DNA. The PCR bands were then visualized by UV transilluminator. Gel for the samples are shown in Fig. 4.4. A,B,C,D,E,F,G,H,I,J and K.

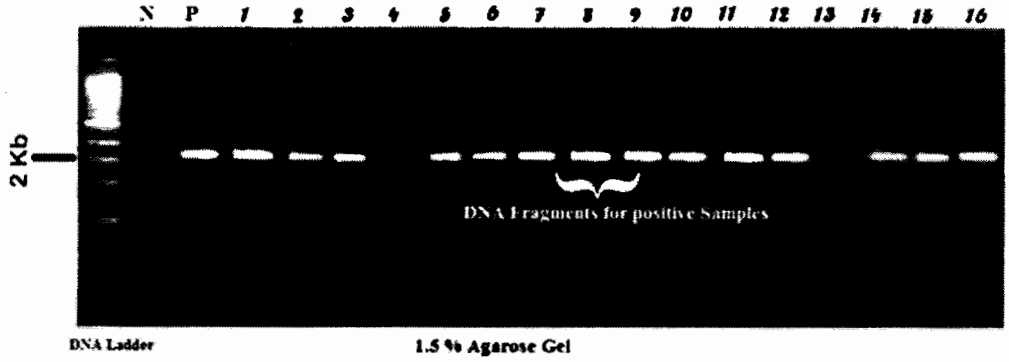


Fig 4.4(A) Positive and Negative Samples with Negative control and DNA ladder

Primer product used for detection of HBV followed by ethidium bromide staining, gel electrophoresis; Lane 1: 1kb DNA ladder, Lane 2: Negative Control. Lane 3: Positive control Well 1-3: Positive samples Lane 4: Negative Samples.

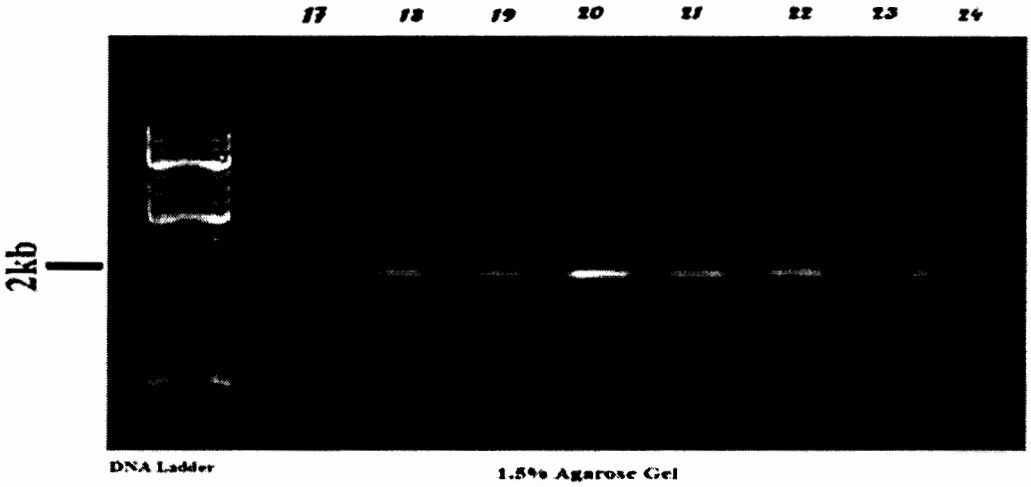


Fig 4.4 (B) Positive and Negative Samples with DNA ladders.

Reported primer product used for detection of HBV followed by ethidium bromide staining, gel electrophoresis; Lane 1: 1kb DNA ladder, wells 17-23: Positive samples, well 24: Negative Samples

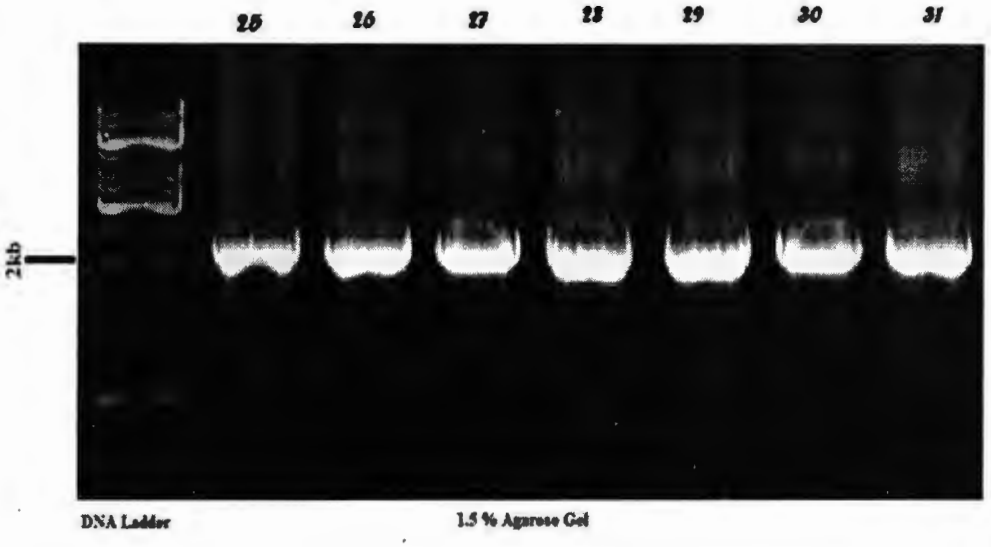


Figure 4.4 (C) Positive and Negative Samples on gel.

Reported primer product used for detection of HBV followed by ethidium bromide staining, gel electrophoresis; Lane 1: 1kb DNA ladder, All are Positive samples

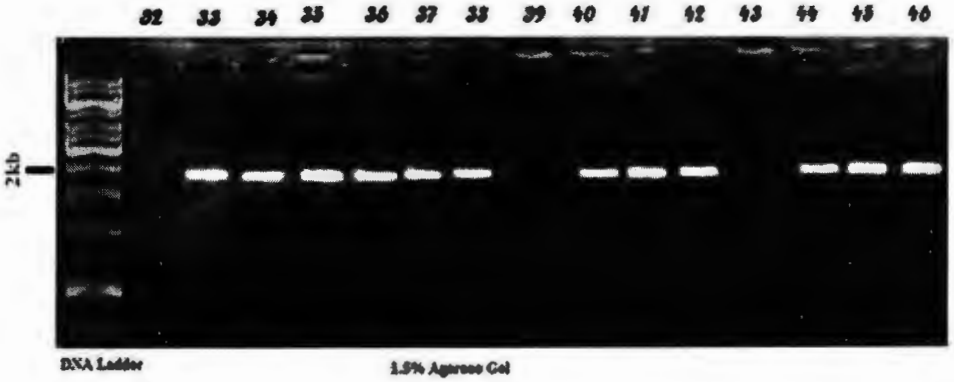


Figure 4.4 (D) Positive and Negative Samples of HBV.

Reported primer product used for detection of HBV followed by ethidium bromide staining, gel electrophoresis; Lane 1: 1kb DNA ladder, wells 33-38,40-42, 44-46: Positive samples, well 32, 39 and 44: Negative Samples

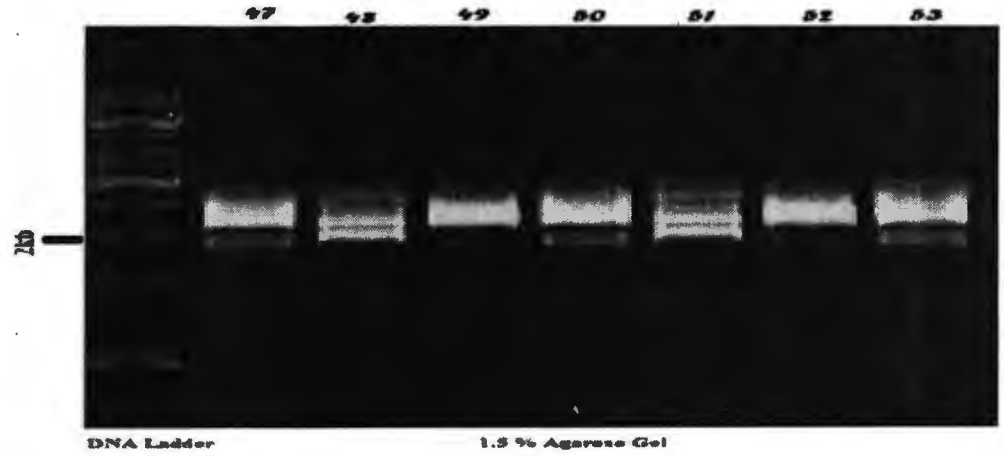


Figure 4.4 (E) Positive and Negative Samples of HBV.

Reported primer product used for detection of HBV followed by ethidium bromide staining, gel electrophoresis; Lane 1: 1kb DNA ladder, all wells: Positive samples.

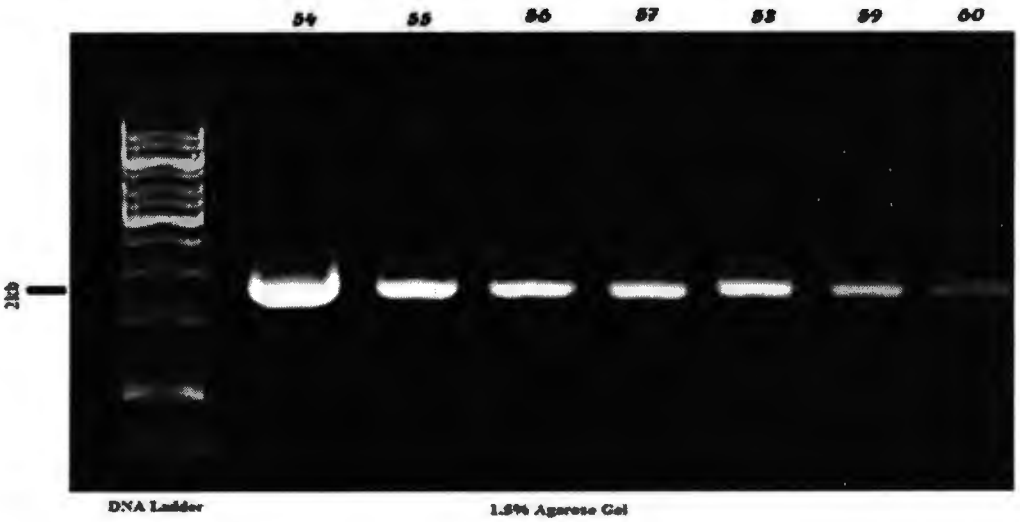


Figure 4.4 (F) Visualization of HBV Positive bands and Negative bands.

Reported primer product used for detection of HBV followed by ethidium bromide staining, gel electrophoresis; Lane 1: 1kb DNA ladder, all wells: Positive samples.

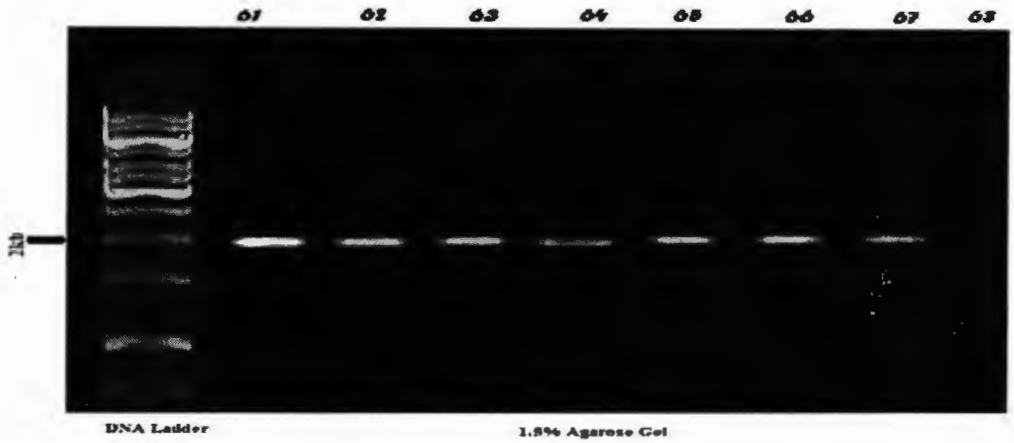


Figure 4.4 (G) Positive and Negative Samples with DNA ladder

Reported primer product used for detection of HBV followed by ethidium bromide staining, gel electrophoresis; Lane 1: 1kb DNA ladder, wells 61-67: Positive samples: well 63: Negative Samples

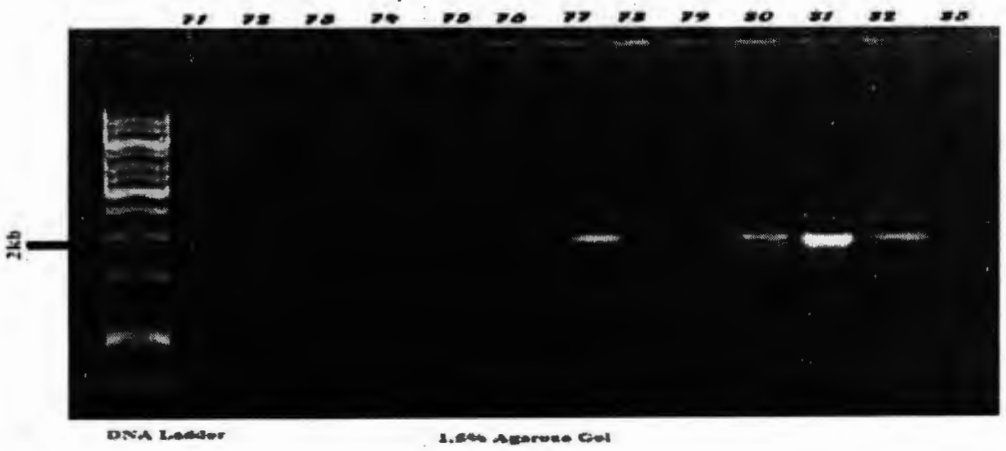


Figure 4.4 (H) HBV DNA Detection on PCR

Reported primer product used for detection of HBV followed by ethidium bromide staining, gel electrophoresis; well 1:1kb DNA ladder, wells 77, 80 and 81: Positive samples Remaining wells: Negative Samples

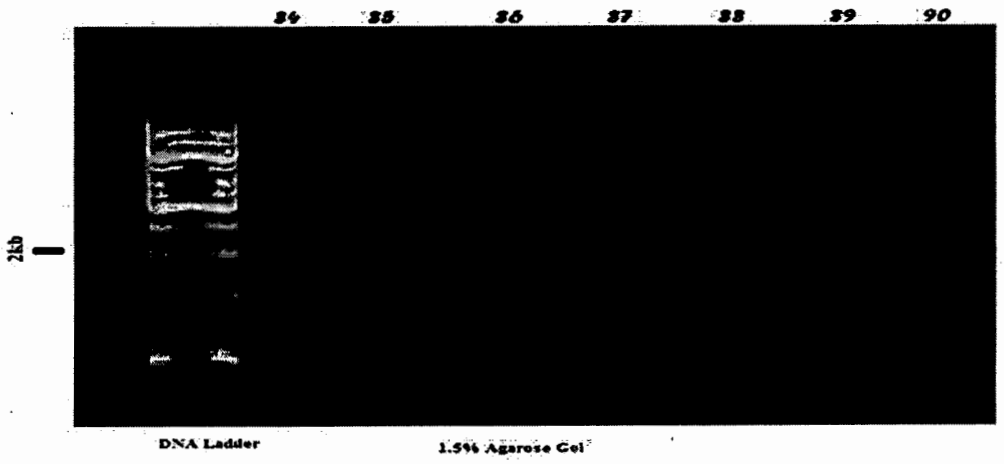


Figure 4.4 (I) PCR Gel shows No bands

Reported primer product used for detection of HBV followed by ethidium bromide staining, gel electrophoresis; Negative Gel, No any bands of HBV detected.

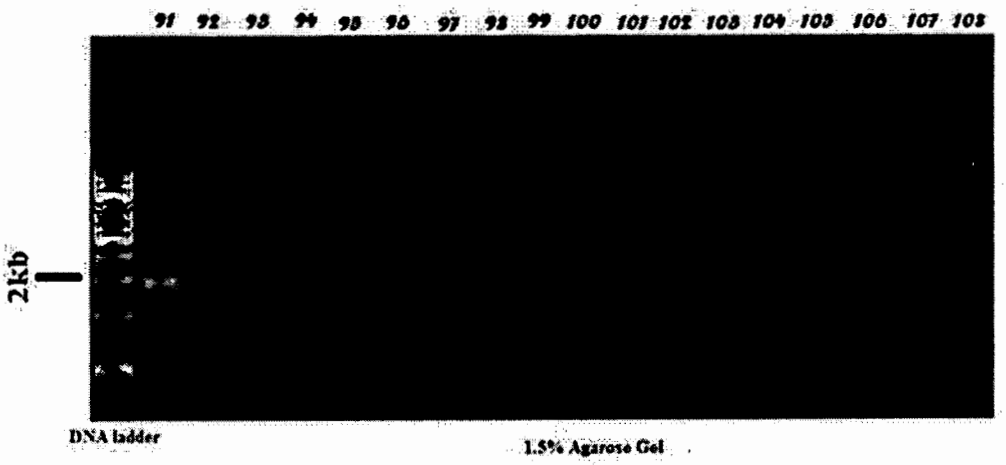


Figure 4.4 (J) Positive and Negative Samples Gel Detected by PCR

Reported primer product used for detection of HBV followed by ethidium bromide staining, gel electrophoresis; Lane 1: 1kb DNA ladder, wells 91, 95 and 104: Positive samples Remaining wells: Negative Samples

4.6. Comparison of HBsAg Qualitative II, Anti-HBc II and PCR

The results of two diagnostic assays HBsAg Qualitative II, Anti-HBc II were tested to check their diagnostic accuracy through paralleling their results with those attained on the PCR. The comparison results of the kit individually with PCR are shown in Fig. 4.4 and Comparison Between HBsAg and HBcAb Assays are shown in Table 4.5.

Test accuracy was measured by positive and negative predicted values/positive negative likelihood ratios sensitivity and specificity. Each measurement of accuracy was used in combination with its complementary measure in following approach:

- Specificity complements sensitivity
- Negative predictive values complement positive predictive values.
- Negative diagnostic likelihood ratio complements positive diagnostic likelihood ratio.

Confidence Interval (CI) was calculated to reflect the statistical significance of each accuracy measure.

4.7. Sensitivity and specificity of kits

The sensitivity of ARCHITECT HBsAg Qualitative II was 98.9% while that of ARCHITECT Anti-HBc II was 89.5%. On the other hand the specificity of the ARCHITECT HBsAg Qualitative II was 98% while the specificity of the ARCHITECT Anti-HBc II was 90.3%. The sensitivity and specificity of the assays are shown in Table 4.6.

4.8. Positive and Negative Predictive Values of kits

The positive Predictive values (PPV) were 90.38% for the ARCHITECT HBsAg Qualitative II while it was 89.5% for ARCHITECT Anti-HBc II. The Negative Predictive value for the ARCHITECT HBsAg Qualitative II was 99.0 % while that of 90.35% for ARCHITECT Anti-HBc II. The positive predictive values and negative predictive values for both assays with are shown in Table 4.7.

Table 4.5 Comparison Between HBsAg and HBcAb Assays

HBsAg Immunoassay	Anti-HBc Immunoassay	Number of outcomes	Total Number of tests	Percentage
Reactive	Non-Reactive	1	197	0.5076%
Reactive	Reactive	93	197	47.20%
Non-Reactive	Reactive	12	197	6.09%
Non-Reactive	Non-Reactive	92	197	46.7%

n=197

Fig 4.5 Comparison of HBcAb, HBsAg and PCR

On the Y-axis Number of the Samples are shown; Results of HBcAb, HBsAg and NAT: In which Positive were indicated with Red color and Negative indicated by Green color.

Table 4.6 Summary of sensitivity and specificity of the HBV assays Evaluations

Assays	Reactive	Non-Reactive	Sensitivity (%)	CI (%)	Specificity (%)	CI (%)
HBsAg	93	104	98.9%	94.2 - 99.9	98	94.7- 99.9
HBcAb	105	92	89.5 %	82.0 - 94.6	90.3	83.3- 95.0

n= 197

Table.4.7. Summary of Positive and Negative Predictive Value of HBV assays Evaluation

Assays	Reactive	Non-Reactive	PPV (%)	CI (%)	NPV (%)	CI (%)
HBsAg	93	104	90.38	94.2-99.2	99.0	94.7-99.9
HBcAb	105	92	89.5	82.0-94.6	90.35	83.3-95.0

4.9. Positive and Negative Diagnostic Likelihood Ratio of Kits

The likelihood ratios are used to assess the values of performing these diagnostic tests for HBV. For positive diagnostic is usually a number greater than one. For the negative diagnostic ratio is usually smaller than one. The positive and Negative Likelihood Ratios are shown in Table 4.8.

4.10 Disease Prevalence

Hepatitis B virus prevalence was measured by giving formula: Results are shown in Table 4.9

$$\text{Disease Prevanlce} = \frac{a + b}{a+b+c+d}$$

4.11. Kappa Agreement for Kits

The measurements of kappa for HBsAg (hepatitis B surface antigen test) were 0.96 and calculations of kappa agreement for HBcAb were 0.88. Values of Kappa are given in Table 4.10 and 2x2 table for Kappa calculation and conditions which are appled are shown in Fig 4.5 and Table 4.10

4.12. Proposed Testing Strategy

The performance of two kits was observed by taking PCR as a Gold standard. The efficacy of the ARCHITECT HBsAg Qualitative II kit was satisfactory and can be used for the further in future to diagnose HBV infection in Pakistani population.

Table 4.8 Positive and negative Likelihood ratio

Parameters	Kits	95% CI		
		Estimated value	Lower Limit	Upper Limit
Positive Likelihood Ratio	**HBsAg**	10.29	14.63	723.77
	HBcAb	9.28	5.27	16.33
Negative Likelihood Ratio	**HBsAg**	0.01	0.00	0.07
	HBcAb	0.12	0.07	0.20

Table 4.9 Hepatitis B virus Disease Prevalence

Disease Prevalence ***Hepatitis B Virus***

Assay	95% confidence Interval		
	Disease Prevalence	Lower Limit	Upper Limit
HBsAg	47.74%	40.63%	54.92%
HBcAb	47.95%	41.17%	54.78%

Fig 4.6 2x2 table for Kappa calculation

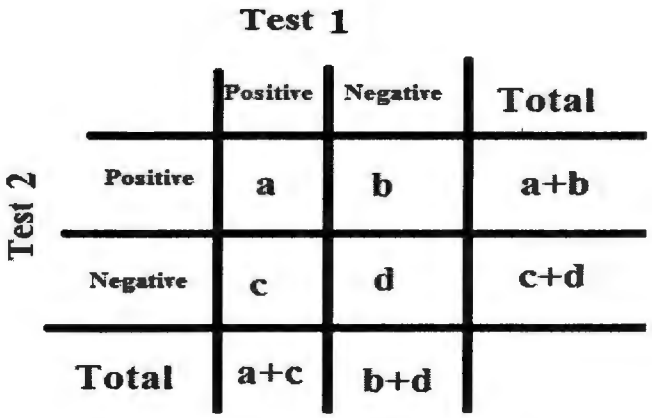


Table 4.10 *Conditions applied for formulas*

Hepatitis B Virus (Disease)

Assay	Present	Denoted by	Absent	Denoted by	Total
Positive	True Positive	a	False Positive	c	a+c
Negative	False Negative	b	True Negative	d	b+d
Total		a+b		c+d	

Chapter 5

Discussion

The chances of TTI (Transfusion transmitted Infection) has been decreased due to advancement in techniques to testing blood and selecting the donors for donation (AuBuchon *et al.*, 1997). In January 1997 usage of PCR for routine testing for HBV was introduced to complete the NAT screening program for the prevention of transfusion transmitted viruses (Roth *et al.*, 2002).

Almost1.5 million of donations are observed in Pakistan annually from which 90% of the collected blood is from the relatives of patients and remaining 10% are charitable donors. The HBsAg, Anti-HBc II and NAT is run parallel on blood of each donor for the sake of achieving blood safety (Zaheer and Waheed, 2014).

Current study shows that 5.5% (105/197) were positive as per anti-HBc assay. Dhawan and colleagues from India reported the core positive rate of 8.4% while in another study the high positivity rate of core reactive i.e. 18.3% was observed by Bhattacharya and group (2004). Donor population study showed 15.9% of Anti-HBc rate (Kumar *et al* ., 2007)

An Iranian Study on healthy blood donors Behbahani and co-workers (2005) reported that 6.55% (12/197) samples were non-reactive to HBsAg and positive for HBcAb while current study results were 6.09% for same parameter indicating a great agreement between the 2 studies.

In the current study there was only 1 sample that was positive for anti-HBc and negative to HBsAg and was found PCR positive for HBV DNA. 5.53 % samples detected is negative by HBsAg not show any PCR bands for the presence of HBV DNA a similar study from india reported these ratio to be 9.17% (Makro *et al.*, 2012). In current research it is observed that HBV infection is endemic and if anti-HBc screening included it would results in discard large unit of blood collected by blood bank.

The total numbers of donors for year 2015 was 27000 annual blood transfusion in Pakistan Institute of medical Sciences (PIMS) out of which 6500 units of blood were utilized by Thalassemia patients and remaining 20500 blood bags for other purposes like Child delivery cases, road accidents, anemia patients and dialysis

patients etc. and from this almost 2100 blood units were discarded on the basis of screening, as shown in Figure 5.1

As current research revealed that if we include Anti-HBc in the screening strategies then transfusion will be followed down fall in the donation rate as core gives false positive results because hepatitis B core antibody have non-specific IgM antibodies which binds to core antigen nonspecifically and cross react with the hepatitis core antigen and reflect false positive results in order to serological response. So it must not take as a parameter for screening to get rid of infection and based on these false positive outcomes large number of blood bags is supposed to be discarded.

It is reported that testing for anti-HBc unanswered problem all over the world they considered unresolved infection felt at large proportion virus production might be present. If these individuals have HBV DNA in their serum then it would be infectious. (Li *et al.*, 2010)

Mostly researchers believed that NAT is useful for the detection of HBV-DNA in regards to obtain more accurate results in window time period. PCR detection of DNA for HBV is considered as constructive where HBV at occurrence is high ratio as in the Pakistani population.

Allian in 2011 suggested that screening for anti-HBc for donor should be restricted special in the area where prevalence rate of HBV is low up to 2% otherwise; the impact on donor deferral would be unsustainable. Countries with high or medium endemicity, and where anti-HBc testing is not routinely done low endemicity rate HBV DNA screening will be more effective (Laperche *et al.*, 2005).

HBV NAT in conjunction with anti-HBc screening would reduce the residual risk of transfusion-transmitted HBV infection (Roth et al., 2002b). Sawke in 2010 suggested as anti-HBc prevalence is high in India therefore screening of HBcAb for donors is not practical and should not be set as discarding criteria. Another study in Egyptian blood donors recommended that policy of testing of blood donors who were positive to anti-HBc may further be submitted to HBV DNA testing analysis. Study proposed a policy of testing blood donors for anti-HBc in addition to HBsAg and those found positive for anti-HBc to be submitted to HBV DNA testing (El-Sherif *et al.*, 2007). On the basis of current study similar actions will be recommended for the Pakistan as well.

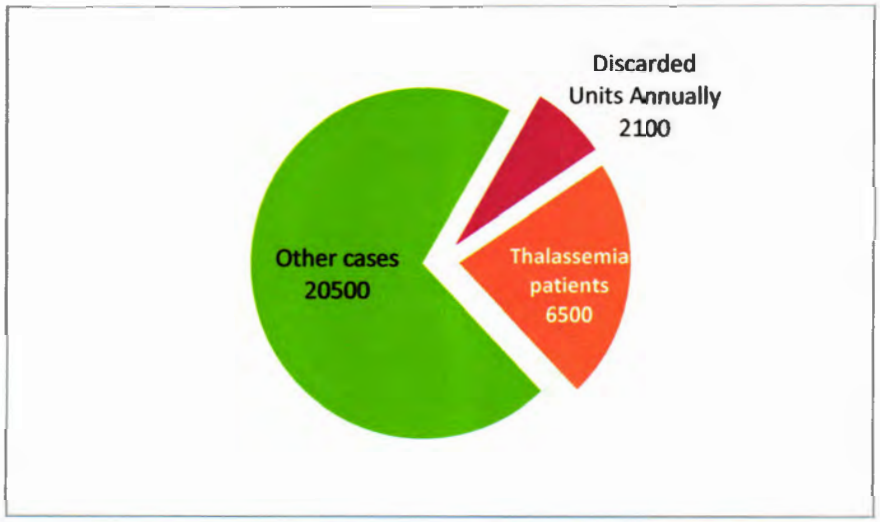


Fig 5.1 *Annual utilization of blood units*

Conclusion

The applicability of anti-HBc is doubtful in this way that if addition of hepatitis B core antibody test is applied in routine along with Hepatitis B surface antigen test may improve the safety but lose huge units of donated blood especially in Pakistan where 5.5% blood bag will be discarded if anti-HBc implemented. The best alternative to include NAT which point out the potentially infectious units of donated blood including window period of donation.

The core antibody of HBV is produced when a person is exposed to acute HBV infection that is first antibody produced at the time of infection to produced immunity in the body of host. The hepatitis B core antibody have non-specific IgM antibodies which binds to core antigen nonspecifically and cross react with the hepatitis core antigen and reflect false positive results in order to serological response. So it must not take as a parameter for screening to get rid of infection. The approach to rejection of anti-HBc in routine screening would return almost 5.5% of blood units to the donor pools.

As different studies suggest that add NAT for better screening in response to HBV infection but it will be expensive approach to developing country like Pakistan so only HBsAg screening could be considered as a best alternative and give satisfactory results at the window time of infection occurs HBsAg results for the reduce risk of HBV infection of transmission are beneficial.

Future Perspective:

The diagnosis of HBV for screening of blood donors alone HBsAg can give enough and satisfactory results as it has similar sensitivity with PCR. It will be cast effective in the way that rather than go toward many other HBV diagnostic test including expensive PCR technique and else other HBsAg alone will helpful in diagnosing HBV.

Chapter 6

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Pakistan Institute of Medical Sciences

Blood Transfusion Service

Interviews of Donors

Name of Donor_____

Blood Group_____

Blood Pressure_____

Age_____ Sex_____

Weight_____

Telephone No._____ Date_____

City_____

Patient Name_____ PCN_____

Questions	Yes	No
Have you donated blood before? If yes… When		
Have you received blood before? If yes… When		
Do you have history of blood pressure and heart disease?		
Do you have history of jaundice?		
Do you have history of T.B?		
Do you have history of Malaria?		
Have you ever had unexplained prolonged fever?		
Any history of significant weight loss?		
Travel abroad…if any please mention country?		
Are you taking any medicine (if yes) mention name?		
Do you have any dental treatment in the past?		
Have you got any recent vaccination?		
Any surgery in the past?		
Medical or diagnostic procedure in the past		
Do you go for shaving to barber		
Are you a drug user?		
Treatment from Homeo or Hakeem?		
Any history of kidney diseases or Epilepsy?		
Any blood disorder or other illness?		
Do you know the patient?		
Are you feeling well today?		

Note: *I consent to donate blood on my own will. I understand the donation process.*

Signature_____

Appendix-I

RECIPES OF SOLUTIONS

1. 2X TBE

- Tris HCl 21.6g (pH 8.0)
- Borate 11g
- EDTA 1.46g

Use as a Chelating agent.

2. 10% SDS

- SDS 10g
- Distilled water 50ml

Use as a Disrupting non-covalent bonds in proteins

3. Bromophenol Blue

- Bromophenol Blue 0.25g
- Glycerol 50ml

Used as a Gel loading dye

4. TE Buffer

- 1M Tris HCl 1ml (pH 8.0)
- 0.5M EDTA 0.2ml

Used as a DNA dissolving buffer and chelating agent

5. 1 µg/ml Ethidium Bromide Staining Solution

- Ethidium Bromide 100 µl of 5 mg/ml
- Deionized or distilled water500 ml

Function as a Staining dye.

Appendix-II

ABBOT ARCHETECHT Kits (HBsAg Qualitative II) Reagents Details

1. **ARCHITECT HBsAg Manual Diluent**

1 Bottle (100 mL) ARCHITECT HBsAg Manual Diluent containing recalcified human plasma;

nonreactive for HBsAg, HIV-1 RNA or HIV-1 Ag, anti-HIV-1/HIV-2, anti-HCV, and anti-HBs. Preservative: Antimicrobial Agent and ProClin 300.

2. **Microparticles**

1 or 4 Bottle(s) (6.6 mL per 100 test bottle/27.0 mL per 500 test bottle) Anti-HBs (Mouse, Monoclonal, IgM, IgG) Coated Microparticles in MES buffer with protein stabilizers. Minimum concentration: 0.0675% solids. Preservative: ProClin 300.

3. **Conjugate**

1 or 4 Bottle(s) (5.9 mL per 100 test bottle/26.3 mL per 500 test bottle) Conjugate: Anti-HBs (Goat, IgG) Acridinium-Labeled Conjugate in MES buffer with protein stabilizers (Bovine and Human Plasma; nonreactive for HBsAg, HIV-1 RNA or HIV-1 Ag, anti-HIV-1/HIV-2, and anti-HCV.) Minimum concentration: 0.25 µg/mL. Preservative: ProClin 300.

4. **ARCHITECT i Pre-Trigger Solution**
   Containing 1.32% (w/v) hydrogen peroxide.
5. **Trigger Solution**

   Containing 1.32% (w/v) hydrogen peroxide

6. **Wash Buffer**

Containing phosphate buffered saline solution. Preservative: Antimicrobial Agent.

Appendix-III

ABBOT ARCHETECHT Kits(HBcAb Qualitative II) Reagents Details

1. **Microparticles**

   1 or 4 Bottle(s) (6.6 mL per 100-test bottle/27.0 mL per 500-test bottle) hepatitis B core (*E. coli*, recombinant) antigen coated microparticles in TRIS buffer. Minimum concentration: 0.08% solids. Preservatives: ProClin 950 and sodium azide.

2. **Conjugate**

1 or 4 Bottle(s) (11.0 mL per 100-test bottle/28.8 mL per 500-test bottle) murine acridinium-labeled anti-human conjugate in MES buffer with protein stabilizers. Minimum concentration: g/mL. Preservatives: sodium alkyl paraben and sodium azide.

3. Assay Diluent

1 or 4 Bottle(s) (5.36 mL per 100-test bottle/23.72 mL per 500-test bottle) assay diluent containing murine protein stabilizers in MOPSO buffer. Preservatives: ProClin 950 and sodium azide.

4. **Specimen Diluent**

1 or 4 Bottle(s) (5.36 mL per 100-test bottle/23.72 mL per 500-test bottle) specimen diluent containing reductant in MOPSO buffer.

5. **ARCHITECT i Pre-Trigger Solution**

Pre-Trigger Solution containing 1.32% (w/v) hydrogen peroxide.

6. **Trigger Solution**

Trigger Solution containing 0.35 N sodium hydroxide.

7. **Wash Buffer**

Wash Buffer containing phosphate buffered saline solution. Preservatives: antimicrobial agents.

Appendix-IV

Donors History

Sample Number	SID	Anti-HBc II (S/CO Values)	HBsAg qualitative II (S/CO Values)	NAT(Results)
1	25841	Reactive 11.56	Reactive 3607.80	Positive
2	24423	Reactive 9.86	Reactive 4202.54	Positive
3	24343	Reactive 10.50	Reactive 4502.89	Positive
4	24419	Reactive 8.10	Reactive 337.86	Negative
5	24358	Reactive 7.38	Reactive 1663.82	Positive
6	448	Reactive 10.03	Reactive 3913.20	Positive
7	208	Reactive 10.45	Reactive 3854.57	Positive
8	26448	Reactive 9.61	Reactive 2319.24	Positive
9	26557	Reactive 10.51	Reactive 2947.20	Positive
10	657	Reactive 10.70	Reactive 3222.38	Positive
11	25764	Reactive 9.78	Reactive 4276.34	Positive
12	26378	Reactive 7.26	Reactive 1886.0	Positive
13	25843	Reactive 8.01	Reactive 1392.29	Positive
14	24324	Reactive 8.86	Reactive 3934.98	Negative
15	24166	Reactive 10.19	Reactive 2060.93	Positive
16	25375	Reactive 9.71	Reactive 2744.23	Positive
17	26243	Reactive 10.10	Reactive 4034.21	Positive
18	2714	Reactive 9.59	Reactive 4139.26	Positive
19	25872	Reactive 9.60	Reactive 2383.20	Positive
20	24321	Reactive 9.72	Reactive 3553.71	Positive
21	2654	Reactive 10.04	Reactive 3102.68	Positive
22	2623	Reactive 10.06	Reactive 1803.89	Positive

23	2594	Reactive 10.29	Reactive 1752.27	Positive
24	1910	Reactive 9.59	Non-Reactive 0.21	Negative
25	2580	Reactive 9.69	Reactive 694.64	Positive
26	2572	Reactive 9.89	Reactive 4659.20	Positive
27	307	Reactive 9.14	Reactive 1397.79	Positive
28	2585	Reactive 10.65	Reactive 4393.11	Positive
29	341	Reactive 9.86	Reactive 4307.44	Positive
30	434	Reactive 10.52	Reactive 3955.43	Positive
31	24864	Reactive 10.44	Reactive 5025.94	Positive
32	1556	Reactive 9.30	Reactive 4539.55	Positive
33	2274	Reactive 9.92	Reactive 2992.44	Positive
34	2250	Reactive 9.71	Reactive 1839.76	Positive
35	2236	Reactive 10.27	Reactive 3195.41	Positive
36	2024	Reactive 10.23	Reactive 1347.70	Positive
37	1858	Reactive 9.07	Reactive 1342.52	Positive
38	2522	Reactive 8.58	Reactive 2578.54	Positive
39	2734	Reactive 8.66	Reactive 2377.42	Non-reactive
40	2697	Reactive 9.97	Reactive 3408.23	Positive
41	3821	Reactive 10.28	Reactive 3339.63	Positive
42	24796	Reactive 10.00	Reactive 4304.67	Positive
43	2523	Reactive 9.00	Reactive 2291.21	Non-reactive
44	2951	Reactive 9.96	Reactive 2548.51	Positive
45	2883	Reactive 10.37	Reactive 2286.80	Positive
46	2824	Reactive 9.46	Reactive 1757.05	Positive
47	1482	Reactive 9.84	Reactive 2060.65	Positive

48	1266	Reactive 10.66	Reactive 4480.30	Positive
49	1437	Reactive 10.21	Reactive 3539.84	Positive
50	1172	Reactive 10.04	Reactive 1531.17	Positive
51	3912	Reactive 10.19	Reactive 4028.79	Positive
52	2992	Reactive 10.51	Reactive 4667.31	Positive
53	4687	Reactive 9.78	Reactive 1953.47	Positive
54	2975	Reactive 8.88	Reactive 3630.31	Positive
55	4462	Reactive 10.17	Reactive 4521.87	Positive
56	3490	Reactive 10.45	Reactive 4159.88	Positive
57	4047	Reactive 8.81	Reactive 3990.02	Positive
58	5144	Reactive 9.32	Reactive 2445.38	Positive
59	7465	Reactive 7.10	Reactive 950.0	Positive
60	6756	Reactive 9.78	Reactive 5104.64	Positive
61	3215	Reactive 9.38	Reactive 4607.00	Positive
62	5023	Reactive 9.03	Reactive 4582.74	Positive
63	6917	Reactive 10.04	Reactive 4560.30	Positive
64	5843	Reactive 9.96	Reactive 1212.10	Positive
65	5522	Reactive 10.37	Reactive 3524.99	Positive
66	5144	Reactive 8.66	Reactive 2445.38	Positive
67	5606	Reactive 9.58	Reactive 4311.55	Positive
68	8397	Reactive 9.87	Reactive 4932.59	Negative
69	5596	Reactive 9.76	Reactive 5297.78	Positive
70	151	Reactive 9.35	Reactive 2758.82	Positive
71	4130	Reactive 8.58	Reactive 4311.55	Negative
72	4107	Reactive 9.35	Reactive 4506.23	Negative

73	4169	Non-Reactive 0.11	Reactive 3975.10	Negative
74	4160	Reactive 10.12	Reactive 1212.10	Negative
75	4168	Reactive 5.70	Reactive 2118.09	Negative
76	4108	Reactive 9.35	Reactive 3534.99	Negative
77	4166	Reactive 4.22	Reactive 4979.71	Positive
78	4173	Reactive 4.22	Reactive 41193.05	Negative
79	4127	Reactive 8.58	Reactive 5298.78	Negative
80	4136	Reactive 4.20	Reactive 4272.15	Positive
81	4140	Reactive 6.10	Reactive 1809.43	Positive
82	4149	Reactive 8.58	Reactive 1803.89	Positive
83	4109	Non-Reactive 0.14	Reactive 4659.20	Negative
84	4120	Reactive 7.58	Reactive 4521.87	Negative
85	4146	Non-Reactive 0.10	Reactive 4582.74	Negative
86	4133	Reactive 3.88	Reactive 1953.47	Negative
87	4122	Reactive 8.58	Reactive 2584.51	Negative
88	4164	Non-Reactive 0.17	Reactive 2700.83	Negative
89	4172	Non-Reactive 0.17	Reactive 1316.98	Negative
90	4177	Non-Reactive 0.42	Reactive 3102.68	Negative
91	4176	Reactive 3.78	Reactive 2992.44	Positive
92	4170	Non-Reactive 0.08	Reactive 2582.56	Negative
93	4134	Non-Reactive 0.11	Reactive 3607.80	Negative
94	4156	Non-Reactive 0.08	Non-Reactive 0.24	Negative
95	4115	Reactive 4.00	Non-Reactive 0.20	Positive
96	4426	Non-reactive 0.02	Non-Reactive 0.29	Negative
97	4397	Non-reactive 0.02	Non-Reactive 0.19	Negative

98	4409	Non-reactive 0.23	Non-Reactive 0.22	Negative
99	4436	Non-reactive 0.29	Non-Reactive 0.17	Negative
100	4435	Non-reactive 0.28	Non-Reactive 0.23	Negative
101	4171	Non-Reactive 0.12	Non-Reactive 0.35	Negative
102	4159	Reactive 7.87	Non-Reactive 0.13	Negative
103	4840	Non-Reactive 0.12	Non-Reactive 0.14	Negative
104	4835	Non-Reactive 0.10	Non-Reactive 0.16	Positive
105	4836	Non-Reactive 0.12	Non-Reactive 0.20	Negative
106	4174	Non-Reactive 0.08	Non-Reactive 0.18	Negative
107	4142	Non-Reactive 0.14	Non-Reactive 0.25	Negative
108	4157	Non-Reactive 0.21	Non-Reactive 0.29	Negative
109	4144	Non-Reactive 0.07	Non-Reactive 0.36	Negative
110	4153	Non-Reactive 0.06	Non-Reactive 0.16	Negative
111	4135	Non-Reactive 0.15	Non-Reactive 0.28	Negative
112	4124	Non-Reactive 0.13	Non-Reactive 0.15	Negative
113	4175	Non-Reactive 0.11	Non-Reactive 0.25	Negative
114	4152	Non-Reactive 0.10	Non-Reactive 0.56	Negative
115	4163	Non-Reactive 0.35	Non-Reactive 0.34	Negative
116	4158	Reactive 8.58	Non-Reactive 0.24	Positive
117	4165	Non-Reactive 0.45	Non-Reactive 0.20	Negative
118	4117	Non-Reactive 0.07	Non-Reactive 0.23	Negative
119	4137	Non-Reactive 0.07	Non-Reactive 0.25	Negative
120	4147	Non-Reactive 0.09	Non-Reactive 0.21	Negative
121	4123	Non-Reactive 0.06	Non-Reactive 0.23	Negative
122	4116	Non-Reactive 0.08	Non-Reactive 0.22	Negative

123	4125	Non-Reactive 0.07	Non-Reactive 0.23	Negative
124	4154	Non-Reactive 0.07	Non-Reactive 0.22	Negative
125	4143	Non-Reactive 0.08	Non-Reactive 0.14	Negative
126	4865	Non-Reactive 0.07	Non-Reactive 0.19	Negative
127	4245	Non-Reactive 0.09	Reactive 4259.97	Negative
128	4447	Non-Reactive 0.10	Non-Reactive 0.22	Negative
129	4471	Non-Reactive 0.09	Non-Reactive 0.18	Negative
130	4466	Reactive 6.04	Non-Reactive 0.14	Positive
131	4870	Non-Reactive 0.07	Non-Reactive 0.18	Positive
132	4832	Reactive 7.66	Non-Reactive 0.19	Positive
133	4862	Non-Reactive 0.09	Non-Reactive 0.17	Negative
134	4884	Non-Reactive 0.13	Non-Reactive 0.17	Negative
135	4892	Non-Reactive 0.11	Non-Reactive 0.24	Negative
136	4886	Non-Reactive 0.09	Non-Reactive 0.17	Negative
137	4866	Non-Reactive 0.73	Non-Reactive 0.19	Negative
138	4854	Non-Reactive 0.07	Non-Reactive 0.17	Negative
139	4874	Non-Reactive 0.11	Non-Reactive 0.35	Positive
140	4901	Non-Reactive 0.06	Non-Reactive 0.23	Negative
141	4847	Non-Reactive 0.10	Non-Reactive 0.18	Negative
142	4885	Non-Reactive 0.08	Non-Reactive 0.18	Negative
143	4958	Non-Reactive 0.10	Non-Reactive 0.24	Negative
144	4888	Non-Reactive 0.08	Non-Reactive 0.21	Negative
145	4845	Non-Reactive 0.07	Non-Reactive 0.21	Positive
146	4861	Non-Reactive 0.10	Non-Reactive 0.19	Negative
147	4145	Reactive 1.73	Non-Reactive 0.22	Positive

148	4110	Reactive 3.80	Non-Reactive 0.22	Positive
149	4119	Non-Reactive 0.06	Non-Reactive 0.21	Negative
150	4912	Non-Reactive 0.06	Non-Reactive 0.29	Positive
151	4430	Non-Reactive 0.47	Non-Reactive 0.23	Negative
152	4439	Non-Reactive 0.07	Non-Reactive 0.20	Negative
153	4438	Non-Reactive 0.12	Non-Reactive 0.22	Negative
154	4429	Non-Reactive 0.08	Non-Rea0.14ctive 0.21	Positive
155	4437	Reactive 5.98	Non-Reactive 0.21	Negative
156	4457	Non-Reactive 0.19	Non-Reactive 0.18	Negative
157	4449	Non-Reactive 0.32	Non-Reactive 0.21	Non-reactive
158	4448	Reactive 4.91	Non-Reactive 0.23	Positive
159	4456	Non-Reactive 0.08	Non-Rea0.14ctive 0.19	Negative
160	4441	Non-Reactive 0.17	Non-Reactive 0.19	Positive
161	4460	Reactive 1.60	Non-Reactive 0.17	Positive
162	4492	Non-Reactive 0.10	Non-Reactive 0.19	Negative
163	4469	Non-Reactive 0.48	Non-Rea0.14ctive 0.13	Negative
164	4468	Non-Reactive 0.12	Non-Reactive 0.19	Negative
165	4467	Non-Reactive 0.16	Non-Reactive 0.20	Negative
166	4464	Non-Reactive 0.13	Non-Reactive 0.19	Negative
167	4454	Non-Reactive 0.09	Non-Reactive 0.20	Negative
168	4463	Non-Reactive 0.15	Non-Rea0.14ctive 0.16	Positive
169	4465	Non-Reactive	Non-Reactive 0.15	Negative
170	4451	Non-Reactive	Non-Reactive 0.16	Positive
171	4482	Reactive 2.00	Non-Reactive 0.16	Positive
172	4461	Non-Reactive 0.08	Non-Reactive 0.19	Negative

173	4452	Non-Reactive 0.15	Non-Reactive 0.20	Negative
174	4453	Non-Reactive 0.08	Non-Reactive 0.18	Negative
175	4445	Non-Reactive 0.13	Non-Rea0.14ctive 0.22	Negative
176	4417	Non-Reactive 0.10	Non-Reactive 0.23	Negative
177	4442	Non-Reactive 0.04	Non-Reactive 0.23	Negative
178	4434	Non-Reactive 0.13	Non-Reactive 0.21	Negative
179	4433	Reactive 5.68	Non-Reactive 0.24	Positive
180	4431	Non-Reactive 0.09	Non-Reactive 0.24	Negative
181	4425	Non-Reactive 0.07	Non-Reactive 0.24	Negative
182	4424	Non-Reactive 0.12	Non-Reactive 0.20	Negative
183	4420	Non-Reactive 0.08	Non-Reactive 0.21	Negative
184	4410	Non-Reactive 0.07	Non-Reactive 0.24	Negative
185	4400	Non-Reactive 0.09	Non-Reactive 0.23	Negative
186	4399	Non-Reactive 0.22	Non-Reactive 0.21	Negative
185	4398	Non-Reactive 0.09	Non-Reactive 0.21	Positive
186	4396	Non-Reactive 0.22	Non-Reactive 0.23	Positive
187	4395	Reactive 6.59	Non-Reactive 0.23	Positive
188	4394	Non-Reactive 0.15	Non-Reactive 0.23	Negative
189	4393	Reactive 8.12	Non-Reactive 0.20	Negative
190	4392	Non-Reactive 0.07	Non-Reactive 0.22	Negative
191	4391	Non-Reactive 0.14	Non-Reactive 0.23	Negative
192	4401	Non-Reactive 0.08	Non-Reactive 0.24	Negative
193	4402	Non-Reactive 0.23	Non-Reactive 0.24	Negative
194	4418	Non-Reactive 0.09	Non-Reactive 0.25	Positive
195	4419	Reactive 3.46	Non-Reactive 0.38	Negative

196	4407	Reactive 5.85	Non-Reactive 0.22	Negative
197	4408	Non-Reactive 0.12	Non-Reactive 0.23	Negative
198	4406	Non-Reactive 0.09	Non-Reactive 0.24	-
199	7576	Non-Reactive 0.08	Reactive 4539.5	
200	7637	Non-Reactive 0.17	Reactive 531.17	-