Prevalence of Torque Teno Virus In Blood Donors of Islamabad

By

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Blood tumors.

Viruses

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"In the name of God, most Gracious, most Compassionate".

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FINAL APPROVAL

It is certified that we have read the thesis submitted by Ms. Nosheen Basharat 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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As a partial fulfillment of requirement for the award of degree of

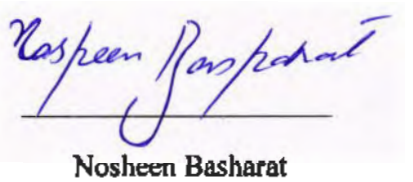
MS in Biotechnology

Dedicated to my Respected Parents, Brother and Sister (late)

Declaration

I hereby declare that the work present in the following thesis is my own effort, except where otherwise acknowledged and that the thesis is my own composition. No part of the thesis has been previously presented for any other degree.

Date 11-Nov-2016



Nosheen Basharat

Contents

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NOSHEEN BASHARAT

LIST OF ABBREVIATIONS

µl	Micro Litre
A	Ampere
s	Second
ALT	Alanine aminotransferase
AntiHCV	Antibody to Hepatitis C virus
CMIA	Chemiluminosense immunosorbent essay
CMV	Cytomegalovirus
DNA	Deoxyribonucleic acid
EDTA	Ethyl diamine tetraacetic acid
EIA	Enzyme immunoassay
ELISA	Enzyme linked immunosorbent assay
GOP	Government of Pakistan
HBV	Hepatitis B Virus
HBsAg	Hepatitis B surface antigen
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus
HRP	Horse Reddish Peroxidase
mM	Milli molar
mRNA	Messenger Ribonucleic acid
nm	Nano meter
ORF	Open reading frame
PCR	Polymerase chain reaction

PIMS	Pakistan Institute of Medical Sciences
PK	Proteinase k
rpm	Revolution per minute
SDS	Sodium Dodecyl Sulphate
TBE	Tris Boric acid Ethyl diamine tetraacetic acid
TMB	3,3',5,5'-Tetramethylbenzidine
TTI	Transfusion Transmitted Infection
TTV	Torque Teno Virus
UTR	Untranslated region
WHO	World Health Organization

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

The safety of blood supply is a multifactorial process. Due to the nature of the factors associated with emerging infections, identification of the epidemic and pandemic status of newly recognized or emerging blood-borne infectious agents in a community is an important issue. Donor screening and testing are two mainstays in blood processing centers. Torque teno virus (TTV) is highly prevalent among the general population throughout the world. TTV was discovered in 1997 in the serum of a Japanese patient with post-transfusion hepatitis of unknown etiology. TTV is a non-enveloped small virus containing a circular single-stranded negative-sense DNA genome. TTV represents the first circovirus-like virus found in humans. To determine the prevalence of TTV in the Pakistani healthy population, we set up ELISA and PCR methods and examined the sera of asymptomatic subjects for the presence of TTV DNA. TTV is also very common in Pakistan: TTV DNA was detected in 27.6% of the subjects. The majority of blood samples (44) belonged to healthy blood donors, whereas 25 blood samples belonged to donors of asymptomatic Hepatitis B virus (HBV) infection and 25 blood samples belonged to asymptomatic Hepatitis C virus (HCV) infection. The results obtained showed that the TTV infection rate in healthy blood donors and those infected with HBV or HCV were 25%, 38% and 22% respectively. There is no possible association between high or low level of alanine aminotransferase (ALT) among TTV positive subjects and TTV negative subjects. ALT level, age, gender, ethnic group has no effect on TTV prevalence in Pakistan. TTV prevalence knowledge in Pakistan helps to develop strategy for virus control and control its transmission in healthy population. This research project provides basis for implication of TTV in daily routine screening in transfusion services for the better health of blood recipients and general population.

Key words: Blood donors, TTV, Pakistan, ELISA

Chapter 1

1. Introduction

Blood transfusions have become a routine process in the hospitals globally. (https://www.nhlbi.nih.gov/health/health-topics/topics/bt). Presently, transfusion practices are critical part of the health practices. Blood transfusion is required in case of severe anaemia, surgery, complication of pregnancy and trauma, etc. Therefore, proper screening of the donor blood is pivotal to reduce the risk of transfusion transmitted infections. All donated blood is screened for Hepatitis C Virus (HCV) antibody, Hepatitis B Virus (HBV) surface antigen, Human Immunodeficiency Virus (HIV), and syphilis. These markers can be transmitted to the recipients at the time of transfusion (Karimi *et al.*, 2013).

Major factors involved in morbidity and mortality of various populations is the rise of different infectious diseases, whose frequency has increased over the time and threaten to increase in near future (Karimi, *et al.*, 2013). One of the emerging threatening infections that are transmitted by the blood and blood products transfusion is torque teno virus (TTV), also named as transfusion transmitted virus (Karimi, *et al.*, 2013).

At present, in healthy population of different countries TTV occurrence is observed, such as in United Arab Emirates (75.0%) (Alfaresi *et al.*, 2006), Iran (13.4%) (Sara *et al.*, 2012), and Alexandria in Egypt (48.4%) (Mazzola *et al.*, 2015).

In Pakistan, a study was conducted with hepatitis B and C positive patient's samples, to check the TTV presence and specific genotype in patients. This study was conducted in 2012. The rates of incidence of TTV in Pakistan among the patients were high (Hussain *et al.*, 2012).

Even after more than a decade of its discovery, role of TTV and relation to a specific disease is still unknown (Jelcic *et al.*, 2004). To solve mystery of how TTV escape from host immune clearance system and how TTV establish long term constant infection, identification of full set of TTV gene products and gene function is required (Kincaid *et al.*, 2013).

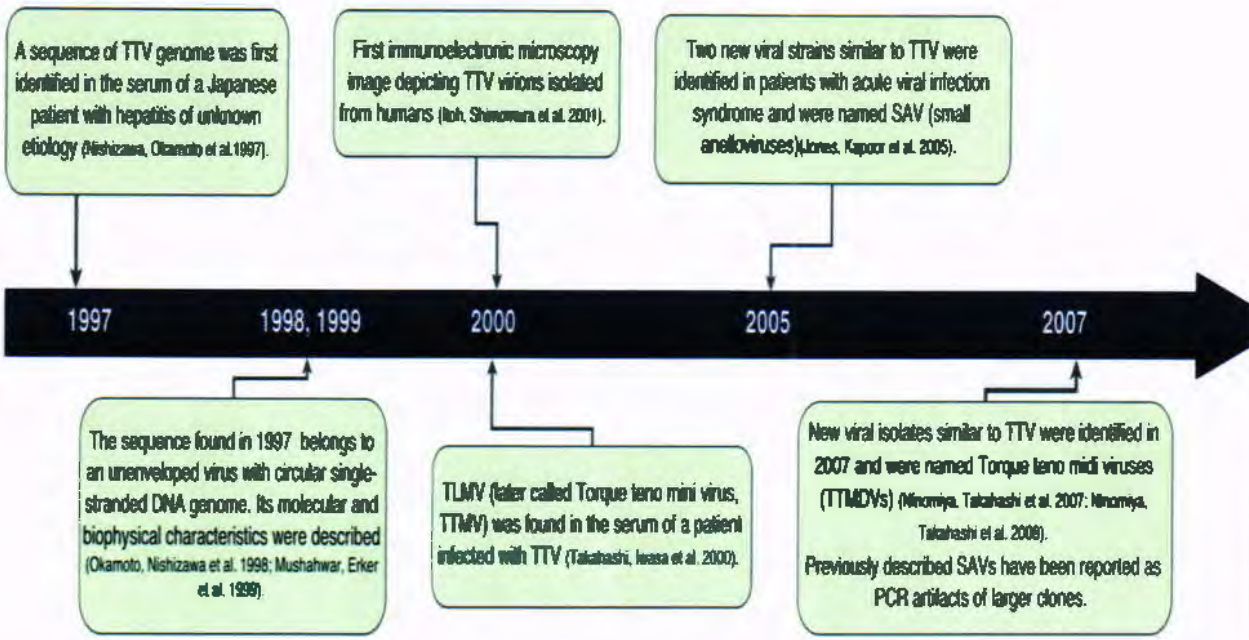


Fig 1-1 Timeline of human anellovirus discovery landmarks. (Spandole *et al.*, 2014)

It was reported after PCR detection that TTV was a DNA virus. Since TTV DNA was not reported from human tissues, so it was concluded that TTV DNA was not originated from humans (Nishiwaza *et al.*, 1997). TTV was reported as a single stranded DNA genome (Mushahwar *et al.*, 1999)

Table 1-1 Reported prevalence of TTV infection in healthy blood donors in some published studies

Country	Total number tested	TTV DNA n (%)	Reference
USA	100	1 (1)	Charlton et al., 1998
USA	250	21 (8.4)	Handa et al., 2000
Brazil	270	198(67-72)	Dewalle *et al.*, 2004; Niel *et al.*, 2001
United Arab Emirates	100	75 (75)	Alfaresi et al., 2006
Egypt	95	46 (48.4)	Hashish et al., 2005
Korea	100	14 (14)	Nakano et al., 1999

New Zealand	413	13 (3.15)	Werno et al., 2001
Turkey	125	21 (16.8)	Kalkan et al., 2005
Taiwan	244	29 (11.9)	Ho et al., 2000
Northeast Thailand	101	28 (28)	Urwijitaroon et al., 2007

Early PCR detection reveals that one third of general population of world has TTV. There is a substantial variation in occurrence of TTV in different countries and also in different part of same country. In particular, moderate prevalence was seen in northern Europe and United State, high in South America and Africa, intermediate in Asia (Pistello *et al.*, 2001).

TTV is asymptomatic when present alone in the patients. Liver had high levels of TTV virion as compared to other organs and tissues. (Singh *et al.*, 2014). TTV occurrence was reported not only in liver but also in other body organs and tissues like memory glands, brain, kidney, bone cells etc. (Ishimura *et al.*, 2010). TTV replication seen in different organs such as spleen, liver tissues, lymphoid tissues etc (Mi *et al.*, 2014).

TTV virion size is 30 – 50 nm. Using electron microscope, virus like particles were seen in serum and fecal samples of patients, with high number of virion (10^8/ml) (Itoh *et al.*, 2000) (Figure 1-2). It uses host machinery for replication. (Spandole *et al.*, 2015). TTV DNA genome is a non enveloped ad single stranded which is covalently circular 3.8kb of size. (Magua *et al.*, 2015).

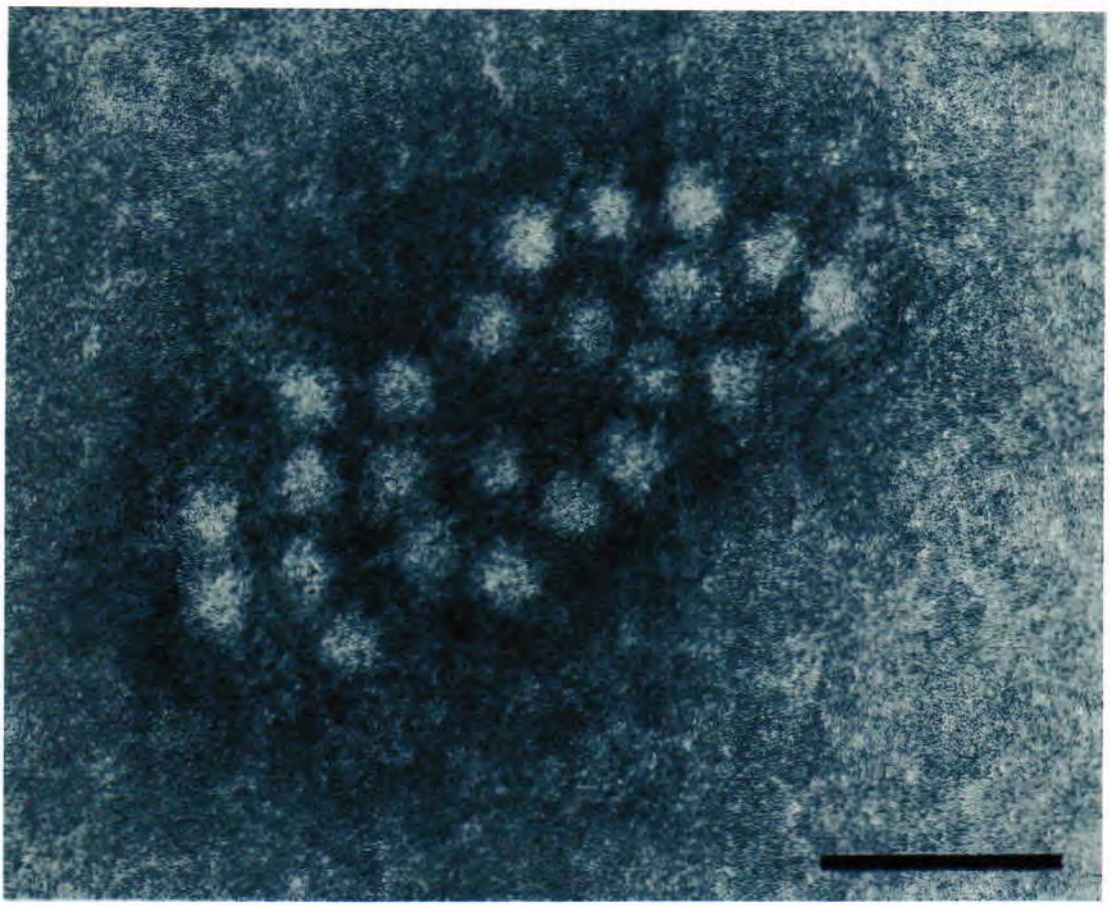


Figure 1-2. Electron microscopy of TTV aggregate in a serum sample with a TTV level of
10^8 copies/ml. Scale bar, 100 nm. (Reproduced from Itoh et al., 2000)

It has UTR and ORF regions which are highly specific and also used to locate the presence of TTV DNA in samples. (de Oliveira., 2015). GC rich region, TATA box and mRNA were also discovered in TTV genome. (Bostan *et al.*, 2013).

Different routes of transmission were observed in TTV transmission into populations, such as parenateral, bile, feces etc (Shaheli *et al.*, 2015). During TTV infection, 4-5 proteins are produced but it is still unclear how TTV cause infection (Bostan *et al.*, 2013). TTV immunocomplexs were also reported in individual having TTV viremia. (Ott *et al.*, 2000).

Immunoelectron microscopy cannot be established because there is no sufficient information on TTV specific and well characterized antibodies. So, TTV specifity of particle is still not been confirmed (Kakkola, 2008)

TTV is not seasonal or epidemic infection. TTV also cause temporary and permanent infections. It was also said that TTV was responsible of high level of ALT in infectious peoples. (Bostan *et al.*, 2013).

Till now, conflicting data has been found on connection of TTV and liver diseases. Major distinction between TTV patient with high level of ALT and patient with normal level of ALT is not identified. Histological test of liver with TTV infection does not expose evident cytopathic effect on liver. TTV and liver disease association is still unsolved mystery (Spandole, *et al.*, 2015).

TTV like viruses have also been discovered in humans. TTV like mini virus, later named as Torque teno minivirus (TTMV), was reported in serum by serendipity; specific PCR and primer condition that helpful in amplification of TTMV not TTV (Takahashi et al., 2000). The genome of TTMV consists also of circular negative sense ssDNA of approximately 2800-2900 nucleotides, i.e. is shorter than that of TTV. The particle size of TTMV has been estimated to be less than 30 nm, going below TTV also in diameter (Takahashi et al., 2000).

TTMV is distributed worldwide among healthy individuals without (apparent) geographical clustering (Biagini et al., 2001; Niel et al., 2001). For example, in sera of blood donors the TTMV DNA prevalence is 62% in France (Biagini et al., 2006), 67-72% in Brazil (Devalle et al., 2004; Niel et al., 2001), and 48% in Norway (Moen et al., 2002). TTMV isolates have been shown to occur in various tissues, such as plasma/serum, faeces, saliva, bone marrow, spleen and cervical swabs, in varying viral loads ($1.3x10^3$-$1.7x10^8$ copies per g of DNA) (Biagini et al., 2001b; Fornai et al., 2001; Thom et al., 2003; Vasconcelos et al., 2002).

These newly identified isolates were termed Torgue teno midiviruses (TTMDV) (Ninomiya et al., 2007a). Upon analyzing more TTMDV sequences, the authors noticed that these formed a large swarm of isolates differing in length (3175-3230 nt) and in sequence (33% divergence at nucleotide level and 60.7% at amino acid level of ORF1). In addition to other TTV-like characteristics, also three Rep-motifs were identified in the ORF1 region, as well as putative stem-loop structures in the GC-rich region. Based on this study, TTMDVs occur in at least 40% of nonsymptomatic individuals (Ninomiya et al., 2008), i.e. apparently ubiquitously worldwide. (Kakkola, 2008)

It has been speculated that these smaller TTVs could be the result of intragenomic rearrangements of full-length TTVs (Leppik et al., 2007).

Worldwide prevalence of TTV infection among healthy donors is observed in many studies. TTV prevalence knowledge in specific regions helps to develop strategy for virus control and control its transmission in healthy population. The aim of the study is to determine the prevalence of TTV in healthy blood donors of Pakistan, as there is a lack of studies showing prevalence of TTV in healthy donors in Pakistan.

1-1 Objectives

- To assess the molecular epidemiology of TTV in healthy blood donors.
- To assess the relationship of TTV with Hepatitis B and Hepatitis C seropositivity.
- To assess the appropriateness of TTV screening as part of routine blood screening.
- To measure the ALT values to assess any relationship of ALT level with TTV pathogenicity.

Chapter 2

2. Review of Literature

2.1 Discovery

In 1997, a patient in Japan with acute post-trnasfusion hepatitis of unidentified etiology was infected by a new infection isolate and named as torque teno virus. TTV was first discovered in 58 years old hepatitis patient with a current history of blood transfusion. TTV DNA was firstly discovered in hepatitis patient blood when liver has a high level of enzyme alanine aminotransferase (ALT). TTV was first characterized as blood-borne virus and thus named transfusion-transmitted (TT) group of viruses. (Nishizawa *et al.*, 1997)

In 2005, the International Committee on Taxonomy of Viruses (ICTV) proposed to classify and TTV into the new genus *Anellovirus*, of the family circoviridae and to assign to the acronym the meaning of Torque teno virus, from the Latin *torque* (necklace) and *tenuis* (narrow), referring to the characteristics of their genome (Manzin *et al.*, 2015).

Intravenous Drug Users and patients suffering from non A-G hepatitis, non A-G cirrhosis, haemodialysis patients, liver transplant recipients and hemophilic patients are common host for TTV (Charlton *et al.*, 1998; Naoumo, *et al.*, 1998; Okamoto *et al.*, 1998; Fujiwara *et al.*, 1998). It is reported that TTV is found in 90% of serum samples, which were taken from healthy population as TTV is present everywhere in nature (Bostan *et al.*, 2013).

Life time viremia of TTV infection is a special character of TTV. 10^6 copies/mL of viral titre in serum of TTV infected healthy population is recorded till now. Possible association of TTV infection with different disease, such as, liver disease, haematological disorders, respiratory diseases, multiple sclerosis and cancer have been reported but an informal straight link between these diseases and TTV infection is still unclear (Mi *et al.*, 2014).

2.2 Epidemiology

Early PCR detection reveals that one third of general population of world has TTV. There is a substantial variation in occurrence of TTV in different countries and also in different part of same country (Fig 2-1). In particular, moderate prevalence was seen

in northern Europe and United State, high in South America and Africa, intermediate in Asia (Pistello *et al.*, 2001).

1-12% donors of blood from different parts of world have been reported of having TTV infection. 18% of the patients, who were gone through blood transfusion process, have TTV infection (Shaheli *et al.*, 2015).

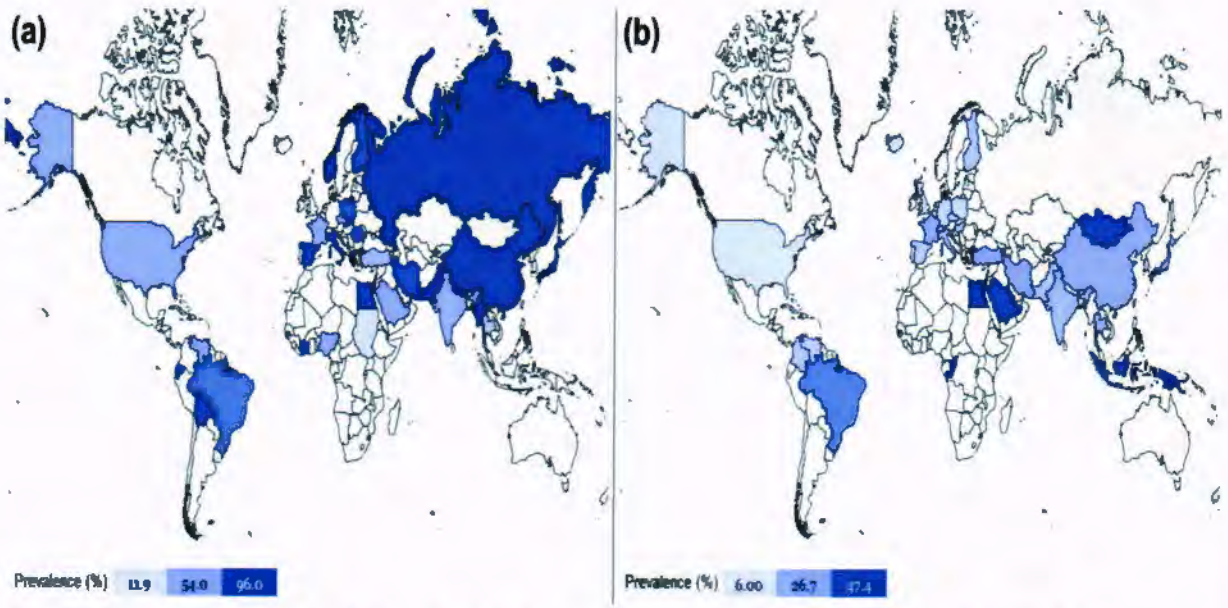


Fig 2-1 Comparative world heat maps of TTV prevalence in humans, detected using UTR- (a) vs. ORF- (b) derived primers. Data (percentages) represent the average prevalence for each country in Online Resource 2, regardless of the number of entries or year. Grey areas represent countries with no data (Spandole *et al.*, 2015).

2.3 Symptoms

In humans, TTV does not cause considerable harm to the tissues, that's why TTV represent no symptoms when present alone in the patients. Primarily, in liver, high level of TTV viremia is observed as compare to blood and other tissues. Certain types of carcinomas are also caused by TTV (Mankotia, D.S., and Irshad, M. (2014). Worldwide distribution, enormously high prevalence and high level of genomic heterogeneity, are the characteristics of TTV (de Oliveira, 2015).

2.4 Occurrence in Body

Liver, mammary glands, kidneys, brain, prostate, peripheral blood mononuclear cells, bone hematopoietic cells and polymorphonuclear cells are said to be damaged by TTV infection (Ishimura, *et al.*, 2010). Breast cancer, lung cancer and gastrointestinal tract cancer and in multiple mylomas, TTV DNA is reported (de Villiers, *et al.*, 2002).

2.5 Replication

Bone marrow and some other organs of the body examination show replicative transitional viral genome of TTV (Jelcic *et al.*, 2004). TTV also replicate in organs or tissues, such as pancrease, liver tissues, spleen, lung tissues and lymphoid tissues (Mi *et al.*, 2014).

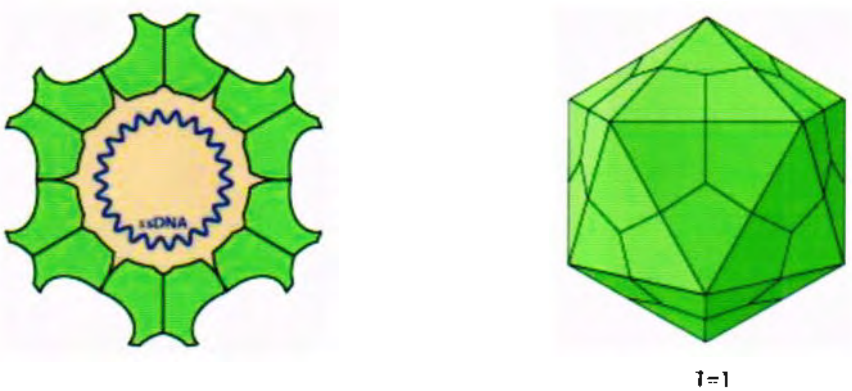


Fig 2-2 Virion. Non-enveloped, T=1 icosahedral symmetry, with a diameter of about 30-32nm (http://viralzone.expasy.org/all_by_species/772.html)

The floating density of TTV virion is 1.31-1.34g/cm^3. Filtration of virion shows 30 to 50 nm particle size of TTV virion (Fig 2-2). TTV use host replicative machinery and use DNA polymerases of host. So, the mutation rate is very low because of polymerase proof reading ability (Spandole *et al.*, 2015).

Virus evade into host cell. Viral ssDNA penetrate into host nucleus by uncoating. Cellular factors involve in converting ssDNA into dsDNA. Viral mRNA is produced by dsDNA transcription. Viral proteins are produced by transcription and translation of viral msRNAs. Replication done in rolling circle manner producing ssDNA genome and replication is mediated by 'rep-like' protein. Newly synthesized ssDNA may be converted to dsDNA and serve as template for transcription/replication. They

may also encapsidated by capsid protein and form virions that are released by host cell lysis (http://viralzone.expasy.org/all_by_species/772.html).

2.6 Genome

It is a single stranded, non-enveloped new DNA virus, having 30-50nm diameter. TTV has covalently closed circular genome of 3.8kbp (Magua *et al.*, 2015). TTV genome comprises of 1.2 kb of untranslated region (UTR) and possible 2.6 kb coding region. UTR is highly conserved region; it helps in regulation of viral replication. ORF1 and ORF2, large open reading frames are present in coding region of TTV genome. ORF encode different length of peptide for different strains of TTV (Fig 2-3) (de Oliveira., 2015).

TTV DNA is of negative polarity. Many TTV isolate have been completely sequenced, showing high divergence among genome of different strains. Evolutionary distance between these genotypes is 0.30 (Niel *et al.*, 2000). Based on phylogenetic analysis, till now group 1-5 of TTV with 39 genotypes have been discovered (de Oliveira, 2015).

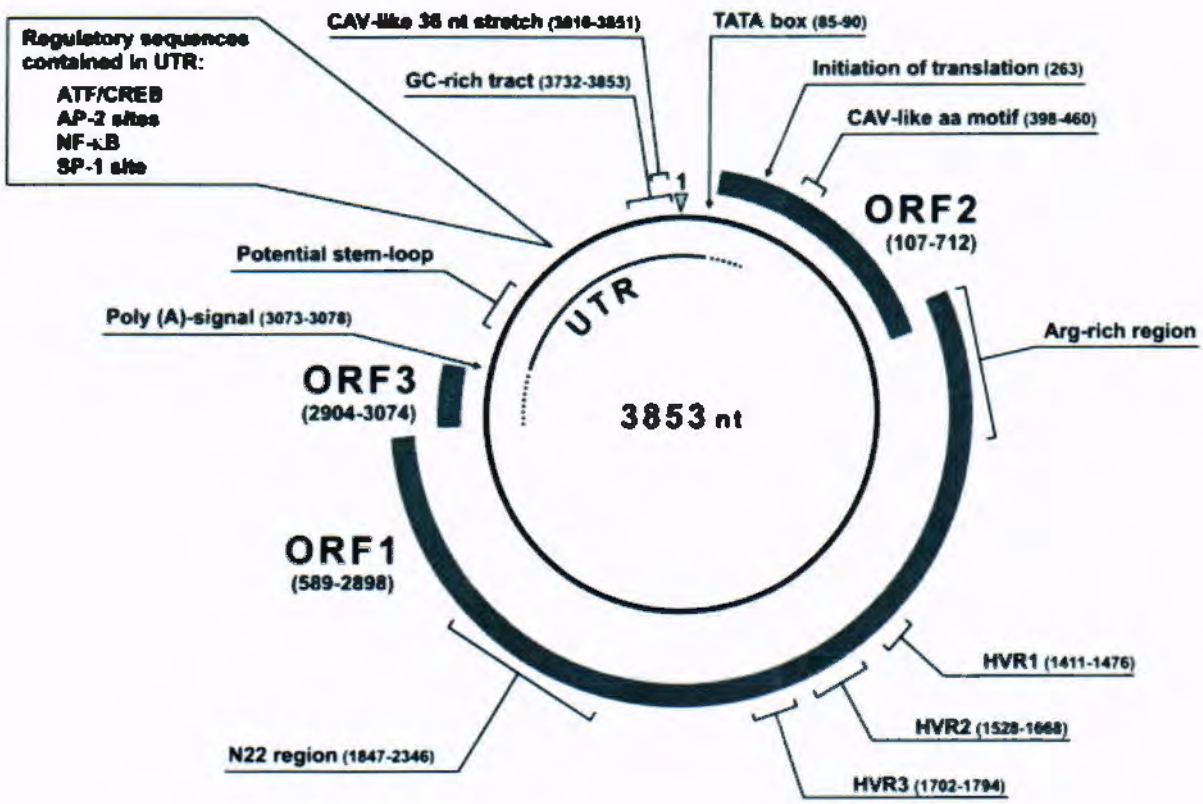


Fig 2-3 Genomic organization of TTV (Adopted from Bostan *et al.*, 2013).

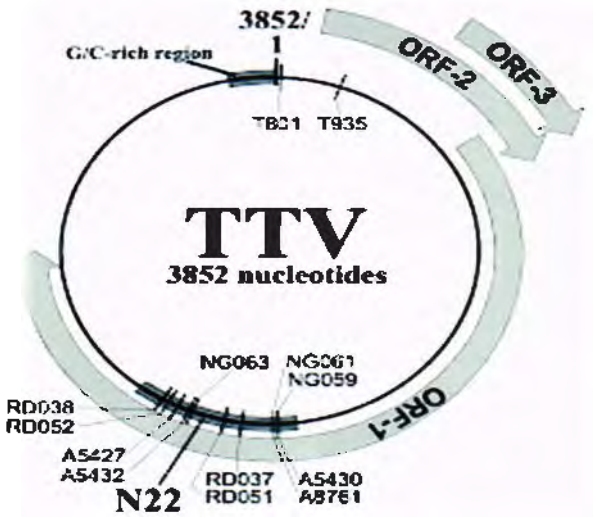


Fig 2-4 Physical map of the genome of TT virus according to the complete TTV genome sequence available under the GenBank number AF122913. (Springfeld *et al.*, 2000).

GC rich regions are conserved regions of TTV genome. GC rich region is 113nt long. Coding region has a downstream poly A sequence and an upstream TATA box. Same TATA box and poly A tail is used by 3 mRNA to start transcription process. These mRNA lengths are 3.0 kb, 1.2 kb and 1.0 kb respectively. By the further splicing of 2nd and 3rd mRNA, two or more ORFs are produced. Thus alternative splicing of TTV genome is confirmed (fig 2-4) (Bostan *et al.*, 2013).

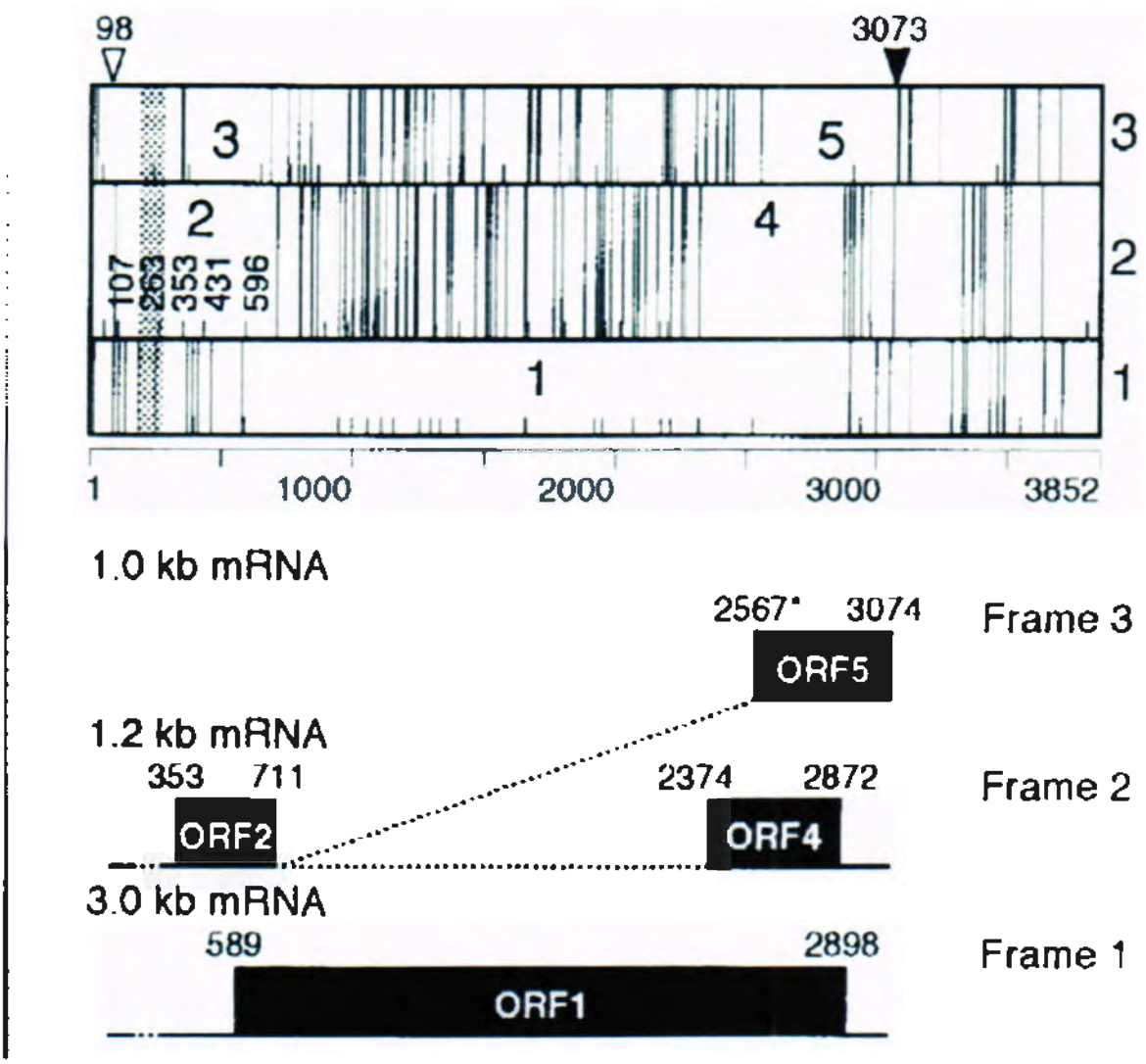


Figure 2- 5 TTV infected cell showing three mRNA. Spliced areas are shown by dashed lines (Kamahora et al., 2000).

Two large open reading frames (ORF) are present in anelloviruses coding region. ORF1, encoding a protein of 770 amino acid residues for TTV (isolate TA278 sequence), 637 amino acid residues for TTMDV (isolate MD1-073 sequence), and 663 amino acid residues for TTMV (isolate CBD231 sequence); and ORF2, encoding of protein of 120, 123 and 91 amino acid residues for TTV (isolate TA278 sequence), TTMDV (isolate MD1-073 sequence) and TTMV (isolate CBD231 sequence) (Spandole, *et al.*, 2015). Peptides of TTV isolates have divergence of 47-70% in the amino acid sequences that they encode (Biagini, *et al.*, 1999). Alternative splicing of pre-mRNA results in protein expression (http://viralzone.expasy.org/all_by_species/772.html).

From humans, TTV complete or partial length genome has been isolated till now. One third part of genome consists of extremely conserved non-coding sequences. Open reading frames (ORF) are present in coding region of genome. ORF1 is responsible for viral capsid protein as well as smaller ORF2, ORF3, and ORF4. Splicing results in additional mRNA (fig 2-5) (Jelcic, *et al.*, 2004).

Some studies confirmed that miRNA are encoded by some TTV variants, which is considered to be non-immunogenic regulatory molecules. It is also found that regulatory molecules may be utilized by TTV to target host cell transcripts (Kincaid *et a.l*, 2013).

5 divergent phylogenetic groups of TTV strains were created on TTV genetic diversity. TA278 strain is placed in group 1, PMV isolate is placed in group 2. 11 genotypes including SANBAN, TYM9 and TUS01 isolates are placed in group 3. KC009 and CT39 are placed in group 4 and 5 respectively. Mainly prevalent genogroups are 1 and 3, followed by 4 and 5. Less prevalent genogroup of TTV is group 2 (Wei *et al.*, 2015).

2.7 Transmission

Different studies on TTV shows different route of transmission. These are feces, saliva, bile juice, breast milk, parenterally, and by blood and blood products. Postnatal transmission is confirmed, but transplacental transmission from mother to child is controversial (Jelcic *et al.*, 2004; Shaheli *et al.*, 2015).

High rate of viral frequency may be direcly linked with form of contamination (Mazzola *et al.*, 2015). Transmission of TTV is also done by clustering of viral genotypes within community resulting in high prevalence of infection (Bostan *et al.*, 2013).

2.8 TTV Immunology

It is still hard to define that how TTV set up constant infection and how immune system is unable to clear TTV viremia (Kincaid *et al.*, 2013). 4-5 TTV proteins are produced by infection, which exaggerate slight immune response. So TTV infection immune response is limited (Bostan *et al.*, 2013). IgM, short term antibody was

appear in TTV infected individual serum, and IgG, long lived antibodies were seen after IgM (Tsuda *et al.*, 2001).

Specific antibodies of ORF1 N and C terminal were found in infected individual blood. TTV also produce immunocomplexes, which have been detected in TTV positive individual serum (Ott et *al.*, 2000). In case of TTV prolonged infection, immunocomplexes have been established but not in acute infection case. Per day, immune system clears about 90% viral particles, but immune system is still unable to wipe out TTV particles. Antibodies that are produce due to TTV infection, donot neutralized, crossed and react (Bostan *et al.*, 2013).

2.9 TTV Pathogenicity

When TTV is discovered, it is thought to be a hepatitis type virus. TTV studies so far conclude that, it is present in 90% of healthy individual. TTV is not an epidemic or seasonal infection. TTV cause permanent or temporary infection. TTV genotype 1 is said to be more pathogenic type than any other TTV type. It is also responsible for high level of transaminase in human serum. TTV is also nominated for making co-infection with other types of viral genotypes. For example, genotype 1 makes worse reaction of thrombocytopenia of chronic hepatitis disorder patients, such as chronic hepatitis B, chronic hepatitis C apart from degree of liver fibrosis (Bostan *et al.*, 2013).

2.10 Clinical Significance of TTV Co-infection with Hepatitis B and C Viruse

Hepatitis viruses (HBV and HCV) and TTV synchronized infection has been widely studied (Spandole *et al.*, 2015). Patients with chronic hepatitis C who devclop hepatocellular carcinoma, have elevated TTV virions (Tokita *et al.*, 2002). ORF3 of TTV genotype 1a encodes a protein which is similar to HCV non-structural protein 5A. This protein plays a major role in IFN-induced antiviral response (Tokita *et al.*, 2002). Death rate of patients with HBV-TTV co-infection is higher as compared to HBV infection alone (Desai MM et al, 2005). Although TTV is frequently detected in patients chronically infected with HBV and HCV, it does not seem to have any influence on the histopathological characteristics of the HBV- or HCV-infected liver (Tokita *et al.*, 2002). Patient's biochemical and clinical profiles with chronic HBV

and HCV co-infected with TTV were not considerably dissimilar from the patients without TTV (Chattopadhyay *et al.*, 2005). However, the possibility that TTV contributes to the progression of liver disease in people infected with HBV or HCV cannot be totally excluded (Spandole *et al.*, 2015).

2.11 Global Prevalence of TTV

Devalle and group reported in 2004 about prevalence of TTV infection. Serum samples collected from 72 Brazilian adults (24 voluntary blood donors, 24 hepatitis B virus (HBV) carriers, and 24 human immunodeficiency virus type 1 (HIV-1)-infected patients) were tested. TTV DNA from at least one genomic group was detected in 11 (46%) blood donors, 13 (54%) HBV carriers, and 24 (100%) HIV-1 patients.

Omar and colleagues in 2006 conducted a study. For which, 84 samples were collected from chronic liver disease patients. 22 samples were collected from hepatocellular carcinoma patients. 80 samples were collected from healthy individual from population. All collected samples were tested for HBV and HCV infection presence. Nested or RT-PCR assays were used to detect TTV infection presence. TTV infection was found in 23.8% of chronic liver disease, 31.8% of hepatocellular carcinoma and 25% of healthy individual.

Masia and fellows in 2001 collected total 317 blood samples to evaluate the presence of TTV infection, in which 57 were anti-HCV positive (12 with HCV-related chronic hepatitis, and 45 samples were without chronic hepatitis), 27 samples have chronic non A-E hepatitis, and 40 samples were HBsAg asymptomatic patients. 57 samples were intravenous drug users, 7 samples were from the patients who undergo a liver transplant for fulminant hepatitis. 137 samples were collected from healthy people from general population. On the whole, TTV infection was observed in 62 samples (19.6%). TTV infection rate in: anti-HCV positive patient was 14% (in 8.3% with chronic hepatitis patients and in 15.5% of without chronic hepatitis patients), 22.2% in non a-E hepatitis patients, 17.9% in HBsAg carriers, 22.8% of intravenous drug users, and 57.1% of fulminant hepatitis patients. 22.4% samples of healthy population were also TTV positive.

Massau and group in 2012 conduct a study, in which, 77 patients and 150 blood donors that require haemodialysis. Samples were investigated for TTV occurrence.

Polymerase chain reaction (PCR) was used for the detection of TTV DNA from the plasma of blood. Occurrence of TTV between patients and blood donors requiring haemodialysis were 68.8% and 73.3% respectively. TTV infection occurrence has no association with sex, education and age, and with history of blood transfusion or haemodialysis duration.

Alavi and fellows in 2012 reported occurrence of TTV from the selected blood samples.That were collected from 90 pediatric thalassemic patients who were getting chronic blood transfusion. 90 healthy volunteer children were also choosed to collect blood samples for the study. HCV AB test was performed in all of the collected blood samples. Semi-nested PCR was used to evaluate the presence of TTV infection in samples. TTV occurrence in thalassemic patients was 64.4%, in which 17.2% of the patients samples were both HCV and TTV positive. Whereas 6.3% patients were HCV Ab positive but found TTV negative. In healthy individual sample, TTV occurrence was 24.4%.

Spandole and group in 2013 reported the prevalence of TTV infection. Total of 113 healthy blood samples were collected from general population. High-resolution melting analysis (HRMA) was used to detect the TTV and its subtypes in blood samples. Then heminested PCR was used to amplify the TTV DNA from positive samples. 52% samples were TTV and its subtypes positive. Subtypes checked in this study were TTMDV and TTMV. This study also showed that HRMA can be used as a competent method for detecting human TTVs.

Feyzioglu and group in 2014 conducted a study for which, blood samples were collected from voluntary blood donors and patients. To detect the TTV DNA infection in collected samples, hybridization probe system was used in real time PCR. TTV infection was found in equally greater part of healthy and patients groups. The occurrence in healthy control was 84.6% (33/39). The occurrence in patients for outpatient control was 94.4 % (18/19), in patients received medical treatment was 94.7% (18/19), in patients with exacerbation needed noninvasive ventilation was 100% (20/20). Patients group and control groups CP values were different from each other.

Wei and fellows in 2015 conducted this study, in which Hospitalized patients of different departments were analyzed for the presence of torque teno virus. Total, 378

serum samples were collected, from which: 15 gastroenteritis patients, 192 tumor patients and 171 cardiovascular disease patients. Samples were analyzed by ELISA and nested PCR for the presence of TTV DNA infection. 64 samples were detected with TTV infection by ELISA and PCR. It is also revealed that patients less than thirty have a higher prevalence rate of TTV infection.

Takemoto and group in 2015 conducted a research on TTV infection prevalence in patients of. Blood samples were collected from 50 renal-transplant patients and 118 dialysis patients. Nested PCR is used to locate the TTV DNA in collected blood samples. Occurrence of TTV was 64% in renal-transplant patients and 53% in dialysis patients. Study also shows no significant involvement with the occurrence of variables and pathogens: blood transfusion, gender, time since transplant, associated presence of hepatitis B, time in dialysis.

El-taher and fellows in 2015, conducted a study to check the frequency of occurrence of TTV infection in hemodialysis patients. 63 and 100 blood samples were collected from hemodialysis patients and healthy individuals respectively, to evaluate the presence of TTV infection. In this study PCR prmiers were derived from conserved region open reading frame of TTV DNA. Prevalence of TTV results showed 19% infection in healthy individual blood samples and 42.9% in hemodialysis patients.

Aboudeh and colleagues in 2015 reported prevalence of torque teno virus by this study, in which, 644 blood samples were collected from different nationalities: 118 Qatari nationals and 526 non-Qatari nationals. All the collected samples were tested for TTV infection presence. 573 blood samples from total samples were belonging to healthy individual from general population. Whereas 54 samples were belong to hepatitis B patients, and 53 samples were belong to hepatitis C patients. The results of TTV prevalence obtained from this study showed that infection rate were 81.4%, 90.75%, and 84.9% in healthy individual samples, hepatitis B patients sample and hepatitis C patients respectively. 5'-untranslated regions were used to detect TTV by PCR assay from collected blood samples.

Mazzola and fellows in 2015 conducted a study to check the prevalence of TTV infection in the 551 healthy blood donors. Set of oligoprimers in nested PCR was used to evaluate the TTV presence in collected samples. Occurrence of TT virus was 69%

in collected samples of 551 healthy blood donors. It was also found that TTV prevalence has a higher rate (74.7%) in 18-24 years old donors.

Magu and fellows in 2015 reported occurrence of TTV infection by taking the sample of 50 chronic hepatitis patients, 50 acute hepatitis patients, and 100 voluntary blood donors. All samples were tested for ALT, serum bilirubin, AST, and alkalaine phosphatase level, and for all available markers for hepatitis. Real time PCR was used for the detection of TTV infection. TTV infection was observed in 77% (10/13) HCV patients, 87.6% (36/41) HBV patients, 77.4& (24/31) in hepatitis A patients, and 72% in volunteer blood donors and 92.8% (13/14) of non-c cases, non-B cases. Considerably, prevalence of TTV infection was higher (92.8%) in non A-E hepatitis group.

Hussain and colleagues in 2012 conducted a study for which blood samples of 116, 100 and 40 HBV infected patients, HCV infected patients and healthy individuals were taken to evaluate the presence of TTV in Pakistan. Primers against 5' UTR was used to detect the presence of infection. 89.7%, 90.0% and 92.5% samples of HBV, HCV patients and healthy individual were positive for TTV infection respectively.

Chapter 3

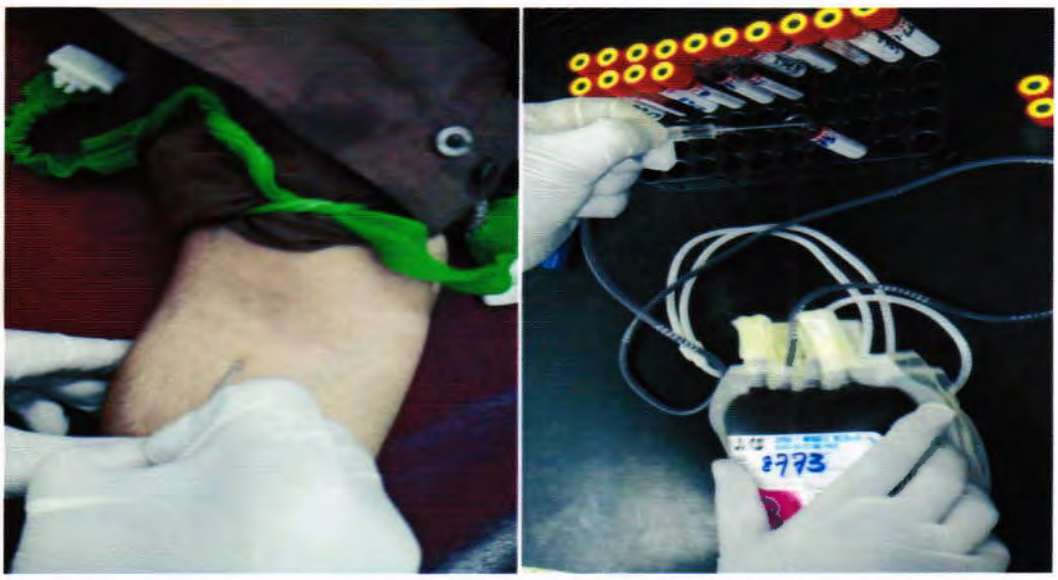
3. Material and methods

3.1 Study approval

Ethical committee of International Islamic University Islamabad gave the approval for this study. Samples of study were collected from PIMS blood bank. Asymptomatic HCV and HBV voluntary blood donors sample was collected for study. All donors come from different geographical areas of Pakistan in PIMS.

3.2 Sample collection

Almost 9000 samples were collected during month of Feb to April (2016) from routine blood donors, from which 25 HBV positive (asymptomatic), 25 HCV positive (asymptomatic) and 44 healthy samples (without HBV, HCV) were choose for study.



Veinpuncture Transfer

Figure 3-1 Sample collection procedure

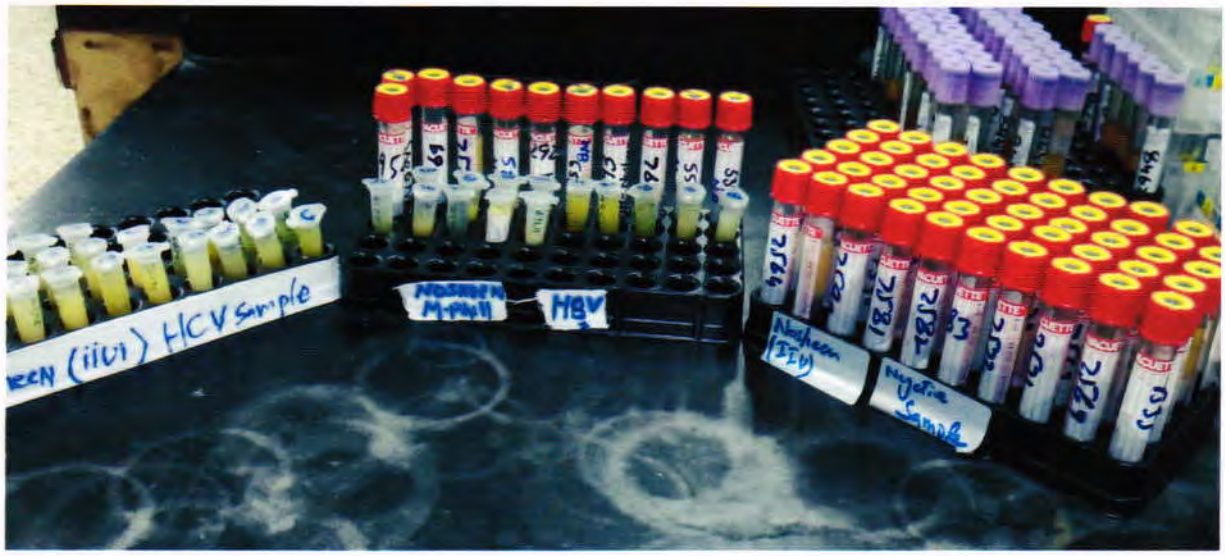


Figure 3-2 Collected

3.3 Centrifuge

Centrifugation was used for the separation of serum/plasma from the whole blood. The sample tubes were centrifuged at 14,000 rpm for 5 minutes. The resulting supernatant was transferred to 1.5ml eppendorf that was used for further serological detection of HBV, HCV and TTV.

Figure 3-3 Centrifuge machine for vacutainers.

3.4 HBsAg Qualitative II

Collected samples were then tested on ARCHITECT HBsAg Qualitative II assay on CMIA technology to detect Hepatitis B presence in volunteer blood donors.

In HBsAg qualitative assay, sample, paramagnetic microparticles coated anti-HBS, and acridinium labeled anti-HBS conjugate create a reaction on combination. They bind with each other and after reaction completion washing with ancillary buffer was done. Two times washing was done and then pre-trigger and trigger solutions were added to reaction. Result on CMIA was measured by relative light unit (RLU). ARCHITECT *i* System optics detect relation between sample HBsAg mount and RLU.

The presence or absence of HBsAg in the sample is determined by comparing the chemiluminescent signal in the reaction to the cutoff signal determined from an active calibration. If the chemiluminescent signal in the specimen is greater than or equal to cutoff signal, the sample is considered reactive for HBsAg.

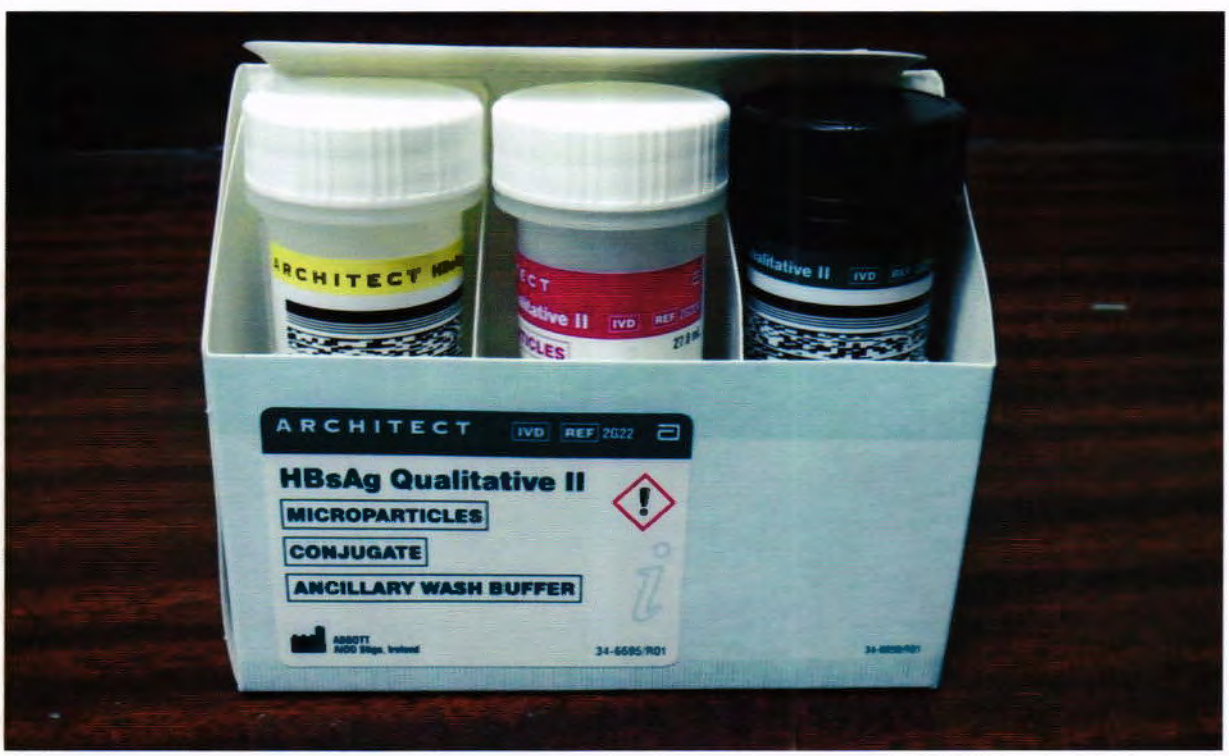


Figure 3-4 HBsAg reagent kit for CLIA (Architect i2000SR)

3.5 Anti-HCVTest

HCV positive samples that were choose, confirmed positive by Anti-HCV test performed on CLIA under the instruction of manufacturer. Assay diluents, sample and paramagnetic particles coated HCV antigen were combined together. Sample having HCV antigen bind to HCV coated microparticles. Then washed with washing buffer. Reaction mixture is formed by addition of acridinium labeled conjugate. Then wash it again. Then pre trigger and trigger solution were added to reaction mixture. The result of chemiluminescent reaction was measured by relative light units (RLUs). Samples were HCV positive or not was determined by cutoff value from calibration. Sample considered positive if the chemiluminescent value is equal or greater to cut off value.

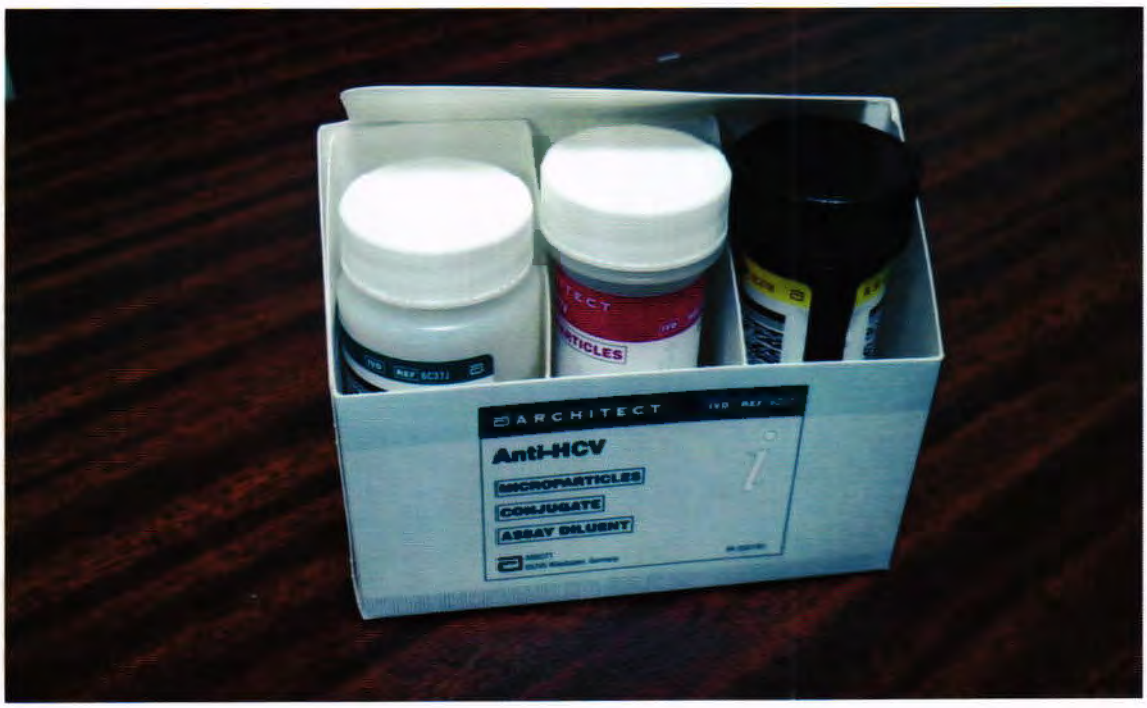


Figure 3-5 Anti HCV reagent kit for CLIA (Architect i2000SR)

Figure 3-6 Chemiluminescence Immunoassay (CLIA) on ARCHITECT *i*2000SR Immunoassay Analyzer provided by Abbott Diagnostics, USA.

3.6 Measurement of ALT level

All collected samples were tested for ALT level measurement in Chemwell machine, manufacturer is Awareness technologies USA. Reagents used in that machine were Pointe scientific USA.

3.7 TTV Antibody Test

For the detection of TTV antigen, automated ELISA kit (Abbexa **abx053944,** Abbexa Ltd., Cambridge Science Park, Cambridge, CB4 0FN, UK) was used, according to manufacturer instruction. The kit used in study was based on ELISA technology. TTV specific antibodies were pre-coated on 96 well plate of ELISA. Controls and samples were added to the wells symmetrically and incubated. After incubation plate was washed by washing buffer so that free components washed away. Then HRP conjugate which is used for detection was added to the all wells. Then TMB substrate is used which is helpful in visualinzing the reaction done by HRP. Then acidic stop solution was added. By the addition of stop solution blue color changed to yellow in TTV virus reactive samples. Optical density was measured at 450nm by spectrophotometre. Kit contained positive and negative controls, and the cut-off values for the

results assay were determined based on 0.15 plus the mean optical density 450 values of the negative control samples.

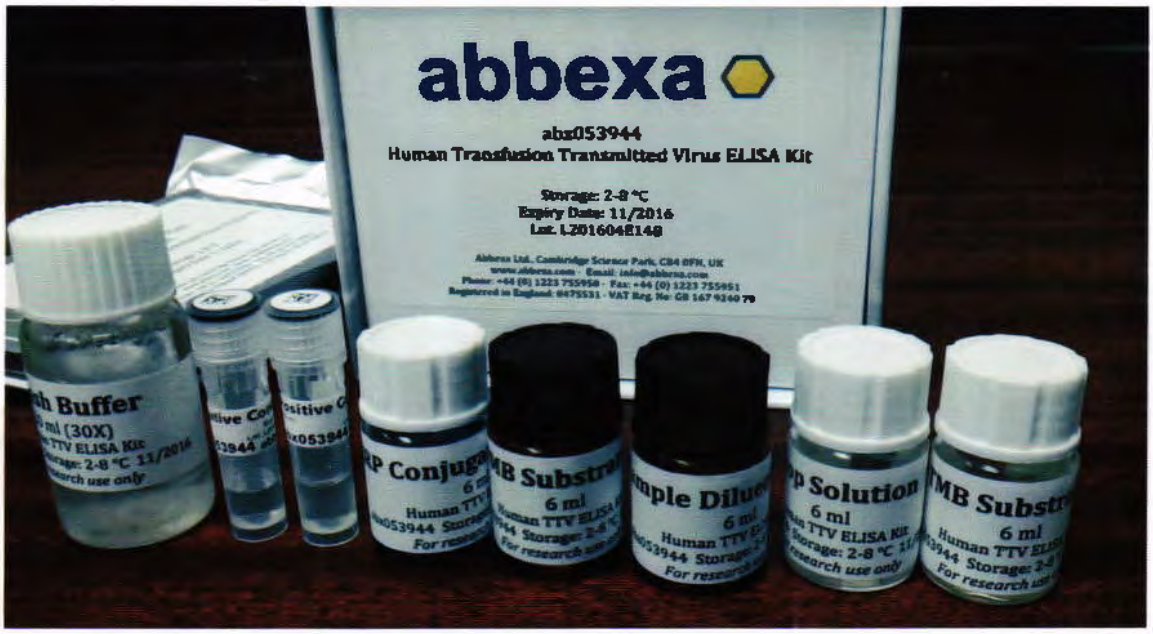


Figure 3-7 TTV ELISA kit reagents for TTV positive sample detection on *eti max* 3000

Figure 3-8 *eti max 3000 (ATCHITECT) ELISA machine*

3.8 DNA Extraction

DNA isolation was carried out by using Sambrook and Russel DNA isolation protocol from serum samples. 50µl serum sample was alliquoted in eppendorf with 350µl of TE buffer of pH 8.0. 0.66%SDS and 1mg/ml of PK was added in eppendorf and incubated for 2 hours at 56°C. Solution was cooled at room temperature and PCI in equal amount was added to mixture afterwards. Until the white precipitate stop appear, above step was repeated. In aqueous layer 0.3M Sodium acetate of pH 5.2 was added. 95% ethanol was added to precipitate out DNA and glycogen is added and stored for 4 hours at -20°C. For the separation of DNA, 15 min centrifugation at 1300rpm was done. Then 70% ethanol was used for washing. Then dried the pellet of DNA and dissolved it in 20µl of TE buffer and extracted DNA was stored at -20°C.

3.9 Gel electropherosis

1 gram of powdered agarose was mixed with 100ml 2x TBE buffer (appendex ii) and heated in microwave for 1-3 minutes till a clear solution was formed. Gel solution was cooled for 25-30 minutes at room temperature. Gel was poured in gel caster plate with 10 well comb. Solution of gel was poured and allowed to set for half an hour at room temperature. Comb was taken out of gel after polymerization. Gel was placed in gel caster that was filled with TBE buffer. 2-3 µl of each ethidium bromide stain and bromophenol blue was added to 5µl DNA sample. In this way mixture was prepared to run on gel. Sample was loaded in wells. For running the gel, optimized conditions were 100 volts for 30 minutes at 23A current. After completing the gel ruing process, the bands were visualized under the UV light using the gel documentation system. DNA bands confirm the presence of TTV DNA in samples.

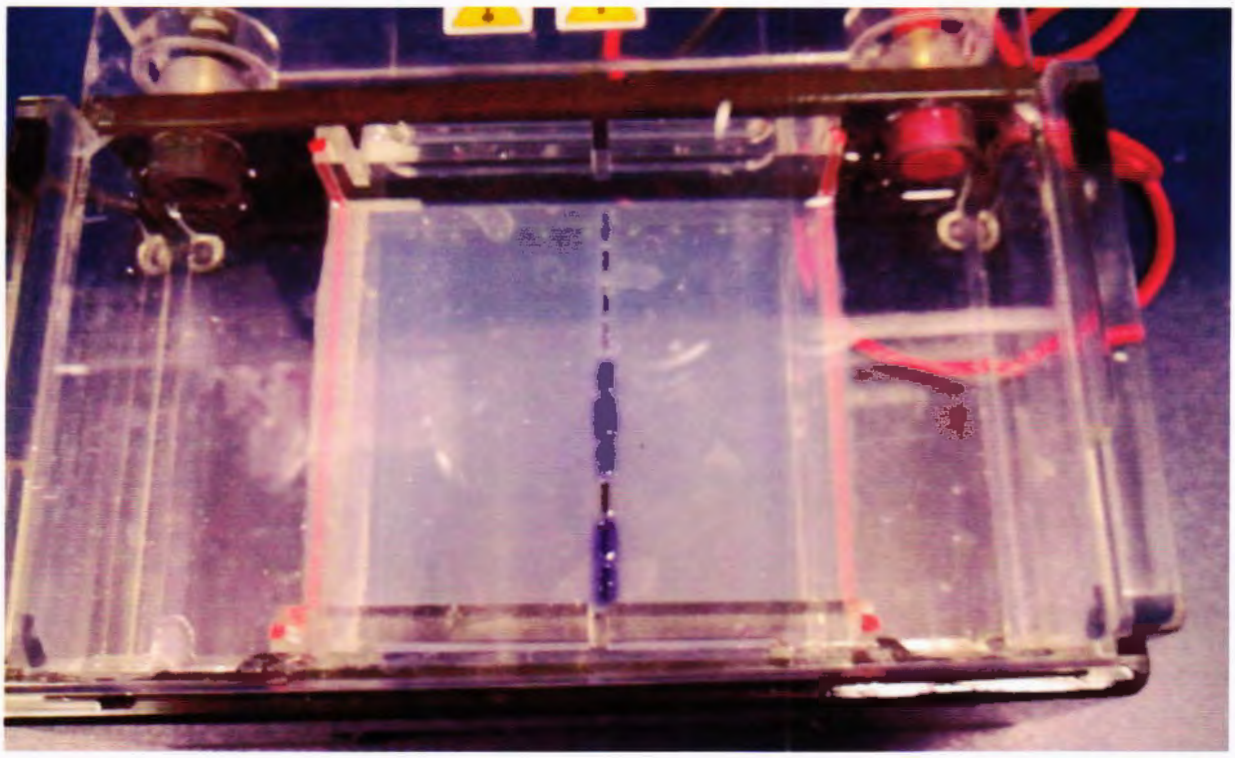


Figure 3-9 Gel caster. DNA is loaded in wells to confirm DNA presence.

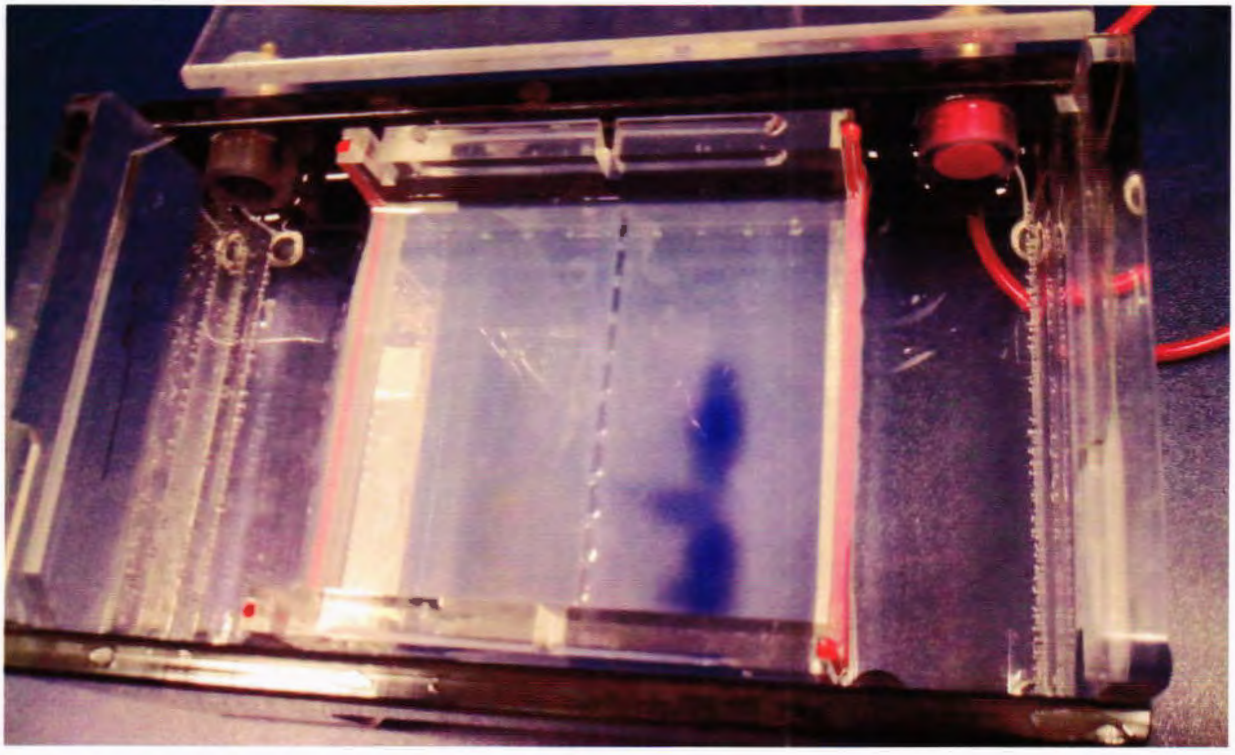


Figure 3-10 DNA move to positive pole.

3.10 Pcr

Primers used in my project were selected from a published study (Ninomiya *et al.*, 2008).

Forward primers: 5- ACA TGT ACC GAA TGG CTG AGT TT – 3
Reverse primers: 3- CCC CTT GACTCG TCG GTG TGT AA – 5

The reaction comprises of 10µl reaction mixture in a PCR tube. Reaction mixture contains 7µl master mix, 1µl forward primers 1µl reverse primers and 1µl genomic DNA. Samples with total volume of 10µl were placed in thermocycler. PCR was optimized at initial denaturation at 94°C for 30s, annealing at 55°C for 30s and extension at 72°C for 30s, with additional 7 minutes in the last cycle. The PCR was performed by using BIO RAD T100™ thermal cycler for 37 cycles. PCR amplified product is then resolved on ethidium bromide stained gel and was observed by visual inspection.

Figure 3-11 PCR Thermocycler.

3.11 Statistical analysis

p-value is calculated by comparing the results of ELISA and PCR by using 1 sample t-test (SPSS 20).

Chapter 4

4. Results

4.1 Sample collection

This research work was carried out in PIMS and IIUI after the final approval from ethical committee of IIUI. For this study, 94 samples were used; out of which 25 were HCV positive, 25 were HBV positive, and 44 were from healthy donors.

4.2 Determination of ALT level

ALT level was measured on Chemwell machine. Complete results of ALT values for the 94 samples are shown as table 4.1. ALT levels difference in samples mostly lies in normal range i.e 40U/L. The average of ALT level found in this project is 35U/L.

4.3 ELISA Test for TTV presence

Samples were run on 96 well ELISA kit (Table 4-2). Collectively ELISA show 26 positive samples from which 5 are from HCV, 9 from HBV and 12 positive results from healthy donors samples. The percentage of TTV infection detected on ELISA among HCV, HBV and healthy donors samples is as 20%, 36% and 24% respectively as shown in the table 4.1.

4.4 TTV PCR results

Samples were run on 96 well PCR thermocycler. Collectively PCR show 26 positive samples from which 6 are from HCV, 10 from HBV and 10 positive results from healthy donor's samples. After PCR samples were checked on 2% agarose gel and results are shown in figure 2(a, b). The percentage of TTV infection detected by PCR by counting gel bands on agarose gel electrophoresis among HCV, HBV and healthy donors samples is as 24%, 40% and 20% respectively (table 4-3).

4.5 Statistical analysis

p-value was calculated using SPSS-20 by 1 sample t-test. The results showed both ELISA and PCR of equal significance. P-value of the results found equal to 1.00. Comparison of both the test (ELISA and PCR) is given as figure 4-3.

Table 4-1: ALT level of collected samples

No	Sample type	TTV results PCR based	ALT value	No	Sample type	TTV results PCR based	ALT level
1	HCV	Positive	27	48	HBV	Negative	27
2	HCV	Positive	26	49	HBV	Negative	29
3	HCV	Positive	39	50	HBV	Positive	61
4	HCV	Positive	30	51	Healthy	Negative	45
5	HCV	Negative	32	52	Healthy	Negative	39
6	HCV	Negative	46	53	Healthy	Negative	58
7	HCV	Negative	31	54	Healthy	Positive	33
8	HCV	Negative	26	55	Healthy	Negative	35
9	HCV	Negative	35	56	Healthy	Positive	28
10	HCV	Negative	28	57	Healthy	Positive	66
11	HCV	Negative	29	58	Healthy	Positive	40
12	HCV	Negative	31	59	Healthy	Negative	33
13	HCV	Negative	63	60	Healthy	Positive	29
14	HCV	Negative	34	61	Healthy	Negative	72
15	HCV	Negative	26	62	Healthy	Positive	34
16	HCV	Negative	30	63	Healthy	Negative	32
17	HCV	Negative	33	64	Healthy	Positive	35
18	HCV	Negative	29	65	Healthy	Positive	49
19	HCV	Negative	26	66	Healthy	Negative	37
20	HCV	Negative	28	67	Healthy	Negative	31
21	HCV	Negative	31	68	Healthy	Negative	68
22	HCV	Negative	34	69	Healthy	Negative	28
23	HCV	Positive	30	70	Healthy	Negative	31
24	HCV	Positive	26	71	Healthy	Negative	70
25	HCV	Negative	25	72	Healthy	Negative	40
26	HBV	Positive	34	73	Healthy	Negative	56
27	HBV	Positive	30	74	Healthy	Negative	33
28	HBV	Negative	32	75	Healthy	Negative	34
29	HBV	Negative	73	76	Healthy	Negative	55
30	HBV	Negative	29	77	Healthy	Negative	39
31	HBV	Negative	30	78	Healthy	Negative	29
32	HBV	Negative	34	79	Healthy	Negative	26
33	HBV	Negative	56	80	Healthy	Negative	65
34	HBV	Negative	33	81	Healthy	Negative	30
35	HBV	Positive	28	82	Healthy	Negative	27
36	HBV	Negative	25	83	Healthy	Negative	31
37	HBV	Negative	34	84	Healthy	Negative	35
38	HBV	Negative	31	85	Healthy	Negative	42
39	HBV	Positive	41	86	Healthy	Negative	56
40	HBV	Positive	29	87	Healthy	Positive	35
41	HBV	Positive	55	88	Healthy	Negative	63
42	HBV	Positive	33	89	Healthy	Negative	27

43	HBV	Negative	27	90	Healthy	Negative	72
44	HBV	Positive	25	91	Healthy	Negative	51
45	HBV	Positive	31	92	Healthy	Negative	33
46	HBV	Negative	33	93	Healthy	Negative	29
47	HBV	Negative	30	94	Healthy	Positive	75

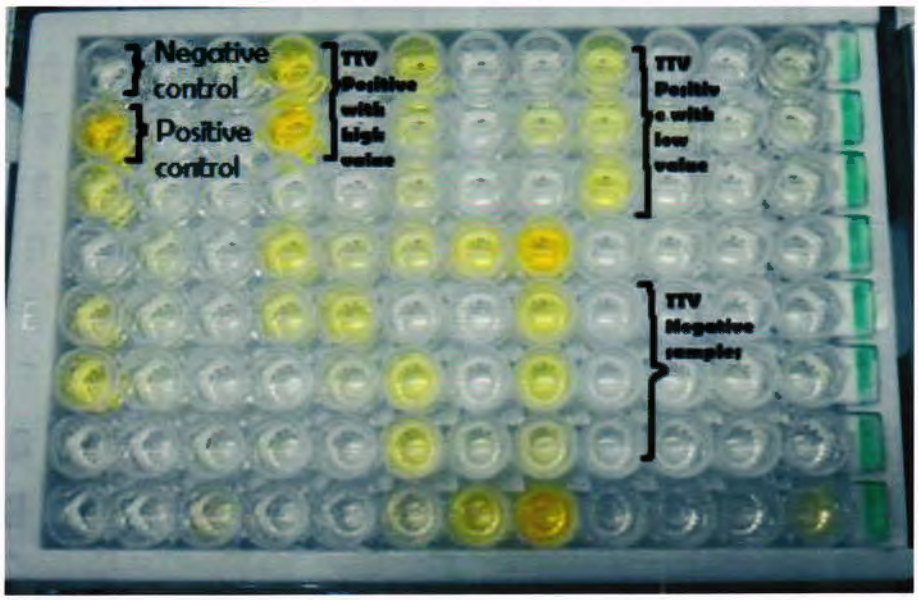


Figure 4-1: ELISA 96 well plate with TTV reactive and non-reactive samples

Table 4-2: Frequency of TTV positive results on ELISA

No	Sample type	Sample size	ELISA result (+ive)	ELISA results (-ive)	%age of +ive results
1	HCV	25	5	20	20%
2	HBV	25	9	16	36%
3	Healthy(without HCV & HBV)	44	12	32	24%

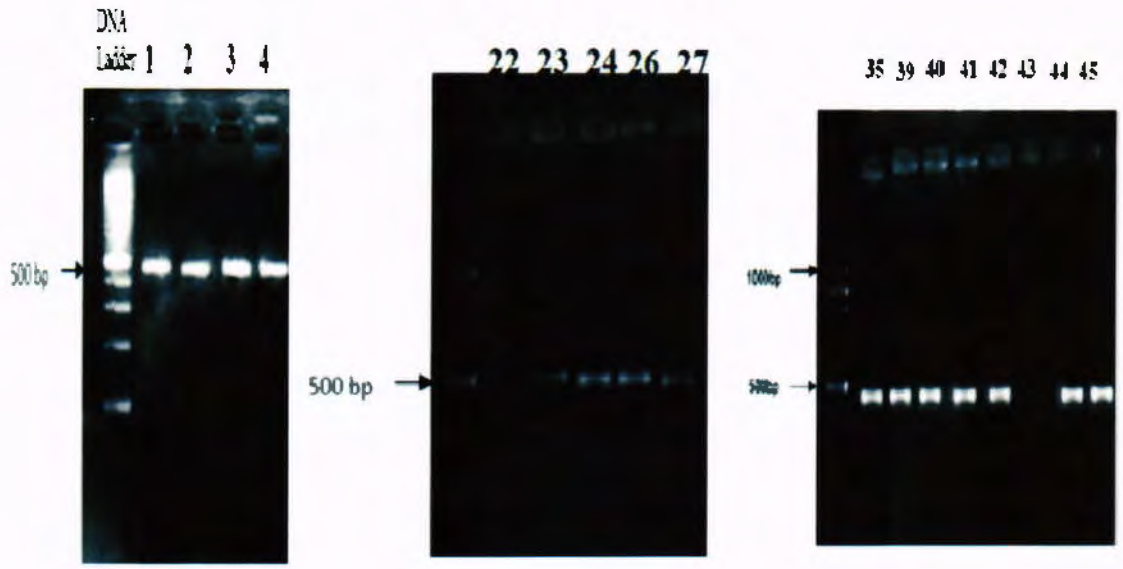


Figure 4-2(a): Agarose gel bands showing PCR results for TTV positive samples band on gel for sample 1 to 48.

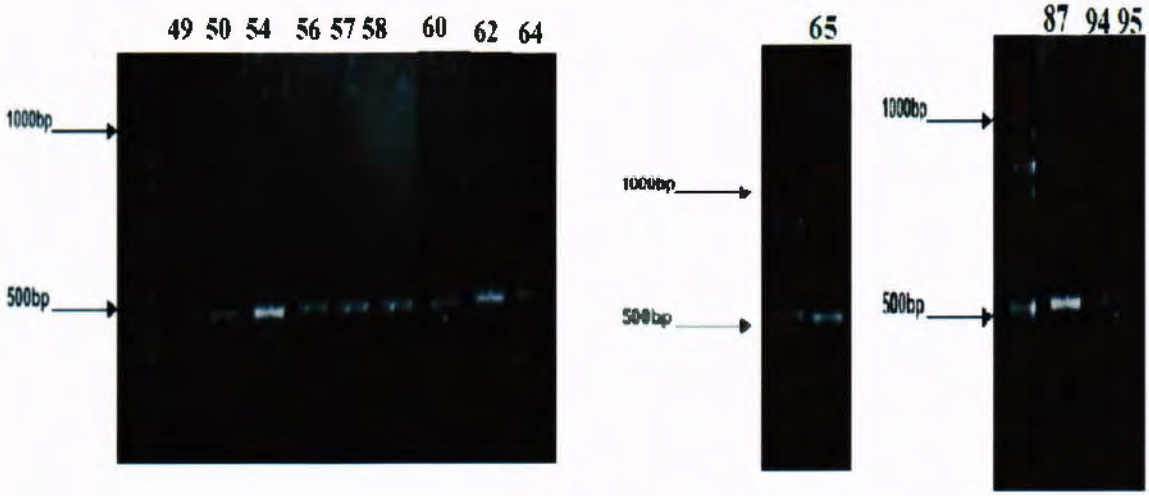


Figure 4-2(b): Agarose gel bands showing PCR results for TTV positive samples band on gel for sample 49 to 96

Table 4-3: PCR test results for TTV on selected samples

No	Sample type	Sample size	PCR result +ve	PCR result -ve	%age of +ve results
1	HCV(asymptomatic)	25	6	19	24%
2	HBV(asymptomatic)	25	10	15	40%
3	Healthy(without HCV & HBV)	44	10	34	22%

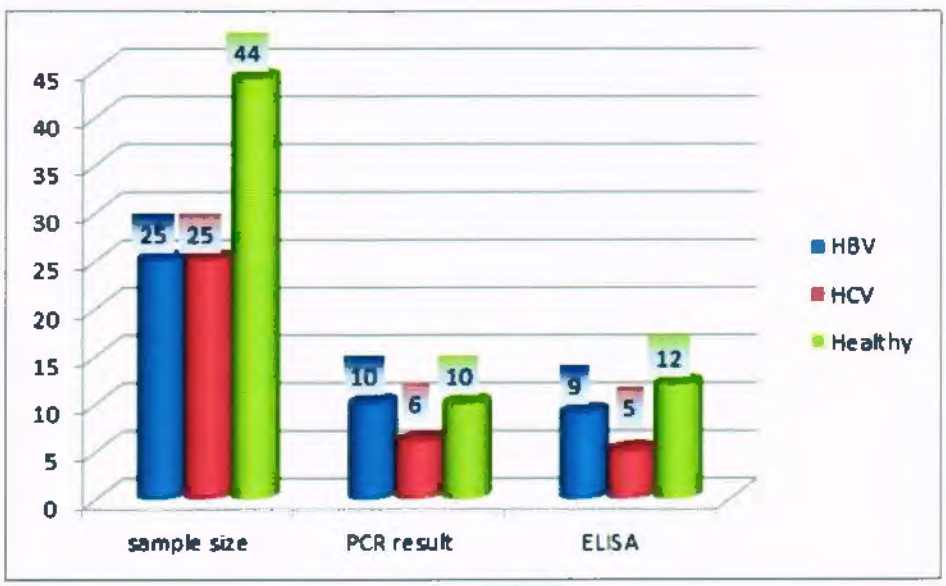


Figure 4-3: TTV Result Comparison of ELISA and PCR

Chapter 5

5. Discussion

Screening for transfusion-transmissible infections (TTIs) is a critical part of the process of ensuring that transfusion is as safe as possible. Unsafe blood transfusion is very costly both from a human and an economic point of view. These Transfusion Transmitted Infections (TTIs) are mainly HIV, HBV, HCV and syphilis, but in Pakistan, malaria, dengue, and in some cases CMV and toxoplasmosis have also been documented to be important as a TTI (GoP, 2013).

TTV DNA was first reported in non A-G hepatitis patient having many blood transfusion reports with unknown etiology in 1997. TTV be capable of be transmitted by blood and blood products (El-Taher *et al.*, 2015). Till today there is no clear evidence on TTV association with liver disease or any other disease (http://www.aabb.org/tm/eid/Documents/158s.pdf#search=TORQUE%20TENO%20VIRUS). Still there have been frequent reports about the TTV viremia in patients of Hepatitis B and C from all over the world but there is severe lack of data on epidemiology of TTV from Pakistan in blood donors. Therefore the present study was designed to clarify the rate of prevalence of TTV in blood donors with asymptomatic HCV, HBV and healthy status.

In the present study, 26 specimens (27.6%) were found positive for TTV, as detected by the human ELISA detection kit and also by PCR detection method but the positive ratio is different in both tests. The prevalence of TTV was 20.0% (5/25) in HCV, 36.0% (9/25) in HBV and 24.0% (12/44) in healthy donors, as checked by ELISA. The prevalence of TTV was 24.0% (6/25), 40.0% (10/25) and 22.7% (10/44) in HCV, HBV and healthy donors, respectively by PCR.

Frequency of TTV has been reported as high as 90% from Hong Kong (Niel *et al.*, 1999) and the lowest reported was 29.7% from Belgium blood donors (Ali *et al.*, 2004). Other studies have reported different percentages as in 2007 Pinto and group reported the prevalence of TTV as 60%, and 62% in northern and southern region of Brazil, respectively. In France 66% ratio of TTV was reported by Baigini and fellows in 2006. Chattopadhyay and fellows in 2005 and Magu and group in 2015 reported TTV prevalence of Indian population as 43% and 72% respectively. In comparison to all these frequencies of TTV occurrence in studied population was found to be lowest

i.e 27.6%. These clearly different frequencies of TTV occurrence around the world and Pakistan are may be due to the different lifestyles in different regions of world, several routes of TTV transmission among different ethnic groups around globe has been reported. Results of this study highlights TTV prevalent nature globally but less prevalent in Pakistani population.

The frequency of TTV in the Russian healthy blood donors was 94%. However, in this study, TTV frequency in healthy blood donors on average of both tests was 23.35 %. Reports of TTV presence in healthy population strengthen the newly reported facts that there are multiple routes of viral transmission present contrary to the initially believed only parenteral route. The variety in routes of transmission may be because of geographical distribution, the methods used for TTV DNA testing, and various demographic, virological features as proposed by Vasilyev and fellows (2009).

UAE study on TTV prevalence reported 97.9, 95.7% of patients with HBV and HCV tested positive for TTV infection which are much higher than our results of 38.0% and 22.0% of HBV and HCV patients testing positive for TTV infection. In India, 87.6%, 77% TTV positive viremia in HBV and HCV which is much higher than our findings (Magua *et al.*, 2015). Many studies had done in Iran to check epidemiology of TTV in blood donors. In Iran, 2.7 to 79.5% ratio has been reported (Karimi , 2013).

Effects of TTV infection on liver was calculated by measuring the liver enzyme, alanine aminotransferase (ALT) values for each sample that were positive and negative for TTV DNA. In the present study, most of the TTV samples had normal level of ALT on average 35U/L. Hence it is observed that the level of ALT doesn't have any effect on pathogenicity of TTV. The same study was reported in saudian blood donors (Al-Mozaini *et al.*, 2006). In contrast to our study, Itoh and fellows reported a higher prevalence of TTV in blood donors with elevated ALT levels than in those with normal levels but our study proved TTV presence was reported in samples having normal ALT level. When first time TTV was reported, researcher found high ALT level up to 2 folds than normal level (Nishiwaza *et al.*, 1997). It was thought that test of ALT may be helpful in early diagnosis of TTV in population. But many studies concluded that ALT level have no effect on TTV.

6. Conclusion

Safe blood supply is a complex process in blood transfusion system. Because of the rising blood borne infections like TTV, epidemic and pandemic status, newly reported infections are important issues. Torque Teno Virus is reported globally and its prevalence knowledge in Pakistan will help to develop strategy for virus control and control its transmission in healthy population. Donor's blood screening is important to maintain general population health because donors show healthy pool of society. TTV is blood transmitted virus like other Transfusion Transmitted Infections. This research project provides basis for implication of TTV in daily routine screening in transfusion services for the better health of blood recipients and general population.

7. Future Consideration

As the TTV is also called as transfusion transmitted virus and it is widely reported and prevailed in blood donors in Pakistan and worldwide. Hence there is a need of legislation and immediate steps to be taken to include TTV screening as routine blood screening in Blood Transfusion Systems or blood banks as other transfusion transmitted infections screening is part of daily screening before donated blood is transfused to recipients. TTV is dangerous as other Transfusion Transmitted Infections. So, preventive steps should be taken for better health of general population as blood donors represent healthy pool of society.

Chapter 6

6. References

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Pakistan institute of medical sciences

Blood Transfusion Services

Interview of Donors

Name of Donor _____

Blood Group _____ Blood Pressure _____

Age _____ Sex _____

Weight _____ Telephone No _____

City _____ Date _____

Questions	Yes	No
Have you donated blood before? When		
Have you received blood before? When		
History of high B.P/Heart disease?		
History of Jaundice?		
History of T.B in past?		
History of Malaria in past?		
Have you ever had unexplained prolonged fever?		
Any history of recent weight loss?		
Travel abroad? Specify country		
Are you taking any medicines? Specify		
Did you have 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 for shaving to a barber?		
Are you a drug user?		
Treatment from Homeo or Hakeem?		
Any history of Kidney disease or Epilepsy?		
Any blood disorder or other illness?		
Are you feeling well today?		

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

Signature _____

Annex-I: Sample detail.

No	Sample type	SSID	CLIA cut off value (SCIO)	ALT (U/L)
1	HCV	547	8.45	27
2	HCV	545	12.87	26
3	HCV	3559	16.05	39
4	HCV	686	15.32	30
5	HCV	26747	5.95	32
6	HCV	26495	14.87	46
7	HCV	26504	8.48	31
8	HCV	4036	16.27	26
9	HCV	26490	15.02	35
10	HCV	26199	4.86	28
11	HCV	27201	11.53	29
12	HCV	697	16.56	31
13	HCV	25917	14.83	63
14	HCV	26313	15.33	34
15	HCV	26012	16.63	26
16	HCV	162	15.05	30
17	HCV	26263	14.63	33
18	HCV	27073	12.40	29
19	HCV	26819	13.35	26
20	HCV	26370	15.86	28
21	HCV	327	12.14	31
22	HCV	562	13.31	34
23	HCV	245	13.94	30
24	HCV	522	13.02	26
25	HCV	26332	12.88	25
26	HBV	5606	4311.55	34
27	HBV	6920	4506.23	30
28	HBV	24754	5053.15	32
29	HBV	5843	1212.10	73
30	HBV	6292	2118.09	29
31	HBV	5522	3534.99	30
32	HBV	6393	4979.71	34
33	HBV	24756	4173.05	56
34	HBV	5596	5298.78	33
35	HBV	5395	4272.15	28
36	HBV	5952	1809.43	25
37	HBV	2623	1803.89	34
38	HBV	2572	4659.20	31
39	HBV	4462	4521.87	41
40	HBV	5023	4582.74	29
41	HBV	4687	1953.47	55
42	HBV	2951	2584.51	33
43	HBV	4628	2700.83	27

44	HBV	2523	1316.98	25
45	HBV	2654	3102.68	31
46	HBV	2274	2992.44	33
947	HBV	498	2582.56	30
48	HBV	26216	2913.79	27
49	HBV	25352	3196.32	29
50	HBV	27211	5324.63	61
51	Healthy	7555	0	45
52	Healthy	7569	0	39
53	Healthy	7571	0	58
54	Healthy	7572	0	33
55	Healthy	7583	0	35
56	Healthy	7582	0	28
57	Healthy	7581	0	66
58	Healthy	7580	0	40
59	Healthy	7575	0	33
60	Healthy	7564	0	29
61	Healthy	7557	0	72
62	Healthy	7560	0	34
63	Healthy	7563	0	32
64	Healthy	7546	0	35
65	Healthy	7553	0	49
66	Healthy	7543	0	37
67	Healthy	7388	0	31
68	Healthy	7540	0	68
69	Healthy	7386	0	28
70	Healthy	7389	0	31
71	Healthy	7390	0	70
72	Healthy	7394	0	40
73	Healthy	7395	0	56
74	Healthy	7402	0	33
75	Healthy	7401	0	34
76	Healthy	7397	0	55
77	Healthy	7396	0	39
78	Healthy	7407	0	29
79	Healthy	7411	0	26
80	Healthy	7413	0	65
81	Healthy	7414	0	30
82	Healthy	7426	0	27
83	Healthy	7420	0	31
84	Healthy	7422	0	35
85	Healthy	7433	0	42
86	Healthy	7434	0	56
87	Healthy	7435	0	35
88	Healthy	7432	0	63
89	Healthy	7428	0	27
90	Healthy	7429	0	72

91	Healthy	7600	0	51
92	Healthy	7486	0	33
93	Healthy	7490	0	29
94	Healthy	7491	0	75
95	Healthy	7494	0	39
96	Healthy	7495	0	27

Annex-II

Recipes of solutions and reagents

S.No	Solutions	Composition	Functions
1.	**2X TBE**	Tris HCl 21.6g (pH 8.0) Borate 11g EDTA 1.46g	Chelating agent
2.	**10% SDS**	SDS 10g Distilled water 50ml	Disrupting non-covalent bonds in proteins
3.	**Bromophenol Blue**	Bromophenol Blue 0.25g Glycerol 50ml 1M Tris 1.00ml (pH 8.0)	Gel loading dye
4.	**TE Buffer**	1M Tris HCl 1ml(pH 8.0) 0.5M EDTA 0.2ml Distilled water 98ml	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	Staining solution
6.	**Microparticles**	1 or 4 bottles anti-HBs coated microparticles in MES buffer with protein stabilizer	Binding
7.	**Conjugate**	1 or 4 bottles anti-HBs and anti-HBs acridinium-labelled conjugate in phosphate buffer with human plasma and protein	Binding
8.	**Ancillary Wash Buffer**	1 or 4 bottles ancillary wash buffer containing MES buffer	Washing
9.	**Pre-Trigger Solution**	1.32% (M/V) Hydrogen Peroxide	Starting
10.	**Trigger Solution**	0.35% N Sodium Hydroxide	Starting
11.	**Wash Buffer**	Phosphate Buffered Saline Solution	Washing