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Safe Blood Transfusion: Screening for Hepatitis B and Hepatitis C Virus Infections in Potential Blood Donors in Rural Southeast Asia

LE VIET

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ACKNOWLEDGEMENT

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Life is good!

Vietnam June 2013

Le Viet

ABBREVIATIONS

ADV	Adefovir
ALT	Alanine aminotransferase
Anti-HBc	Antibodies to Hepatitis B core antigen
Anti-HBc IgG	IgG antibody to hepatitis B core antigen
Anti-HBc IgM	IgM antibody to hepatitis B core antigen
Anti-HBe	Antibodies to Hepatitis B envelope antigen
Anti-HBs	Antibodies to Hepatitis B surface antigen
Anti-HCV	Antibodies to Hepatitis C
BCP	Basal Core Promoter
cccDNA	Covalently Closed Circular DNA
CHC	Chronic hepatitis C
CMIA	Chemiluminescent Microparticle Immunoassay
EIA	Enzyme Immunoassay
ETV	Entecavir
FDA	Food and Drug Administration
HBcAg	Hepatitis B Core Antigen
HBeAg	Hepatitis B Envelope antigen
HBIG	Hepatitis B Immunoglobulin
HBsAg	Hepatitis B surface antigen

HBV	Hepatitis B virus
HBV DNA	Hepatitis B virus DNA
HCC	Hepato-cellular carcinoma
HCV	Hepatitis C virus
HIV	Human Immunodeficiency Virus
ICBS	International Consortium for Blood Safety
IFN-α	Interferon-alpha
IRES	Internal Ribosome Entry Site
LdT	Telbivudine
LVD	Lamivudine
MHL	Major Hydrophilic Loop
MU	Million Units
NAT	Nucleic Acid Amplification Technology
NCR	Non-Coding Region
ng	Nanogram
NRTIs	Nucleoside Reserve Transcriptase Inhibitors
NRVRD	Non-Remunerated Voluntary Repeat Donors
OBI	Occult Hepatitis B infection
Peg-INF	Pegylated interferon
PEI	Paul-Ehrlich Institute

RLUs	Relative Light Units
RNA	Ribonucleic Acid
RT	Reverse Transcriptase
RT-PCR	Real Time - Polymerase Chain Reaction
STD	Sexually Transmitted Disease
SVS	Sustained Viral Response
TDF	Tenofovir
Total anti-HBc	Total Hepatitis B Core Antibody
TTID	Transfusion-Transmitted Infectious Diseases
WHO	World Health Organization
WP	Window Period

LIST OF PAPERS

Bjoerkvoll B, Viet L, Ol HS, Lan NTN, Sothy S, Hoel H, et al. Screening test accuracy among potential blood donors of HBsAg, anti-HBc and anti-HCV to detect hepatitis B and C virus infection in rural Cambodia and Vietnam. The Southeast Asian Journal of Tropical Medicine and Public Health 2010; 41:1127–35.

Viet L, Lan NTN, Ty PX, Björkvoll B, Hoel H, Gutteberg T, et al. Prevalence of hepatitis B & hepatitis C virus infections in potential blood donors in rural Vietnam. Indian J Med Res 2012; 136:74–81.

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CONTENTS

BACKGROUND

Safe blood and blood products should be offered to recipients in need for blood transfusion; however, safe blood transfusion remains a problem in developing countries where resources are limited and blood transmitted diseases are endemic [1]. Among transfusion-transmitted infections, hepatitis B virus (HBV) infection is regarded as the most common. The risk of transfusion-related infection with hepatitis B and hepatitis C viruses (HCV) and HIV-1 is reported as 1: 63,000; 1:103,000; and 1: 493,000 transfused-units respectively in a study conducted in five blood centres in different parts of the United States where prevalence of HBV is low [2]. In the area where hepatitis B is endemic including Vietnam and Cambodia the risk of HBV transmitted transfusion is probably higher and the infection occurs in part due to improper testing [3,4]. Blood donor screening for HBV surface antigen (HBsAg) is in place also in low-income countries. However, HBV transmission may still occur during the initial sero-negative-window period of an acute infection, upon improper testing and also during late stages where virus is still present (HBV-DNA positive) though HBsAg is negative, so-called occult hepatitis B infection (OBI) [5,6]. OBI may originate from recovered infections with persistent low level viral replication, from escape mutants blocking export of antigen, or from reduced HBV replication after co-infection with HCV; HBsAg may or may not be present [7,8].

HBV and HCV share the common routes of transmission and can be transmitted by sexual intercourse, contact with body fluids from infected persons and from infected mothers to their babies. The most frequently risk factors of HCV transmission are blood transfusions from infected donors, injections of drugs, unsafe therapeutic injections and other practice related to health care [9]. Blood contact is also identified as the most important means of HBV transmission among three main identified modes of HBV transmission [10]. The risk of HBV transmitted transfusion is associated with blood donations collected in window period (WP), false negative test results or from donors with Occult Hepatitis B infection (OBI) [4] characterized as the presence of HBV DNA in blood or tissues in HBsAg negative patients with or without antibodies to hepatitis B core antigen (anti-HBc) or hepatitis B surface antigen (anti-HBs). Transmission of HBV infection from hepatitis B surface antigen (HBsAg) negative- anti-HBc positive donors to recipients has been reported [11]. However, WP donations are more likely to transmit HBV than donations collected from OBI donors [12].

Testing strategies for HBV infection in blood donors varies globally depending on the prevalence of HBV infection in a given country. Screening tests for HBsAg are performed to avoid transmission of HBV infection by blood or blood products in most countries [13] including Southeast Asian countries. The anti-HBc testing is used as a surrogate test in some countries such as United State and Japan in order to prevent blood donations from HBsAg negative infectious donors [14]. Under this screening strategy, any blood donor positive either of the tests is excluded due to on-going HBV infection or potential OBI. This combined strategy helps to eliminate HBV transmission from donors in the widow period (WP) with the absence of detectable HBsAg and the presence of anti-HBc and/or HBV DNA [2,15]. However, anti-HBc screening is not practical in intermediate and endemic HBV prevalence countries where up to 90% of adults are exposed to either past or on-going HBV infection [16]. As a result, vast numbers of blood donors are excluded. For this reason, some Southeast Asian countries including Taiwan, Vietnam, and Cambodia perform the screening tests for HBsAg in blood donors in order to

avoid a large exclusion of blood donors, ensuring reasonable blood stocks, but bearing the residual risk of post-transfusion HBV infection, particularly in those blood donors who are in WP or potential OBI.

In addition, the infectivity of OBI is not clear though several studies report that exclusion of anti-HBc positive donors regardless of anti-HBs titre probably decreases the rate of HBV transmission by blood transfusion [17,18]. One should take into account that many studies of transmission risks may have methodological flaws that make it hard to interpret the findings [4]. Still there are clear indications that both the viral load and the immune status of the recipient must be taken into consideration when assuming that the risk for transmission of virus is higher in low-income countries where large populations have deranged immune capacity from chronic malnutrition and endemic diseases. It is thus urgent to get at scientific estimates of the infectivity of OBI in blood donations [19].

Accurate detection not only of HBV and HCV carriers, but also of anti-HBc-positive donors is an urgent issue in order to set standards for safe blood transfusion where HBV infections are endemic. ELISA test is considered as standard test for testing HBV and HCV in developing countries. However, the tests are expensive, require complex instrumentation, and are not feasible in rural remote district hospitals in low-income countries. Rapid tests may be feasible tools for blood donor screening in poor communities. It is well established that rapid tests may yield false test outcomes due to the prozone effect due to imbalance between antibodies and antigens. In addition, the rapid test-accuracy claimed by the producers is normally based on seroconversion test panels which do not necessarily reflect the antibody spectrum in the population studied. It is thus possible that accuracy tests on pre-arranged test panels may yield falsely high performance indicators.

There seems to be large local variations in HBV prevalence rates in South East Asia. Previous studies report prevalence rates of HBV infection in Cambodia of 8% and HCV of 6.5% [20], and in Vietnam in the range of 8% to 25% [21,22]. Also studies in Thailand report large prevalence variations among different groups of the population [23]. However, the Southeast Asian populations so far studied have been relatively small; consequently the prevalence estimates are imprecise.

HEPATITIS B VIRUS

CLASSIFICATION AND CHARACTERISTICS

Hepatitis B virus belongs to the Hepadnaviridae family of the viruses. The entire virus is spherical particle with a diameter of 42nm, consists of an outer protein envelope and an inner 28 nm icosahedral core known as nucleocapsid. The outer envelope is composed of several proteins known as hepatitis B surface protein (HBs) which encase the nucleocapsid. The inner protein shell contains hepatitis B core protein.

GENOMIC STRUCTURE OF HBV

Hepatitis B genome is a single molecule of partially double-stranded circular HBV DNA and viral DNA polymerase. Its genome is a relaxed circular DNA of approximately 3,200 nucleotides consisting of a full-length negative strand and a shorter positive strand. The 5' end of the negative strand is covalently linked to the viral reserve transcriptase. The 5' end of the positive strand is linked to oligoribonucleotides [24].

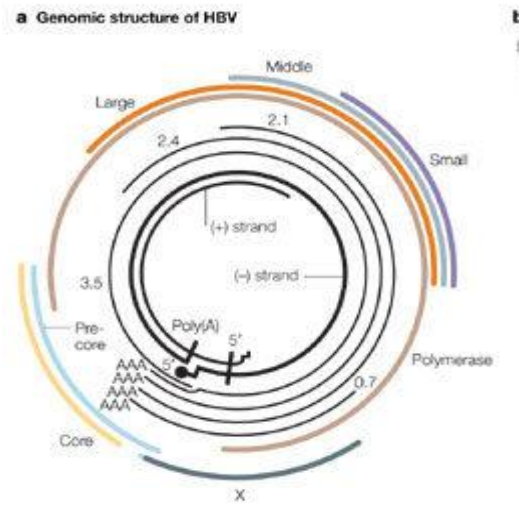

Figure 1: Genomic structure of hepatitis B virus

Rehermann B, Nascimbeni M. Immunology of hepatitis B virus and hepatitis C virus infection. Nat Rev Immunol 2005; 5: 215–29 with permission.

Figure 1 shows the genomic structure of hepatitis B virus (HBV). The inner circles represent the full-length negative strand (with the terminal protein attached to its 5' end) and the incomplete positive strand of the HBV genome. The thin black lines represent the 3.5, 2.4, 2.1 and 0.7 kB mRNA transcripts, which are all terminated near the poly(A) (polyadenylation) signal. The outermost coloured lines indicate the translated HBV proteins: that is, large, middle and small HBV surface proteins, polymerase protein, X protein, and core and pre-core proteins.

When hepatitis B virus enters the body, it encompasses the immune system and infects the liver cell. Firstly, virus attaches to the liver cells membrane, before it enters the liver cell. After virions enter hepatocytes, by an as-yet-unknown receptor, nucleocapsids transport their cargo – the genomic HBV DNA – to the nucleus, where the relaxed circular DNA is converted to covalently closed circulation DNA (cccDNA). The cccDNA serves as the template for the transcription of four viral RNAs (Figure 1), which are exported to the cytoplasm and used as mRNA for translation of HBV proteins. The longest (pre-genomic) RNA also functions as the template for HBV replication, which takes places in nucleocapsids in the cytoplasm. Some of the HBV DNA and polymerase-containing capsids are then transported back to the nucleus where they release the newly generated relaxed

circulator DNA to form additional cccDNA. The blood of HBV infected patients contains 20-nm spheres that consist of HBsAg and host-devired lipids [24].

Unlike retroviruses, hepadnaviruses bind polymerase proteins into a stem-loop formation, subsequently packaged by core proteins in the golgi and secreted via exocytosis into the blood stream, where it can contact other liver cells and continue replication [24]. In some cases, all HBV DNA can accumulate in DNA of liver cell. The virus transcription may stop or take place slowly; only hepatitis B antigen (HBsAg) is produced, not producing the entire virus.

GENETIC HETEROGENEITY OF HBV

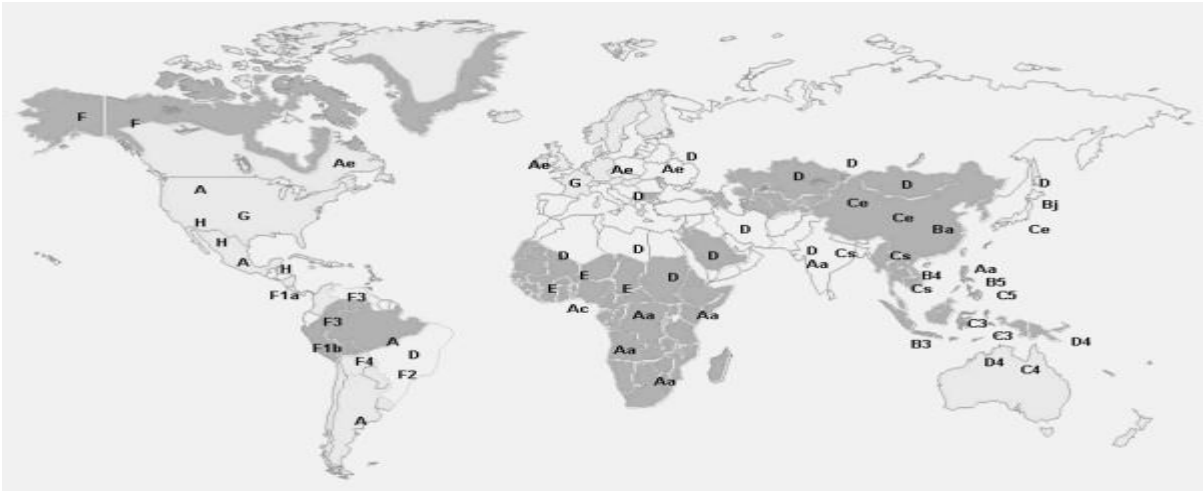

Figure 2: Worldwide distribution pattern of HBV genotypes and subgenotypes [25]

Datta S. An overview of molecular epidemiology of hepatitis B virus (HBV) in India. Virology Journal 2008; 5:156.

Based on the divergence over the entire genome sequence of more than 8% among HBV strains, eight genotypes of HBV have been identified namely A, B, C, D, E, F, G, and H [26,27]. With extensive phylogenetic analysis of HBV genome, sub-genotypes of genotypes A, B, C, D, F based on more than 4% intra-genotypic divergence have been found. So far, 5 sub-genotypes for each genotype A,B, C, D have been identified while 4 sub-genotypes of genotype F have been well documented [28]. Having evolved distinctly in specific geo-ethnic populations, HBV genotypes/subgenotypes have a distinct geographical distribution pattern (Figure 2), which shows the distribution of HBV genotypes and geno-subtypes globally.

Basically, HBV strains were classified by the existence of two pair of mutually exclusive serotype determinants ‘d’/ ‘y’ and ‘w’/ ‘r’ in the HBsAg along with the main antigenic determinant ‘a’, therefore, 4 serotypes of HBV strains have been identified as adw, adr, ayw, or ayr. There is also documented that 9 serotypes as ayw1, ayw2, ayw3, ayw4, ayr, adw2, adw4, adrq+ and adrq- [29]. Genotypes of HBV have specific geographic distribution. Genotype A is found predominantly in Northwest Europe, North America, Central and sub-Saharan Africa; genotype B and C in Southeast Asia, China and Japan; genotype D in the Mediterranean, the Middle East, and

India; Genotype E in Africa; genotype F in America, Polynesia, and Central and South Africa; genotype D in the United State and genotype H in Central America [27,30].

Toan et al. (2006) reported in their study that genotype C, D and A was detected in Vietnam at 25.1%; 20.3%; and 18.1% respectively. Genotype A was significantly more frequent in asymptomatic and non-hepatocellular carcinoma (HCC) carriers while genotype C is significantly more frequent in HCC and asymptomatic patients [30].

A study done by Norder et al. (2004) analysing the sequences of 234 complete genomes and 631 HBsAg genes to assess the worldwide diversity of HBV, reported that sub-genotypes B and C distributed in different geographic regions, with B1 dominating in Japan; B2 in China and Vietnam; B3 in Indonesia; B4 in Vietnam, all strains contains specifying subtype ayw1. Sub genotype C1 was predominant in Japan, Korea, China; C2 in China, Southeast Asia and Bangladesh; and C3 composing specifying adrq- [31].

Genetic heterogeneity of HBV has clinical significance as some studies have shown that HBV genotypes and/or sub-genotypes can influence mutation escape, HBeAg seroconversion rates that could eventually influence the variances in clinical symptoms and even response to antiviral therapy [32–34]. The basal core promoter (BCP) double mutations $1762^{T}/1764^{A}$ down regulate HBeAg production and are associated with chronic HBV infection leading to HCC [35], occur more often among patients who are infected with genotypes A, C and F [28]. Genotype C was observed more in patients with cirrhosis [36,37]. HBV genotype B is associated with a higher rate of IFN-induced HBeAg clearance compared to genotype C [38]. Escape mutants is also a matter of concern when considering the efficacy of HBV vaccine in a given population. Regarding this, efficacy of HBV vaccine depends on HBV genotype prevalence in a given population [39].

SEROLOGIC MARKERS OF HEPATITIS B AND ITS SIGNIFICANCE TO DIAGNOSTIC CRITERIA

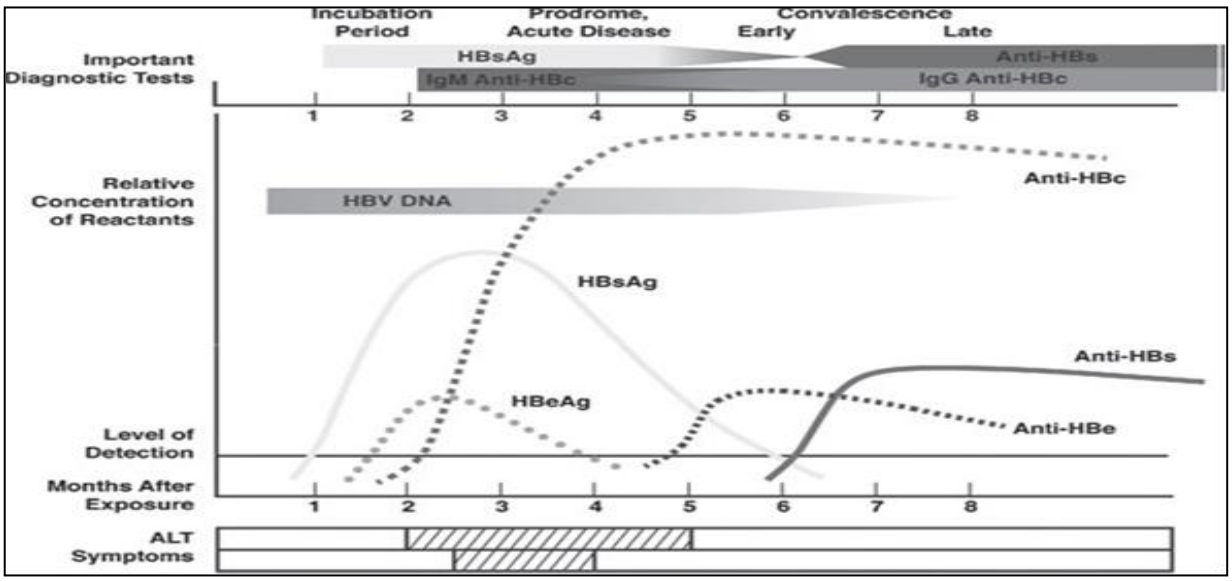

Figure 3: The serologic and clinical patterns observed during acute infection [4].

Hollinger FB. Hepatitis B virus infection and transfusion medicine: science and the occult. Transfusion 2008; 48:1001–26 with permission.

Serological testing to diagnose HBV infection involves the measurement of a variety of distinct HBV specific antigens and antibodies that the host reacts to these antigens after initial HBV infection. Figure 3 shows the different serologic markers that appear in acute HBV infection.

HEPATITIS B DNA (HBV DNA)

HBV DNA can be detected very early after HBV infection (Figure 3) and generally indicates active viral replication. The presence of HBV DNA is a direct evidence of HBV in bloodstream. Quantitative test of HBV DNA can be used as an indicator of disease progression.

HEPATITIS B SURFACE ANTIGEN (HBSAG)

HBsAg is the first viral antigen to be detected appearing in plasma of patients with acute HBV infection before symptoms appear. The incubation of the Hepatitis B Virus (Hepatitis B) (time from the acquisition of HBV to the onset of clinical symptoms) is typically between 8 to 12 weeks (Figure 3). The first serologic marker to appear is hepatitis B surface antigen (HBsAg), which can initially be detected in serum from 1 to 12 weeks (average, 30 to 60 days) after infection. The HBsAg level increases when symptoms appear and decreases after 2-3 months (Figure 3). The presence of HBsAg in plasma proves the presence of HBV DNA virus in hepatocyte. Testing HBsAg is an indicator of HBV infection. The presence of HBsAg for more than six months generally indicates chronic HBV infection. HBsAg is not detectable in patients with resolved HBV infection.

A negative test for HBsAg in some acute HBV infectious patients might suggest that the current assay does not detect a very low level of HBsAg or HBsAg is neutralised by anti-HBs antibodies.

HEPATITIS B E ANTIGEN (HBEAG)

HBeAg develops one week after HBsAg is detectable. HBeAg usually disappears about 3 weeks before HBsAg disappears. The presence of HBeAg in serum of patients indicates a chronic HBV infection. The presence of HBeAg generally correlates with a higher degree of infectivity. Therefore, HBeAg-positive patients are potential HBV carriers to transmit the disease to others because the presence of HBeAg means that HBV is replicating. The risk of perinatal transmission of HBV is about 85-90% if the mother is both HBsAg-positive and HBeAg -positive.

HEPATITIS B CORE ANTIGEN (HBCAG)

The HBcAg is an intracellular antigen synthesized within infected hepatocytes. HBcAg is not detectable in plasma. Anti-HBc antibodies can be detected in the sample of hepatocytes taken after a liver biopsy due to immunization upon sampling.

TOTAL HEPATITIS B CORE ANTIBODY (TOTAL ANTI-HBC)

The first detectable antibodies to appear around 8 weeks after infection with HBV are antibodies to HBV core protein (Figure 3). Anti-HBc appears 5 to 14 days after HBeAg appears and can be detected shortly before HBsAg in acute infection.

The initial antibodies are classified as IgM and IgG and generally appear after the appearance of HBsAg, but often before alanine aminotransferase (ALT) elevations. Anti-HBc IgM is present in the first weeks of the disease indicating current HBV infection. Anti-HBc IgG appears later and persists longer. Anti-HBc may persist months to years in convalescent period after acute HBV infections and persist longer in chronic HBV infections (Figure 3). Antibodies to HBcAg do not neutralise the virus and anti-HBc is not protective against HBV re-infection [17,40].

HEPATITIS B E ANTIBODY (ANTI-HBE)

Anti-HBe is usually detectable between 12 and 16 weeks, when HBeAg disappears (Figure 3). Anti-HBe is not detectable until the immune system has cleared most of the HBe antigens from the blood. The presence of anti-HBe generally indicates a good immune response to HBV infection.

ANTI-HBS (ANTI-HBS)

Anti-HBs antibodies appear after three-month of infection with HBV and normally at that time HBsAg disappears. Anti-HBs neutralize the HBsAg and is protective for re-infection. IgM anti-HBs is present in the acute period, IgG anti-HBs appears later and persist longer. The presence of anti-HBs is an indicator of recovery. Anti-HBs play an important role to protect patients from HBV re-infection, therefore, anti-HBs is a component to be used to produce HBV hyper-immune plasma. When vaccinated with HBV vaccine, anti-HBs is the only antibody present in bloodstream.

IMMUNE RESPONSE TO HBV INFECTIONS

SEROLOGIC RESPONSE TO ACUTE HBV INFECTION

Figure 4 shows the immune response in acute HBV infections, followed by clinical recovery. After recovery, neutralizing anti-HBs and HBV-specific T cell persists for life [24].

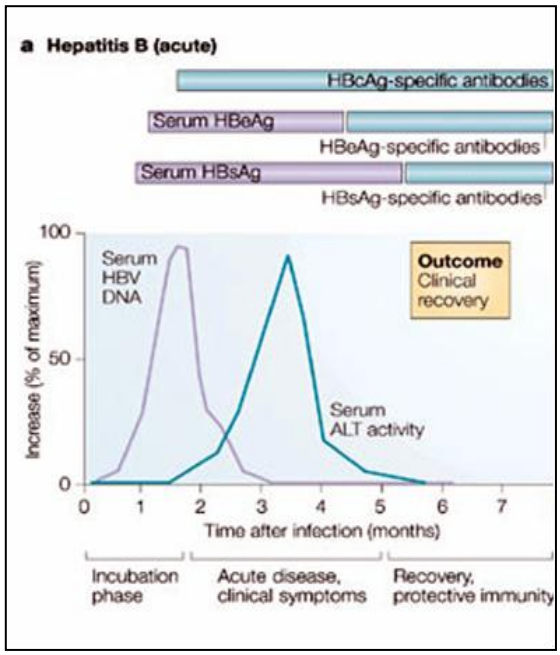

Figure 4: immune response in acute HBV infections. Rehermann B, Nascimbeni M. Immunology of hepatitis B virus and hepatitis C virus infection. Nat Rev Immunol 2005; 5: 215–29 with permission.

The incubation phase defined as time from HBV infection to the onset of clinical symptoms is about 8 -12 weeks [41]. During acute infection, HBsAg is the first serologic marker to appear and can be detectable from 1 to 12 weeks after initial infection. Soon after, HBeAg can be detected [42,43]. With onset of clinical symptoms, alanine aminotransferase level increases that reflect hepatic injury [44]. About this time anti-HBc IgM appears and then decline to undetectable levels within 6 months while anti-

HBc IgG can last much longer.

In a typical case of acute HBV infection, HBV DNA can be detectable in the circulation using PCR technique within one month of infection, but it remains at the relative low level of 10^{-2} – 10^{-4} genome up to six weeks before HBV DNA, HBeAg, HBsAg increases to their peaks. Approximately 10-15 weeks after infection, serum alanine aminotransferase (ALT) concentration starts to rise (Figure 4) [24].

SEROLOGICAL RESPONSE WITH RESOLVED HBV INFECTION

Following acute infection, the progress of serologic markers depends on the outcomes of the host immune response. Approximately 90% of adults will resolve while up to 90% infections in childhood develop chronic infection [24]. In resolved patients, HBsAg disappears in about 3-6 months, following the presence of anti-HBs that indicates recovery and protective immunity against re-infection. In the meantime, the disappearance of HBeAg occurs and development of anti-HBe becomes evident. In resolved HBV patients, anti-HBc persists for life.

SEROLOGIC RESPONSE IN CHRONIC HBV INFECTION

Chronic HBV patients have the similar serologic response in the acute phase as the resolved HBV patients. Persistence of HBsAg for more than 6 months indicates chronic HBV infection. In chronic HBV infection, HBsAg and anti-HBc IgG generally persist for life and HBV DNA can be detected by nucleic acid amplification. The presence of HBsAg and the absence of IgM anti-HBc also indicate chronic HBV infection. The presence of HBeAg indicates high HBV DNA and greater infectivity.

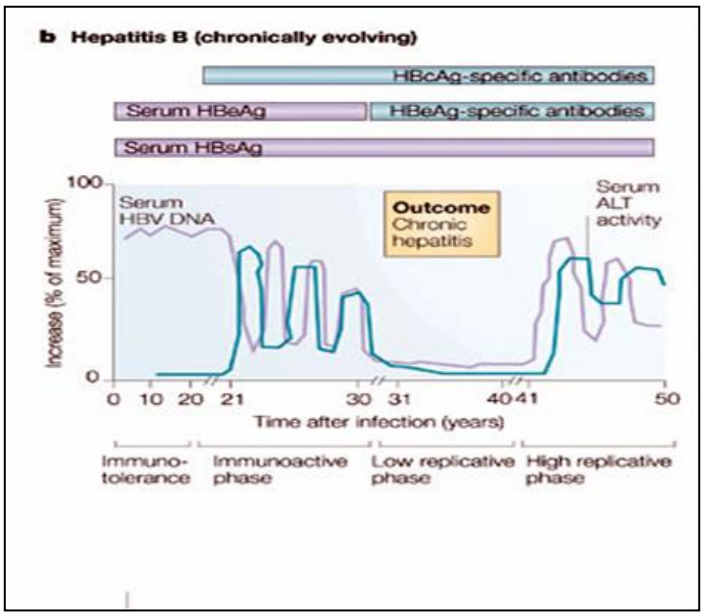

Figure 5: Chronically evolving hepatitis B results from vertical transmission. Rehermann B, Nascimbeni M. Immunology of hepatitis B virus and hepatitis C virus infection. Nat Rev Immunol 2005; 5: 215–29 with permission.

Chronic hepatitis B infection is most commonly observed through vertical transmission from HBV infected mothers to neonate. The course of the disease includes several phases with different lengths. The immune-tolerant phase, which can last for decades, is characterized with high circulating HBV DNA and HBV e antigen (HBeAg) and normal alanine aminotransferase levels (Figure 5). Then it can transit to an immune-active phase with lower HBV DNA level detected, but liver diseases may be severe and progress to liver cirrhosis. The immuno-active phase can transit to a low replicative phase with the clearance of free HBeAg from the serum and appearance of HBeAg-specific

antibodies. In this phase, HBV DNA might not be detectable; alanine aminotransferase is at normal level and necro-inflammatory liver diseases improve [24].

EPIDEMIOLOGY AND TRANSMISSION OF HBV

EPIDEMIOLOGY

Hepatitis B is a serious public health problem globally and a major cause of chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC). There is estimated that about two billion people worldwide have infected with HBV and more than 350 million are chronic HBV carries, of whom 75% live in Asia and Western Pacific [45,46]. It is estimated that 15%-40% of chronic HBV patients develop cirrhosis, liver failure or HCC [47].

Prevalence of chronic HBV infection is classified as high where prevalence is more than 8% such as Southeast Asia, China, sub-Saharan Africa and the Amazon Basin; as intermediate where prevalence is 2%-7% including Eastern and Southern Europe, Middle East, Japan, and part of South America; and as low where prevalence is less than 2% in North America, Northern and Western Europe and Australia. In high endemic areas, 70%-90% of the population has a past or on-going serologic evidence of HBV infection and most infections were observed in infancy or childhood. In intermediate areas, 10%-60% of the population shows evidence of HBV infection and 2%-7% are chronic carriers. Many infections occur in adolescent and adults, but infection during infancy and children still contribute at high rate. In low HBV prevalence areas, 5%-7.5% of population has evidence of serologic HBV infection, of which 0.5%-2% are chronic carriers. Most HBV infections occur in adolescent and young adults in high risk groups such as injection drug use, homosexual males, healthcare workers, patients given blood transfusion [48].

A study on prevalence of HBV infection in potential blood donors in rural Cambodia reported that the overall prevalence of HBsAg positive in the study population was 7.7% (95% CI: 6.2%-9.3%) and the prevalence of anti-HBc sample was 58.6% [1]. The prevalence of HBV infection in blood donors in Thailand declined from 7.14% in 1978 to 2.63% in 2009 resulting from an effective expanded immunization program against HBV [49]. In 13,897 first time blood donors in Lao during 2003 to 2005 the prevalence of HBsAg was reported to be 8.7%; with a higher level in males (9.7%) than in females (6.2%) [50].

A retrospective study conducted in Malaysia on 44,658 voluntary blood donors between 2000 to 2004, revealed that the mean prevalence of hepatitis B infection among first time and regular blood donors were significantly different,1.8% and 0.4% respectively. Prevalence of HBV infection in male blood donors was at 1.2% compared to 0.4% in female donors [51].

TRANSMISSION OF HBV INFECTION

HBV can be transmitted through contacts with body fluids from infected HBV patients. Blood is the most important route of HBV transmission, but other body fluids such as semen and saliva have been reported to be the source of transmission. So far, three main modes of HBV transmission have been identified: perinatal mode from infective mothers to their babies, sexual intercourse and parenteral/percutaneous routes.

Transmission of HBV from HBV infected mothers to their babies is the most important factors in high endemic HBV prevalence such as China and Southeast Asia. The transmission can occur during the perinatal period through three main routes: Trans-placental transmission of HBV in utero transmission during delivery; and postnatal transmission during care or breast milk [48]. For a child less than one year old who is perinatally infected with HBV, the risk of chronic HBV development is 90% due to the immature immune system [24].

In high endemic HBV areas, HBV is predominantly transmitted among young children through HBV infected mothers to their babies [52]. Infants born to chronic HBV infected mothers, especially positive HBeAg mothers are at high risk of becoming infected with HBV at birth. In East and Southeast Asia 35 to 50% of the women who are HBsAg positive are also HBeAg positive [53]. It is estimated that 65% to 90% of their infants will become infected, develops chronic HBV carriers; perinatal transmission results in 30-50% of all chronic HBV infections in high endemic countries [54].

Transmission of HBV infection though sexual contacts has been reported as a major source of transmission globally, particularly in low endemic HBV prevalence countries. The highest risk of HBV as a sexually transmitted disease (STD) is considered to be where men have sex with men, resulting in 70% HBV infections in homosexual men. Sexual contacts of injection drug users and of sexual workers are at high risk of HBV acquisitions [16,55].

Injections of drugs, blood transfusions, acupuncture, casual accident in healthcare setting, tattooing and household contacts are also vehicles of HBV transmission. Although screening for HBV infection in blood donors has contributed considerably to the reduction of transfusion transmitted HBV infection, HBV infection after blood transfusion is still a matter of concern. Insufficient testing is probably the main cause of HBV TTID and blood donors with the presence of HBV DNA and absence of HBsAg, the so-called “Occult Hepatitis B Infection - OBI” can be infective [11]. This will be described in depth in the section “Occult hepatitis B and blood transfusion” in this thesis.

PREVENTION AND TREATMENT

PREVENTION

There are several approaches in prevention of HBV infection including: safe blood products, behaviour change to prevent disease spread; passive immune-prophylaxis in those who have been exposed to HBV and active immunization.

Deferral of blood donors with risk behaviour and improved screening have contributed to the reduction of HBV infection transmitted by blood transfusions. Use of condoms during sexual intercourse is commonly recommended not only for HIV prevention but also for HBV prevention. Increasing sensitivity of HBV assays also plays an important role in the management of HBV spread. Behaviour changes also involve activities such as health education for the public as well as targeting high risk groups.

Administration of Hepatitis B Immune Globulin (HBIG) is a passive immune-prophylaxis for prevention of HBV infection in those who may have been exposed. HBIG is made from human plasma from selected donors who already have a high level of antibodies to HBV. HBIG is recommended in four situations: new-borns of HBV infected mothers; after needle stick exposure; after sexual exposure; and after liver transplantation [48]. HBIG is recommended for all infants born from HBsAg positive mother immediately after delivery or within 12 hours after birth in combination of recombinant vaccine against HBV. It is reported that up to 90% has protective levels of antibodies protecting against perinatal acquisition of HBV [56]. HBIG mono-therapy at a high dose can prevent recurrence of HBV in from 60% to 80% of patients who have undergone liver transplantation [57].

Universal HBV vaccination programs

Active immunization (HBV vaccination) is an important approach to decrease the risk of chronic HBV infection and the complications. The World Health Organization (WHO) recommended that vaccination against HBV should be included in national vaccine programs in all countries with HBV prevalence of 8% by 1991, more than 8% by 1995 and all countries by 1997. The HBV vaccination program had been introduced in 154 countries by May 2002 [58] and 168 countries by the end of 2006 [59]. The effectiveness of universal infant HB vaccination is significant and reduction or eradication of chronic HBV infection has been recognized in many countries; however, there are challenges to achieve the goal of the universal immunization programs due to poor immunization delivery infrastructure, low coverage as well as sustainable financial situation [48].

Hepatitis B vaccination is given for all infants at birth with three doses to ensure early protection. In neonates and infants, the result of vaccination is 98-100% protective anti-HBs levels equal or larger than 10 IU/L one month after completion of three doses of the HBV vaccine. Most children vaccinated at birth retain immunologic memory to hepatitis B vaccine for 15 years [59].

The impact of vaccination programs in Taiwan is illustrated as one of the most successful and effective public health programs to prevent chronic hepatitis B infection. Controlled randomized clinical trials on hepatitis B immunoglobulin and vaccine in Taiwan revealed an 80– 90% protective effect among infants of either HBsAg positive or HBeAg positive mothers. The prevalence surveys on infants born before and after the launch of the national vaccination program found a steady reduction in seroprevalence of hepatitis B surface antigen in Taiwan, with 78–87% effectiveness after the national vaccination program was implemented. Studies on the secular trend of liver disease risk also indicated a 68% reduction in mortality from fulminant hepatitis in infants and a 75% decline in the incidence of hepato-cellular carcinoma in children 6–9 years old after the national vaccination program began [60]. A review by Lee et al, revealed that the combination of vaccine plus HBIG is superior to vaccine alone in term of prevention of HBV infection [61]. The universal vaccination of newborn was introduced in Taiwan in 1983-1985. The impact of this program is that the HBsAg prevalence in children younger than 15 years decreased from 9.8% in 1984 to 0.7% in 1999, and further to 0.7% in 2004 [62]. In Malaysia, a cross-sectional study in school children aged 7-12 years from 1997 to 2003 showed a steady decline of the HBsAg prevalence from 2.5% for children born in 1985 to 0.4% among school children born in 1996 after the implementation of a universal new-born vaccination program in 1989 [63].

Universal infant HBV vaccination was implemented in Vietnam in 2003 [22] with the coverage of more than 98% annually. Vaccination against HBV for new-borns within 24 hours after delivery has been preferably integrated in universal national immunization programs in healthcare settings particularly in hospitals.

TREATMENT

Treatment of chronic HBV patients is a broad issue beyond the scope of our current work; however, information provided in this section is an attempt to describe antiviral therapy approved worldwide and in Asia countries for chronic HBV management, briefly review some studied results regarding response and resistance of antiviral therapy.

It is known that active replication is the key driver of liver injury and disease progress; therefore, viral suppression plays a very important role in chronic HBV management [64]. The primary goal of treatment of chronic HBV infection is to permanently suppress HBV replication. The suppression helps to reduce infectivity and pathogenicity of HBV. The decreased pathogenicity leads to the reduced hepatic necro-inflammation. Clinically, the short-term treatment goal is to obtain initial response in terms of HBeAg seroconversion and/or HBV DNA suppression, ATL normalization, and prevention of hepatic decompensation; to ensure sustained response to reduce hepatic necro-inflammation and fibrosis during/after antiviral therapy. The ultimate long-term goal of treatment is to achieve durable response to prevent the progression to cirrhosis and /or HCC, and prolong survival [65].

Most antiviral drugs approved by Food and Drug Administration (FDA) for treatment of HBV infection are intended to target the reverse transcriptase (RT) and classified as nucleoside RT inhibitors (NRTIs) that suppress the viral replication. It is reported that HBV genotypes diversity affects NRTIs resistance. Also due to the S surface antigen and P genes overlapping in the large reading frame, genetic differences that affect the hepatitis B surface may change the viral polymerase sequence, function and drug susceptibility [66]. Currently six antiviral drugs have been approved the U.S. Food and Drug Administration for chronic HBV treatment including IFN-α, pegylated IFN-α, lamivudine, adefovir, dipivoxil, entecavir, and telbivudine. IFN-α (and pegylated formations) is the only drug that eliminates the covalently closed circular DNA (cccDNA) from hepatocytes and thus potentially curative [67]. IFN-α, lamivudine, adefovir, entecavir, telbivudine and PegIFN-α-2a have been currently licensed globally. Clevudine has been approved in Korea and Thymosin α1 has been approved in many countries in Asia [65].

IFN-α treatment has been used for chronic HBV infection for more than twenty years. Several studies found that response to IFN-α treatment was observed to be higher in patients infected with genotype A (70%) compared to patients infected with genotype D and E (40%) [68]; and in patients infected with genotype B (41%) and with genotype C (15%) [36,69]. Interferon therapy had a higher rate of HBeAg seroconversion in patients infected with genotype A than in patients infected with genotype D or C [70,71]. A four to six month course of IFN-α treatment at a dose of 5 million units (MU) daily or 10MU three times a week obtained HBeAg loss in nearly 33% of HBeAg patients compared with 12% in control group. Small dose (5-6 MU three times weekly) has been used in Asian patients with similar efficacy. Retreatment in relapse patients with IFN-α treatment showed a response rate of 20-40% and when HBeAg seroconversion attained, it is sustained in more

than 80% of cases [72]. IPN-α treatment resulted in end-of-treatment biochemical and virological response in up to 90% HBeAg negative patients; however, sustained response rate was low: 10-15% with 4-6 month treatment; 22% with 12 month course; and 30% in 24 month treatment [65]. Main advantages of IFN-α include a course of finite duration with modest response, long-term benefits and no resistance, but having side effects such as influenza-like symptoms, fatigue, neutropenia, thrombocytopenia and depression [65,72].

A study in Asian patients showed that a 24-week treatment of weekly pegylated IFN-2α (40kD) achieved a higher HBeAg seroconversion than IFN-α-based therapy (33% vs. 25%; $p>0.05$). Several studies using Pegylated IFN-2β showed similar efficacy [72]. Pegylated IFN-2β was safe and effective in HBeAg positive chronic HBV patients with advanced fibrosis or cirrhosis as those with early state of fibrosis [73] Patients with chronic HBV infection who are lamivudine refractory and those who are lamivudine naïve response similarly to Pegylated IFN-2β [74].

Lamivudine (LVD) was the first safe, effective, and well-tolerated oral medication for the treatment of HBV infection. LVD resistance has been seen in approximately 20% of HBeAg seroconversion patients (a marker that is usually associated with a reduction in viral replication) after one year and up to 70% after five years [75,76]. The HBeAg seroconversion rate found similar in patients with HBV genotype B or C.

Adefovir (ADV) has been approved by FDA only for the treatment of HBV infection. After a 5-year period treatment, it was estimated that 29% of ADV-treated patients were reported to develop ADV resistance as compared to 70% for LVD [77]. However, other studies documented that as many as 50% of ADV treated patients fail to obtain adequate viral suppression [78] and that high levels of ADV resistance occurrence were seen after 1-2 years of treatment [79,80]. Liu et al. indicated that patients with LVD-resistant mutations treated for 2–5 months with combination therapy of ADV and LVD obtained improved rates of viral suppression but did not improve biochemical indicators of liver health [81]. A study by Chan et al. (2007) demonstrated that virological suppression by ADV is not ideal in the majority of LVD-resistant patients. However, early treatment by ADV when HBV DNA is low played an importance to retain virological suppression [82].

Tenofovir (TDF) is used for the treatment of HIV infections and is known also to inhibit HBV polymerase. Jain et al. showed that combined LVD/TDF therapy suppresses synthesis of HBV DNA more effectively than mono-therapy of either LVD or TDF alone [83]. More patients infected with HBV genotype A responded to TDF-based treatment better than the patients infected with non-A genotype HBV, regardless of therapeutic regimen or compliance, or prior antiretroviral treatment for those with HIV co-infection [83]. In vitro drug combination studies have revealed that TDF has an additive effect when combined with LVD, ETV, or LdT [84]. However, in Jain et al. the patients were HBV/HIV co-infected and so far LVD/TDF combination is not recommended as first-line therapy in HBV mono-infected patients [83].

Entecavir (ETV) has several distinct advantages over LVD and ADV. ETV is known as the most potent inhibitor of HBVRT. It not only inhibits both wild-type and LVD-resistant HBV but also not associated with any major adverse effects. In addition, ETV has limited potential for development of resistance [85].

Telbivudine (LdT) is an orally administered nucleoside analogue, approved for the treatment of chronic hepatitis B, with good tolerance, lack of mitochondrial toxicity, and no dose-limiting side effects. In clinical trial, LdT gave more potent HBV suppression than LVD and ADV [65].

Although anti-viral therapy for HBV chronic management is approved; many of the drugs is not affordable to the average HBV patient, especially for those who live in developing Asian countries where hepatitis B infection is endemic and resources are limited [86]. The cost of the treatment has been a matter of concern not only for the patients but also for public health policies for decades. The universal infant vaccination program against hepatitis B virus proved one of the most successful and effective public health programs to prevent chronic hepatitis B infection globally; therefore, it should be encouraged with high coverage in all countries.

SCREENING TESTS FOR HBV IN BLOOD DONORS (HBSAG, ANTI-HBC, HBV DNA)

The screening programs for HBV infection in blood donors vary worldwide depending on the prevalence of HBV infection and financial situation in a specific country. Screening tests for hepatitis B antigen (HBsAg) are performed to prevent transmission of HBV infection by blood or blood products in addition to monitor the status of the patients in combination with other serological HBV markers in most countries [13]. HBsAg appears in infected patients from weeks to months after onset of infection and before symptoms starts. Some infected patients never have HBsAg positivity, but generally produce anti-HBc to respond to hepatitis B core antigen. The fact that there are some false negative for HBsAg is the reason for the performance of anti-HBc testing in some countries. However, determination of HBsAg negative/anti-HBc positive individual has been problematic for blood donor collection facilities [14]. In low HBV infection prevalence countries such as United States and Japan, screening for both HBsAg and anti-HBc is integrated into screening program for blood donors [87,88]. Under this regimen, any blood donor positive for either of the tests, were excluded because of current HBV infection or potential OBI. However, this combined strategy is not practical in intermediate and endemic HBV prevalence where up to 90% of adults' population exposed to either past or on-going HBV infection [16] leading to a vast exclusions of blood donors. For this reason, some Asian countries including Taiwan, Vietnam, and Cambodia perform the screening tests for on-going HBV infection (HBsAg) in blood donors, not for past HBV infection (anti-HBc). This HBsAg screening program avoids a large exclusion of blood donation, maintaining reasonable blood stocks, but bearing the residual risk of post transfused HBV infection, particularly in those donors who are in WP or potential OBI.

As HBV testing was improved and more sensitive after introduction of nucleic acid amplification technology (NAT), HBV DNA has been identified in HBsAg negative, anti-HBc positive blood donors. In low HBV prevalence areas, HBV DNA was found in less than 5% of HBsAg negative and anti-HBc positive blood units [89] whereas serum HBV DNA was found in 4-25% of HBsAg negative and anti-HBc positive individuals in high HBV prevalence [90–93]. It is reported that in high endemic countries, most HBV infections are transmitted through perinatal routes or early in childhood, therefore, a higher fraction of infected adults have late chronic HBV with the absence of HBsAg resulting in a higher rate of OBI in anti-HBc positive individuals in these regions [5].

As mentioned above, OBI may derive from healthy chronic carriers without any serologic markers of HBV infection other than HBV DNA. Over time, antibody markers may become undetectable leaving HBV DNA the

only marker of the infection. In all cases, the viral load in OBI is usually low, often below 100 IU/ml. At these levels, HBV DNA measurement using NAT in pools is likely to be largely ineffective [7]. The efficacy of anti-HBc approach has been evaluated in low prevalence areas where a few seropositive samples contained HBV DNA. Data from 10 studies in seven Asian countries revealed that the prevalence of anti-HBc is from 7% to 43%, and about 5% (range: 0 -18%) of anti-HBc samples contained HBV DNA [17,94,95]. It can be concluded that the efficacy of anti-HBc screening program was relatively high in these regions where NAT is infeasible due to limited resources.

In addition, current knowledge shows that anti-HBc testing has the potential of disqualifying majority of OBIs, leaving only the probably rare cases with HBV DNA alone undetected. Currently available HBV DNA assays with sensitivity of 20-50 UI/ml could only detect OBI with > 320-800 IU/ml when sample was diluted by 16 as it is when testing mini-pools of samples. However, many cases of OBIs in blood donors are below that viral load, therefore, enhancement of NAT sensitivity in Asia becomes a critical issue [5]. NAT HBV DNA assays have not eliminated the necessity for serological assays for HBV infected donors. It is hoped that NAT testing would reduce WP donors; identify low viral levels of HBV; provide another mechanism for re-entry of HBsAg false negative donors; and replace serological testing [14].

Raimondo et al. (2010) stated that HBsAg negative, HBV DNA positive blood have to be considered infectious and may account for HBV transfusion-transmitted infection. More importantly, HBV-DNA (NAT) is considered to be the only reliable diagnostic marker of OBI [96]. Blood donor screening for anti-HBc and NAT testing have been implemented in some developed countries in order to avoid OBI. However, anti-HBc testing is not practical in countries with endemic HBV prevalence. More importantly, the NAT technology is also not feasible in low-income countries, especially in low financial resource settings due to its high cost.

OCCULT HEPATITIS B AND BLOOD TRANSFUSION

Recently there has been much concern about “Occult Hepatitis B infection (OBI)” in blood transfusion settings. OBI is characterized as the presence of HBV DNA in blood or tissues in HBsAg negative patients with or without antibodies to hepatitis B core antigen (anti-HBc) or hepatitis B surface antigen (anti-HBs) [11]. Allain 2004 indicated several clinical conditions where OBI is found: a) at the time of recovery from past infection characterized with detectable anti-HBs; b) in individuals with chronic hepatitis B with surface antigen escape mutants that are not detected by current assays; c) in individuals with chronic hepatitis B carriers without any serologic markers other than HBV DNA; d) in individuals with chronic hepatitis at the healthy carriage state indicated by the presence of anti-HBe [97].

In 2008 International workshop on OBI, experts from the European Association for the Study of the Liver defined OBI as the ‘presence of HBV DNA in the liver (with detectable or undetectable HBV DNA in the plasma) of individuals testing HBsAg negative by currently assays’. The experts in the meeting also introduce an OBI cut-off for HBV DNA of less than 200 IU/ml [98]. OBI individuals are also classified as either sero-negative with the absence of both anti-HBs and anti-HBc or seropositive with the presence of anti-HBc with or without anti-HBs [99].

EPIDEMIOLOGY OF OBI

The prevalence of OBI varies greatly between geographic areas as well as among patients tested with different assays for routine serologic or NAT screening [4,97]. The prevalence of OBI is correlated with the prevalence of HBV infection in a given population [100,101]. Patients from highly endemic HBV prevalence areas are more likely to develop OBI [102] as most patients in these areas are infected during perinatal or during childhood responsible for high proportion of OBI in anti-HBc positive populations [5].

Prevalence of OBI was observed in 0.1 to 2.4% in HBsAg negative anti-HBc positive blood donors in Western countries where only 5% of the population has evidence of exposure to HBV infection. Meanwhile, up to 6% of OBIs were identified in endemic areas where 70% -90% of population have prior exposure to HBV [4]. In Western countries, OBIs are observed in range of 1:2,000 to 1: 20,000 donation collected and are more frequently found in male over 50 years old with normal ALT and low viral DNA. Most OBI donors are anti-HBc positive or absence of anti-HBs [103–106].

The prevalence of OBI is reported 16% in general population with normal ALT level in Korea [107], 10.6% in HBsAg negative healthy people in China [108]. The rate of detected HBV DNA was observed highest in patients positive for anti-HBc alone; average in those positive with both anti-HBc and anti-HBs; and lowest in those whose sera are negative [101]. Allain (2004) reported that HBV DNA was observed 0% to 7.7% from either blood donors or in the general population in Northern Euro and North America with low HBV prevalence [97]. Study in Taiwan reported that HBV DNA is detected in 7.5% among 147 stored donated blood samples [3]. The review by Allain and Candotti (2012) on OBI prevalence from blood donors in different studies in China, reported a range between 1:600 and 1: 21,000 blood units with a mean about 1: 1,000 blood donors [109]. OBI prevalence in blood donors in Taiwan was reported approximately 1: 1,000 blood units [110,111].

There are several possible explanations for the mechanism of OBI. Mutations in regulatory regions of HBV genome that prevent HBsAg production and viral replication may be the first possible explanation. Any mutations in the pre-S/S region may cause the change of HBsAg antigenicity and inhibition of anti-HBs production [11]. Mutations in the pre-S1 region may terminate the induction of HBV large HBV protein, decline the formation of HBV virions, and avoid interaction of HBV in hepatocytes. Current studies demonstrated the evidence of numerous mutations and deletions in OBI genome, but the overall locations of mutations are similar in occult and non-occult blood samples. Differences in the methylation pattern between occult and non-occult blood samples also were identified in a study done by Vivekanandan [112]. The study done by Weinberger (2000) indicated that the major hydrophilic loop (MHL) is the area of increasing genetic variability. The frequency of mutation in MHL of OBI patients (22.6/1000 amino acid) was significantly higher compared in non-MHL (9.4/1000 amino acid)[113].

Another possibility is the persistence of immune complexes consisting of HBsAg bound to anti-HBs. A study in 11 Japanese patients showed that the level of free and Ig-bound HBV is equal in acute phase, Ig-bound HBV is dominated in WP in spite of the presence of free HBV, and free HBV is not detectable after sero-conversion. The authors predicted that immune complexes that occur after sero-conversion are not infectious and HBV reservoir likely takes place in the liver or peripheral blood monocular cells [114].

Viral interference in co-infection with another virus is another explanation for the existence of OBI. Study of OBI in patients co-infected with HCV shows that HBV specific transcripts and HBV antigens declined two to fourfold by the presence of HCV gene structure and the secretion of HBV viral particles was suppressed by 20 fold. The authors speculated that the core protein of HCV serves as a gene-regulatory protein in this case [115].

Another possible explanation for the existence of OBI is the presence of a block to secretion of free HBsAg resulting in the only secretion of Dane particles without increased HBsAg in the serum. Change in the Pre S domain may be the reason to limit virion excretion [116].

CLINICAL SIGNIFICANCE OF OBI IN BLOOD DONATION

OBI after acute HBV infection

Recovered patients from acute HBV infection might carry HBV genome for many years without any clinical or biochemical evidence of liver diseases [114,117]. Whether these patients with this disorder are at risk for transmitting HBV infection to others is a matter of concern. It is reported that in immune-competent patients who have developed anti-HBc and anti-HBs, no transmission of HBV has been observed in blood donations [4].

OBI in Blood transfusion and Infectivity

It is generally accepted that not all OBI donors transmit HBV infection to recipients. Anti-HBc is the first antibody to appear in HBV infection and remains persistence for life. This antibody test may be positive without the presence of both HBsAg and anti-HBs antibodies; during window period; before the appearance of anti-HBs antibodies; or in resolved HBV patients. It is documented that anti-HBc antibodies are not protective [17,40] whereas a certain amount of anti-HBs antibodies are protective by neutralizing the antigen and avoiding infectivity. Patients with anti-HBs antibodies may neutralize a certain amount of virus upon re-infection thereby not being infected upon transfusion of OBI blood donors.

There are also other possible explanations why not all recipients of HBsAg negative HBV DNA positive blood donors develop HBV infection upon transfusion. These explanations include: “1) vaccination or prior disease induce immunity to HBV; 2) concurrent infusion of anti-HBs in another blood components; 3) presence of immune complexes; 4) inocula below the minimum infectious doses; 5) presence of defective or replication-incompetent virions; and 6) viral interference from another pathogen” [118].

Like other viral infections, HBV infectivity depends on three main factors: the infectious dose, the level of neutralizing antibodies and the immuno-competence of the host. It is documented that blood components from HBV OBI donors were not infectious in immuno-competent recipients, but infective in immuno-suppressed recipients. It has been accepted that when HBV DNA is present, infectivity may occur, however, the viral load needed to infect has not been documented in human so far [5,14].

Post-transfusion HBV from OBI blood donors is detected in 1: 60,000 transfused units in low prevalence areas [2,17], while in hepatitis B endemic areas transmission rates are probably much higher and occur in part due to

improper testing [3,4]. Residual transfusion transmitted HBV risk in repeat-donors is estimated to be 0.69-8.69; 7.5-15.8; and 30.6-200 per million donations in low, moderate and high HBV prevalence, respectively [119].

The residual risk of transfusion-transmitted HBV is mainly associated with blood donations with the absence of detectable HBsAg that have been drawn either in a window period (WP) defined as time between infection and detection of viral antigen or antibody markers, or during later stages of infection [4,119]. WP donations are more likely to transmit HBV than donations collected from chronic OBI [12]. The risk of transmission is high in blood donors with absence or low level of anti-HBs. Blood components containing anti-HBs even at a certain level, do not appear to transmit HBV, only 10% of blood with low anti-HBs were infectious [7]. The risk of transmission is not significant with the presence of anti-HBs in the blood regardless of anti-HBc levels. However, caution should be taken when immune-deficient patients receive anti-HBc-positive, anti-HBs-positive donations [119].

Studies in Europe and North America revealed that nearly 90% of blood donors who have recovered from an HBV infection are anti-HBc antibody and anti-HBs antibody positive. Donors with anti-HBs ≥ 100IU/L are presumable non-infectious, those with no anti-HBs or anti-HBs <100IU/L are potentially infectious [119]. Study in Japan showed that no HBV infection was observed in recipients of HBsAg negative-HBV DNA -positive OBI with anti-HBs antibodies present. The risk of post transfusion infection seems to be negligible when blood donors carried anti-HBs antibodies at a certain level of 100-200 IU/L. Some other studies confirm that there is a high risk of HBV post transfusion when anti-HBs negative blood from OBI donors is transfused to susceptible recipients [12]. Anti-HBs in the recipient of OBI blood may also be protected from HBV infection.

OBI with HCV infection

HCV and HBV infections share the similar transmission routes; therefore, co-infection of HBV and HCV occurs. There is more association between the prevalence of anti-HCV antibodies and the prevalence of anti-HBc antibodies compared to the presence of HBsAg. HCV infected patients appear to have higher prevalence of OBI. It is reported that HBV DNA is detected in one-third of HBsAg negative HCV carriers in Mediterranean basin and even higher in Far East Asian countries [120,121].

HEPATITIS C VIRUS

In the middle of 1970s, there were epidemiological evidences that some patients, who had been given blood transfusion, developed liver disease several years after transfusion. In order to distinguish this kind of disease with known HBV and HAV, called “non-A, non-B Hepatitis, this pathogen was identified in 1989 and named Hepatitis C virus [122].

CLASSIFICATION AND CHARACTERISTICS

Hepatitis C virus is a single strand RNA virus. It is a member of family Flaviviridae, responsible for acute and chronic hepatitis C infection in about 3% of human population [123]. Based on the analysis of nucleic acids of its genome, six major genotypes 1-6 and a large diversity of subtypes have been identified and distributed in different regions in the world [124].

Clinically, patients with acute hepatitis C infection often are asymptomatic in exception for some cases that demonstrate jaundice. About 80% - 85% of acute HCV infections develop into chronic infections. The liver failure in chronic cases is not only caused by the virus, but also from autoimmune pathology. It has been estimated that HCV accounts for 27% of cirrhosis and 25% of hepatocellular carcinoma globally [125]

GENOME STRUCTURE

HCV is classified in the Hepacivirus genus belonging to the Flaviviridae family. HCV has a positive strand RNA genome containing 9,460 ribonucleotides. It’s genome is composed of a 5’ non-coding region (NCR), which includes an internal ribosome entry site (IRES), a large open reading frame that encodes at least 10 structural and non-structural proteins, and a 3’-NCR. The structural proteins of hepatitis C virus include the core protein and the envelope proteins, E1 and E2. The non-structural proteins include the p7 ion channel, the NS2-3 protease, the NS3 serine protease and RNA helicase, the NS4A polypeptide, the NS4B and NS5A proteins and the NS5B RNA-dependent RNA polymerase (RdRp) [126].

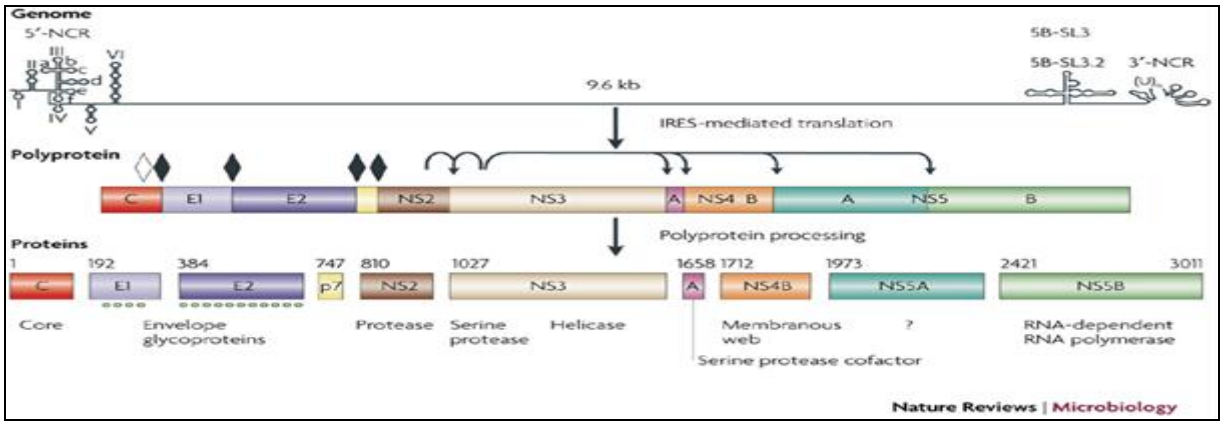

Figure 6: Genetic organization and poly-protein processing of hepatitis C virus (HCV)[126]

Moradpour D, Penin F, Rice CM. Replication of hepatitis C virus. Nature Reviews Microbiology 2007; 5:453–63 with permission.

The 9.6-kb positive-strand RNA genome is schematically depicted at the top (Figure 6). Simplified RNA secondary structures in the 5'- and 3'-non-coding regions (NCRs) and the core gene, as well as the NS5B stem-loop 3 cis-acting replication element (5B-SL3) are shown. Internal ribosome entry site (IRES)-mediated translation yields a poly-protein precursor that is processed into the mature structural and non-structural proteins. Amino-acid numbers are shown above each protein (HCV H strain; genotype 1a; GenBank accession number AF009606). Solid diamonds denote cleavage sites of the HCV poly-protein precursor by the endoplasmic reticulum signal peptidase. The open diamond indicates further C-terminal processing of the core protein by signal peptide peptidase. Arrows indicate cleavages by the HCV NS2–3 and NS3–4A proteases. Dots in E1 and E2 indicate the glycosylation of the envelope proteins (4 and 11 N-linked glycans, respectively, in the HCV H strain) [126].

GENETIC HETEROGENEITY OF HCV

There are six major HCV genotypes (genotypes 1-6). It is reported that around 60% of HCV infections are caused by type 1a and 1b [127]. Genotypes 1, 2, and 3 of the Hepatitis C virus (HCV) are widely distributed throughout Western countries and the Far East (Japan, China, Taiwan, and Thailand). Types 5 and 6 are mainly confined to South Africa and Southeast Asia, respectively. In contrast, type 4 is predominant in the Middle East and Central Africa [128]. Genotype 6 viruses are predominant in Southeast Asia including Vietnam, Indonesia, Thailand, Cambodia, Myanmar [129]. Currently 17 subtypes (6a-6q) of genotypes 6 HCV have been identified based on complete genome analysis [130]. Subtype 6r and 6s existence was recently proposed according to sequence analysis of core/E1 and Ns5B from HCV strains of Cambodian immigrants in Canada [131]. HCV genotypes 7, 8, 9 have been identified only in Vietnamese patients [132] whereas HCV genotypes 10, 11 are found in Indonesian patients [133].

HCV subtypes 1a and 1b are most commonly distributed in The United State and predominant in Europe [128,134–136]. Subtype 1b is found in Japan accounting for up to 73% of HCV infections [137]. While HCV subtypes 2a, 2b are relative common in North America, Europe, and Japan; HCV subtype 2c is commonly identified in North Italia. HCV 3a is found mainly in intravenous drug users in Europe and the United State [138].

IMMUNE RESPONSE TO HCV INFECTION

HCV concentration reaches high serum level within 1 week of infection [139,140]. Adaptive cellular immune responses are postponed by at least one month and humoral immune responses are delayed by at least 2 months. After the first weeks of infection, the rate of acceleration of viral titre occurs slowly. Approximately 8-12 weeks after HCV infection, HCV RNA concentration decreases when serum ALT level reaches its peak (Figure 7). HCV specific antibodies might become detectable around this time. Most acute HCV infected patients develop chronic hepatitis C. Some acute HCV patients recover and have negative test for HCV RNA in current assays. HCV specific antibodies titre reduces and might be absent completely 10-20 years after recovery, therefore, complete clearance of HCV RNA might be obtained by at least a group of patients [141,142].

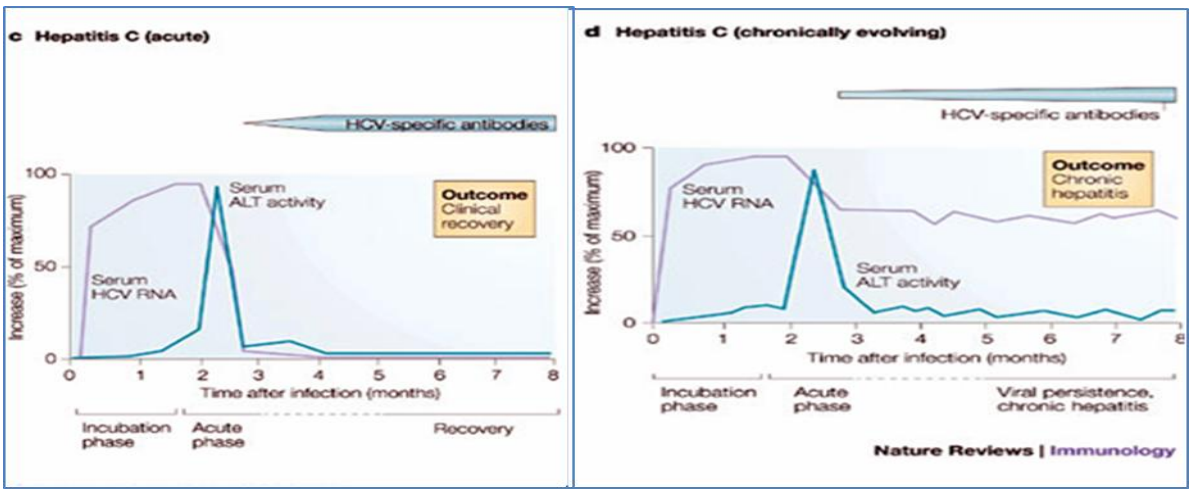

Figure 7: Immune response of HCV infection [24].

Rehermann B, Nascimbeni M. Immunology of hepatitis B virus and hepatitis C virus infection. Nat Rev Immunol 2005; 5: 215–29 with permission.

The figure demonstrates the immune response in acute HCV infections. The development of HCV specific antibodies may vary and the clearance of the virus might occur either before development of a measurable humoral response or even in the absence of development of a detectable antibody response. Incubation phase and acute phase describe alanine aminotransferase (ALT) levels and have no implication of clinical symptoms. Most cases of HCV infections are asymptomatic.

The figure illustrates also immune response in chronic HCV infections. HCV RNA titres decline by 2-3 logs after the ALT levels reach its peak, but then remain steady during the chronic HCV infection.

EPIDEMIOLOGY AND TRANSMISSION OF HCV

Up to 170 million people worldwide are currently infected with HCV infection, which is causing chronic liver diseases, inflammation, and long-term complications. HCV infection is a public health problem in developing countries including Asia and Africa [123]. The estimated prevalence of HCV infection in Southeast Asian countries are 2.0 to 3.8% for general population [23,143]; 12.5% for patients with chronic liver diseases [144] and more than 90% for injecting drug users [145]. HCV affects approximately 32 million people in Southeast Asia making the HCV disease burden is higher than a total of 22 million HCV infected persons from Europe, North America, and South America in combination [146,147].

In Vietnam, the prevalence of HCV infection was reported at 2-2.9%, blood transfusion was identified as the predominant risk factor and the most common genotype are 1 and 6 [148]. A study in rural Thai Binh province, Vietnam, reported that HCV prevalence in general population was 1% (95% CI 0.4%-1.9%) with no significant association between genders. There is a significant association between HCV infection and hospital admission and tattoos practice [149]. In Thailand, prevalence of HCV in blood donors increased from 1.6% in 1991 to 1.86% in 1994, thereafter it was reported to decline to 0.51% in 2009 [49]. A study on 13,897 first time blood donors in Lao PDR from 2003 to 2005 shows that the prevalence of anti-HCV positive blood donors was 1.1% [50]. A recent study on HCV infection in potential blood donors in rural area conducted in Samlot and Pailin

communities, Battambang province, Cambodia reported an overall prevalence of HCV infection was 14.7% (95% CI: 12.7%-16.7%) in the study population, 17% in Samlot and 12.3% in Pailin [1]. Higher prevalence of HCV infection in Cambodia compared to neighbouring countries is interesting, but difficult to explain.

A systematic review of HCV epidemiology in Asia, Australia and Egypt from 690 relevant articles among 7,770 articles done by Sievert W and co-authors estimated that 49.3 to 64.0 million adults in Asia, Australia and Egypt are anti-HCV positive. While a relatively high prevalence of HCV infection observed in Egypt (15%); Pakistan (4.7%) and Taiwan (4.4%), most other countries had prevalence from 1 to 2% of HCV infection. Blood transfusions and nosocomial infections are common risk factors in the region. Genotype 1 was common in China, Australia, Taiwan and other countries whereas genotype 6 was identified in Vietnam and other southeast countries [148].

HCV is usually transmitted by infected blood, sexual intercourse, contact with body fluids of infected HCV persons and from infected HCV mother to their babies [9]. The most frequently risk factors for HCV transmission are blood transfusions from unscreened donors; injection drug use; unsafe therapeutic injections; and other improper practice related to health care [123]. In developed countries, drug use is the primary cause of transmission for HCV infection, such as in USA and Australia where prevalence of HCV infection in drug users is reported 68% and 80% respectively [150,151]. Contaminated injection equipment was identified as the major risk factor for HCV infection in several countries such as Egypt, India. Blood-borne transmission is the most common transmission route of HCV infection worldwide [123]. The risk of transfusion-related infection with hepatitis C, hepatitis B, HIV-1 was reported as 1:103,000; 1: 63,000; 1: 493,000 transfused-units respectively in a study conducted in five blood centres in different parts of the United States where prevalence of HBV is low [2].

Classification of HCV infection as acute or chronic depends on the duration of virus' existence in the host body [9]. Acute HCV infection occurs during six months after exposed to HCV. The acute state of HCV infection is characterized with elevated serum alanine aminotransferase (ALT) level and jaundice which accounts for up to 25% of HCV cases [127]. Approximately 80% of acute HCV infections are asymptomatic and difficult to diagnose [152]. The remaining 20% of acute cases are symptomatic with poor appetite, abdominal pain in right upper abdominal quadrant, joint and muscle pains, nausea, vomiting and fever [122,127,152].

Acute HCV infection becomes chronic HCV disease if HCV-infected person is not able to clear the virus within 6 months of infection without any therapeutic intervention. Most chronic HCV infected persons show no symptoms until serious complications appear generally 20 years after initial infection. These complications include hepatocellular carcinoma, liver failure, and cirrhosis [9]. About 10% to 20% of individuals with chronic HCV infections regardless of country of origin or risk factors, develop cirrhosis, resulting in up to 5% development of hepatocellular carcinoma (HCC) within 30 years after initial HCV infection [127].

HEPATITIS C DIAGNOSTIC ASSAYS

Diagnostic tests for HCV infections include serologic assays for HCV antibodies and molecular test for viral particles. Screening tests for HCV antibody detection was introduced in 1989 and have resulted in the progress in early detection of HCV infection and contributed to the reduction of transfusion-related infections. The first serologic assay for HCV infection was an enzyme immunoassay for antibody detection. The currently used 2nd and 3rd generation enzyme immunoassays have a higher sensitivity containing core protein as well as non-structural proteins 3 and 4 (non-structural protein 5 in 3rd generation) and can detect antibodies within 4 to 10 weeks after initial infection [153]. The recombinant immune-blot assay has been used to confirm antibody assays. It uses antigens similar to those for the enzyme immunoassay but in an immuno-blot format so that responses to the individual proteins can be identified. A positive assay is defined by the detection of antibodies against two or more antigens; and negative assay by the detection of antibodies against a single antigen [153]. The use of a recombinant immuno-blot assay to confirm results is normally recommended only in low risk setting for example for blood donors [154]. Recently, molecular assays for HCV RNA detection have been produced as qualitative and quantitative tests. PCR technique for qualitative HCV RNA assays has a lower limit of detection of 100 copies of HCV RNA per millilitre [155]. There tests are of choice for confirmation of viraemia and assessment of treatment of response. A qualitative PCR assay can also be used in HBV-inspected patients with negative results on enzyme immunoassay, in HBV-infected patients without identifiable course, and in patients with known reasons for false negative results on antibody assays [153].

Recently there was a new enzyme immunoassay developed for the simultaneous detection of HCV core antigen and anti-HCV antibodies (Monolisa HCV Ag/Ab ULTRA; Bio-Rad). In terms of sensitivity, the performance of this test was not as high as that observed by HCV core Ag assays (73.8% and 96.9% of positive samples for HCV core Ag blood screening assay and trak-C assay respectively). However, this HCV combined assay reduces the window period to 21.6 days and 30.1 days compared with that of the most sensitive assays and Monilisa HCV Ab assay respectively, and provides a notable improvement for early detection of HCV infection during window period, especially in the early phase when antibodies are undetectable compared with previous anti-HCV assays. This combined assay also provides a new alternative to directly detection of viraemia with NAT or an HCV specific assay particularly with regard to cost, organization, emergency, and logistic difficulty. It could be performed for blood screening in low resource settings where NAT or HCV core antigen assays are not affordable and/or infeasible [156].

Routine HCV screening is performed in blood banks in Asia including Vietnam. The most widely used anti-HCV screening tests are enzyme immunoassays. Real Time Polymerase Chain Reaction (RT-PCR) is used for detection of HCV RNA. In acute HCV infections, anti-HCV is positive after two months of initial infections; 60% - 80% of HCV acute infections become positive after six months. The presence of HCV RNA in serum is the most reliable indicator of HCV infection, however, the presence of anti-HCV antibodies in serum or elevated ALT level at least 10 fold of upper limit of normal range can also be indicator of HCV infection [122,127]. Chronic HCV infection is identified by the presence of anti-HCV and the elevation of ALT levels for more than 6 months [122].

PREVENTION AND TREATMENT

Currently, there is not an effective vaccine to prevent HVC infection. It has been difficult to develop a single effective vaccine against all types of the virus. The main reasons include the genetic diversity among HCV genotype and the mutation rate of HCV virus [9]. Therefore, immunotherapy is given as HCV treatment.

The goal of HCV treatment is to avoid development of chronic infection, increase the quality of life for patients and prevent morbidity and mortality. Antiviral therapy based on the combination of pegylated interferon and ribavirin has obtained an acceptable response rate [157]. The type and duration of therapy administered for HCV patients depend on a number of factors such as genotype of the virus, age, gender, the state of fibrosis, body mass index, insulin resistance, co-infection with another hepatitis virus or with human immune-deficiency virus (HIV) [9,158]. The viral genotype is the most important factors affecting the type and duration of therapy and treatment outcomes; therefore, it is recommended that the genotype of a specific infection should be confirmed in order to plan an appropriate therapeutic regime. Eleven HCV genotypes have been identified so far, but only genotypes 1,2,3 are distributed globally and about 60% of HCV infection are caused by type 1a and 1b [127]. It is reported that Interferon (IFN) treatment is more effective against HCV genotype 2 and 3 than genotype 1, it is recommended that it is sufficient to implementation of a 48-week regime of treatment for genotype 1 and a 24 week therapy for genotypes 2 and 3 [122].

Two kinds of pegylated interferon-alfa (peg-IFN-α) are available, known as peg-IFN-α-2a and peg-IFN-α-2b being used as mono-therapy to treat HCV infections. In a review by Aman, 2012, it is shown that one week administration of peg-IFN-α-2a is more efficient than IFN-α-2a though peg-IFN-α treatment has some side effects including headache, weakness, haematological disorder, and injection site reaction [9]. Ribavirin is not recommended as mono-therapy for HCV treatment because it is more efficient in combined therapy. The highest sustained viral response (SVS) has been obtained by the combination therapy of peg-IFN-α-2a and ribavirin. This regimen is known as a current standard of care for chronic HCV patients who have not been treated previously [157]. Among 6 genotypes of HCV, 48 week combination therapy is recommended a standard treatment for genotype 1 and 4; 24 week combination regimen is proposed for genotypes 2 and 3 [159].

The cost of antivirus therapy against HCV is expensive. The wholesale price of a 48-week therapy of peg-IFN plus ribavirin is about $30,000, while a comparable regime of non-pegylated IFN plus ribavirin costs between $15,000 and $20,000 [9]. The high cost of treatment of HCV patients makes it unaffordable for HVC patients in Asian countries located in the highest reported HCV prevalence rate [123]. Therefore, none-therapeutic treatment is another recommended approach to prevent HCV patients from developing liver diseases. After diagnosis of HCV infection, patients should change lifestyle to limit disease development and control further health consequences. These changes involve regular physical exercises; adopting healthy eating habits, and more importantly maintaining a healthy weight. It is believed that obesity in HCV patients plays a crucial role in fibrosis progression [160,161]. Patients should also avoid drinking alcohol and smoking as alcohol is reported as one of the major risk factor for liver diseases [162,163] and smoking may contribute to the development of diseases and increase the possibility of hepatocellular carcinoma progression [164].

Approaches to control spread of HCV infection include primary and second prevention activities. Primary prevention activities are intended to reduce the risk of contracting HCV through main transmission routes. These involve virus inactivation in blood products; testing organs and blood donors; and providing counselling on risk reduction such as the use of contaminated drug needles, sexual intercourses with HCV infected partners and percutaneous exposure to infected blood in health care settings. Secondary prevention activities including counselling, identifying and testing people at risk; and treating HCV-infected patients, are aimed at reducing the risk for liver and other chronic diseases in HCV-infected patients [9].

COMPLICATIONS TO CHRONIC HBV AND HCV INFECTIONS

The hepatitis B virus (HBV) still remains a major global public health problem. It is estimated that more than one million HBV-infected patients die annually of HBV-related liver disease or hepatocellular carcinoma. The prolonged immunologic response to HBV infection leads to the development of cirrhosis, liver failure, or hepatocellular carcinoma (HCC) in up to 40% of patients [165,166]. It is estimated that 15% - 40% of chronic HBV carriers develop severe liver disease complications during their lifetime and need treatment [167]. This is not only a problem in poor-resource countries, but also in developed countries such as France [168]. Long term risk of HCC and cirrhosis development is directly correlated with viral replication and serum HBV DNA level [169,170]. The role of viral genotype on the risk of HCC development is still identified [171]: in children, 80% of HCCs are present in cirrhosis genotype B patients, whereas young adults with HCC are mostly non-cirrhotic [36,172]. Furthermore, in adult, genotypes C and F increase the risk of HCC development [36,173,174]. Chronic HBV patients with persistent HBeAg seropositivity have a higher incidence (3.5% per year) of cirrhosis [175]. A long-term follow-up study in Korea involving 52 HBeAg negative and 136 HBeAg posivitive patients showed that age and persistent ALT elevation are independent factors for the development of cirrhosis decompensation, and HCC [176]. HCC develops at annual incidence of 3%-6% in individuals with cirrhosis and far less frequently in noncirhotic patients [177–179]. HBeAg seropositivity and HBV DNA level more than 2,000 IU/ml are significant factors for the development of cirrhosis and HCC, even in asymptomatic patients with chronic HBV infection [169,174,180,181].

Nguyen et al. 2008 estimated the burden of HBV-related liver diseases in Vietnam and indicated that chronic HBV prevalence is likely reduce in Vietnam over the next two decades due to the implementation of universal infant HBV vaccination; however, the burden of HBV-related liver diseases will continue to increase. The estimated incidence of HBV-related liver cirrhosis increased 67% from 21 900 cases in 1990 to 36 500 cases in 2005 and projected to be 58 600 cases by 2025. It is also predicted that HBV-related HCC incidence increased from 9 400 cases in 1 990 to 15 600 cases in 2005 and projected to be 25 000 cases in 2025. The projected annual HBV-related mortality increased from 12 700 cases in 1990 to 23 300 cases in 2005 and 40 000 cases in 2025 [22].

Up to 170 million people worldwide are currently infected with HCV infection. Hepatitis C causes significant liver-related morbidity and mortality due to hepatic decompensation and development of hepatocellular carcinoma [182]. The most burden of HCV infection is recognised through the development of chronic liver disease and HCC, which is reported increasing incidence not only in developed countries, but also in developing

countries [123]. It is estimated that approximately 80% of HCV-infected individuals develop chronic infections and 20% - 30% of HCV-infected individuals are expected to clear the virus within six months of infection. Serious complications from HCV infection does not occur until 20 years after infection and 20% of chronic HCV carriers develop life-threatening cirrhosis and another 20% develop liver cancer [127,183]. The proportion of evolution to chronic HCV carriers after acute exposure to HCV was reported 92% in patients exposed to HCV genotype 1b infection compared with 35% to 50% in patients exposed to other HCV genotype [184]. Most HCV-infected patients will die before onset of HCV-related complications, which are important public health problem, even though they occur in only a fraction of HCV-infected patients [123].

TEST ACCURACY: SENSITIVITY AND SPECIFICITY

Screening tests must qualify high sensitivity with a maximum specificity while exhibiting a low initial reactive rate , which may result in time saving and improve cost and effectiveness [13]. High test sensitivity plays a fundamental role in blood testing because it can minimise the false negative test results which may contribute to the risk of post transfusion infections. This section attempts to provide information on current test sensitivity for detection of HBsAg, anti-HBc and HBV DNA.

Three are different units in literature regarding to express the test results. Positive test results are expressed in different ways either as international units (IU) or nanogram (ng) according to different standards. It is useful to know that one international unit (IU) is equivalent to 5.6 Abbott ng, 1.9 French ng according to WHO international standard [12]. One ng of HBV protein is equivalent to approximately 2×10^8 22 nm sub viral particles and to approximately 5×10^7 HBV particles with assuming that the virus particles have four times larger surface. Current assays for HBsAg vary in detection limits ranging from 0.04 to 0.62ng/mL. At a detection limit of 0.04ng/mL, there must be approximately 2 million particles in blood for a positive result test, and the excessive presence of sub viral particles can improve detection [40]. It is clear that screening blood donors for HBsAg even with highly sensitive assays does not eliminate the post transfusion of HBV transmission. There are some possible explanations for false negative results in current HBsAg assays. Firstly, blood donors with HBsAg mutant may escape detection by screening assays [185,186]. Secondly, virus diversity yields sequences that are not detected by antibodies employed in the assays [187]. Thirdly, the concomitant presence of anti-HBs resulting in the formation of circulating immune complexes not or poorly displaced by HBs Ag capture antibody [188].

A recent study on the efficacy of 70 HBsAg assays bought from open markets around the world were conducted by the International Consortium for Blood Safety (ICBS), on plasma units collected from blood banks around the world. The study showed the range of sensitivity between the most sensitive assays and the least sensitive HBsAg test kits was 0.021 IU/ml to more than 2.33 IU/ml in Paul-Ehrlich Institute (PEI) standard and 0.013 IU/ml to more than 4 IU/ml in ICBS panel. This reflected a >300 fold difference between the most and the least sensitivity of the current assays for HBV detection. The study also documented that seventeen HBsAg EIA assays with high analytic sensitivity <0.13 IU/ml prove 100% diagnostic sensitivity and were even sensitive to the various HBV variants tested. Another six EIA assay demonstrated high sensitivity < 0.13 IU/ml, but missed HBsAg mutant and/or showed reduced sensitivity to some certain HBV genotypes. Twenty EIAs for HBsAg had

sensitivity of 0.13 – 1 IU/ml. Another eight EIA assays and 19 rapid tests had analytical sensitivity of 1 – > 4 IU/ml. Those assays with sensitivity > 1 IU/ml significantly reduce the length of the HBsAg positive period resulting in less reliable HBsAg detection in asymptomatic HBV infection. These assays also showed lower analytical sensitivity to certain genotype. It should be noted that laboratories should take into account the analytical sensitivity for HBsAg test kits and check for the relevant HBV variants for a given population [189].

Many studies have been conducted to determine the efficacy of anti-HBc assays by testing anti-HBc positive blood samples for the presence of HBV DNA. As mentioned earlier, anti-HBc seems to have the potential of disqualifying majority of OBIs, leaving only the probably rare cases with HBV DNA alone undetected. However, anti-HBc tests demonstrate some limitations. For many years, the specificity of anti-HBc assays is far below the performance of anti-HIV and anti-HCV assays as well as the lack of confirmatory assays [88]. As required, assays for anti-HBc approved for blood screening must have a specificity of more than 99%, but the existence of confirmatory assays still remains a problem [190].

The sensitivity of HBV DNA also has a crucial impact on results in a given population as the concentration of viral genome in anti-HBc samples was mostly less than 100 IU/mL The gold standard for OBI diagnosis is intended to explore the extracted DNA from liver or blood requiring a very specific and sensitive assay. Experts recommended assays with detection limit of 10 copies of HBV DNA per reaction [98]. Current assays for HBV DNA detection are nested PCR, real-time PCR, and transcription based mediated amplification. These assays can decline the lower detection limit of less than 5 copies/ml of HBV DNA. This is especially important in OBI due to the fact that HBV DNA varies from less than 10 to 425 copies/mL [40]. Detection of OBI requires the assays with the highest sensitivity and specificity with lower limit of detection less than 10 IU/mL for HBV DNA and <0.1 ng/mL for HBsAg [12].

KNOWLEDGE GAPS

HBV and HCV infections are public health problem in developing countries and a leading cause of chronic liver diseases, cirrhosis, and hepatocellular carcinoma. Asian countries including Cambodia and Vietnam are classified as high prevalence areas of HBV and HCV infections. HBV and HCV share the common modes of transmission and can be transmitted horizontally and vertically. Blood transfusion is identified a frequent risk factor for both disease transmission. Screening test for HBsAg and anti-HCV in blood donors is in place in South East Asia including Cambodia and Vietnam whereas anti-HBc screening in blood donors is implemented in developed countries such as United States and Japan. It is documented that HBV transmission can be spread from HBsAg negative and anti-HBc positive blood donors to recipients when blood collected during the initial sero-negative window period of an acute HBV infection or during late stage where HBV DNA is present through HBsAg is negative, the so called ”OBI”. Anti-HBc screening helps to eliminate HBV transmission in window period, but is not practical in intermediate and endemic HBV prevalence where half of population carries anti-HBc, resulting in the exclusion of vast number of blood donors. NAT tests are accurate, but not feasible due to high cost. In the current context of Vietnam and other countries in Southeast Asia, we have to find other mean of estimating the risk of transfusion-transmitted hepatitis B.

Rapid tests may be a feasible tool for detection of HBV infection in blood donors in poor communities. With the rapid test, one can run a single sample and the result can be available in a short time. However, it is reported that rapid tests may yield false negative results due to prozone effects when used in endemic HBV population. Enzyme Immunoassay (EIA) is considered as a standard test for HBV and HCV detection in South East Asia. However, the EIA test is expensive, not practical in rural remote district hospitals due to the fact that the EIA test is normally designed to run with a total of 96 blood samples including control samples in a single run. EIA tests for detection of HBV and HCV is regulated as a standard test in national guideline in Vietnam. However, the rapid tests for HBV and HCV detection were already in use in some parts in Cambodia. One may question how accurate the rapid test results are and what the potential risk of transmitted transfusion infection diseases may be under current screening for blood donors in Southeast Asian.

STUDY OBJECTIVES

The overall objective of the thesis was to study the overall pattern of HBV and HBC infections in Southeast Asia in order to give advice to the public sector on how to avoid transfusion-transmitted HBV and HCV. This was done through the following specific aims:

1. To examine the accuracy of rapid tests for detection of HBsAg, anti-HBc and anti-HCV in potential blood donor in rural Vietnam and Cambodia (paper 1)

2. To estimate more accurate prevalence rates of HBV and HCV infections among potential blood donors in a rural area of Vietnam and to examine the accuracy of enzyme immunoassay (EIA) technique used for HBsAg, anti-HBc, anti-HCV testing of blood donors at a local laboratory in Vietnam (paper 2)

3. To estimate the risk of transfusion-transmitted hepatitis B in Vietnam based on the available data on HBV prevalence and the estimated prevalence of OBI in blood donors established from current knowledge of OBI (paper 3).

MATERIALS AND METHODS

STUDY POPULATION

A cross-sectional epidemiological study on prevalence of hepatitis B and hepatitis C in potential blood donors in rural Cambodia and Vietnam was conducted in a multicentre design. The study was carried out in February-June 2007 in Battambang and Pailin Provinces, Cambodia; and Quang Tri Province, Vietnam. In order to detect the prevalence differences between two populations in each country of at least 10% with a significance level of 5% with two sided testing; and a test power of 90%, a total of 1,200 samples were required for the study in each country. Stratified sampling was applied to assess local prevalence variations in two equal subsamples, 600 samples in each subsample in each country.

The study population was the potential blood donors in the study area. Eligible participants were individuals aged 18 to 55, not vaccinated against HBV and living permanently in the study areas. Before collecting blood samples, villagers were informed by local health authorities that the study linked up to the introduction of safe blood transfusion service for the local population; that participation was voluntary and free of charge; that all participants would be informed of the test outcome and get medical advice and counselling accordingly. After the informed consent forms were signed with the witness of blood trained collectors, participants were asked to fill in and sign registration forms before inclusion.

The study subjects for paper 1 include a total of 2,400 blood samples in both countries to examine the accuracy of one rapid test for detection of HBsAg, anti-HBc and anti-HCV; 1,200 blood samples collected from Vietnam were used for paper 2 to estimate the prevalence and identify the risk factors; and existing data from paper 2 was used for the risk assessment model in paper 3. Sample collection procedures, screening tests used; and validation of the test accuracy is done according to the same study protocol in both countries.

STUDY SAMPLES

Paper 1: The study samples consisted of 2,400 female and male potential blood donors from rural Cambodia and Vietnam, 1,200 from each country. The mean age of the study population was 33.4 years (SD 9.5); 41 % were males. The ages did not differ significantly between the subjects from the two study countries, but the rate of female participants was slightly higher in Vietnam. The study participants in Cambodia included 677 females and 523 males; the mean age was 32.8 years, with a range of 18 to 52 years. The voluntary participants in Vietnam were consisted of 730 females and 470 males with the mean age of 34.6 years (SD: 8.6) with a range of 18-55 years.

Paper 2: In order to assess local prevalence variations the blood samples were collected with a stratified sampling procedure. Samples were collected with a stratified sampling procedure: 600 samples (mean age 35 years, SD=8.4; female/male: 329/271) were collected from all villages in one remote rural area, Trieu Trach community (a total population of 6,801 persons, 3,510 female) with less developed health infrastructure, and 600 samples (mean age 34.2, SD=8.8; female/male: 401/199) from all villages in an area with more developed

health infrastructure, Cam Thuy community (a total population of 4,994 persons, 2,557 female). Within each area, sampling sites at village level were established, and blood samples collected from the consenting individuals. With this sampling design, potential clustering of results at village level had to be considered and adjusted for in the prevalence estimates.

Paper 3: A stochastic Monte Carlo models were used to estimate the probabilities of HBV transmission from OBI in different scenarios. Inputs to the model were based on data from the prevalence study in paper 2; literature review; and expert opinions. An existing database from a cross-sectional epidemiological study in a reference population of potential blood donors in rural areas of Vietnam was used as baseline data (Viet L et al., 2012). The study, hereafter named "the 2007 prevalence study", was carried out in February-March 2007 in the Quang Tri province of Vietnam.

EXPERT PANEL ESTIMATES OF OBI PREVALENCE

As no certain estimates of OBI prevalence existed, this information for input into the simulation models was obtained by two independent international recognized experts in the field of our study, Dr. JP Allain (a) and Dr. R. Reddy (b). Both were asked to give their best qualified assumptions of OBI prevalence in a population with high, intermediate and low endemic HBsAg prevalence. This was used to produce an expert-based estimate on the OBI prevalence in Vietnam to be used in the simulation model.

According to the expert panel, a wide range of OBI prevalence in blood donors has been reported; between 1:6 and 1:50,000 globally, depending on the sensitivity of the NAT and HBsAg assays used, the prevalent genotype in a given population; and the efficacy of the host immune system (Allain JP, personal communication, 2011, [190]. In Vietnam, where HBV genotypes B and C are prevalent, the prevalence of OBI is likely around 1:1000 – similar to the situation in Taiwan and South China. The review by Allain and Candotti (2011) on OBI prevalence from blood donors in different studies in China, reported a range between 1:600 and 1: 21,000 blood units with a mean about 1: 1,000 blood donors [191]. OBI prevalence in blood donors in Taiwan was reported approximately 1: 1,000 blood units [110,111]. In South Africa where HBV prevalence in the population is 8-10%, the OBI NAT yield rate in first time donors is 1:3,545 (Ravi Reddy Expert panel, Personal communication, 2011). Because data on OBI prevalence in Vietnam is not available, we used the estimated prevalence of OBI of 1:1,000 reported from China and Taiwan and also given by the expert panel as reference. The reasons for this decision are twofold: The genotypes B and C are prevalent in China, Taiwan and Vietnam [48,192–194] and both Taiwan and Vietnam are categorized as high-HBV prevalence countries according to the classification by WHO [5,22].

MONTE CARLO SIMULATION MODELLING

Stochastic models are used to bring randomness and uncertainties into the final estimates and are often used when decisions under uncertainties are requested. To estimate the probabilities of HBV transmission in different scenarios, we established a stochastic Monte Carlo simulation model using the @Risk software (ver 5.7, Palisade Corp. Ithaca, NY) add-in to Excel™. Inputs to the model were based on data related to HBsAg and

anti-HBc on actual analysis done by the authors and controlled in a Norwegian accredited microbiology laboratory from the 2007 prevalence study, literature review, and the expert panel estimates. The number of blood donors for the simulation was set at 1,000,000 corresponding to the expected number of donated blood units needed for health care system in Vietnam in 2012. The age structure of the population by gender was estimated based on data from the National Vietnam population and the housing census performed in Vietnam in 2009 using 18-29; 30-39; and equal and more than 40 as age groups.

The crucial inputs of the model were the test sensitivities and specificities, first as specified by the producer, modelled using the beta function in @Risk, where e.g. a sensitivity with x/n positive is modelled as a Risk-Beta (x+1, n-x+1), and later modified with reducing the sensitivity by increasing the x by 2 and 5 respectively, to reflect a more realistic scenario when a test is used in many laboratories under various conditions where a reduced sensitivity must be expected. These estimates were used as inputs in various Risk-Binomial functions in the model. The true prevalence (TP) of the population was based upon results of apparent prevalence (AP) from the 2007 prevalence study calculated by the formula from Dohoo et al (2009), pooling gender/ age group estimates form this study and using as the starting point for further modelling:

$$TP = p(D+) = \frac{[AP - (1 - Sp)]}{1 - [(1 - Sp) + (1 - Se)]} = \frac{AP + Sp - 1}{Se + Sp - 1}$$

The Risk Triangular function was used for the population probability-distribution of OBI at three scenarios based upon the expert panel with a fixed lower value for OBI estimated prevalence of 0.0005, an expected (middle) value of 0.001, and upper values of 0.0015 (scenario I), 0.002 (scenario II) and 0.003 (scenario III) respectively.

Thus, the model represents a combination of three OBI scenarios and three HBsAg EIA sensitivities, giving nine different scenarios for comparison. The simulation model first produced age/ gender groups from 1,000,000 donors, and then calculated the true prevalence and the number in each of four groups based on of EIA test results. In the model, all HBsAg positive samples were removed and not used for transfusion. A main concern was to distinguish between HBsAg false-negative blood donors by EIA due to limited sensitivity and potential OBI defined as study persons with true negative HBsAg test and anti-HBc true positive and anti-HBc false negative.

SAMPLE COLLECTION

One blood sample of 5 ml was collected from each voluntary participant by trained laboratory technicians, set aside to be coagulated for 30 minutes, then centrifuged and cooled to 4 ºC in portable cooling boxes. The serum samples were then taken for analysis either at Battambang Blood Transfusion Centre in Cambodia or at Quang Tri Provincial Preventive Medicine Centre, Vietnam. Analysis was done within three days after sampling. All samples were processed according to manufacturers' instructions and included in the study sample.

SCREENING TESTS

RAPID TESTS

The rapid tests in this study were already in use for blood donor screening in some parts of Cambodia. Three qualitative chromatographic immunoassays for HBsAg, anti-HBc, anti-HCV were used: ACON® HBsAg one step ultra, no. IHBsg-U302; ACON® anti-HBc one step, no. IHBcb-302; and ACON® anti- HCV one step, no. IHC-302 (Acon Laboratories, San Diego, CA). The HBsAg test is made by a combination of monoclonal and polyclonal antibodies to selectively detect elevated levels of HBsAg in serum and plasma. The test can detect a limit of 0.5 ng/ml for HBsAg for both ad and ay subtypes at concentrations from zero to 300 ng/ml. The anti-HBc test utilizes a combination of monoclonal antibodies and antigen to selectively detect elevated levels of anti-HBc in serum or plasma. The anti-HCV test utilizes a combination of protein A coated particles and recombinant HCV proteins to selectively detect antibody to HCV in plasma or serum. The recombinant antigen used in the test was encoded by genes for both structural and non-structural proteins. All three tests had an internal control (Acon laboratories, n.d.). The rapid tests were carried out according to the manufacturers' instructions. The rapid test outcomes were compared to the test properties claimed by the manufacturer. Agreement analysis was undertaken to compare the overall rapid test outcomes with enzyme immunoassay (EIA) as a reference test.

EIA TESTS

The EIA reference test (Monolisa EIA Assay® BioRad; Monolisa® HBsAg ultra, no. 72348; Monolisa® anti-HBc plus, no. 72316; Monolisa® anti-HCV plus, no. 72318; BioRad Diagnostics, 92430 Marnes-la-Coquette, France) was carried out correspondingly either at Battambang Blood Transfusion Center in Cambodia or at the Quang Tri Provincial Preventive Medicine Center in Vietnam. The tests were carried out according to the manufacturers' instructions. The actual EIA test has sensitivity and specificity for HBsAg of 100% and 99.94%; for anti-HBc of 99.53% and 99.5%; and for anti-HCV of 100% and 99.8%, respectively as claimed by producers. The EIA test measures a numerical ratio (S/CO) for classification of test-positives and test-negatives. Ratios lower than 1.0 were classified as negative results; ratios higher or equal to 1.0 were classified as positive results; units with a ratio in the range of 0.9-1.0 were classified as equivocal and reanalysed.

Principle of the EIA assays

Monolisa™ HBs Ag ULTRA assay is a one-step enzyme immunoassay based on the principle of the "sandwich" type using monoclonal antibodies and polyclonal antibodies selected for their ability to bind themselves to the various subtypes of HBs Ag now recognized by the WHO and the most part of variant HBV strains in human serum and plasma. The Monolisa™ HBs Ag ULTRA solid phase is coated with monoclonal antibodies and its conjugates are based upon the use of monoclonal antibodies from mouse and polyclonal antibody from goat against the HBs Ag. These antibodies are bound to the peroxidase. The sensitivity limit of the assay for HBsAg detection has been estimated < 0.060 ng/ml during the evaluation with the French SFTS 2001 panel of HBs antigen. The limit of detection has been estimated less than 0.130 IU/ml by testing the WHO 2nd International

standard NIBSC code 00/588 and found at 0.025 IU/mL CI95% [0.019 – 0.037 IU/mL]. The following sub-types from SFTS 2001 panel: adw2, adw4, adr, ayw1, ayw2, ayw3, ayw4, ayw5, and ayr, were all found positive with a ratio larger than 5 with Monolisa™ HBs Ag ULTRA.

MONOLISA® anti-HBc PLUS is an enzyme immunoassay for the simultaneous detection of total antibodies to hepatitis B virus core in human serum or plasma. MONOLISA® anti-HBc PLUS is based upon the use of a solid phase prepared with recombinant HBc antigen.

Steps of the MONOLISA® anti-HBc PLUS manipulation include as follow:

Firstly, the sera to be tested and the control sera are added to the wells. The presence of antibodies to HBc in the sera will bind to the antigens fixed on the solid phase. Secondly, the peroxidase-labelled antibodies to human IgG and IgM are added after a washing step. Then they in turn bind to the specific antibodies captured on the solid phase. Thirdly, after removal of the unbound enzymatic conjugate, the antigen-antibody complex is revealed by addition of substrate. Finally, after the reaction has been stopped, the absorbance values are measured using a spectrophotometer at 450/620-700 nm. The absorbance measured for a sample allows the presence or absence of antibodies to HBc to be determined. The colour intensity is proportional to the quantity of anti-HBc antibodies bound on the solid phase.

When performing the evaluations, the sensitivity of the MONOLISA® anti-HBc PLUS was evaluated using the Paul Ehrlich Institute IgG and IgM standards, and the limit of detection was estimated at 0.5 U PEI/ml and 8 U PEI/ml for the IgG and IgM respectively.

MONOLISA® anti-HCV PLUS Version 2 is an indirect immuno-enzymatic technique for the detection of anti-HCV serum or plasma of HCV-infected patient. MONOLISA® anti-HCV PLUS Version 2 is based upon the use of a solid phase prepared with purified antigens: 3 recombinant proteins produced by *E. coli* from clones selected in the non-structural area (NS3 and NS4) and in the structural area of the hepatitis C virus genome. Detection is with goat anti-human IgG antibody purified by affinity chromatography and coupled to peroxidase.

The performance of the MONOLISA® anti-HCV PLUS Version 2 includes the following reaction step:

Firstly, the samples to be tested and the control sera are added to the wells. If antibodies to HCV are present, they will bind to the antigens fixed on the solid phase. Secondly, the peroxidase-labelled antibodies to human IgG are added after a washing step. They in turn bind to the specific antibodies captured on the solid phase. Thirdly, after removal of the unbound enzymatic conjugate, the antigen-antibody complex is revealed by addition of substrate. Eventually, after the reaction has been stopped, the absorbance values are read using a spectrophotometer at 450/620- 700 nm. The absorbance measured for a sample allows the presence or absence of antibody to HCV to be determined. The colour intensity is proportional to the quantity of antibody to HCV bound on the solid phase.

VALIDATION OF TEST ACCURACY

The EIA test outcome was blindly validated with the Automated Chemiluminescent Microparticle Immunoassay Technique (CMIA; Architect® HBsAg, ref 6C36; Architect® anti-HBc, ref 7C17; Architect® anti-HCV, ref 6C37; Abbott GmbH & Co. KG, 65205 Wiesbaden-Delkenheim, Germany). A subset of 640 serum samples including 321 samples from Vietnam and 319 samples from Cambodia (*n* =240 for each category, HBsAg, anti-HBc, and HCV) was randomly selected for validation at the Department of Microbiology, University Hospital of North Norway.

The subset sample size (*n* = 240) was estimated in order to detect test indicator differences of more than 5% with 95% confidence, the subset being selected to get at a balance of 2/3 assumed test-positive units versus 1/3 assumed test-negative units. With the CMIA testing of HBsAg in Norway, samples with values less than 0.05 IU/ml were classified negative and those with values greater than or equal to 0.05 IU/ml were classified positive. CMIA analysis of anti-HBc and anti-HCV was based on the ratio of the signal to the cut-off value (S/CO). A ratio less than 1.00 was defined as negative, and a ratio greater than 1.00 was defined as positive. Ratios in the range of 0.90-1.00 were considered as equivocal and re-analysed.

In paper 1, a high agreement between the EIA and CMIA test outcomes were reported with kappa values higher than 0.8 for HBsAg, anti-HBc, and anti-HCV detection. Based on these results, the EIA test outcomes for HBsAg, anti-HBc, and anti-HCV in the total study population (n = 2,400) were used as a reference for evaluation of rapid test accuracy in the actual study.

Principles of the assays:

The ARCHITECT HBsAg assay is a Chemiluminescent Microparticle Immunoassay (CMIA) for the quantitative determination of HBsAg in human serum and plasma. In the first step assay performance, sample and anti-HBs coated paramagnetic micro particles are combined. HBsAg present in the sample binds to the anti-HBs coated micro particles. After washing, acridinium-labeled anti-HBs conjugate is added in the second step. Following another wash cycle, Pre-Trigger and Trigger Solutions are added to the reaction mixture. The resulting chemiluminescent reaction is measured as relative light units (RLUs). A direct relationship exists between the amount of HBsAg in the sample and the RLUs detected by the ARCHITECT i*optical system. The concentration of HBsAg in the specimen is determined using a previously generated ARCHITECT HBsAg calibration curve. If the concentration of the specimen is greater than or equal to 0.05 IU/mL, the specimen is considered reactive for HBsAg.

The ARCHITECT anti-HBc assay is a Chemiluminescent Microparticle Immunoassay (CMIA) for the qualitative detection of antibody to anti-HBc in human serum and plasma. In the first step, sample, assay diluent, and HBc antigen coated paramagnetic micro particles are combined. Anti-HBc present in the sample binds to the HBc antigen coated micro particles. After washing, anti-human acridinium-labeled conjugate is added in the second step. Following another wash cycle, Pre-Trigger and Trigger Solutions are added to the reaction vessel. The resulting chemiluminescent reaction is measured as relative light units (RLUs). A direct relationship exists

between the amount of anti-HBc in the sample and the RLUs detected by the ARCHITECT i* optical system. The presence or absence of anti-HBc in the specimen is determined by comparing the chemiluminescent signal in the reaction to the cut-off signal determined from a previous calibration. If the chemiluminescent signal in the specimen is greater than or equal to the cut-off signal, the specimen is considered reactive for anti-HBc.

The ARCHITECT Anti-HCV assay is a chemiluminescent micro particle immunoassay (CMIA) for the qualitative detection of antibody to hepatitis C virus (anti-HCV) in human serum and plasma. In the first step, sample, recombinant HCV antigen coated paramagnetic micro particles and Assay diluent are combined. Anti-HCV present in the sample binds to the HCV coated micro particles. After washing, anti-human acridinium-labeled conjugate is added in the second step. Following another wash cycle, Pre-Trigger and Trigger Solutions are added to the reaction mixture. The resulting chemiluminescent reaction is measured as relative light units (RLUs). A direct relationship exists between the amount of anti-HCV in the sample and the RLUs detected by the ARCHITECT i* System optics. The presence or absence of anti-HCV in the specimen is determined by comparing the chemiluminescent signal in the reaction to the cut-off signal determined from a previous ARCHITECT Anti-HCV calibration. If the chemiluminescent signal in the specimen is greater than or equal to the cut-off signal, the specimen is considered reactive for anti-HCV.

STATISTICAL PLATFORM

Continuous variables were expressed as mean value with 95% confidence intervals constructed by the Student procedure. Categorical variables are presented in contingency tables with 95% confidence intervals, and the chi Square was used to test the difference between two groups. The Kappa (κ) analysis was used to express agreement between test methods; κ-values of 0.4-0.6 were classified as having acceptable agreement, values of 0.6-0.8 as having high agreement, and values of 0.8-1 as having very high agreement. In paper 2, a multivariable logistic regression model was established to test if observed differences between areas were due to sampling biases. Model fit was assessed using the Hosmer-Lemeshow test and Receiver Operating Statistics Curves (ROC). Different statistical techniques were employed using the software JMP® for Windows (ver 8.0, SAS Institute Inc.) or Stata (SE/11 for Windows, StataCorp. College Station, TX).

ETHICAL CONSIDERATIONS

Informed consents were obtained after orally and written information was given by local health authorities. Medical counselling according to the test outcomes was granted to all voluntary participants. The consent form was signed on-site in fields before the blood samples were collected. Written files of demographical and laboratory data were stored in locked steel shelves at the Quang Tri Preventive Medicine Centre, Vietnam; and at the Trauma Care Foundation head office in Battambang, Cambodia. Access to non-anonymous data was restricted to members of the research team. The study protocol was ethically approved by the Quang Tri Health Service, and by Quang Tri Provincial People's Committee, Vietnam (Decision No. 2472/QD-UBND, 20/12/2006); and by the Cambodian Committee for Research Ethics (ref 023 NECHR, 2/4/2007). All data were stored and processed by permission from the Norwegian Social Science Data Service, Norway (ref. no. 13702).

MAIN RESULTS

PAPER 1

The actual rapid tests were observed with high specificity for all three HBsAg, anti-HBc and anti-HCV tests as claimed by the manufacturer. The test sensitivity was found significantly lower than that claimed by the producers: 86.5% for HBsAg; 86.6% for anti-HBc; and 76.4% for anti-HCV compared to >99%; 96.3%; and 96.8% respectively as claimed by the producers. There were large and significant variations in test performance between Vietnam and Cambodia, particularly for HBsAg detection. The sensitivity was significantly lower and the rate of false-negatives significantly higher for HBsAg and anti-HBc in the Vietnamese subsample compared to the Cambodian subsample. For the anti-HCV test, the sensitivity was observed low in Cambodia; inter-country comparison could not be done because few HCV serum samples were positive for anti-HCV in Vietnam. The low sensitivity of the actual rapid tests for HBsAg, anti-HBc and anti-HCV make them useless for blood donor screening in rural Southeast Asia.

PAPER 2

In the study population, the prevalence of HBsAg was 11.4% (95% CI 9.6 – 13.2) and the prevalence of anti-HBc was 51.7% (95% CI 48.8 – 54.5). The prevalence was found higher in males than females. The prevalence of anti-HCV was observed at 0.17%. The test agreement between the EIA and CMIA techniques was found to be high both for HBsAg detection ($\kappa = 0.91$; 95% CI 0.83 – 0.99) and for anti-HBc detection ($\kappa = 0.89$; 95% CI 0.81 - 0.97). The positive and negative predictive values of the EIA tests compared to CMIA tests were observed to be 94.9% (95% CI 87.5 – 98.6) and 97.5% (95% CI 86.8 – 99.9) for HBsAg respectively; and 92.4% (95% CI 84.2 – 97.2) and 100% (95% CI 91.2 – 100) for anti-HBc.

PAPER 3

With 1,000,000 blood donors running in the model, the potential OBI ranged from 658 to 747 blood units at 5 percentile and from 1,342 to 2,507 blood units per million at 95 percentile resulting in the risk for post-transfusion hepatitis ranging from 66 to 250 blood units per million assuming that risk of post-transfusion from potential OBI is 10%. Using the manufacturer's HBsAg sensitivity, the mean rate of blood units per million donations having false-negative HBsAg results was 298 (5-95 percentile: 14-893). When the test sensitivity was set lower and more in accordance with the local testing results, false-negative tests was observed at a mean of 1,087 per million (5-95 percentile: 762-3220). The fraction of potential OBI was found to increase with increasing age groups in both male and female. In the male group, this fraction was found to be 0.33; 0.44; 0.70 whereas 0.29; 0.45; 0.61 in female in age group less than 29; 30-39; and larger than 40 years old, respectively.

GENERAL DISCUSSIONS

METHODOLOGICAL CONSIDERATIONS

In a cross-sectional study some errors related to the sampling of study participants may exist. Though the study was aimed at potential blood donors we cannot exclude the possibility of having included some individuals with risk behaviour such as drug users. In order to control this, local health workers as well as local authorities were mobilised to recruit voluntary participants according to the inclusive criteria for the establishment of local blood transfusion services. Therefore, we believe that such bias if any should be moderate and without systematic or significant effect on the main outcome variables.

Another concern is that selection bias due to spontaneous recruitment may have occurred in the study groups. Thus, we observed an imbalance of gender participants in Vietnam. As we can see that rate of female/male in Trieu Trach (3510/3291=1.07) is similar to rate of female/male in Cam Thuy (2557/2437=1.05); however, the sex ratio in the actual study population is 740/470= 1.57. We could not control the sex ratio of the study samples as participation was voluntary. We used only one community health centre in each area in Vietnam to collect blood samples, hence any person turning up and meeting the selection criteria was included, irrespective of gender. We could have met a sex distribution of 1:1 by refusing women to entry the study when 300 women had been included at each site. However, this would also have meant a biased selection. We tried to adjust for this in the statistical analyses by detection of potential clustering phenomena. Our prevalence estimates were adjusted according to the geographical design. The multivariable model allowed us to adjust for the potential bias linked to age and gender, showing that there was no statistically valid difference between areas, and that estimates were influenced by the age/ gender in the sampled population. In conclusion, we maintain, that we do not expect our conclusions to be much influenced by potential bias linked to the study design.

Next, failures regarding the technical sampling and processing in field may have happened. To control for this, all laboratory technicians collecting the blood sample in field were trained and all procedures were performed strictly according to the protocol under close supervision of the research team. No accidental events were reported and we feel confident that there have not been technical errors of significant impact.

Also, one should scrutinize the accuracy of laboratory analysis. Only one experienced microbiological technician was assigned to process blood samples in field and analyse the serum samples in the laboratory. During the EIA analysis, all samples with test outcome close to the EIA cut-off level were re-analysed before test results were registered in the database. Also the EIA technique used for analysis was validated blindly by CMIA analysis at a high-tech Norwegian medical laboratory. A very high agreement between the two test outcomes (EIA and CMIA) was observed; therefore we hold that the actual prevalence estimates are reliable.

In the Monte Carlo model, the inputs in the model were based on rather limited data related to HBsAg and anti-HBc prevalence conducted in Quang Tri Vietnam; literature review; and the expert panel estimates. The inputs are not evidence-based and one may question that the estimated risk of transfusion-transmitted HBV in

Vietnam may be imprecise. It is clear that the estimate relies on the input parameters. Although our estimates are consistent with the residual risk of HBV post-transmission reported recently [119,195] in other high endemic HBV prevalence, we still need further studies of OBI in blood transfusion in order to more precise estimate the risk for future strategy of transfusion settings.

DISCUSSIONS OF MAIN RESULTS

THE ACCURACY OF RAPID TESTS

Paper 1 reveals three main findings. First, the accuracy of the rapid test for qualitative detection of HBsAg, anti-HBc and anti-HCV in potential blood donors in rural Southeast Asia, is low. For detection of HCV the false - negative rate was about 20%. Second, there was a significant difference between the observed and producer - claimed test sensitivities for all three test assays. Third, significant differences in test sensitivity between two sub-populations in Cambodia and Vietnam were observed.

One may question the reliability of the local reference test used for assessment of rapid test performance. As stated in the first part of the discussion, during EIA analysis in Cambodia and Vietnam, all study blood samples with a test outcome close to the EIA cut-off levels were re-analysed before test results were registered in the database. EIA analysis was also validated blindly by CMIA technique in a representative subset of the study at a Norwegian medical laboratory of high standard demonstrating high agreement ($\kappa > 0.8$) between the reference test (EIA) and the CMIA test outcomes for HBsAg, anti-HBc, and anti-HCV. Therefore, there should be no reason to doubt the accuracy of the reference test used in the study.

The study was conducted among potential blood donors in rural populations in Vietnam and Cambodia where hepatitis is endemic and both antigen and antibody levels may be high. Therefore a prozone effect may be one reason why some true positive results turned out to be negative with the rapid tests. Cross reacting antibodies may also influence the test results. There may also be test flaws due to genotype variations that may affect test sensitivity. For both HBV and HCV there are genotype differences between Western countries and Southeast Asia. HCV is an extremely variable virus with six different genotypes and more than 70 subtypes; HCV genotype 6 is found to be dominant in Southeast Asia, with large subgenotypes differences between countries, *e.g.* Vietnam: 6a and 6d/e, Cambodia: 6q, Thailand: 6f, 6i/j, 6m and 6n [196–198]. For HBV, the genotypes B and C are the most common in Southeast Asia, while genotypes A and D are dominant in Europe [31,32].The low sensitivity and the large difference in sensitivity between countries, especially for HCV, can be related to deficient detection of genotypes and/or subtypes with the tests. Therefore, test accuracy in one study population or in one study country does not mean good performance in another population.

The relatively good rapid test specificity, but the low sensitivity with many false negative rapid tests having reference test results far above the cut-off level by itself, indicates the poor rapid test performance in the study population is real. Previous study to evaluate the accuracy of HBsAg and anti-HCV assays in blood donors in Equador documented there was significantly higher false-negative rate for rapid test in comparison with EIA test outcomes [199]. A study conducted by Lien et al. 2000 to evaluate the three different rapid tests for

detection of HBsAg in 117 Vietnamese participants (not including ACON test) showed the tests performed well, but one test had a false-negative rate of 3-4% [200]. In our study with larger sample size we found significant difference in test performance; there may be some uncontrolled variables that may influence the test outcomes such as prozone effects and genotype difference. This means that the use of rapid tests must be questioned. One can predict that the use of rapid test have resulted in transfusion of infectious blood to patients.

THE PREVALENCE ESTIMATES

The actual study documents high prevalence of HBV infection in the rural study area in Vietnam and thus confirms the findings in previous smaller epidemiological studies conducted in rural as well as urban Vietnamese populations from which prevalence rates of HBV infection were reported in the range of 8% to 25% [21,22]. Due to the size of our present study samples, we believe that the prevalence estimates are solid with narrow confidence limits for the studied population of potential blood donors. Although there was a gender bias in the main sample, the actual study indicates that gender is a risk variable, men having a higher risk of HBV infection among studied population than women.

The study also reports a high prevalence of anti-HBc (51.7%) in the study area consistently with previous survey estimates from Thanh Hoa province in Vietnam (49.7%) [21]. Blood samples being HBsAg negative and anti-HBc positive (42.2%) indicate that HBV infections have been endemic in the study area for some time. We also found higher prevalence of anti-HBc in the less developed community. However, the difference was moderate (10%) and hardly of medical significance. One may question the risk of HBV transmission from HBsAg negative and anti-HBc positive blood donors in the study population. The accurate estimates cannot be given because the present study participants could not be tested for HBV DNA due to limited resources. The rate of HBV DNA detected in HBsAg negative samples is reported to vary with the prevalence of HBV infection: less than 5 per cent in low HBV infection such as European countries and as high as 24 per cent in high HBV prevalence areas [4,17,201].

Out of 1,200 studied samples, only two were anti-HCV positive with EIA technique. Of the two, one was also positive for both HBsAg and anti-HBc; the other was HBsAg negative and anti-HBc positive. Two more anti-HCV positive samples were found with CMIA technique in Norway. The finding of HCV infection in our study area corresponds well with prevalence estimates at 0.4% HCV infection reported in two different districts of Thanh Hoa province, Vietnam [149]. The two clearly missed anti-HCV positive blood samples by EIA picked up by CMIA analysis may be due to test properties. There are HCV genotype differences between Western countries as East Asia. Genotype C6 of HCV is found to be dominant in South East Asia, China and South Africa whereas it is less common in other part of the world [67]. The test in use was developed by a Western company, and it may be that this test did not fully catch the antibodies produced by the genotypes in the actual study population.

An important problem is the risk of virus transmission from OBI donors and/or blood donors collected during window period where HBsAg is undetectable and cases where there is a false negative test result. In the actual study population the rate of HBsAg negative, anti-HBc positive participants was high (42.2%) which raises the question of how many of these are also OBI cases and potential transmitters of HBV infection? Accurate estimates cannot be given because the actual study participants due to resource restrictions could not be tested for HBV-DNA. The rate of HBV DNA detected in HBsAg-negative samples is reported to vary with the prevalence of HBV infection. Less than 5% of HBsAg negative, anti-HBc positive blood donor samples are reported to have detectable HBV DNA in European countries with low HBV prevalence, whereas the corresponding rate is as high as 24% in areas with high HBV prevalence [4,17,201].

We report a method to estimate the OBI in Vietnam by a stochastic Monte Carlo model approach to perform risk assessment for transfusion-transmitted hepatitis B. The model estimates OBI from HBsAg negative and anti-HBc positive blood donors in Vietnam at the range from approximately 660 to 2500 per million blood units using the producer-specific test information. In Vietnam around 50% of the population is anti-HBc positive. If we assume that individuals in this subpopulation of blood recipients are immune-competent, the risk of HBV transmission is probably not higher than 10% (Allain JP, 2011, personal communication). Assuming that the risk of post transfusion HBV from OBI donors is 10% as maximum, we then estimate the risk of post transfusion HBV to be 66 to 250 per million, which is consistent with the residual risk in transfusion-transmitted HBV transmission reported in other high endemic HBV prevalence areas [119]. The prevalence of HBsAg in first time blood donors in Hong Kong (approximately 8%) was similar to that found in our potential first time donors in the actual study in Vietnam; however, over the last decades the prevalence of HBsAg in Hong Kong has been reduced to 1.83% (Ultrio screening period 2007-2009) and 1.13% (Ultrio Plus screening period 2009-2011). In these periods the Ultrio and the more sensitive Ultrio Plus assay detected OBI yield rates of 130 and 335 per million first time donations, respectively [195]. When adjusted for the HBsAg carrier rate (114 /1200=9.5%) in our present study one would expect a (8: 1.83) x (9.5:8) = 5.2 (Ultrio screening period) and (8: 1.13) x (9.5:8) = 8.4 (Ultrio Plus screening period) higher OBI yield rate in Vietnamese first time donors compared with Hong Kong first time donors. When the HBsAg carrier rate indeed corresponds linearly to the OBI rate in South East Asia one would predict an OBI yield rate of 130 x 5.2= 676 per million for the Ultrio assay and equivalent to 335 x 8.4= 2814 for the more sensitive Ultrio Plus assay. These data is corresponding to the estimates of the Monte Carlo model based on an estimated OBI rate of 1:1000.

A recent multicentre look back study estimated 24% probability of OBI transmission by red blood cells (RBC), 85%-100% by fresh frozen plasma (FFP), 51% by platelet concentrates (PCs), and 48% for all blood products from OBI donors that are anti-HBs positive (50% OBI carriers are anti-HBs positive with concentration more than 10 mIU/mL) [202]. If half of the estimated 676 per million OBI donors (based on extrapolations from Ultrio Hong Kong data) were potentially infectious (anti-HBs negative) and 50% of recipients in Vietnam would be susceptible (anti-HBc positive/anti-HBs prevalence unknown) one would predict that 0.50 x 0.5 x 0.24 x 676 = 41 OBI transmissions per million RBC recipients would occur in Vietnam. If the Ultrio Plus instead of the Ultrio

OBI yield rate in Hong Kong first time donors is used for the projections, the transmission rate would be 0.5 x 0.5 x 0.24 x 2814 = 169 OBI transmissions per million RBC recipients. These estimates are somewhat lower than the 60-250 cases estimated by the Monte Carlo modelling. However, taking the OBI transmission from all blood product (48% vs. 24%), OBI yield rate would be double (82 and 338 transmission per million respectively for Ultrio and Ultrio Plus assays). These estimates would be higher for PCs and FFP recipients in Vietnam. It is clear that the estimate depends on the input parameters and further study on OBI transmission in Vietnam is recommended.

More importantly, false negative HBsAg blood samples are observed at a mean of 298 (5-95 percentile: 14-893) blood units per million in the model at producer-claimed sensitivity If more realistic test properties are taken into consideration, the mean rate of false negative HBsAg is found to be 1,087 per million blood units (5-95 percentile: 762-3220) as lower test sensitivity at local laboratories was reported in Cambodia and Vietnam [203,204]. This shows the importance of test sensitivity and demonstrates that low HBsAg test sensitivity is the main cause of transfusion-transmitted HBV infections in Vietnam.

It is documented that HBV transmission can spread through HBsAg negative and anti-HBc positive blood donors to recipients. Under the current context of Vietnam, HBsAg screening is a routine practice in blood banks, anti-HBc screening and/or NAT can be performed, but in a limited scale in capable laboratories under the approval of health authorities. Data on OBI prevalence in Vietnam is limited. It is essential to perform some pilot studies on the prevalence of anti-HBc in HBsAg negative blood donors and testing for HBV DNA on those potential OBI blood donors to get precise estimate of OBI in blood setting for future blood safety.

CONCLUSIONS AND RECOMMENDATIONS

Our study demonstrates that low sensitivity of the actual rapid tests for HBsAg, anti-HBc and anti-HCV make them useless for blood donor screening in rural Southeast Asia. Rapid tests may be useful screening tools in blood transfusion services in low-resource settings, but tests should be carefully validated locally before being used for screening purposes since test performance varies by location.

The prevalence study shows that hepatitis B virus infection is endemic in rural areas of Vietnam and that almost half the population is or has been infected with HBV. Hepatitis C infection is rare, but false negative test results cannot be ruled out. The results also indicate that the EIA performance in blood donor screening in Vietnam may be sub-optimal, missing 2.5% of hepatitis B virus carriers and falsely excluding more than 7% of blood donors. As the prevalence of hepatitis B infection is high, occult hepatitis B infection may represent a threat to safe blood transfusion. We previously reported that Nucleic Acid Amplification Testing for HBV should be considered for blood donor screening in Vietnam in the future. However, using Monte Carlo modelling to develop a solid risk estimate of transfusion transmitted HBV from OBI we found that the dominant risk of post transfusion comes from HBsAg false negative blood donors and blood donors with OBI. In order to reduce the risk of HBV infection after transfusion, the focus should be to implement the best possible locally validated HBsAg test rather than spending resources on expensive NAT testing. Implementation of anti-HBc screening is not feasible at present since 50% of the donors would be deferred.

RECOMMENDATIONS FOR FUTURE STUDIES

In Vietnam and some other countries in Southeast Asia, EIA test for HBsAg is compulsory to perform for blood donors in addition to anti-HCV, HIV, malaria, syphilis; however, current EIA test for HBsAg detection of blood donors in Southeast Asia documents that false-negative test for HBsAg due to low sensitivities imposes a dominant risk of post HBV transmitted transmission. In the meantime, best possible locally validated HBsAg EIA assays are optimal for HBsAg detection than anti-HBc tests as well as costly NAT in Southeast Asia in general and in Vietnam in particular. It is recommended that key laboratories at national level and at regional level should continuously monitor the test quality and take into account the analytical sensitivity for HBsAg assays including EIA and rapid tests; check the relevant HBV variants circulating for a given population. It is preferable to select more sensitive EIA test for HBsAg detection as low sensitive HBsAg assays are the predominant cause of transmitted-transfusion HBV in the region.

Rapid tests can be used for first-line screening for blood donors to exclude all positive test results in remote areas. Then the accumulated negative blood donors in some remote areas should be gathered and confirmed by EIA tests in high laboratories to identify the false negative test results. Not all blood donors in Southeast Asia are first time donors, and repeat testing in duplicate in first time and/or regular donors not only declines the potential infectivity among blood donors, but is also likely to increase the amount of eligible blood products. This may even be more cost-effective because the cost of discarding positive blood bags is likely to be higher than the reagent cost of extra testing.

The present study attempts to extrapolate information from rather limited studies in Quang Tri province and relevant OBI prevalence to form a national pattern for estimating risk for transfusion-transmitted hepatitis B in Vietnam as currently we have limited data elsewhere from Vietnam. Further studies of OBI in Vietnam are recommended in blood transfusion settings in order to more precisely estimate the OBI prevalence for future strategy of transfusion safety. Our simulation model can be used and developed for this purpose, as more data will be made available.

It is documented that transfusion-transmitted infectious diseases are severe side effects of blood transfusions. There are several solutions to make blood transfusion safer. First of all, transfusions must be given to patients on strict indications and there is a room for improvement on evidence-based use of blood and blood components. Secondly, blood products from non-remunerated voluntary repeat donors (NRVRD) are safer than from family and replacement donors. Vietnam government has made efforts to build up Regional and Sub-regional Blood Transfusion Centres across the country to increase number of NRVRD and should work toward 100% NRVRD which always must be kept in mind for politicians and health care workers. Also, donor selection may be a challenge in areas endemic for hepatitis, malaria and dengue fever. What is considered proper donor selection in Vietnam may result in the exclusion of too many donors and the lack of sufficient blood supply in the country. Lastly, pathogen reduction of all donated blood units should also be considered. This method is not available for whole blood yet and is in general too expensive for low-resource countries. However, in the future, pathogen reduction may be a measure that will further increase the safety of blood transfusion.

HBV vaccines have been integrated in National Universal Immunization Program of infants in Vietnam since 2003. Vietnamese blood donors can be currently divided into two groups: a) non-vaccinated and non-infected, and b) non-vaccinated but being infected or potential OBI cases. Recipients can be grouped as a) non-vaccinated and non-infected; b) non-vaccinated but infected or being potential OBI cases; and c) vaccinated children less than 10 years old. Blood from non-vaccinated and non-infected donors is safe for all three groups of recipients. Blood transfusion from non-vaccinated but sero-positive donors may include OBI blood. If donors have a level of anti-HBs≥ 100 IU/ml, the transfusion is considered safe for all three groups of recipients. Quality control studies to evaluate the effect of an infant vaccination program on transfusion safety should be performed.

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