Frequency of Hepatitis B, C and HIV infections among transfusion-dependent Thalassemia patients in Bangladesh

A Dissertation Submitted to the Department of Mathematics and Natural Sciences, BRAC University in Partial Fulfillment of the Requirement for the Degree of Bachelor of Science in Biotechnology

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Dedicated to

My family and well-wishers

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List of Abbreviations

TTI	Transfusion transmitted infection
TD	Transfusion dependent
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
AIDS	Acquired immunodeficiency syndrome
ICT	Immunochromatographic
HbE	Hemoglobin E
HBsAg	Hepatitis B surface antigen
HbA	Hemoglobin A
B Thal	Beta thalassemia
NAFLD	Non-alcoholic fatty acid
ALD	Alcoholic liver disease
DFV	Dengue fever virus
vCJD	Virus Creutzfeldt-Jakob disease
BSL	Bio safety level
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IgA	Immunoglobulin V
nm	Nanometer
µL	Microlitre
ELISA	Enzyme linked immunosorbent assay
BMI	Body mass index
EBT	E beta thalassemia
BTM	Beta thalassemia major
DAA	Direct acting antigen
RBV	Ribavirin
IFN	Interferon
RNA	Ribonucleic acid
NAT	Nucleic acid testing
RIBA	Recombinant immunoblot assay
PCR	Polymerase chain reacton
DNA	Deoxyribo nuceic acid

Abstract

Transfusion transmitted infections (TTI) have become a major problem in patients with thalassemia who have to undergo regular transfusion. Though effective screening system and proper donor selection have lowered the rate of infections, still the multitransfused patients are not risk free. In this study, a total of 148 transfusion-dependent (TD) patients with Beta Thalassemia was screened and among them infected cases with HCV, HBV and HIV were 12.83%, 3.37% and 0%, respectively. Moreover, 2.02% patients were found to be co-infected with both HBV and HCV. Immunochromatographic (ICT)-based rapid test kits are usually used to screen these infections in the donors' blood before transfusion. However, the traditional ICT kits are not sensitive enough to detect infections. So, combination of both Nucleic Acid testing (NAT) and serological testing may be done to significantly reduce the risk of viral infections during blood transfusion. Besides, although HCV infections are most prevalent among maultitransfused patients, an effective vaccination system may reduce the rate of occurrences.

Chapter-1

Introduction

1.1 Background

Thalassemia is an inherited blood disorder in which the body makes an abnormal form of hemoglobin. Hemoglobin is the protein molecule in red blood cells that carries oxygen. This disorder results in excessive destruction of red blood cells, which leads to anemia, that is, it is a condition in which our body doesn't have enough normal, healthy red blood cells. Thalassemia is inherited, meaning that at least one of your parents must be a carrier of the disease. It is caused by either a genetic mutation or a deletion of certain key gene fragments [1]. Thalassemia is one of the common and major health problem causing morbidity and mortality [2]. Thalassemia can be classified into two types- beta thalassemia and alpha thalassemia. Beta thalassemia is caused when beta globin genes are affected, two genes one from each parent is inherited and alpha thalassemia is caused when alpha globin genes are affected, 4 genes, 2 genes from one of each parent is inherited. According to the World Health Organization (WHO), approximately 240 million people are chronically infected with HBV worldwide, while 150 million people are infected with HCV [3].

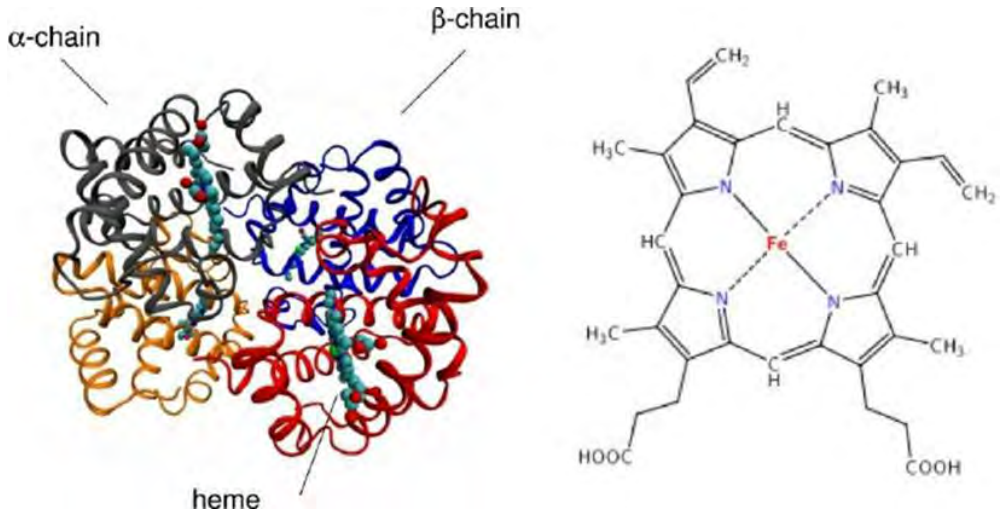


Figure-1: The quaternary structure of hemoglobin and its oxygen carrier heme

This inherited hemoglobin disorders are becoming a global public health concern. An estimated 320,000 babies are born each year with a clinically significant hemoglobin disorder. Nearly 80% of these births occur in developing countries [4]. Most conservative estimates suggest that at least 5.2% of the world population (over 360 million) carry a significant hemoglobin variant and in excess of 100 million beta thalassemia carriers with a global frequency of 1.5%. Hemoglobinopathies are mostly seen in certain malaria prone parts of the world including Africa, all Mediterranean countries, the Middle East, the Indian subcontinent and Southeast Asia. In each year, over 50,000 new patients are born with a severe form of thalassemia (beta-thalassemia major and HbE beta thalassemia) worldwide. Due to high rate of international migration, thalassemia is spreading to non-endemic parts of the world. In many Asian countries, the most common form of thalassemia results from the coinheritance of beta thalassemia and HbE. In the eastern parts of Indian subcontinent, Bangladesh and other Southeast Asian countries, HbE is the most prevalent hemoglobin variant [4].

Bangladesh is one of the most densely populated countries in the world, with a population of over 160 million people [4]. Over 70% of the population lives in highly resource-constrained rural areas, while most tertiary hospitals are located in big cities, notably in Dhaka, the capital city. Public hospitals are often overcrowded and lack resources (such as specialized and basic medical equipment, healthcare professionals and essential drugs). On the other hand, some private clinics and hospitals are relatively resourceful but these are not accessible to the mass population as it is expensive. The treatment drop-out rate among a population plagued by poverty is expected to be very high, and is presumably driven by lack of access, either due to lack of awareness or income of patients seeking care on the demand side, or inadequate expertise, facilities, knowledge, and infrastructure from the supply side of health care.

1.2 Beta Thalassemia

1.2.1 Beta Thalassemia Major:

Beta Thalassemia Major or Cooley's anemia is a serious anemia affecting people who have inherited the two unusual beta thalassemia genes, one from each parent. That is, it is caused when the beta genes are missing. People with beta thalassemia major do not produce enough healthy, mature red blood cells, therefore, they become very pale and anemic. From about the age of 3 months children with this condition become very pale, lethargic, lose their appetite and soon fail to thrive, and usually die between 1 and 10 years of age. But with proper medical care they can have long life [5].

1.2.2 Thalassemia Intermedia:

Beta thalassemia minor occurs when an individual inherits one usual beta gene from one parent and one unusual beta gene from the other (Hb A bThal). It is found in areas of the world where malaria is, or was, common. That is, it develops because of alterations in both beta globin genes. People with beta thalassemia minor have red blood cells, which are smaller, paler, and have less red pigment, but this does not cause any health problems. Generally, their red blood cells are still able to carry oxygen around the body as efficiently as someone with the two usual b genes. Because these individuals are themselves healthy, they will not know that they have this unusual trait unless they have a special blood test. If a couple both have b thalassemia minor, each time they expect a child there is a 1 in 4 chance that their child could inherit a serious blood disease called b thalassemia major.

1.3 Alpha Thalassemia

1.3.1 Hemoglobin H:

Hemoglobin H is caused when a person is missing three alpha globin genes or experiences changes in these genes. In this condition the patient usually makes sufficient hemoglobin to allow for normal life. However, the red blood cells have less red pigment than usual [6]. On occasions, this condition may need medical treatment; for example, blood transfusion or medication. This disease can lead to bone issues. The cheeks, forehead, and jaw may all overgrow.

1.3.2 Hydrops fetalis:

Hydrops fetalis is an extremely severe form of thalassemia that occurs before birth. Most individuals with this condition are either stillborn or die shortly after being born. This condition develops when all four alpha globin genes are altered or missing.

1.4 Iron overload

Repeated transfusions represent the major cause of iron overload in thalassemia major. Each unit of blood represents 200-250 mg of iron. Considering that total body iron stores are approximately 4 grams, and that normal daily iron losses are of the order of 1-2 mg (with a very limited capacity for the body to regulate these losses), one can understand that, when a given individual needs for instance one unit of blood every 2 weeks, body iron overload develops rapidly[7]. Thalassemia causes iron to accumulate in the body. There are two main ways in which patients with thalassemia absorb iron: from the diet, and from transfused blood. If this excess iron is not removed, it can cause damage to important organs such as the liver and heart. Thalassemia patients must therefore use special drugs called chelators, which remove iron from the body. In

thalassemia major, the body attempts to compensate for the patient's severe anemia by absorbing significantly more iron from the gut than normal (2-5g/year compared to 0.0015g/year in healthy individuals), in order to make more red blood cells. How much more iron is absorbed depends on the severity of the anemia. Other factors may also play a part in determining the amount of iron absorbed by the gut. For example, the presence of vitamin C increases the amount of iron absorbed, while tea and some cereals lead to a decrease. The main source of iron overload in patients receiving transfusions however is blood transfused. In fact, the amount of iron the patient absorbs through blood transfusions is far greater than that absorbed from the diet through the gut. It is therefore important that patients on regular blood transfusions, use iron chelators that bind with iron and remove it from the system [5]. The clinical symptoms of iron overload generally appear around the age of 10, although evidence of the toxic effects of iron has been found in the liver of much younger children. Heart disease is one of the most frequent causes of death in thalassemia major -has also been reported within 10 years of the start of a transfusion regime, although heart failure does not usually occur until after 15 years or more.

Table-1: Effect of excess iron deposition in the body [5]

Consequences excess iron	
Heart	Biventricular failure Arrhythmia
Pituitary	Hypogonadotrophic-hypogonadism Osteoporosis
Endocrine gland	Diabetes Hypothyroid Hypoparathyroid
Liver	Fibrosis Cirrosis

1.5 Epidemiology

Geographically the thalassemia belt includes the Mediterranean passing through West and Central Asian countries like Turkey, Iran, Afghanistan onto Pakistan & India and passes on to the South East Asian countries like Indonesia, Burma and Thailand, Vietnam and Cambodia. This makes it most common in African, Greek, Italian, Middle Eastern and Southern Asian populations [6]. Thalassemia affects approximately 4.4 of every 10,000 live births throughout the world. It causes males and females to inherit the relevant gene mutations equally because it follows an autosomal pattern of inheritance with no preference for gender. The widespread of two types of thalassemia is given below: [8]

Alpha thalassemia around the world:

- America: 0-5% of the population has a thalassemia trait, up to 40% may be a genetic carrier
- Eastern Mediterranean: 0-2% of the population has a thalassemia trait, up to 60% may be a genetic carrier
- Europe: 1-2% of the population has a thalassemia trait, up to 12% may be a genetic carrier
- Southeast Asia: 1-30% of the population has a thalassemia trait, up to 40% may be a genetic carrier
- Sub-Saharan Africa: 0% of the population has a thalassemia trait, up to 50% may be a genetic carrier
- Western Pacific: 0% of the population has a thalassemia trait, up to 60% may be a genetic carrier

Beta thalassemia around the world:

- Americas: 0-3% of population is affected by a gene mutation
- Eastern Mediterranean: 2-18% of population is affected a gene mutation
- Europe: 0-19% of population is affected a gene mutation
- Southeast Asia: 0-11% of population is affected a gene mutation

- Sub-Saharan Africa: 0-12% of population is affected a gene mutation
- Western Pacific: 0-13% of population is affected a gene mutation

Thalassemia being an inherited disease is usually treated with blood transfusion, iron chelation therapy and bone marrow transplantation depending on the severity of the disease. After being treated the patients are not able to lead a complete healthy life. It is seen that almost all the patients die at the age of 30-35years. The main cause of their death is due to heart problems. Due to constant blood transfusion the amount of iron in the patient increases and excess of iron deposition causes this heart problem. Though excess iron is removed using iron chelating therapy but it is seen that different chelating drugs remove iron from different parts of the body, that is, the drugs are unable to remove iron from all over the body at a time. The second cause of the death of the patients is infection. They get infected by HIV, Hepatitis B and Hepatitis C. In Bangladesh, it is seen that the patients get infected mostly by HBV and HCV. Poor quality of life is one of the reasons of this infected being transmitted. Though the blood is tested before it is being transfused to the patient but the screening test that is performed is not up to the mark or is not sensitive enough to detect the presence of the virus. It can also happen that while the screening of the blood the amount of HCV or HBV is relatively low that could not get detected. On the other hand, there is vaccine available for hepatitis C. The cost of treatment varies according to age, body weight and severity of the disease. The most conservative direct medical cost ranges from BDT 127,000 to BDT 309,000per year [4]. There is neither a national insurance system nor subsidized or free treatment from the government health facilities. It is expected that patients must pay for their treatment and it is difficult for most of the families to afford proper treatment. Over 72% of the patient's monthly household income was between BDT 10,000to BDT 20,000 suggesting a huge economic burden that could render seeking treatment for most thalassemia patients unviable in Bangladesh.

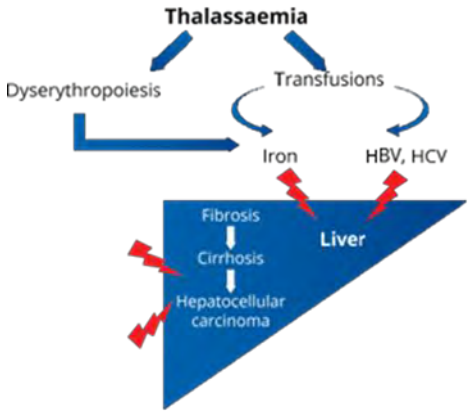


Figure-2: Main causes of hepatic iron damage in thalassemia. NAFLD, ALD, HBV, and HCV

1.6 Treatments for Thalassemia

Over the last three decades, clinical observations and research have established that thalassemia major is a treatable condition. Studies have shown that regular transfusion therapy with safe and appropriately processed blood, combined with regular and effective iron chelation tremendously increase patients' survival and quality of life. But these patients are seen to die due to cardiac arrest and liver problems at the later stage of treatment. Though cardiac arrest is the main cause of death but the second cause of their death is infections-HIV, HBV and HCV.

1.6.1. Blood transfusion:

Regular blood transfusions greatly contribute to the quality and length of life of patients with thalassemia major, and have been a central aspect of the treatment of thalassemia. Patients with thalassemia major lack red blood cells. Therefore, patients receiving blood transfusion therapy should ideally receive only red blood cells, which contain none of the other components of whole blood - e.g. plasma, white blood cells and platelets. If a patient receives whole blood, there is a risk that the body's circulatory

system will be overloaded, developing complications such as heart failure and pulmonary edema. The removal of white cells and platelets from whole blood also decreases the risk of unwanted effects such as fevers during and after the blood transfusion [6]. Although such symptoms can be treated, every effort should be made to avoid any complications by providing only that component of blood the patient requires. And during this blood transfusion, the patient gets attacked by either hepatitis B or hepatitis C virus. Though, before transfusion screening is done but it is seen that patients are suffering from hepatitis. This is because, in our country the screening test is not performed properly and if performed the machines used are not sensitive enough to detect the amount of the virus present in the donor's blood.

1.6.2. Iron chelation therapy:

As the body has no effective means of removing iron, the only way to remove excess iron is to use drugs called iron chelators (iron binders), which form a compound with iron that can be excreted from the body through the urine and/or stools. As a general rule, patients should begin iron chelation treatment once they have had 10-20 transfusions, or when ferritin levels rise above 1000□g/l [5].

1.7 Infection

After the heart problem the second cause of death of the thalassemia patients is infection. Parvovirus B19, Dengue fever virus (DFV), Babesia microti, Plasmodia species, Leishmania, Brucella and Creutzfeldt-Jakob disease (vCJD) prions [9]. Among these infections, HIV, hepatitis B and C are the most common. Though the donors blood is tested before it is transfused to the patient. But still it is commonly seen in the blood transfused patients that in their later stage they suffer either from hepatitis B or hepatitis C. HBV and HCV are among the principal causes of severe liver disease, including hepatocellular carcinoma and cirrhosis-related end-stage liver disease. The World Health

Organization (WHO) estimates that Hepatitis B to result in 563,000 deaths and hepatitis C in 366,000 deaths annually [10].

Hepatitis B is a chronic infection and at the initial stage it has no symptoms. Some develop a rapid onset of sickness with vomiting, yellowish skin, tiredness, dark urine and abdominal pain. Often these symptoms last a few weeks and rarely does the initial infection result in death. It may take 30 to 180 days for symptoms to begin. In those who get infected around the time of birth 90% develop chronic hepatitis B while less than 10% of those infected after the age of five do. Most of those with chronic disease have no symptoms but however, cirrhosis and liver cancer may eventually develop. These complications result in the death of 15 to 25% of those with chronic disease. Vaccination is available for this and it is recommended to vaccinate a child after birth. But in Bangladesh, the under privileged people are not well aware about the fact and also they cannot afford the cost of this vaccine due to their low income. On the other hand, this vaccine requires 3 dosage after certain intervals of time and most of the time it is seen that people after the first dosage usually ten to miss or delay the next dosage.

Hepatitis C is another chronic disease that primarily affects the liver. During the initial infection people often have mild or no symptoms. Occasionally a fever, dark urine, abdominal pain, and yellow tinged skin occur. The virus persists in the liver in about 75% to 85% of those initially infected. Over many years however, it often leads to liver disease and occasionally cirrhosis. In some cases, those with cirrhosis will develop complications such as liver failure, liver cancer, or dilated blood vessels in the esophagus and stomach. Due to lack of effective vaccines against HCV and inadequate infection control strategies, HCV is considered as major public problem in low to middle-income countries. Approximately 180.5 million people are infected by HCV in the world, of which 54.4 million is in South Asia. A number of studies have reported the higher prevalence of HCV among multi-transfused thalassemia patients, ranging from 3 to 67.3%. Increased risk of HCV infection in β-thalassemia patients is mainly associated with median age, duration, and mean amount of blood transfused[11]. HCV is considered as major public problem in Bangladesh.

AIDS (acquired immunodeficiency syndrome) is a syndrome caused by a virus called HIV (human immunodeficiency virus). The disease alters the immune system, making people much more vulnerable to infections and diseases. This susceptibility worsens if the syndrome progresses. HIV is found throughout all the tissues of the body but is transmitted through the body fluids of an infected person (semen, vaginal fluids, blood, and breast milk) [12]. Children with thalassemia are susceptible to HIV because they receive multiple blood transfusions. Prevalence of HIV infection in thalassemia varies greatly worldwide, from less than 1% to more than 20%. The risk of transfusion transmission of HIV may be alarming due to high seroprevalence of anti HIV–1 viz; 0.5% in blood donors [13].

1.8 Aim of the study

The aim of the study is to determine the frequency of the chronic infections- hepatitis B, hepatitis C and HIV in thalassemia patients who are going through regular blood transfusion.

Chapter-2

Materials and Methodology

2.1 Working Place

All the laboratory works were performed at ideSHi laboratory, Mohakhali, Dhaka. The laboratory has been setup to meet the international BSL-2 requirements in order to carry out molecular, immunological and genetic studies.

2.2 Duration of the study

The study was conducted for 6 months (January- July).

2.3 Study population

The samples were collected from Dhaka Thalassemia Shomity Hospital after a written informed consent was obtained from adults as well as legal guardians of the children and those who refused to give consent were excluded from the study. All the samples were collected from patients who were going through blood transfusion. Ethical clearance was also taken before conducting the study.

2.4 Sample collection

5ml of blood were collected from each patient before transfusion using the standard venipuncture method. The vacutainer tubes containing the collected blood were immediately transferred to ideSHi laboratory and kept in 4^{o}C freezer.

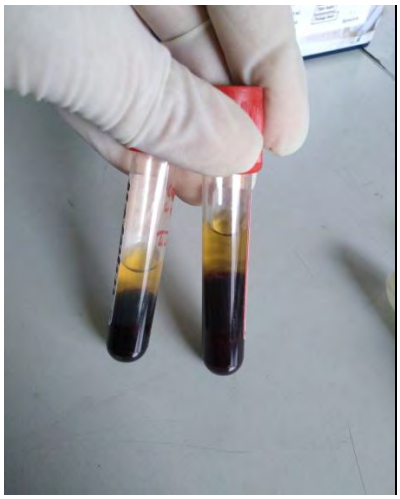


Figure-3: Sample from the patients

2.5 Serum separation:

After collection of the whole blood, it was allowed to clot by leaving it undisturbed for 15-30 minutes at room temperature. The clot was removed by centrifuging at 3000 x g for 10 minutes in a refrigerated centrifuge. The resulting supernatant is designated serum and stored for testing further.

2.6 Bioelisa HbsAg

Bioelisa HbsAg is a direct immunoenzymatic method of the «sandwich» type in which guinea pig anti-HBs antibodies coated to microplate wells act as the capture antibody and goat anti-HBs antibodies marked with peroxidase serve as conjugate antibodies. The sample to be analysed is incubated in one of the antibody-coated wells. If the sample contains HBsAg, the antigen will bind to the antibody on the plate. After washing to eliminate any unbound material, goat anti-HBs conjugate to peroxidase is added to the well and allowed to react with the
antigen-antibody complex formed in the first incubation. After a second incubation and subsequent washing, an enzyme substrate containing a chromogen is added. The substrate

will develop a blue colour if the sample is positive for HBsAg. The blue colour changes to yellow after blocking the reaction with sulphuric acid. The intensity of the colour is proportional to the amount of HBsAg in the test specimens.

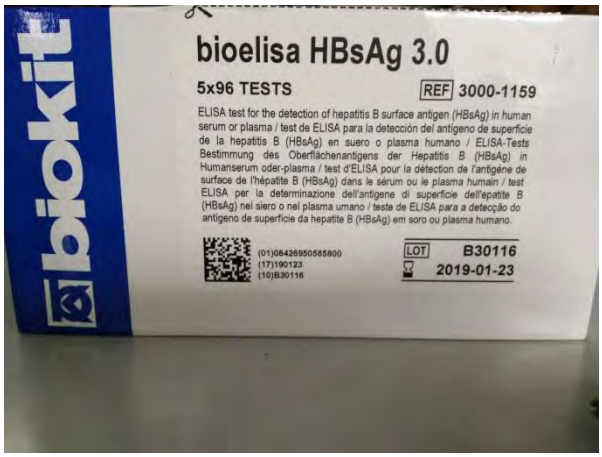


Figure-4: Bioelisa Kit for HBV detection

The procedure is as follows:

- 100ul of sample and controls were pipetted in each well.
- These were then incubated for 1hour at 37^{o}C in an incubator.
- It was then washed 4 times with the washing solution.
- 100ul of conjugate was pipetted in each well.
- Again it was incubated for 30 minutes at 37^{o}
- It was then washed 4 times with the washing solution.
- 100ul of substrate was then added to each of the well.
- At room temperature it was again kept for incubation for 30 minutes.
- Lastly, 100ul stopping solution was added in each of the wells.
- The wells were read at 450nm in the ELISA reader.

2.7 Bioelisa HCV:

Bioelisa HCV 4.0 is an immunoenzymatic method in which the wells of a microplate are coated with recombinant antigens representing epitopes of HCV: Core, NS3, NS4 and NS5. Serum or plasma samples are added to these wells. If antibodies specific for HCV are present in the sample, they will form stable complexes with the HCV antigens on the well. Excess sample is removed by a wash step and a rabbit anti-human IgG conjugated with peroxidase is then added and allowed to incubate. The conjugate will bind to any antigen-antibody complexes formed. After a second wash, a solution of enzyme substrate and chromogen is added. This solution will develop a blue colour if the sample is positive. The blue colour changes to yellow after blocking the reaction with sulphuric acid. The intensity of colour is proportional to anti-HCV antibodies concentration in the sample. Wells containing negative samples remain colorless.

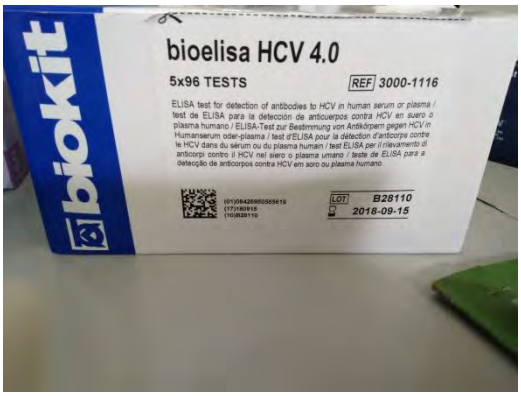


Figure-5: Bioelisa Kit for HCV detection

The procedure is as follows:

- 200ul of sample diluent was pipetted in each well.
- 10ul of sample was pipetted in each well.
- 200ul of control was then added in each of the well.

- This was then incubated for 1hour at 37^{o} in the incubator.
- It was then washed 6 times with the washing solution.
- 100ul of conjugate was pipetted in each well.
- Again it was incubated for 30 minutes at 37^{o}
- It was then washed 6 times with the washing solution.
- 100ul of substrate was then added to each of the well.
- At room temperature it was again kept for incubation for 30 minutes.
- Lastly, 100ul stopping solution was added in each of the wells.
- The wells were read at 450nm in the ELISA reader.

2.8 Bioelisa HIV

The HIV 1/2/O Antigen/Antibody EIA Test Kit is a solid phase qualitative enzyme immunoassay based on a sandwich principle for the detection of HIV-1 P24 antigen and total antibodies (IgG, IgM and IgA) to HIV-1, HIV-2, and/or Subtype O in human serum or plasma. The microwell plate is coated with HIV monoclonal antibodies and recombinant antigens. During testing, the specimens are added to the antibody/antigen coated microwell plate and then incubated. If the specimen contains HIV-1 P24 antigens and/or antibodies to HIV-1, HIV-2, and/or Subtype O, it will bind to the antibodies/antigens coated on the microwell plate to form immobilized antigen- antibody complexes. If the specimens do not contain HIV-1 P24 antigens and antibodies to HIV-1, HIV-2, and/or Subtype O, the complexes will not be formed. After initial incubation, the microwell plate is washed to remove unbound materials. The enzyme-conjugated HIV polyclonal antibodies and recombinant antigens are added to the microwell plate and then incubated. The enzyme-conjugated HIV polyclonal antibodies and recombinant antigens will bind to the immobilized antigen- antibody complexes present. After the second incubation, the microwell plate is washed to remove unbound materials. Substrate A and substrate B are added and then incubated to produce a blue color indicating the amount of HIV antigens/antibodies present in the specimens. Sulfuric acid solution is added to the microwell plate to stop the reaction producing a color change from blue to yellow. The

color intensity, which corresponds to the amount of HIV antigens/antibodies present in the specimens, is measured with a microplate reader at 450/630-700 nm or 450 nm.

Figure-6: Bioelisa Kit for HIV detection

The procedure is as follows:

- Working Wash Buffer was prepared by diluting the concentrated wash buffer 1:25 with deionized water. The working wash buffer must be stable for 2 weeks at 15-30^{0}C.
- The unused strips from the microwell plate were removed, and store in the original resealable pouch at 2-8°C.
- 50 µL specimen diluent was added in respective wells including negative control, positive control, blank and specimen wells.
- The microwell plate was mixed by gentle swirling on a flat bench for 30 seconds.
- The microwell plate was covered with the plate sealer and incubated in a water bath or an incubator at 37^o C± 2^{o}C for 60 minutes ± 2 minutes.
- Each well was washed 5 times with 350µL of working wash buffer.
- 100µL of conjugate was added to each well except for the blank well.
- The microwell plate was covered with the late sealer and incubated at 37°C for 30 min.
- Each well was again washed 5 times with 350µL of working wash buffer.

- 50µL of Substrate A and 50µL of Substrate B was added in each well.
- The microwell plate was mixed and covered with plate sealer and incubated at 37°C for 30 min.
- 50µL of stop solution was added to each well.
- The wells were read at 450nm in the ELISA reader.

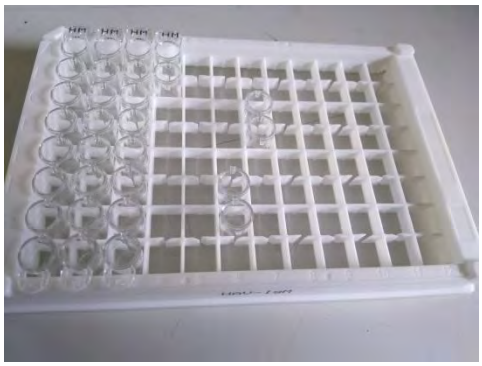
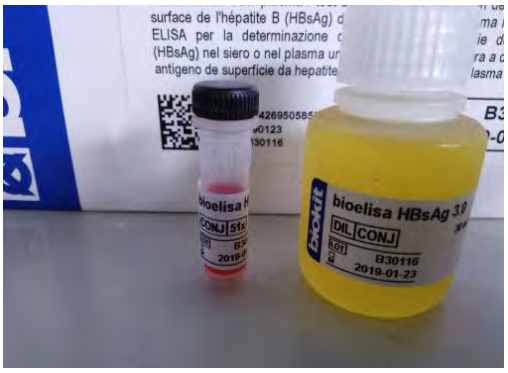
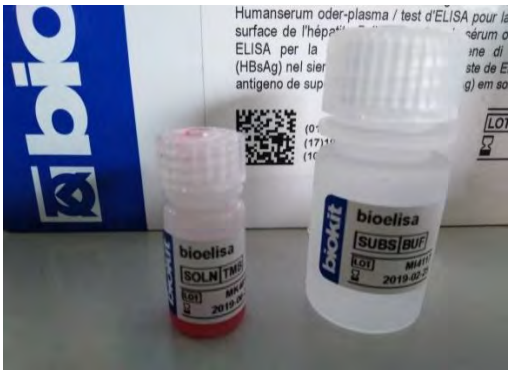


Figure-7: Microplate with 96 wells

Figure-8: Strips from microplate

A 

B 

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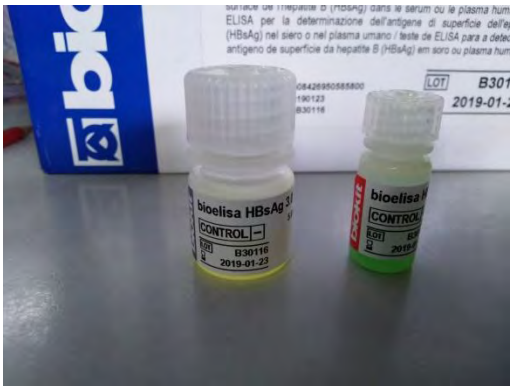
D 

Figure-09: Chemicals use: (A) Conjugates; (B) Substrates; (C) Wash buffer; (D) Controls

A 

B 

Figure-10: Machines use: (A) ELISA Reader; (B) Incubator

2.9 Interpretation of ELISA reader

For HBV, Cut off value = Negative control +0.040; Sample absorbance/cut off value =result

If result is >_ 1.0 then it is positive

If result is < 0.9 then it is negative

For HCV, Cut off value =Lowest positive control x 0.9; Sample absorbance/cut off value = result

If result is >_ 1.0 then it is positive

If result is < 0.9 then it is negative

For HIV, Cut off value = Negative control + 0.160;

If sample OD is > cut off value then it is positive

If result is < cut off value then it is negative

Chapter-3

Result

Table-2: Socio-demographic data of the participants

Categories	Patient
Gender	Male=79 (53.38%) Female=69 (46.62%)
Age(years)	17.15± 9.33
BMI	17.39±3.32
Transfusion interval (days)	33.68±29.98
Transfusion number	228.32±189.85
Splenomegaly(cm)	6.94±3.09
Splenoctomy	21 (13.37%)
Thalassemia type	EBT=83 (60.58%), BTM= 54 (39.41%)

This chart shows some details about the patients those who had taken part in the project. Out of 148 patients 79 of them were male and 69 of them were female their age on average were from 7-26 years with average BMI of 17.39. The time interval of their blood transfusion on average 4-63 days. These patients on average had taken 39-419 times. The normal size of human spleen is 11cm but in these patients it was seen that the size increased up to 10cm. Out of 148 patients, 21 patients already had to remove their spleen. 83 patients had EBT and 54 patients had BTM.

Table-3: Infection data of the enrolled participants

Thalassemia type	Infection				Total infected
	HBV	HCV	HIV	Both HBV and HCV	
EBT	3 (3.61%)	11(13.25%)	0	1 (1.20%)	14 (16.86%)
BTM	2 (3.70%)	8 (14.81%)	0	2 (3.70%)	10 (18.51%)

The above chart shows the total people infected by HBV, HCV, HIV and both HBV and HCV. In case of EBT, 3 out of 83 patients were infected with HBC, 11 patients were infected with HCV and 1 patient was infected with both HBV and HCV. On the other hand, in case of BTM, 2 out of 54 patients were infected with HBV, 8 patients were

infected with HCV and 2 patients were infected with both HBV and HCV. But in both the cases no patient was infected with HIV.

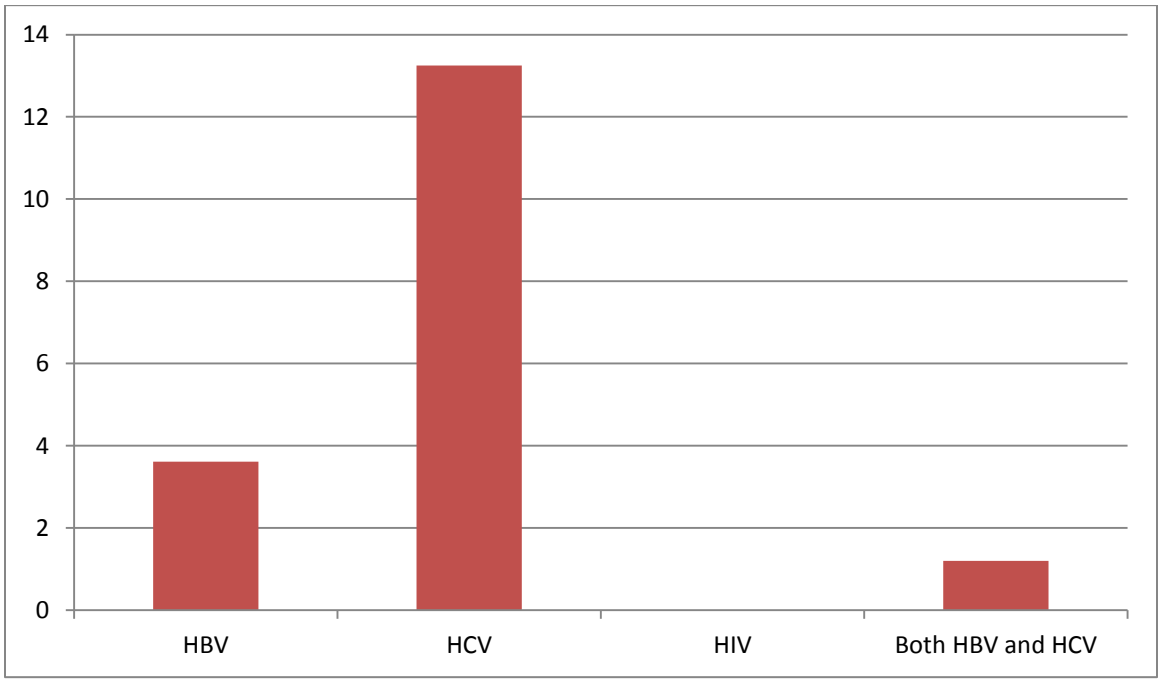


Figure-11: Percentage of HBV, HCV and HIV in EBT patients.

This graph represents that, less than 4% has been infected by HBV, more than 12% has been infected by HCV and 1.20% has both HBV and HCV among all the people suffering from EBT. But no trait of HIV has been found.

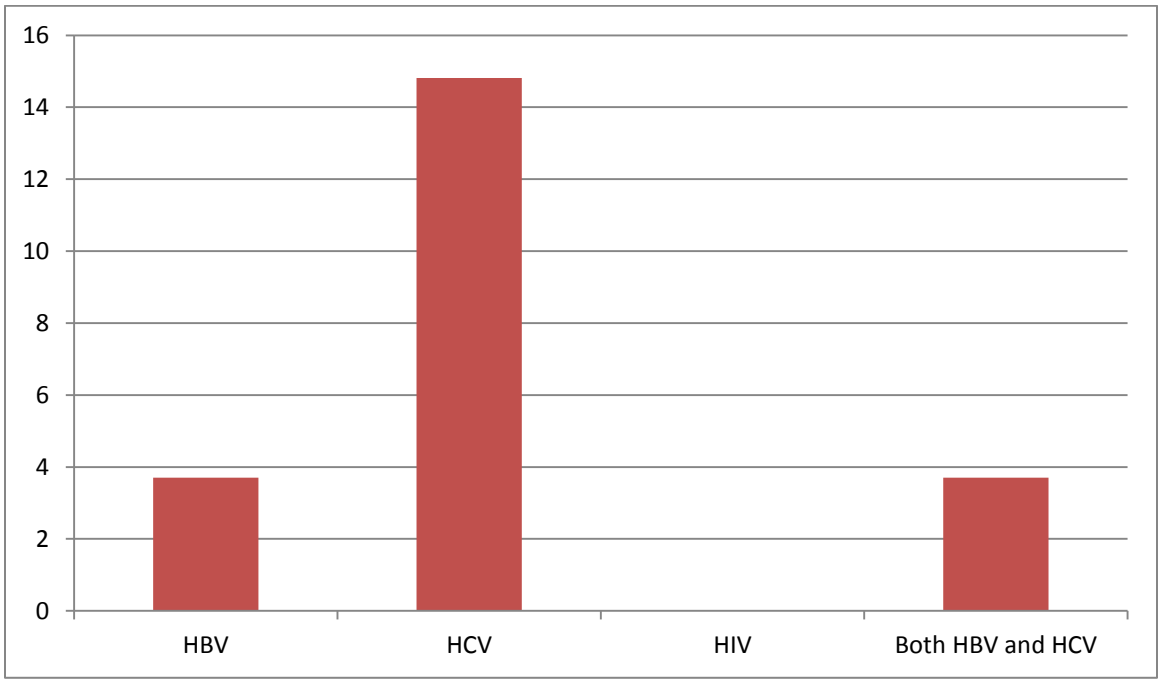


Figure-:12 Percentage of HBV, HCV and HIV in BTM patients.

This graph represents that, almost 4% has been infected by HBV, less than 15% has been infected by HCV and around 4% has both HBV and HCV among all the people suffering from BTM. But no trait of HIV has been found. In this case, percentage of patients infected with both HBV and HCV is more compared to EBT patients.

Chapter-4

Discussion

Blood cannot be manufactured and there is no substitute for it. It is a constant demand for thalassemia worldwide. It is necessary to develop societies of young blood donors who commit to donate regularly to save lives. Thalassemia major children are not able to regulate normal hemoglobin level so they need regular blood transfusions. Without regular blood transfusions support about 85% of children with β-thalassemia major will die by 5 years of age. Therefore it is constant need of their lives to provide in time safe and match transfusion regularly to reduce complications [2].

Hepatitis B prevalence is highest in the WHO Western Pacific Region and the WHO African Region, where 6.2% and 6.1%, respectively of the adult population are infected. In the WHO Eastern Mediterranean Region, the WHO South-East Asia Region and the WHO European Region, an estimated 3.3%, 2.0% and 1.6%% of the general population are infected, respectively [16]. On the other hand, the studies carried out by the researchers in India, Pakistan, Iran and many other developing countries it was seen that the prevalence of HBV is comparatively less in thalassemia patients then HCV. And the reason is the availability of vaccine for HBV. Bangladesh has 2 types of vaccine for HBV one for infants and the other for the adults which are being manufactured by 5 different pharmaceutical companies [31]. But currently no vaccine is available for HCV.

Efforts to develop a hepatitis C vaccine started more than 25 years ago, when the hepatitis C virus was identified. Since then, researchers have studied more than 20 potential vaccines in animals. A few of these vaccines, developed mainly in the past decade, have undergone limited testing in people [14]. HCV treatment of patient with thalassemia with interferon-alpha (IFN) was challenging not only due to its unfavorable safety and tolerability profile but also due to necessary combined use of ribavirin (RBV) and the subsequent hemolysis and increased need for blood transfusions. The introduction of the current direct acting antivirals (DAAs), which can be used in IFN free and RBV-free regimens, has dramatically improved the management of all HCV patients including those with thalassemia. This is because DAAs target specific steps in the HCV viral life cycle and some of these crucial particles, or enzymes, include the NS3/4A protease, the NS5B RNA-dependent RNA polymerase, and the NS5A protein. According to all current

international guidelines, thalassaemia patients do not represent a special group for the current HCV treatment and can be treated with the same indications and regimens used for patients without haemoglobinopathies. However, in countries which still prioritize the use of DAAs according to the severity of liver disease, thalassaemia patients are often excluded from such prioritization and have access to DAAs therapy regardless of their fibrosis severity [15].

A study conducted in Pakistan where it was seen that out of the total patients 49.47% were infected with anti-HCV and 4% with HBV [17]. In another study conducted in Bandar Abbas of Iran, where it was seen that out of the total patients 2.5% were infected with HBV and 15% with HCV. In this case more accurate result was found because PCR was performed as well [18]. Another study in Iran showed that 19.3% patients were HCV and 1.5% were HBV out of the total patients [19]. In a study conducted in India showed 1.0% prevalence of HBV and 5.0% of HCV among the total thalassemia patients [2]. In another study conducted in West Bengal showed that 41% were infected with HCV and 50% with HCV out of the total patients [20]. A study conducted in Egypt showed that HCV antibodies were detected in 20.7% of patients and 5% were HBV positive. RIBA was performed to conform HCV [21]. Thalassemia children are at high risk of transfusion related infections such as HIV and CMV, hepatitis B and C viruses. A study from Egypt showed that out of 97 beta thalassemia major pediatric patients, 36 (37.11%) had positive serology for HCV, 4 (3.88%) patients were positive for each HbsAg and CMV IgM antibodies, 2 (1.94%) patients had both HCV and HBV and 1 patient had both HCV and CMV infections while none had HIV infection10**.** A study done in India showed that hepatitis B surface antigen, anti-hepatitis C antibodies and human immunodeficiency virus antibodies were positive in 1 of 96 (1.04%), 24 of 96 (25%) and 1 of 96 (1.04%) respectively in beta thalassemia major patients who had received multiple blood transfusions. A study has been found in Pakistan,, carried on 160 patients with thalassemia major, the seroprevalence of HBV, HCV and HIV was 21 (13.1%), 2 (1.25%) and 0 (0%) respectively[22]**.**

From all the studies done earlier it was seen that little or no amount of HIV infected patient was found. This is because HIV transmission through donated blood has become very rare after testing became mandatory for HIV-1 on 1989 and HIV-2 on 1993[13].

In spite of the availability of the HBV vaccine in Bangladesh, a large number of thalassemia patients get infected from HBV as we found in our present study. From this study we also found out that HCV is more prevalent then HBV in transfusion dependent thalassemia patients. But the question still arises that why thalassemia patients got infected by hepatitis who took blood transfusion for several times. It is also known to us that before a donor donates blood they undergo several extensive screening tests including tests for hepatitis. The first reason is that, though HBV vaccines are available in our country but due to low standard of life among the poor people they are unable to afford the cost of the vaccine. As a result they remain unvaccinated. Another reason is the unawareness among the people that is, the vaccine requires 3-4 dosage at certain interval but most of the time it is seen that they forget to take the next dosage and as a result the vaccine does not work in their body. Another major reason is the lack of proper screening process. In case of HBV, there are some asymptomatic donors who are in the 'window period' (i.e. the early infectivity period when an immunologic test is non-reactive) without any expression of HbsAg. Also, people with occult HBV may remain undetected by traditional ICT kits. Moreover, these kits might not detect certain prevalent serotypes of HBV in any particular region. Previous study also showed that the sensitivity of ICT-based rapid tests was not higher enough to detect hepatitis status of a donor [23]. In our study, the numbers of patients infected with HCV were higher than that of HBV. It should be noted here that although there were HBV vaccine coverage to some extent in Bangladesh, however, there were no HCV vaccination program in Bangladesh. This explains why there was more thalassemia patients infected with HCV than HBV. In this present study, none of the patients was found to be infected with HIV. In Bangladesh Thalassemia Samity Hospital, although ELISA is done as a confirmatory test after the cases are suspected as positives by ICT tests, previous study has shown that ELISA only detects HbsAg, whereas real-time PCR represents infection status by detecting HBV-DNA [24] and RIBA (recombinant immunoblot assay) has more accuracy in detecting

HCV [25]. Moreover, the multitransfused patients with thalassemia face increased immune dysfunction in the presence of iron overload following splenectomy, which makes them more susceptible to infections [26]. Also, in case of emergency, there is a common practice in Bangladesh of risky blood donations from professional donors without any kind of testing who are mostly drug abusers [27]. Although nowadays an effective awareness in safe blood transfusions and iron chelation therapy have made the morbidity rate of transfusion-dependent thalassemia patients lower, new complications like hepatocellular carcinoma are taking hold in the patients with thalassemia, which may be due to carcinogenicity of iron overload and chronic infections [28]. Specially, the thalassemia patients who become co-infected with both HBV and HCV are at a greater risk of cirrhosis and hepatocellular carcinoma compared to the mono-infected patients [29].

So, there is no alternative to blood transfusion process safer by proper selection of voluntary healthy blood donors, along with nucleic acid testing (NAT) which reveals viral agents earlier in the 'window period' than other immunoassays. Additionally, screening of HBV-DNA and HCV-RNA needs to be practiced regularly to avoid any kind of risks during blood transfusions [26]. However, in case of very low levels of viremia, NAT might not be effective enough to detect the infections. Despite the limitations, to ensure safe blood transfusion, combination of both NAT and serological testing may significantly reduce the risk of viral infections during transfusion [30] and thus may improve the quality of life of the thalassemia patients.

Chapter-5

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Appendix

List of the important equipment used throughout the study.

Name	Manufacturer
bioelisa HBsAg 3.0	Biokit
bioelisa HCV 4.0	Biokit
HIV 1/2/O Antigen/Antibody EIA Test Ki	Foresight
Refrigerator	Electra, Samsung (+4°C); Vestfrost (+4°C)
Incubator	Memmert
Micropipette	(2-20µl)- Gilson and Costar® (20-200µl)- Gilson and Costar® (200-1000µl)- Gilson
Bio-safety cabinet	ESCO Class-II Type-A2 Labculture® Biological Safety Cabinet
ELISA reader	EON