การตรวจหา ดี เอ็น เอ ของเชื้อไวรัสตับอักเสบ บี ในซีรัมที่ให้ผลบวกเฉพาะ Anti-HBc โดยวิธี Ultrasensitive PCR

นางสาวนิรมล ธรรมาเจริญราช

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาจุลชีววิทยาทางการแพทย์ (สหสาขาวิชา)
บัณฑิตวิทยาลัย จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2547
ISBN 974-53-1161-8

DETECTION OF HEPATITIS B VIRUS FROM PATIENTS WITH ANTI-HBC AS THE ONLY MARKER OF HBV INFECTION BY ULTRASENSITIVE PCR

Miss Niramol Thammacharoenrach

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Medical Microbiology (Inter-Department)
Graduate School
Chulalongkorn University
Academic Year 2004

ISBN 974-53-1161-8

Thesis Title	Detection of Hepatitis B virus from patients with Anti-HBc as the only marker of HBV infection by ultrasensitive PCR
By	Miss Niramol Thammacharoenrach
Field of study	Medical Microbiology
Thesis Advisor	Thaweesak Tirawatnapong, Ph.D.

Accepted by the Graduate School, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

…..…….. Dean of the Graduate School
(Assistant Professor M.R. Kalaya Tingsabadh, Ph.D.)

THESIS COMMITTEE

…….. Chairman
(Associate Professor Somatat Wongsawang, Dr. Med. vet.)

…….……. Thesis Advisor
(Thaweesak Tirawatnapong, Ph.D.)

…….. Member
(Associate Professor Wasan Chantratita, Ph.D)

นิรมล ธรรมาเจริญราช : การตรวจหา ดี เอ็น เอ ของเชื้อไวรัสตับอักเสบ บี ในซีรัมที่ให้ผลบวกเฉพาะ Anti-HBc โดยวิธี Ultrasensitive PCR (DETECTION OF HEPATITIS B VIRUS FROM PATIENTS WITH ANTI-HBC AS THE ONLY MARKER OF HBV INFECTION BY ULTRASENSITIVE PCR)อาจารย์ที่ปรึกษา : ดร.ทวีศักดิ์ ตีระวัฒนพงษ์ 74 หน้า ISBN 974-53-1161-8

ปัจจุบันการตรวจวินิจฉัยการติดเชื้อไวรัสตับอักเสบ บี (HBV) ใช้วิธีทาง Serological diagnosis ซึ่ง marker ที่สำคัญ คือ HBsAg, Anti-HBs และ Anti-HBc ซึ่งสามารถวินิจฉัยได้ว่ามีหรือไม่มีการติดเชื้อไวรัสตับอักเสบ บี การให้ผลบวกเฉพาะ Anti-HBc เพียงอย่างเดียวนั้น ส่วนใหญ่แปลผลว่าเคยได้รับเชื้อมาก่อน แต่ได้มีการศึกษาทางชีวโมเลกุล พบว่าบางรายตรวจพบเชื้อไวรัสตับอักเสบ บี ได้ สาเหตุที่ผลทาง Serology ไม่สามารถตรวจพบ HBsAg ได้นั้นอาจเกิดขึ้นจากมีเชื้อไวรัสปริมาณน้อยเกินกว่าที่จะตรวจพบ, มีการติดเชื้อไวรัสตับอักเสบ ซี ร่วมด้วย และการเกิด Mutation ของ S-gene โดยเฉพาะตำแหน่ง “a”determinant ซึ่งเกี่ยวข้องกับการสร้าง HBsAg ดังนั้นการศึกษาในครั้งนี้ จึงนำเทคนิค nested PCR และ Ultrasensitive PCR มาช่วยในการตรวจหาเชื้อไวรัสตับอักเสบ บี ในสิ่งส่งตรวจที่ให้ผลบวกเฉพาะ anti-HBc เพียงอย่างเดียว สิ่งส่งตรวจจากการศึกษานี้ได้มาจากผู้ป่วยที่เข้าตรวจและรักษาในโรงพยาบาลจุฬาลงกรณ์ ระหว่างเดือน กรกฎาคม 2546 ถึง พฤษภาคม 2547 จำนวน158 ตัวอย่าง ผลการศึกษาพบ สิ่งส่งตรวจ 8 ราย (5.06%) ที่ให้ผลบวกเมื่อตรวจโดยใช้เทคนิค PCR ซึ่ง 4 ราย (50%) ของการตรวจพบ HBV DNA มาจากการใช้เทคนิค ultrasensitive PCR และเมื่อนำไปตรวจวัดปริมาณไวรัสด้วย Cobas Amplicor HBV Monitor™ Test (Roche diagnostic, USA) ได้ค่า <200 copies/ml แสดงว่า มีปริมาณไวรัสน้อยจนไม่สามารถตรวจพบ HBsAgได้ ซึ่งการใช้เทคนิค ultrasensitive PCR สามารถเพิ่มประสิทธิภาพในการตรวจเชื้อ HBV ได้ สำหรับการตรวจ Anti-HCV พบผลบวกต่อ Anti-HCV ในกลุ่มตัวอย่างจำนวน 10 ตัวอย่าง(6.33%) ซึ่งทั้ง 10 ตัวอย่างตรวจไม่พบ HBV DNA แสดงว่า การติดเชื้อไวรัสตับอักเสบ ซี ไม่มีผลเกี่ยวข้องต่อการสร้าง HBsAg ส่วนตัวอย่างที่ตรวจพบ HBV DNA นำมา sequence ส่วนของ S gene เพื่อหา mutation ที่ตำแหน่ง “a” determinant พบตัวอย่าง 5 ใน 8 ราย เกิด amino acid substitution ที่ตำแหน่งแตกต่างกันไป ดังนั้นการเกิด mutation ในส่วนของ “a” determinant อาจเป็นสาเหตุหนึ่งที่ทำให้ตรวจไม่พบ HBsAg แต่สาเหตุหลักที่น่าจะเป็นไปได้ คือ ไวรัสมีปริมาณน้อยเกินกว่าที่จะสามารถตรวจด้วยวิธีทาง serological assays ได้

สาขาวิชา จุลชีววิทยาทางการแพทย์ (สหสาขาวิชา)... ลายมือชื่อนิสิต.....

ปีการศึกษา ...2547..... ลายมือชื่ออาจารย์ที่ปรึกษา.....

4589100420 : MAJOR MEDICAL MICROBIOLOGY
KEY WORD : HEPATITIS B VIRUS / ULTRASENSITIVE PCR / ANTI-HBC /MUTATION
NIRAMOL THAMMACHAROENRACH : DETECTION OF HEPATITIS B VIRUS FROM PATIENTS WITH ANTI-HBC AS THE ONLY MARKER OF HBV INFECTION BY ULTRASENSITIVE PCR
THESIS ADVISOR: THAWEESAK TIRAWATNAPONG, Ph.D., 74 pp
ISBN 974-53-1161-8

The serological diagnosis is the major method to determine hepatitis B virus (HBV) infection. The major serological markers for HBV are HBsAg, Anti-HBs and Anti-HBc. The presence of anti-HBc as the only serological marker for HBV is usually interpreted as follow; low titer of HBV, interference of HBsAg synthesis by coinfection with HCV and mutation of S gene especially in “a” determinant in HBsAg and the past history of infection with undetectable anti-HBs. In order to test this hypothesis we develop ultrasensitive PCR for HBV DNA detection. The clinical specimens are from patients in King Chulalongkorn Memorial Hospital during July 2003 to May 2004. Of the 158 specimens, 8 (5.06%) were HBV DNA positive. Half of the HBV DNA positive samples were detected by ultrasensitive PCR method. All samples have viral load <200 copies/ml when detected by Cobas Amplicor HBV Monitor™ Test (Roche Diagnostic,USA). The ultrasensitive PCR can increase the detection sensitivity up to 4 fold. Interference of HCV infection is not involved in our study. The mutation in “a” determinant were found in five samples with amino acid substitution in many positions. According to our finding, the most probable reason of undetectable HBsAg in only anti-HBc positive marker is the very low virus titer which is below the detection limit of the serological assays.

Field of study…Medical Microbiology…. Student’s signature……..
Academic year…2004……... Advisor’s signature…….

ACKNOWLEDGEMENTS

I would like to express my sincere appreciation and gratitude to the following whose gave me the possibility to complete my thesis.

Thaweesak Tirawatnapong,Ph.D., my thesis advisor at the Division of Immunology, Department of Microbiology, Faculty of Medicine, Chulalongkorn University, for his guidance, general suggestion, discussion, contractive criticism, kindness and strong encouragement throughout the course of this study.

My grateful appreciation is extended to Associated Professor Dr. Sommatat Wongsawang and Associated Professor Dr. Wasan Chantratita, my thesis committee, for their valuable discussion and suggestions.

I sincerely thank Abbott Diagnostic (Thailand) to supported MEIA kits (AxSYM® HBsAg[V2], Abbott Diagnostic, Germany)

Special thank are also extended to all member in inter-department of Medical Microbiology, staffs and personnel in the Department of Microbiology, Faculty of Medicine, Chulalongkorn University and Biomolecular Laboratory (Thailand) for providing of facilities needed, and strong encouragement.

I am deeply indebted to my family and my friends for their support, patience, help, cheerfulness, encouragement, and understanding.

Finally, I am grateful to Graduate school, Chulalongkorn University, for giving me opportunity to be a part of this program and funding this research.

CONTENTS

Page

CONTENTS

Page

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

ABBREVIATIONS

HBV	=	Hepatitis B Virus
HCV	=	Hepatitis C Virus
HBsAg	=	Hepatitis B surface antigen
Anti-HBs	=	Antibodies to HBsAg
HBcAg	=	Hepatitis core antigen
Anti-HBc	=	Antibodies to HBcAg
HBeAg	=	Hepatitis e antigen
Anti-HBe	=	Antibodies to HBeAg
Anti-HBcIgM	=	Antibodies to HBcAg IgM class
Anti-HCV	=	Antibodies to hepatitis C virus
DNA	=	Deoxynucleic acid
dNTPs	=	Deoxynucleotidetriphosphate
mg/L	=	Milligram per liter
min	=	Minute
sec	=	second
hr	=	hour
ml	=	Milliliter
mM	=	Millimolar
pmole	=	Picomole
PCR	=	Polymerase Chain Reaction
µl	=	Microliter
ug	=	Microgram
rpm	=	Round per minute
UV	=	Ultraviolet
ng	=	Nanogram
nm	=	Nanometer
bp	=	Base pair
kb	=	Kilobase pair

Arg (R)	=	Arginine
Gly (G)	=	Glycine
Cys (C)	=	Cysteine
Pro (P)	=	Proline
Met (M)	=	Methionine
Val (V)	=	Valine
Ile (I)	=	Isoleucine
Thr (T)	=	Threonine
Asn (N)	=	Asparagine
Leu (L)	=	Leucine
Glu (E)	=	Glutamic Acid
Phe (F)	=	Phenylalanine
Tyr (Y)	=	Tyrosine
Ser (S)	=	Serine
Asp (D)	=	Aspartic Acid

CHAPTER I

INTRODUCTION

Hepatitis B virus (HBV) is the member of hepanaviridae family, which is the smallest DNA virus. It's genome is 3.2 kb in length and partially double-stranded. The hepatocytes are the major cell types in the liver which are the primary site of viral infection. Hepatitis B viral infection is the major cause of acute and chronic hepatitis, cirrhosis and hepatocellular carcinoma. Most of the acutely infected adults recover spontaneously and completely from acute HBV infection but about 5% of HBV infected healthy adults become chronic carriers and progressive liver disease leading to hepatic failure. The member of HBV carriers was reported to be more than 350 million worldwide. The mode of HBV transmission and their relative importance vary in different regions of the world. In highly endemic regions (>7% HBsAg prevalence),transmission occur mainly perinatally or in early childhood. By contrast, most infections are acquired during early adult life in low endemic regions (<2% HBsAg prevalence).

The major serological markers for HBV are HBsAg, Anti-HBs, Anti-HBc, Anti-HBcIgM, HBeAg and Anti-HBe, The markers can be used to interprete the different stages of HBV infection from the specific patterns of these markers. Samples with negative HBsAg but positive Anti-HBc by conventional serological assays are able to transmit the infection, both after transfusion and after organ transplantation. The presence of Anti-HBc alone is not clear. The interpretations for this only marker are low level of HBV replication inside the hepatocyte with undetectable HBsAg, window phase of acute HBV infection, interference of HBsAg synthesis by HCV infection and variation in the S region, especially in "a" determinant which is exposed at the surface of HBV particles and recognized by Anti-HBs.

In this study, we used molecular technique to detect viral DNA from patients with only Anti-HBc as the serological marker. The conventional nested PCR and the ultrasensitive PCR are also used in this study. The ultrasensitive PCR is the modification of conventional nested PCR in the nucleic acid preparation step, by using 1 ml of serum and ultracentrifuged at 50,000 rpm for 1 hour. This additional step is intended to concentrate virus that presence in very low amount in order to detect the mutation in “a” determinant. The samples which are positives by PCR will be quantitated by Cobas Amplicor HBV Monitor^{TM} Test (Roche Diagnostic, USA) to validate their low copy number and the S region will be sequenced and compared with wild type virus.

CHAPTER II

OBJECTIVES

1. To detect HBV by molecular technique from serum with anti-HBc as the only marker of HBV infection
2. To detect low-level HBV by ultrasensitive PCR
3. To study HCV infection involved with HBsAg synthesis
4. To analyze mutation in genomic sequence of S gene in the “a” determinant

CHAPTER III

LITERATURES REVIEW

HISTORY

Viral hepatitis remains a globally important disease. It was first described in the fifth century BC. When Hippocrates described epidemic jaundice, he was undoubtedly referring to persons infected with acute hepatitis B virus (HBV) as well as other agents capable of infecting the liver. Epidemics of jaundice have been described throughout history and were particularly common during various wars in the 19th and 20th centuries. While many of these outbreaks were due to hepatitis A virus, it was likely that the epidemic transmission of hepatitis B virus also occurred in settings where the use of blood-containing products was common [1].

The recognition of a form of hepatitis that was transmitted by direct inoculation of blood or blood products was first documented by Lurman in Bremen, Germany, in 1883, during a smallpox immunization campaign. Thousands of people received vaccine that had been prepared from human lymph. Of 1,289 shipyard workers who received this vaccine, 191 (15%) developed jaundice several weeks to 8 months later; jaundice did not occur among unvaccinated workers. In the first part of the 20th century, outbreaks of “long-incubation” hepatitis were described in a variety of risk groups including persons who attended clinics for venereal diseases, diabetes, and tuberculosis; patients who received blood transfusions; persons inoculated with mumps or measles convalescent-phase serum; and military personnel who received yellow fever vaccine during World War II. The outbreak in yellow fever vaccine recipients was linked to a specific lot of vaccine that contained human serum. A follow-up study in the 1980s demonstrated that 97% of recipients of the serum-containing vaccine had serologic evidence of HBV infection as compared to 13% of persons who received yellow fever vaccine that did not contain human serum, confirming that HBV was the cause of this outbreak[1].

In 1947, MacCallum and Bauer proposed the current nomenclature of hepatitis A for infectious hepatitis and hepatitis B for “homologous serum” hepatitis. At that time, it was known that the epidemiology of the two diseases differed. Hepatitis A was transmitted by the fecal-oral route, had an incubation period of 2 to 6 weeks, and was primarily a disease of younger children. In contrast, hepatitis B was transmitted by percutaneous exposure to blood products, had a longer incubation period (ranging from 2 to 6 months), and occurred more often in adults[1].

VIROLOGY

The viral etiology of hapatitis B was established by electron microscopy (referred to as Dane particles) that reacted with antisera to Australia antigen. So, it was demonstrated that the Dane particle was HBV. Hepatitis B virus is a double-stranded, enveloped DNA virus of the Hepadnaviridae family,Orthohepadnavirus genus.The hepadnaviruses (*hepa* from *hepa*totropic, *dna* from their *DNA* genome) share with retroviruses the property of encoding RT and replicating via an RNA-to-DNA step. However, they package DNA in the virion. The process of reverse trancription shares features that have been described for the retroviruses but difference in many important details are also documented.

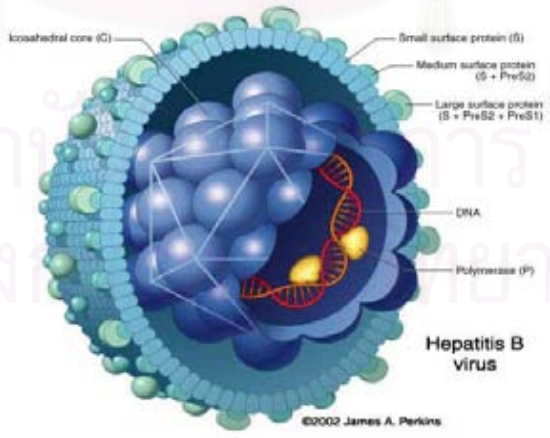


Figure 1. Hepatitis B virus

The hepadnavirus replicates in the liver and causes hepatic dysfunction. It is the smallest DNA virus known: it has only 3200 bp in its genome and about 42 nm in diameter, partly double stranded and circular pattern (Fig1). HBsAg is found on the surface of the virus and is also produced in excess amounts. The free HBsAg are circulating in the blood as 22 nm spherical and tubular particles (Fig2).

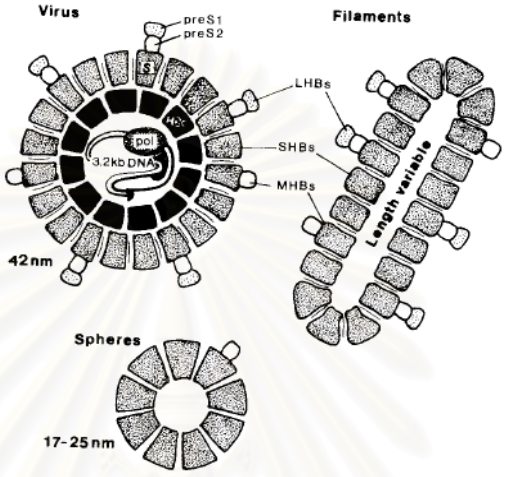


Figure 2. Schematic diagram of hepadnavirus particles

The minus (-) strand of the DNA is almost a complete circle and contains overlapping genes that encode both structural proteins (pre-S, surface and core) and replicative proteins (polymerase(P) and X protein). The plus (+) strand of the DNA is shorter and is variable in length (Fig3)[1,2].

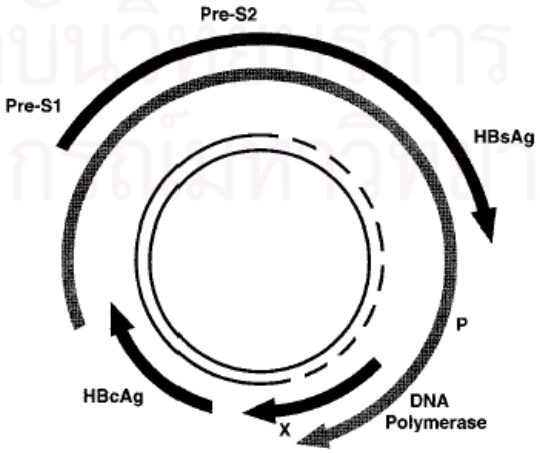


Figure 3. Structure and organization of the HBV genome

Hepatocytes are the major cell type in the liver, it is also expected that they are the major target of infection by a hepato-tropic virus such as HBV. After HBV infection to a cell, the virion core enters the cytoplasm, viral DNA is imported into the nucleus and repaired to produce a covalently closed circular molecule(called cccDNA). The hepadnaviral DNA is not normally integrated into the host's genome. However, cccDNA persists in the nucleus as an autonomous episome from which viral RNAs are transcribed by the host cell RNA polymerase II. Four mRNA transcripts of known function have been identified as being involved in HBV transcription and translation. The longest genome (3.5 kb) is the template for genome replication and the expression of precore/core and polymerase proteins. A 2.4 kb transcript encodes pre-S1, pre-S2 and HBsAg, while a 2.1 kb transcript encodes only pre-S2 and HBsAg. The smallest transcript (0.7 kb) encodes the X protein. P protein provides enzymatic and other activities required for reverse transcription. Following reverse transcription, the DNA-containing nascent core particles can follow one of two pathways. The first pathway happens late in infection, when the cisternae of the endoplasmic reticulum contain an abundance of viral envelope glycoprotein, they can be bud into the endoplasmic reticulum and eventually be secreted as progeny virions. Alternatively, if they do not become enveloped, the core particles can be recycled to the nucleus, where they give rise to additional copies of covalently closed circular DNA. The amplification pathway predominates at early times after infection when there is little envelope protein available. Eventually, 10 to 20 covalently closed circles accumulate in the nucleus and, a steady-state balance of DNA and cytoplasmic virion protein component is maintained [1,2].

The core and polymerase genes are essential for viral DNA replication. The envelope proteins, which consists of two or three subspecies depending on the hepadnavirus, are essential for envelopment of nucleocapsids. Two additional gene products that are expressed during natural infections (hepatitis X protein (HBx) and hepatitis e-antigen (HBeAg)) are of unknown function. The core gene encodes the viral capsid protein, known as hepatitis B virus core protein(HBcAg).

The surface/pre-S gene encodes for the virus envelope. The major protein that forms the HBsAg particles is the smallest gene product (SHBs). The middle protein (MHBs) which contains the pre-S2 component, and the large surface protein (LHBs) which contains pre-S1, are also incorporated into HBsAg particles but are found in larger proportions in the intact virus particles. The specialized functions of these proteins have been the subject of intense study. It is suggested that the pre-S proteins play an important role in the attachment of HBV to hepatocytes. Liver-specific attachment sites have been identified in vitro for pre-S1 and pre-S2. In addition, pre-S2 attaches to artificially polymerized human serum albumin.

All three envelope components are glycosylated. Type II transmembrane proteins that can form multimers are stabilized by disulfide bridges formed by cysteine residues present in the S domain. L and M are present in roughly equal amounts in Dane particles and together constitute approximately 30% of the envelope protein content. S, by itself, and together with the larger envelope proteins, also forms filamentous and spherical “surface antigen” particles that are secreted from infected cells in at least 100-fold excess over virions. These spheres and filaments can be accumulated to concentrations of several hundred micrograms per milliliter in the blood of HBV-infected patients. Complexes of these particles with their cognate antibodies are probably responsible for the immune complex syndromes that sometimes occur during transient infections. Antibodies to surface antigen particles composed of S protein alone are sufficient to provide protection against HBV infection. However, there is good reason to believe that the pre-S1 domain is, at least in part, the substrate for the still elusive viral receptor. Epitopes in pre-S1 displayed on the outside of surface antigen can also elicit virus-neutralizing antibodies and alter the host range of the virus upon genetic recombination[2].

Figure 4. Schematic diagram of two loops of the "a" determinant of HBS protein.

The surface gene encodes for two loops that located at external surface of the virus. These exposed loops are amino acid 124-137 and 139-147. Which are important to induce the antibodies(Anti-HBs) referred to as "a" determinant (Fig4)[3]. The "a" determinant is now considered to be within a larger antigenic area called the major hydrophilic region (MHR). Two other determinants of HBs have been described. One determinant has either a, d or y specificity, and the other has w or r. There are four major subtypes, termed adr, adw, ayw and ayr. Either lysine(K) or arginine (R) at position 122 and 160 (Fig5) is the major in defining an antigenic subtype [4].

Prior to the definition of the genotypes, HBV strains were distinguished by serological analysis into nine hepatitis B surface antigen (HBsAg) subtypes designated *ayw1*, *ayw2*, *ayw3*, *ayw4*, *ayr*, *adw2*, *adw4q–*, *adrq +*, and *adrq–*[5,6]. The molecular bases for the *d*/*y* and *w/r* variations were both shown to depend on Lys/Arg substitutions at residues 122 and 160, respectively . By sequencing codons 101–180 of 44 small S genes of HBV strains with known subtype, residue 127 was found to be important for the *w1–w4* variations, and strains specifying *w1*/*w2*, *w3,* and *w4* encoded Pro, Thr, and Leu, respectively, at this position. The expression of *w1* also depends on Arg122, Phe134, and/or Ala159 . Another determinant designated *q* is expressed by most HBV strains apart from strains encoding *adw4* and some strains encoding *adr*. Residues that are important for the expression of *q* are suggested to reside at residues 177 and 178 [5]. The identification of the critical residues for subtype expression enables subtyping of most strains by sequencing. However,

subtypes with new specificity combinations remain putative until confirmed by serological typing.

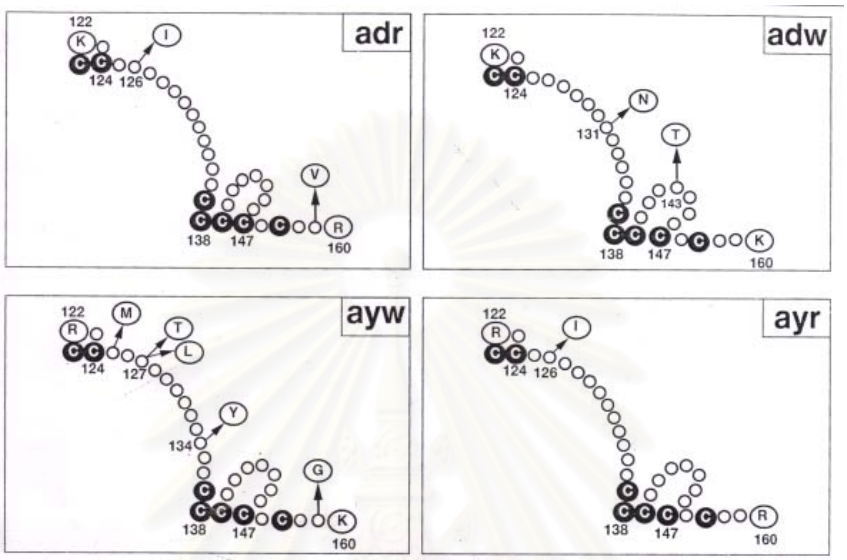


Figure 5. Subtype variability in the region between amino acid 122 and 160

Recently, HBV has been classified into eight genotype , A to H. There is a correlation between subtypes and HBV genotypes, although several subtypes are encoded by more than one genotype. Strains specifying *adw2* occur mainly in genotypes A, B, and G, but may also be specified by genotype C and D strains, and genotype A and B strains may also specify *ayw1*. All strains specifying *adr* and *ayr* belong to genotype C. Strains specifying *adw4q–* occur only in genotypes F and H. Strains specifying *ayw2* and *ayw3* are only found in genotype D, while strains specifying *ayw4* occur in genotype E [5].

HBV genotypes show characteristic distribution: genotype A is pandemic but most prevalent in northern Europe, North America and central Africa, The strains comprising genotype B originated mainly from China, Japan, and Southeast Asia(Vietnam, Thailand and Indonesia). Genotype C can be found in eastern Asia, Korea, China, Japan, Polynesia and Vietnam. Genotype D is also predominant in the Mediterranean area, the Middle East and India. Genotype E is typical for Africa. Genotype F is found in American natives and in Polynesia. Genotype G can be found in western Europe and North America and genotype H is predominant in Central America. The most common subtype and genotype of Thai people with HBV infection are adr (~60%), adw2 (~40%) and genotype C (60-70%), B (30-40%), A (<5%). HBV infection in Thailand is an intermediate endemic level, with 8-10% HBV of males infected and 6-8% of females infected [7,8,69].

HBV surface component was designated hepatitis B surface antigen (HBsAg). The core component contains endogenous DNA and hepatitis core antigen (HBcAg). The antibodies to HBsAg (Anti-HBs), Antibodies to HBcAg (Anti-HBc) were used to classify patients as having acute or chronic infections. IgM class antibodies to HBcAg (Anti-HBcIgM) is related to early infection. Hepatitis e antigen (HBeAg) is related to viral infectivity.

TRANSMISSION

HBV is transmitted by percutaneous or permucosal exposure to infectious body fluids e.g. blood, semen, breast milk and in some circumstances, saliva. People most at risk include: anybody who has unprotected sexual intercourse; IV drug users who share needles and syringes; health care workers in contact with potentially contaminated blood or body fluids; babies born to mothers with the virus; anyone in intimate contact with the infected person. Many cases of acute hepatitis B occur sporadically with no known source and studies have shown that prior unrecognised infection is common.

Chronic infection acquired at birth is thought to be the major mechanism by which the virus persists in nature. Up to 90% of babies born to mothers who are acutely or chronically infected with HBV and positive for HBeAg will be infected by HBV, and most of these will become chronically infected. In the United States, there are an estimate of 1.2 million carriers of HBV, and there are an estimate of 350 million. Worldwide the fraction of the population chronically infected with HBV varies from 0.1-0.5% in developed countries to 5-15% in Southeast Asia and sub-Saharan Africa.

The incubation period of hepatits B is average to be 120 days (range 45-160 days). Constitutional symptoms such as malaise and anorexia may precede jaundice by 1-2 weeks. Clinical symptoms and signs include nausea, vomiting, abdominal pain and jaundice. Skin rashes, joint pains and arthritis may occur. The case-fatality rate is approximately 1%. Acute HBV infection causes chronic (long-term) infection in 30-90% of persons infected as infants or children and in 6-10% of adolescents and adults. Chronic infection can lead to chronic liver disease, liver scarring (cirrhosis) and liver cancer.

Liver cancer causes more than 500,000 deaths a year worldwide, and about 90% of primary malignant tumors of the liver are hepatocellular carcinoma (HCC). HCC is more common in men than women, by 4 to 1, and is in the top 10 in frequency of cancers in humans. HCC is more common in regions that exhibit high chronicity for HBV. The association of HBV with HCC is clearly shown by data such as the finding that in areas in which chronic infection occurs in 5-10% of the population, 50-80% of HCC patients are chronically infected with HBV. It seems reasonable to conclude that chronic HBV infection contributes to a large fraction of HCC cases.

DIAGNOSTIC

Hepatitis B surface antigen is one of the most important serological markers used to diagnose an HBV infection. It is a polypeptide of varying size and is a component of the external envelope of the hepatitis B virus particle (HBV). The blood of persons infected with HBV contains, in addition to intact infectious HBV particles, smaller non-infectious “empty” envelope particles, which are formed in great exceed and also contain the hepatitis B surface antigen. The HBsAg determinant a, against which the immune response is mainly directed, is common to all HBsAg particles. In addition, either the main determinants d or y and w or r are present. The detection of HBsAg in human serum or plasma indicates an infection by the hepatitis B virus.

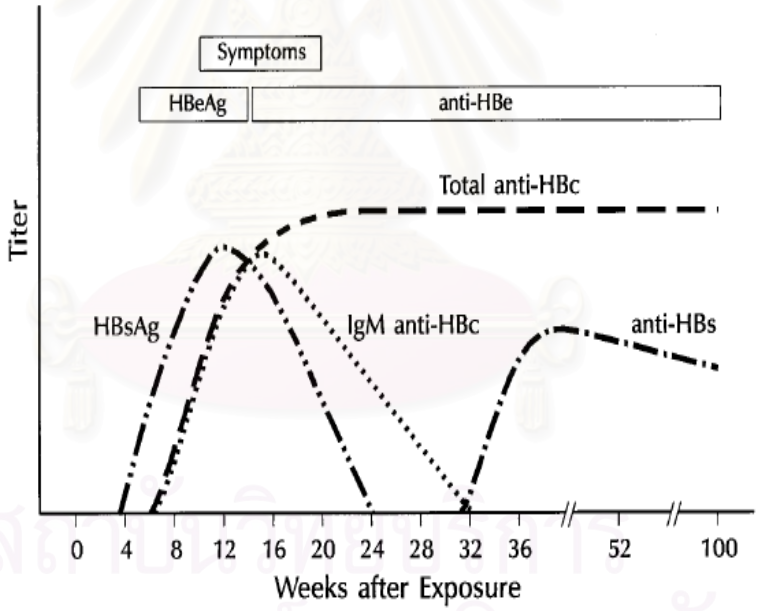


Figure 6. Characteristics of acute hepatitis B with recovery

Acute HBV infection is characterized by the presence of HBsAg in serum and the development of IgM class antibody (anti-HBcIgM). The antibody against the core component of the Dane particle (anti-HBc) is generally detected in the sera of individuals with acute type B hepatitis and in chronic HBsAg carriers. HBeAg is also detectable during acute infection (Table1). During convalescence, HBsAg and HBeAg are cleared , and anti-HBs, anti-HBc and anti-HBe develop (Fig6). Anti-HBs is a protective antibody that neutralizes the virus. The presence of anti-HBs following acute infection indicates recovery and immunity from reinfection. Immunoassays for detection of total anti-HBc involve both IgM and IgG class antibody to the core protein and indicate current or past exposure to virus and viral replication. Anti-HBcIgG appears shortly after HBsAg among persons with acute disease and generally persists for life. In persons with chronic HBV infection , HBsAg remains persistently detectable. HBeAg is variably present, and anti-HBcIgM generally becomes undetectable 6 months after acute infection (Fig6) [1].

Table 1. Interpretation of available serologic test results for HBV

patthern	HBsAg	HBeAg	Anti-HBc	Anti-HBcIgM	Anti-HBe	Anti-HBs	Interpretation
1	-	-	-	-	-	-	Not infection of HBV
2	-	-	-	-	-	+	Response to hepatitis B vaccine
3	+	+	-	-	-	-	Acute HBV infection
4	-	-	+	-	+/-	+	Past HBV infection, recovered
5	+	+/-	+	-	-	-	Acute or chronic HBV infection or carrier
6	+	-	+	-	+	-	HBV can't replicate in the presence of Anti-HBe
7	+	-	+	+	-	-	Early HBV infection
8	-	-	+	-	-	-	Not clear, several explanations have been suggested (low level HBsAg, genetic variability etc.)

EPIDEMIOLOGY

The prevalence of chronic HBV infection is low (< 2%) in general population in Northern and Western Europe, North America, Australia, New Zealand, Mexico and Southern South America. The prevalence of chronic HBV infection is intermediate (2-7%) in South Central and Southwest Asia, Israel, Japan, Eastern and Southern Europe, Russia, most areas surrounding the Amazon River basin, Honduras and Guatemala. The prevalence of chronic HBV infection is high (>8%) in all socioeconomic groups in certain areas: all of Africa; Southeast Asia including China , Korea, Indonesia and Philippines; the Middle East, except Israel; south and Western Pacific island ; the interior Amazon basin; and certain areas of Caribbean (Haiti and the Dominican Republic)

In some countries with a high prevalence of HBV infection, such as Japan, exclusion of all anti-HBcAg-positive plasma units would result in a drastic reduction in the number of blood units available for transfusion. Additional testing, like determination of the level of anti-HBcAg antibody , has been included to enable the transfusion of some of the anti-HBcAg-positive plasma units. On the other hand, anti-HBcAg testing is not mandatory in some countries. More rational criteria for discarding HBcAg-positive blood units may help these countries to adopt this additional testing[9,10].

In Japan, studies in two population of anti-HBc positive plasma units were observed: one with low antibody titers in the monoclonal inhibition assay (positive only undiluted, 56% of the total plasma) and the other with titers equal to or greater than 1/100 (31%). They found the presence of HBV-DNA in a low number of anti-HBc positive sample in 8 of 167 (4.8%) plasma samples by the low volume extraction procedure. Two additional positive plasma samples (10 of 167 [6%]) were detectd by a procedure which allowed the analysis of higher volume of plasma and reduced the risk of false-negative results by inhibition of PCR [9]. So, studies are needed to evaluate the possible usefulness of detecting this acute-phase marker among anti-HBc positive samples in blood donors, to discard possible donations from patients during the window period of infection.

Transmission of HBV infection from donors who are negative for HBsAg but positive for anti-HBc has been reported [11-15]. The risk for a recipient to develop HBV infection from an anti-HBc+ donor has been estimated to be 33% to 78%. However, these results were mostly derived from studies performed in geographic regions with a low prevalence (3% to 4%) of anti-HBc positivity in the liver donor population. In Spain, the prevalence of HBsAg in adult population ranges from 1.2% to 1.7% and the prevalence of anti-HBc positivity in the general population is greater than that reported in areas of low prevalence of HBV infection, that found in 10%. So, the transplantation experience between 1995 and 1998; HBV developed after receiving a liver from an anti-HBc+ donor in 15 of 30 recipients (50%) and only 3 of 181 recipients (1.7%) of liver from anti-HBc- donors [11]. The prevalence of acquired hepatitis B after liver transplantation during the study period greater than the 2% to 3% reported in previous investigations. Main factor that may account for this difference include, the high incidence of anti-HBc+ donors in this area. However, anti-HBc+ donors represented only 12.3% of the transplanted livers, 83% of acquired HBV infections could be ascribed to these donors. The difference in incidence of HBV infection between recipients of livers from anti-HBc+ and anti-HBc- donors (50% and 1.7%) suggests that other sources were not important in HBV acquisition.

In USA, the transplantation experience of four centers between 1989-1994 was reviewed. In donors that were negative for HBsAg but reactive for anti-HBc, they were believed to be low level carrier of HBV that were undetectable by conventional immunoassay. This study found that hepatitis B developed in 18 of 23 recipients of livers from anti-HBc+ donors (78%) is compared to only 3 of 651 recipients of anti-HBc- donors (0.5%) [12].

The incidence of post-transfusion hepatitis B has been greatly reduced by screening blood donors for HBsAg. However, HBV still accounts for a certain number of post-transfusion hepatitis. Residual risk of post-transfusion HBV infection due to seroconverting donors has been estimated to be 1 per 63,000 units. It was found that the addition of anti-HBc screening helped in preventing some cases of HBV transmission.

Molecular method can be used to demonstrate the presence of the virus in patients with anti-HBc alone with a frequency varying from 0-90%[16]. In Brazil, HBV-DNA positivity ranges from 0.8-23.4% among individuals attending outpatient clinics in the state of Sao Paulo. In Northeast Brazil, the high prevalence of anti-HBc-positive blood donors has resulted in a very high level of donation rejection, with obvious consequences in the setting of blood scarcity and very high costs. From the serological markers used in Brazilian blood transfusion centers, one could not assure the safety of a blood transfusion from anti-HBc-positive donor [16, 17]. In their institution, 3.49% of the collected blood units are discarded due to anti-HBc reactivity, whereas overall discard for HBsAg is only 0.31%. However most of the discarded anti-HBc positive blood units are associated with anti-HBs, about 10% of them present isolated anti-HBc, the meaning of which is still unclear [17].

The diagnosis of hepatitis B virus (HBV) infection is based on the detection of hepatitis B surface antigen (HBsAg) in sera. When the patient's serology becomes HBsAg negative, it had been interpreted as previous HBV exposure without ongoing viral infection. Several investigators, however, have demonstrated that the HBV genome is frequently detected in serum, liver, and peripheral blood mononuclear cells of individuals with acute hepatitis B long after the disappearance of HBsAg . These findings suggest that HBV infection persists for a long period in the liver tissue of individuals previously exposed to HBV. They recently demonstrated transmission of HBV from donors with antibodies to the HBV core antigen (anti-HBc) to the recipient through living related liver transplantation (LRLT), suggesting that healthy individuals who are positive for anti-HBc, even at low titer, have persistent HBV infection in their liver tissues. Moreover, cadaveric donors with anti-HBc but not HBsAg are recognized to be a high-risk source of HBV transmission to recipients through liver transplantation [18].

Antibody to hepatitis B core antigen (anti-HBc) is the most sensitive marker of previous hepatitis B contact. It appears in acute phase of HBV infection and usually persists after virus clearance. Diagnostic problems may arise when anti-HBc is found without HBsAg and anti-HBs. Introduction of HBV-DNA detection by Polymerase Chain Reaction (PCR) has implementd the confirmation of HBV infection in carriers with isolated anti-HBc [10, 16-28]. In India, the presence of HBV DNA was shown in 40 of 147 (27.21%) donors reactive for only anti-HBc[19]. In Saudi Arabia, they screened blood donors in one center for a safety transfusion, they found 1.2% of HBV DNA by PCR from HBsAg negative /anti-HBc positive blood donors[20]. This result are in agreement with another study which showed HBV DNA in HBsAg negative blood sample of apparently healthy individuals [21,24,29]. With the advantage of genomic amplification methods, the potential use of HBV DNA detection was raised but has not yet been implemented in any country for blood screening. It was anticipated that the extreme sensitivity of this technology would potentially be effective in identifying donations capable of transmitting HBV in the absence of detectable HBsAg, either early in infection or following clearance of HBsAg when anti-HBc is present in the absence of high titer of Anti-HBs. Amplification technique is extremely sensitive method for detection of nucleic acid. The advantage of PCR technique in diagnosis of HBV is the ability to detect the presence of HBV genome. The PCR can be modified to use for the diagnosis of low level virus by ultrasensitive manaul protocol. Virus particles in plasma will be concentrated by high-speed centrifugation [30-33]

Hepatitis C virus (HCV) is the main blood-borne non-A,non-B hepatitis virus which plays a major role in the development of chronic hepatitis and hepatocellular carcinoma. The viral genome has been completely cloned and shown to be a positive-strand RNA approximately 9.4 kb. The most important of HCV infections is that the prevalence of HCV infection in patients with hepatitis B virus infection has been examined in several studies [21,34,35,37]. Interestingly, HCV superinfection in patients with chronic HBV infection might exert a viral interference effect that suppresses or terminates the HBV carrier state [21,34-38]. In animal studies, it have been shown that HCV superinfection can interfere HBV replication in HBV carrier chimpanzees[34].

In Japan, They cotransfected the full-length or truncated version of HCV structural gene (core and envelope 1) together with the cloned HBV DNA into a human hepatoma cell line (HuH-7). Expression of HBV-specific major transcripts (3.5 and 2.1 kb), as well as HBV antigens was reduced about two to four-fold by the presence of the HCV structural genes. In extension, the secretion of HBV viral particles, including the viral nucleocapsid and mature virion, was drastically suppressed about 20-fold. Analysis of the intracellular HBV core protein-associated nucleic acid indicated that the encapsidated HBV pregenomic RNA was similarly reduced about 14-fold [34]. So, they demonstrated that the HCV structural proteins, in particular the core protein, may interfere expression and replication of HBV genome in the absence of host factors.

There was a report that studied whether coinfection with HCV influence the chance of detecting HBV DNA in sera with markers of past hepatitis B. This report suggested an association of HCV with the serological status of HBV infection, based on the high coincidence of anti-HCV and anti-HBc as the only HBV marker. Their study suggests an association between a positive HCV serostatus, indicating past or ongoing HCV infection, and the presence of HBV DNA in serum of anti-HBc positive/HBsAg negative patients [36].

MUTATION OF HEPATITIS B VIRUS

Hepatitis B virus (HBV) is a hepatotrophic DNA virus, but have DNA/RNA cycle during replication using reverse transcription enzyme like retrovirus. Hence HBV can develop many point mutations similar to RNA viruses. HBV has four open reading frames, including the preS/S gene, preC/C gene, P gene, and X gene. Hepatitis B surface antigen (HBsAg) is coded for by the S gene and the common antigenic epitopes of all subtypes of HBsAg are found in the same 'a' determinant (amino acids 124 to 147) [39]. Antibodies against these epitopes are used in standard assays for HBsAg. Patients infected with HBV are primarily diagnosed by detection of HBsAg in their sera. While most patients with some mutations in the 'a' determinant are positive for HBsAg [40-49], a few patients infected with HBV are negative for HBsAg in spite of a positive test for HBV-DNA or HBV polymerase chain reaction[50,51].

The surface protein of the virus, HBsAg is the established serological marker for diagnosis of acute or chronic HBV infection. Moreover, HBsAg screening is often the only measure to prevent transmission of HBV by transfusion. Serological assays for detection of HBsAg have been under constant pressure for improved sensitivity to reduce the diagnostic window and reliably detection of HBsAg in chronic infection where levels may be very low. Possible explanations of false-negative results in commercial assays could be HBsAg levels are below the detection limit or the existing of virus variants that are not recognized by the antibodies employed in the assays. These mutants cause changes in the common "a" determinant of the surface antigen in such a way that they may become undetectable by certain assays. There were reports in Thailand about a number of serum samples with a combination of HBsAg and anti-HBs positive results over the last couple decades during routine HBV testing services at a large hospital [52]. They explored the presence of HBsAg mutant in clinical samples, using two assays with different antibody capture formats. The HBsAg+/anti-HBs+ results have been suggested as an indicator of HBsAg mutation. The first kit was a sandwich ELISA kit with color detection whereas the second was a sandwich MEIA kit with fluorescence determination (AxSYM® HBsAg[V2], Abbott Diagnostic, Germany). They were found discordant by negative ELISA and positive MEIA and HBV DNA was detected in positive samples by MEIA. Direct sequencing

of these samples showed a glycine substitution with arginine at codon 145 (G145R), but this phenomenon was not found in another sample [52,53]. Other report showed subtitution at amino acid 120 (Pro120Gln), 131 (Thr131Iie), 135 (Pro135Ser), 141(Lys141Glu), 142(Pro142Leu/Ser); These recombinant proteins containing mutations were detected by all current Abbott HBsAg assays [53,54].

In some report, they described a rare variant of HBV isolated from a patient whose serum was negative for HBsAg by both ELISA and RIA but positive for HBV-DNA by dot blot hybridization. Sequencing result revealed substitutions at positions 129 (glutamine to asparagine) and 145 (glycine to alanine) in the 'a' determinant within the S gene and PreS/S deletion mutants[55]. In this case, glutamine at position 129 and glycine at position 145 were replaced by alanine and asparagine, respectively. Collectively, almost all reports about mutant viruses described substitutions and/or insertion within the "a" loop or surrounding areas. Although most of them revealed that their cases showed positive results for HBsAg, some of their cases and one reported by Protzer-Knolle were negative for HBsAg in spite of positive for HBV-DNA. They found that substitution at position 129 introduces a new asparagine-linked (N-linked) glycosylation site. It has been recently reported that the mutation in simian immunodeficiency virus, with newly introduced glycosylation, allowed the virus to escape neutralization by antibodies that could neutralize the parental virus. Therefore, the created putative glycosylation site in the mutant HBV may change the antigenicity of HBsAg, leading to escape of detection by standard HBsAg assays [39].

The most common point mutation that has been reported in immunized children causes a substitution of Arg for Gly at position 145 of HBsAg [56]. Other reports about substitutions in HBsAg such as 120, 121, 126, 129, 131, 133, 141, 144 were found repeatedly[57]. Some cases had discovered a mutation at nt551 A-to-G of HBV genome, resulting in the alternation of aa133 from Met to Val of HBsAg[58,59]. In Japan, there was a could report showing that point mutation converting Gly-145 to Arg-145, or Ile (Thr)-126 to Asn-126, abolish the expression of group-specific HBsAg determinant. These mutation can escape the vaccination or therapeutically administered monoclonal anti-HBs and prevail in circulation [59].

HBV infection with molecular variants of the virus has been found in vaccinated persons in Italy, Singapore, the Gambia and the United States and in liver transplant recipients who received HBIG for prophylaxis of relapse of HBV infection [60-66]. It has been proposed that these variants contain HBsAg that is not recognized by vaccine induced antibodies and that acute HBV infection occurs in the presence of protective levels of anti-HBs. Several investigators have reported a mutation in the genome that causes a change in one amino acid in the “a” determinant of the HBs protein which is the proposed conformational epitope essential for recognition and neutralization by anti-HBs antibodies. The most common alteration described is a replacement of glycine by arginine at amino acid 145, but other mutations such as replacement of aspartic acid by alanine at amino acid 144 have also been described. Similar variants have also been found in unvaccinated persons with chronic HBV infection, suggesting that they occur naturally. The incidence of infection with variant strains of HBV among vaccinated infants has been reported in a population-based study of 1,092 infants born to HBeAg-positive pregnant women between 1981 and 1993. Overall, 94 (8.6%) of the infants became infected and HBV variant strains were isolated from 22 of the infected children. As found in other studies, the most common amino acid changes occurred at positions 143 to 145. However, mutations were also found across most of the region. In addition, mixed infections involving wild-type and mutant viruses were often detected in both mothers and infants, suggesting that infants acquire their mutant strain from the mother.

At present, the public health importance of the HBV molecular variants is debatable. Studies of vaccinated household members living with persons chronically infected with variants have not demonstrated intrahousehold transmission of the variant [68]. In addition, preexposure vaccination of chimpanzees with currently licensed vaccines (not containing pre-S epitopes) conferred protection following intravenous challenge with the 145-HBV mutant [67]. Further studies and enhanced surveillance to detect the emergence of these variants are remain at high priorities in evaluating the effectiveness of current immunization strategies.

Interestingly, the reason for the lack of HBsAg in only anti-HBc positive individuals is not clear but several explanations have been suggested. HBsAg synthesis may be downregulated by coinfection with HCV. Variations in the pre-S region or mutations in the surface antigen itself, especially in the “a” determinant, which is recognised by anti-HBs that are may render HBsAg undetectable by conventional assays. Concentrations of antigen are being considered as other causes below the detection limit of available serological tests or non infectious HBV.

In this study, we used the conventional nested PCR and ultrasensitive nested PCR to study the potential HBV infectivity of anti-HBcAg-positive, HBsAg-negative and anti-HBs negative plasma and retest HBsAg (AxSYM® HBsAg[V2], Abbott Diagnostic, Germany) that detected wild type as well as mutant forms of HBsAg.

CHAPTER IV

MATERIALS AND METHODS

Methodology Scheme

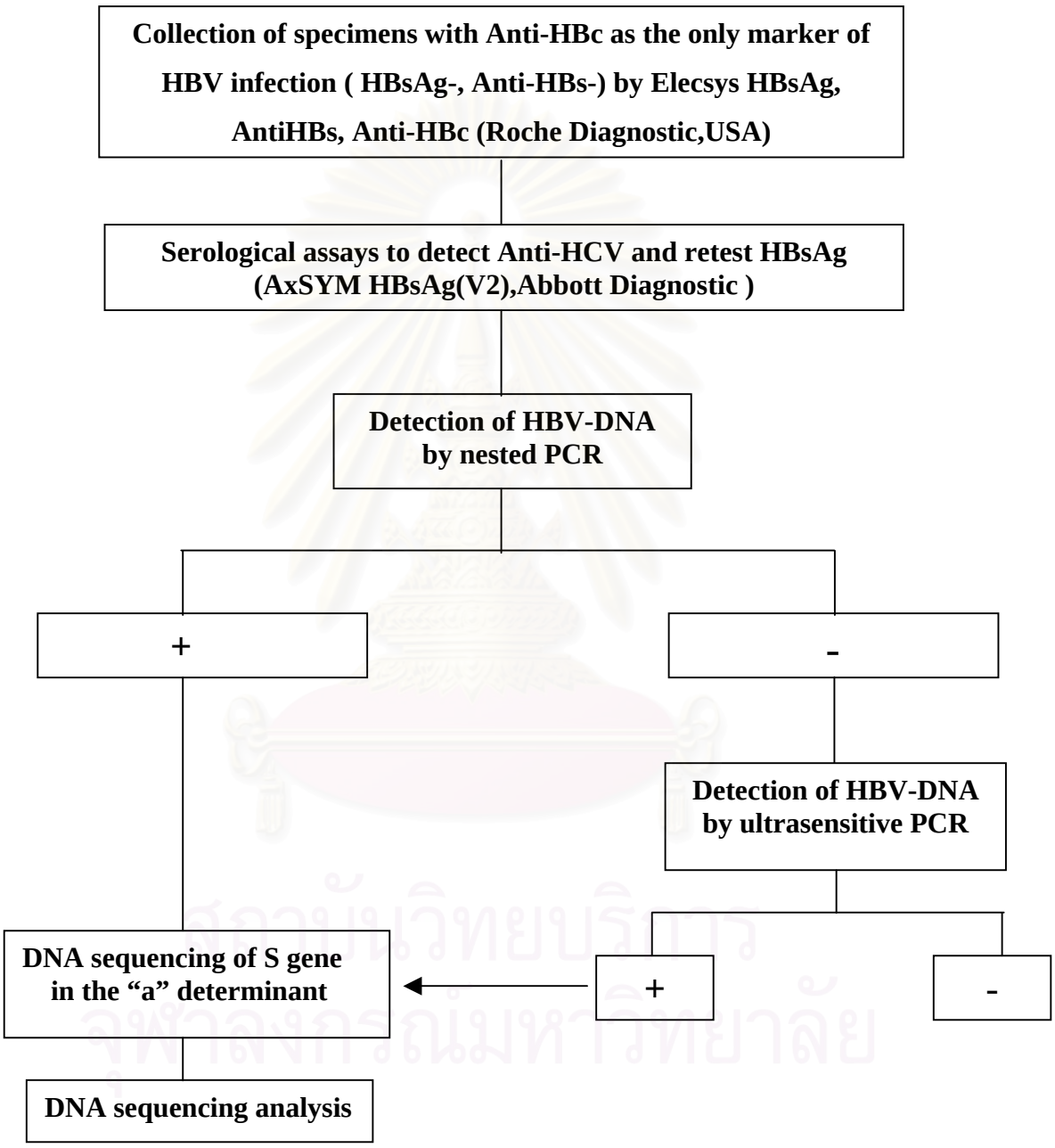


Figure 7 : Methodology scheme

PART I : Collection of sample

1. Serum samples

In this study, the sera were selected from Department of Microbiology, King Chulalongkorn Memorial Hospital. Whole blood was collected in stunted tubes with no anticoagulant and was stored at room temperature for no more than 6 hrs. The serum was then separated by centrifugation at 1,600xg for 10 min at room temperature. The clinical specimens for studies were chosen from routine diagnostics for Hepatitis profile by using fully automated electrochemiluminescence Immunoassay "ECLIA" on the Roche Elecsys 2010. The specimens with presence of anti-HBc as only serological marker will be aliquoted and store at $-20^{o}C$ or lower.

2. Samples size

$n = Z_{\alpha}{}^{2}PQ/d^{2}$

$Z_{\alpha} = Z_{0.05/2} = 1.96$

$P =$ Prevalence $= 0.11$

$Q = 1-0.11$

$d =$ acceptable error $= 0.05$

$n = (1.96)^{2}(0.11)(1-0.11)/(0.05)^{2}$

$= 151$

According to the calculation above, the sample size in this study was 151 samples

PART II : Serological assays

Sera found to be positive for anti-HBc only were retested for HBsAg by another commercial serological assay, AxSYM HBsAg(V2) (Abbott Diagnostics,Germany) which uses microparticle enzyme immunoassay. The microparticles coated with monoclonal anti-HBs and another polyclonal detection reagent for the detection of HBsAg are used to determine whether it is repeatedly negative or positive. AxSYM HBsAg(V2) have been used to detect wild type and mutant sample in other experiments(recombinant and natural mutant). The specificity of AxSYM HBsAg(V2) is 99.95% and sensitivity is 0.23 ng/ml. Screening for anti-HCV was performed by an indirect solid phase enzyme immunoassay (Cobas Core Anti-HCV EIA II, Roche Diagnostics,USA).

PART III : Amplification and Detection of HBV DNA by nested PCR

Sample presence of anti-HBc only were tested for HBV-DNA by nested PCR using specific primers in the core region of HBV genome.

1. DNA Extraction

DNA was extracted from serum using the QIAamp DNA Blood Kit (Qiagen, Hilden, Germany) with modification. Viral DNA was isolated from 200 μl of serum with 200 μl Buffer AL and 20 μl proteinase K. Following incubation at 56^{o}C for 10 min, 200 μl ethanol (96-100%) was added to the sample. DNA was silica gel filtration purified as described by the manufacturer protocol (Qiagen). The viral DNA was eluted by 50 μl Buffer AE.

2. Amplification and Detection of HBV DNA by nested PCR

The eluted DNA was amplified for HBV DNA by Nested Polymerase Chain Reaction technique. Primer sequences are specific for HBV core region. The primers represent highly conserved regions of the core region and the neighbouring sequences are identified after an alignment of 10 complete HBV genomes (Table2). Each amplification reaction was done in a 50 μl assay. First reaction containing 10 μl of template and 40 μl reaction mixture. The second reaction containing 2 μl template and 48 μl reaction mixture. The reaction mixture comprises of 2.0 units *Taq* DNA Polymerase, 0.2 mM dNTP(each), 1.5 mM $MgCl_2$, 10xbuffer and 50 pmol primers(each). PCR was run in a 2400 thermal cycler(Applied Biosystems, USA) with the following thermal profile: 95^{o}C for 3 min; 30 cycles with 95^{o}C for 30s (denaturation), 55^{o}C for 30s (annealing) and 72^{o}C for 30s (extension) ; final extension at 72^{o}C for 5 min; hold at 4^{o}C.

3. Detection of HBV-DNA by ultrasensitive PCR

The sensitivity of PCR can be increased by modification of DNA preparation procedure. By using 1 ml of serum (instead of 200 μl by conventional PCR) and ultracentrifuge in polycarbonate centrifuge tube (Beckman, USA). The 1 ml serum was centrifuged at 50,000 rpm for 1 hr at 4^{o}C in the Optima^{TM} TLX Ultracentrifuge (Beckman, USA) with rotor model TLA-120.2. To reduce the volume of the sample, 800 μl of serum were discarded. Then the remaining serum including pellet will be used for DNA extraction by QIAamp DNA Blood Kit(Qiagen, Hilden, Germany) and then followed by conventional nested PCR.

4. Quality control

To minimize the risk of contamination, DNA extraction, PCR amplification, and electrophoresis were performed in separate area with containment. Each area had its own set of pipettes, gloves and disposable racks; and its own UV hood in some area. The aerosol resistant pipette tips were used throughout the procedure. All reagents used were sub-divided into small aliquots for each PCR run. To further avoid contamination, negative control, master mix control and positive control were included in every run.

5. Analysis of PCR Products

2% agarose gel (GIBCO; Grand Island, N.Y.USA) in Tris-acetate (TAE) buffer pH 8.00 containing 0.5 ug of ethidium bromide (Sigma, MO, USA) per ml was prepared. 10 μl of DNA product was separated by electrophoresis with the current of 100 volts until the bromphenol blue indicator in the loading buffer had migrated to bottom of the gel. The amplified products were then visualized on UV light transilluminator. 100 bp ladder DNA marker(Promega, USA) was used as reference size marker. The presence of amplicon 255 bp in length is considered as positive result for HBV if all controls react as expected.

6. Sensitivity of HBV-DNA by nested PCR and ultrasensitive PCR

To determine the sensitivity of our nested PCR and ultrasensitive PCR assays for the detection of very low levels of HBV DNA, known viral titer from Cobas Amplicor HBV Monitor™ Test(Roche diagnostic, USA) was also included. The 400 copies/ml of serum was serially diluted with buffer to a concentration of 200, 100, 50, 25 and 12.5 copies/ml. Then the diluted standard was subjected to DNA extraction according to our protocol. The extract DNA was then amplified by conventional PCR and ultrasensitive PCR for 10 runs in each concentrations. The sensitivity of nested PCR was determined to be 100 copies/ml and the sensitivity for ultrasensitive PCR was 25 copies/ml. Therefore the sensitivity can be increased 4 folds by ultrasensitive PCR.

7. HBV-DNA Quantitation

Samples which were positive by nested PCR and ultrasensitive PCR were subjected for HBV DNA quantitation by Cobas Amplicor HBV Monitor™ Test. This test is an in vitro nucleic acid amplification for the quantitation of HBV DNA in human serum or plasma on the Cobas Amplicor™ Analyzer based on four major processes; sample preparation; PCR amplification of target DNA using HBV specific complementary primers; hybridization of the amplified products to oligonucleotide probes specific for the target(s); and detection of the probe-bound amplified products by colorimetric determination.

Cobas Amplicor HBV Monitor™ Test permits simultaneous PCR amplification of HBV target and HBV Quantitation Standard DNA. The master mix reagent contains a primer pair specific for both HBV DNA and HBV Quantitation Standard DNA. The quantitation of HBV viral DNA is performed using the HBV Quantitation Standard. The HBV Quantitation Standard is a non-infectious linearized plasmid that contains the identical primer binding sites as the HBV DNA target and a unique probe binding region that allows HBV quantitation standard amplicon to be distinguished from HBV amplicon. The HBV quantitation standard is incorporated into each individual sample at a known copy number and is carried through the sample

preparation, PCR amplification, hybridization and detection steps along with the HBV target. The Cobas Amplicor Analyzer calculates the HBV DNA levels in the test samples by comparing the HBV target signal to the HBV quantitation standard signal for each sample and control. 100 μl serum was extracted by Cobas Amplicor HBV Monitor Kit and eluted by 200 μl buffer and run on Cobas Amplicor™ Analyzer.

Table2 : Sequences of the oligonucleotides used as primers for amplifying and sequencing the S-gene in the "a" determinant of HBV (Accession number AF461043)

Specific for	Primer	Sequence 5'→ 3'	Length	Position
Core region	HBVC1	CTT TGT ACT AGG AGG CTG TAG GC	23	1766-1788
Core region	HBVC2	GGA GGA GTG CGA ATC CAC ACT	21	2289-2268
Core region	HBVC3	GCT TTG GGG CAT GGA CAT TGA C	22	1891-1912
Core region	HBVC4	CTA CTA ATT CCC TGG ATG CTG G	22	2156-2135
S region	HBVS1	CTC GTG GTG GAC TTC TCT CA	20	127-146
S region	HBVS2	CT(G/A) CGA ACC ACT GAA CAA AT	20	581-560
S region	HBVS3	CTG GTT ATC GCT GGA TGT GT	20	237-256
S region	HBVS4	AAC CAC TGA ACA AAT GGC ACT	21	576-556

PART IV : HBV-DNA Sequencing of S-region in the “a” determinant

1. Amplification of the S-region

Samples that were positive for HBV-DNA by nested PCR and ultrasensitive PCR were amplified with primers specific for S-region which cover fragments of “a” determinant and were subjected to sequencing. The primers represent highly conserved region of the s region and the neighbouring sequences identified after an alignment of 10 complete HBV genomes (Table2). Each amplification was amplified in a 50 μl assay. First reaction contains 10 μl of template and 40μl reaction mixture. The second reaction contains 2 μl template and 48 μl reaction mixture. The reaction mixture comprises of 2.0 units *Taq* DNA Polymerase, 0.2 mM dNTP(each), 1.5 mM $MgCl_2$, 10xbuffer and 50 pmol primers(each). PCR was run in a 2400 thermal cycler (Applied Biosystems, USA) with the following thermal profile: 95^{o}C for 3 min; 35 cycles with 95^{o}C for 30s (denaturation), 55^{o}C for 30s (annealing) and 72^{o}C for 30s (extension); final extension at 72^{o}C for 5 min; hold at 4^{o}C. PCR product was analyzed by electrophoresis on ethidium bromide stained agarose gels in TAE buffer.

2. Purification of PCR Products

The amplified products of S-region were purified by adsorption to silica gel membranes in spin columns (QIAquick PCR Purification Kit, Qiagen, Germany). DNA was absorbed to the silica-membrane in the presence of high salt while contaminants pass through the column. Impurities were efficiently washed away and the pure DNA was then eluted with Tris buffer.The concentration of DNA was measured by spectrophotometer (BIO RAD, Smart Spec^{tm} 3000, USA) and adjusted to 50 ng/μl for preparation as DNA template for sequencing

3. Sequencing Reaction Preparation

Both strands of DNA were sequenced using forward and reverse primers in separate reactions. Approximately 50 ng of DNA sample was sequenced using HBVS3 and HBVS4 primer (Table2) with ABI Prism Big Dye terminator cycle sequencing ready reaction kit (Perkin Elmer Corporation, ABI prism , Applied Biosystem , Foster city, USA). The sequencing reaction required 4 μl of BigDye terminator as described by manufacturer, with 4.0 pmole of primer and 50 ng of purified PCR product template in a total volume of 15 μl. The sequencing cycle was performed using 25 cycles of

Denaturing step : at 96^{o}C for 10 sec
Annealing step : at 50^{o}C for 5 sec
Extension step : at 60^{o}C for 4 min

4. Extension Product Purification

Amplified sequencing product was purified by ethanol/sodium acetate precipitation; 80 μl of ethanol acetate was added to sequencing reaction, mix and incubate at RT for 15 min, centrifuge at 16,400 rpm for 20 min; discard supernatant and then 250 μl of 70% ethanol was added, mix and then centrifuge at 16,400 rpm for 5 min, dry in vacuum for 15-20 min. The precipitated product was resuspended in loading buffer and then analyzed by ABI prism 310 automated sequencer (Perkin Elmer Corporation, ABI prism, Applied Biosystem, Foster city, USA).

5. Sequence Analysis

Sequence analysis and Sequence Navigator (Applied Biosystem, USA) were used for analyzing and comparing forward and reverse sequences. The nucleotide sequence and the protein sequence were analyzed with the software available over the internet at the National Center for Biotechnology Information (**http://www.ncbi.nlm.nih.gov**). The multiple sequence alignment and comparisons analyzed by Chromas, Genedoc and ClustalW program, version 1.74. The nucleotide sequence and genotype were compared with the database available from **http://www.ncbi.nlm.nih.gov**.

CHAPTER V

RESULTS

PART I : Serological assays

All 158 samples from Department of Microbiology, King Chulalongkorn Memorial Hospital which presence of anti-HBc only marker of HBV (HBsAg-, anti-HBs-) were used in this study. Ninty-six samples were from male patients and 62 from female patients (Table3). All 158 samples were negative for HBsAg by AxSYM (detected wild type and mutant forms of HBsAg) and 10 samples (6.33%) were positive by enzyme immunoassay (EIA) for anti-HCV (Roche Diagnostic, USA) (Table4). All 10 samples were HBV DNA negative by nested and ultrasensitive PCR.

Table 3 : The number of sample in this study.

sex	No.of sample
Male	96
Female	62
Total	158

Table 4. Results of HBsAg by AxSYM HBsAg[V2], Anti-HCV by Cobas Core Anti-HCV EIA II and HBV DNA by Nested and Ultrasensitive PCR from serum of anti HBc only marker.

No.	HBsAg(AxSYM)		Anti-HCV(EIA)		HBV PCR (nested and Ultrasensitive)	
	Negative	Positive	Negative	Positive	Negative	Positive
158	158	-	148	10	150	8

Table 5. Results of HBsAg by AxSYM HBsAg[V2], Anti-HCV by Cobas Core Anti-HCV EIA II and HBV DNA by Nested and Ultrasensitive PCR from serum of anti HBc only marker with HBV DNA positive

Code No.	HBsAg by AxSYM	Anti-HCV (EIA)	HBV PCR	
			Nested PCR	Ultrasensitive PCR
H002	-	-	+	ND
H018	-	-	-	+
H041	-	-	+	ND
H069	-	-	+	ND
H087	-	-	-	+
H103	-	-	+	ND
H128	-	-	-	+
H148	-	-	-	+

Abbreviations: - Negative; + Positive; ND = Not done

PART II : Detection of HBV DNA by PCR Amplification

1. Sensitivity of PCR Amplification

The sensitivity of nested PCR and ultrasensitive PCR was determined by testing serial dilution of stock serum with known HBV viral titer from Cobas Amplicore HBV Monitor™ Test (Roche Diagnostic, USA). The nested PCR and the ultrasensitive PCR assays were able to detect 100 and 25 copies/ml of HBV DNA respectively. The sensitivity of both nested PCR and ultrasensitive PCR were shown in Figure 8.

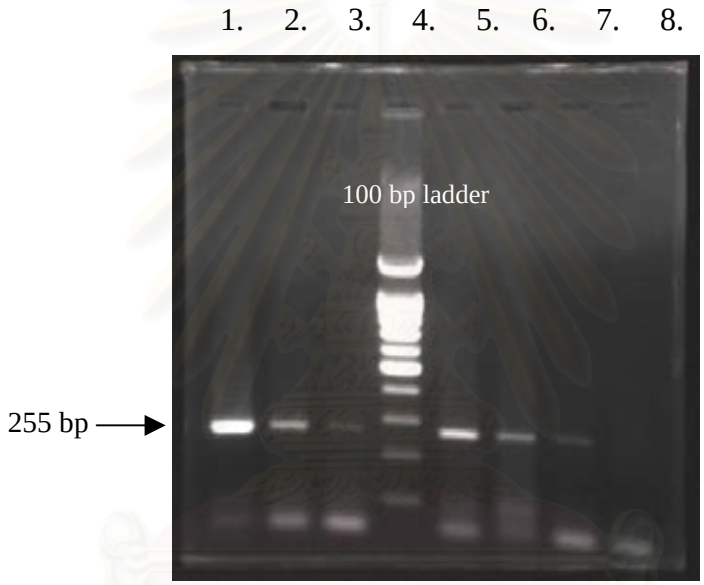


Figure 8 : Sensitivity of HBV DNA of serial dilution of stock serum containing HBV DNA. The stock serum were serially diluted and subjected to nested PCR and ultrasensitive PCR. The PCR products were electrophoresed on 2% agarose gel and detected under UV light.

Lane1 : HBV 400 copies/ml
Lane2 : HBV 200 copies/ml
Lane3 : HBV 100 copies/ml
} Nested PCR

Lane4 : Marker 100 bp DNA ladder

Lane5 : HBV 100 copies/ml
Lane6 : HBV 50 copies/ml
Lane7 : HBV 25 copies/ml
Lane8 : HBV 12.5 copies/ml
} Ultrasensitive PCR

2. Detection of HBV DNA in anti HBc only marker

All specimens were tested for the presence of HBV DNA in serum by nested PCR using core gene specific primers. The core region was chosen because this region was specific to hepatitis B virus and are conserved in every HBV genotype with less variation. Eight samples (5.06%) were positive for HBV DNA in serum; four samples were positive for HBV DNA by nested PCR and additional four samples were positive for HBV DNA by ultrasensitive PCR (Table5). The HBV DNA was found in 6 samples from male patients and 2 samples from female patients. The results were tabulated in figure 9.

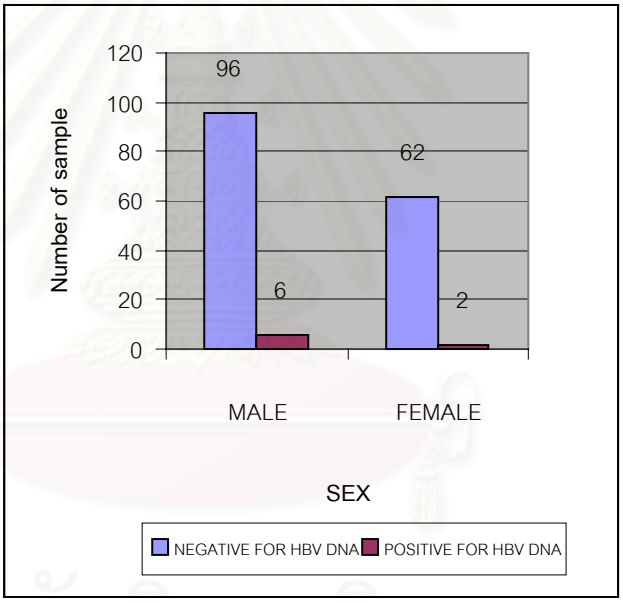


Figure 9 : Number of patients from anti-HBc only marker and number of patients with HBV DNA positive by PCR

3. HBV DNA Quantitation

The viral DNA titer in PCR positive samples can be quantitated by using commercial assays available from Cobas Amplicor HBV Monitor™ Test(Roche Diagnostic, USA). All samples had a viral load of below 200 copies/ml. This assay confirmed the low virus concentration in the Anti-HBc only marker. The results were shown in Table 6.

Table 6 : HBV DNA Quantitative PCR from samples positive by nested PCR and ultrasensitive PCR

Code No.	Viral load (Copies/ml)
H002	<200
H018	<200
H041	<200
H069	<200
H087	<200
H103	<200
H128	<200
H148	<200

PART III : HBV DNA sequencing and data processing

The HBV s gene was sequenced by using primers HBVS1, HBVS2, HBVS3 and HBVS4. These primers cover special locations of “a” determinant. The DNA product was 340 bp that encompasses nucleotides 237-576. The PCR product was shown in Figure 10.

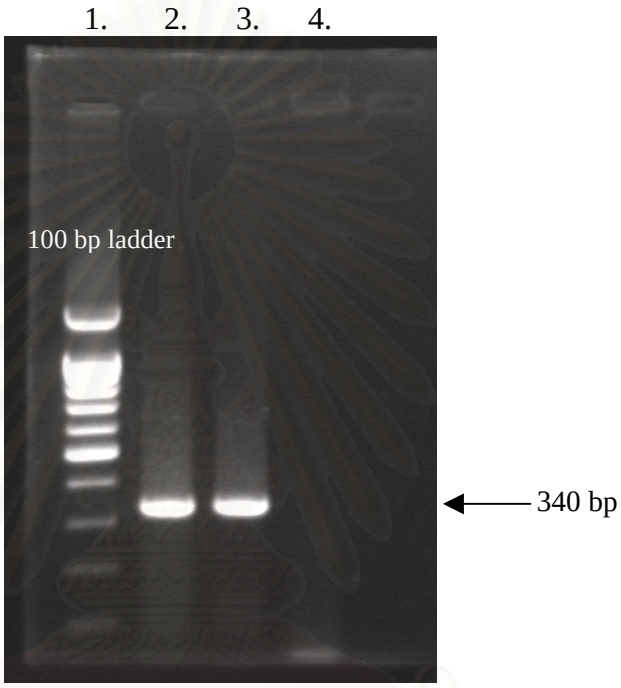


Figure 10 : Agarose gel electrophoresis of HBV PCR product of s region (340 bp). The PCR product was analyzed by 2% agarose gel electrophoresis and detected under UV light.

Lane1 : Molecular weight marker (100 bp DNA ladder)
Lane2,3 : PCR Product of S-region
Lane4 : Negative control

1. Subtype/Genotype identification

The “a” determinant of the s gene of isolates from 8 HBV carriers that were positive for only anti-HBc were sequenced and analyzed by freeware from NCBI (http://www.ncbi.nlm.nih.gov). The samples are subtypes adr, adw, ayw and genotypes C and B. The results were shown in Table 7. All wild type controls were genotype C, subtype adr (most common in Thailand).

Table 7. Subtype/Genotype identification by sequencing of S region

Code No.	Subtype	Genotype
H002	ayw	B
H018	adw	B
H041	adw	B
H069	adr	C
H087	adr	C
H103	adr	C
H128	adr	C
H148	adw	B

2. Mutation of HBV DNA in “a” determinant

All 8 isolates of HBV DNA positive were sequenced and aligned according to s gene in “a” determinant by freeware from NCBI (http://www.ncbi.nlm.nih.gov) with wild type control form HBsAg positive sample and published sequences that are available in Database which found in Thailand (AB073830, AB074755, AB074756 and AF068756; genotype B and C) and other subgenotype in published sequences as shown in Figure 11, 12. Five of these isolates had mutated nucleotide leading to amino acid changes such as sample code H069, H128 and H148 had substituted at one position, H018 had substituted at two positions and H087 had substituted at five positions. These amino acid changes in the surface determinants might prevent recognition of HBsAg by the serological assays. The results of amino acid substitutions were tabulated in Table 8 and Table 9.

Table 8 : Substitution of nucleotide in “a” determinant (S gene)

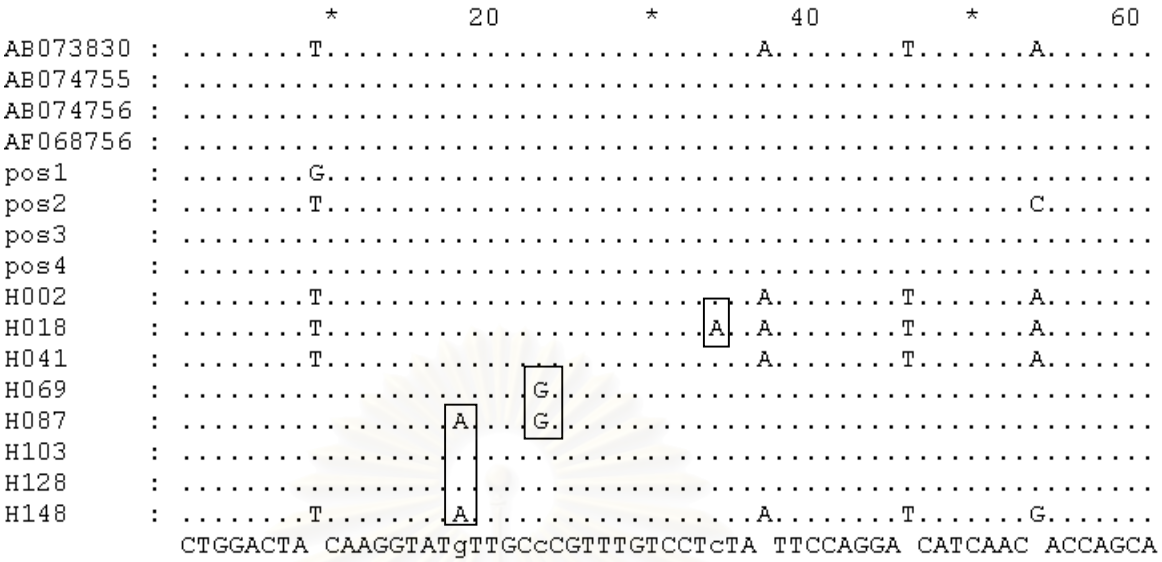
Code	Position of nucleotide sequences						
	G463	C468	C479	T532	T554	C582	C586
H002	-	-	-	-	-	-	-
H018	-	-	A	-	A	-	-
H041	-	-	-	-	-	-	-
H069	-	G	-	-	-	-	-
H087	A	G	-	G	G	T	-
H103	-	-	-	-	-	-	-
H128	-	-	-	-	-	-	A
H148	A	-	-	-	-	-	-

Abbreviations : A, adenosine; T, thymine; C, cytosine; G, guanine

Table 9 : Substitution of amino acid in “a” determinant (S gene)

Code	Position of amino acid sequence						
	M103	P105	L109	T/I126	F134	S143	D144
H002	-	-	-	-	-	-	-
H018	-	-	I	-	I	-	-
H041	-	-	-	-	-	-	-
H069	-	R	-	-	-	-	-
H087	I	R	-	M	V	L	-
H103	-	-	-	-	-	-	-
H128	-	-	-	-	-	-	E
H148	I	-	-	-	-	-	-

Abbreviations : M, methionine; P, proline; L, leucine; T, threonine; I, isoleucine; F, phenylalanine; S, serine; D, aspartic acid; R, arginine; V, valine; E, glutamic acid



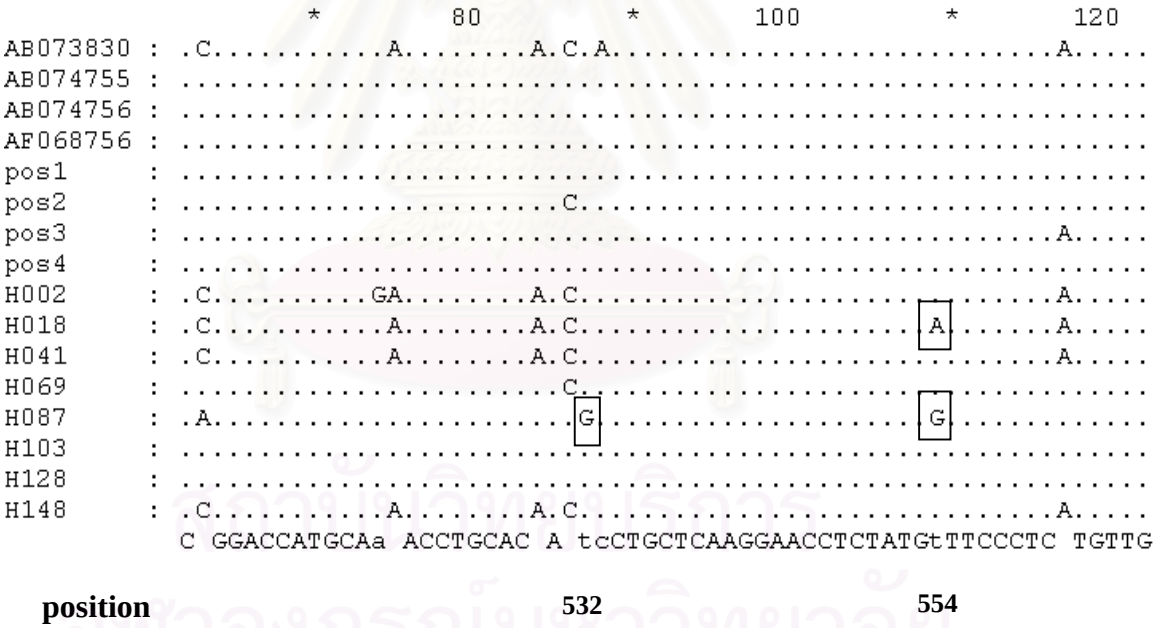


Figure 11 : Alignments of the nucleotide sequences of HBV for S gene in "a" determinant ; GenBank (found in Thailand) AB073830(GenotypeB), AB074755(GenotypeC),AB074756(GenotypeC)and AF068756(GenotypeC) ; Wild-type (GenotypeC) were pos 1, 2, 3 and 4 respectively; Samples were H002, 018, 041, 069, 087, 103, 128 and 148. Nucleotide substitution of samples at position 463, 468, 479, 532, 554, 582 and 586 are indicated.

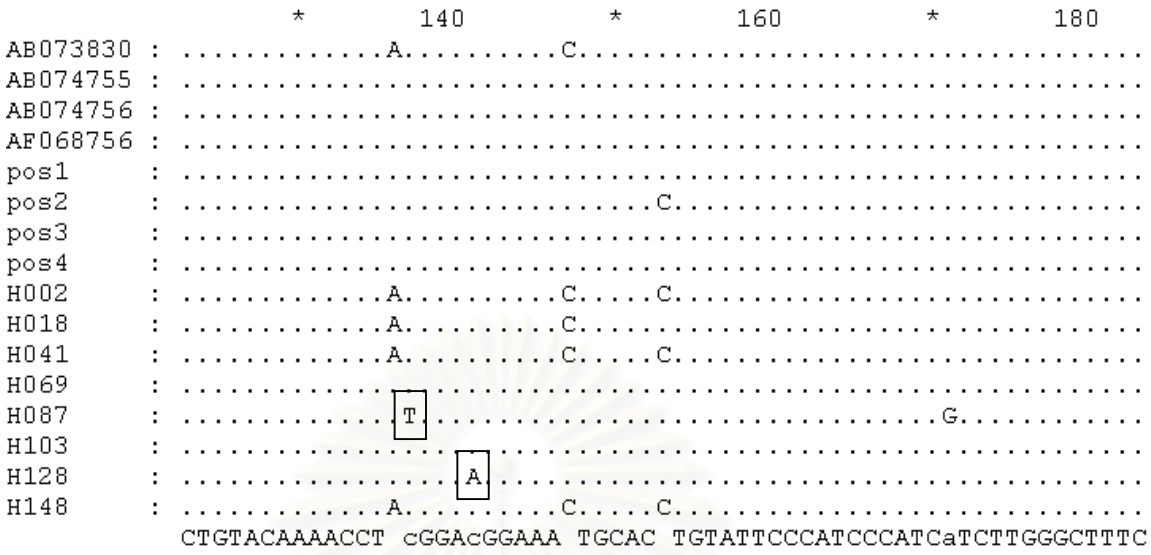


Figure 11(Cont.): Alignments of the nucleotide sequences of HBV for S gene in "a" determinant ; GenBank (In Thailand) AB073830(GenotypeB), AB074755 (GenotypeC), AB074756 (GenotypeC) and AF068756(GenotypeC) ; Wild-type (GenotypeC) were pos 1, 2, 3 and 4 respectively; Samples were H002, 018, 041, 069, 087, 103, 128 and 148. Nucleotide substitution of samples at position 463, 468, 479, 532, 554, 582 and 586 are indicated.

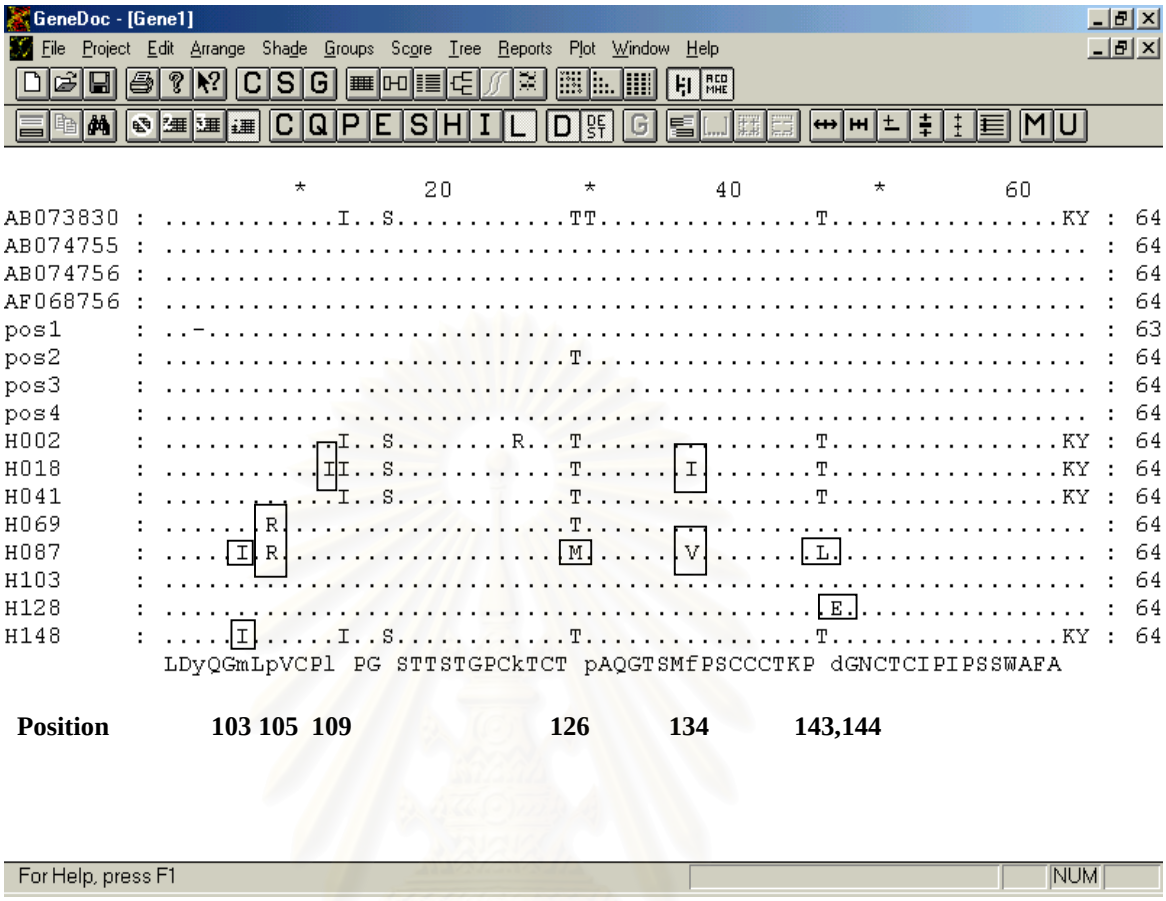


Figure 12: Alignments of the amino acid sequence of HBV for S gene in "a"determinant ; GenBank (In Thailand) AB073830(GenotypeB), AB074755(GenotypeC),AB074756(GenotypeC) and AF068756(GenotypeC); Wild-type (GenotypeC) were pos 1, 2, 3 and 4 respectively; Samples were H002, 018, 041, 069, 087, 103, 128 and 148. Amino acid substitution of samples at position 103, 105, 109, 126, 134, 143 and 144 are indicated.

CHAPTER VI

DISCUSSION

Determination of HBsAg, anti-HBs and anti-HBc in the serum allows the discrimination of a variety of different states of HBV infection (Table1). The most difficult in the interpretation of different states of HBV infection remains in individuals who are serologically anti-HBc positive but HBsAg and anti-HBs negative. The presence of anti-HBc as the only serological marker for HBV is usually interpreted as past hepatitis B infection or HBV carriers. These individuals may transmit hepatitis B when donating blood and organ transplantation. The reason for this unusual serological pattern is not clear but several explanations have been suggested. HBsAg may be hidden as circulating immune complexes. HBsAg synthesis could be downregulated by co-infection with HCV[34,35,38]. The variations in the pre-s region especially in its “a” determinant which is exposed at the surface of HBsAg particles and recognized by anti-HBs, may render the particles undetectable by conventional immunological assays. The simplest interpretation is the remainder of a past hepatitis B infection where anti-HBs has fallen below detectable level or it is a “core window” period. The anti-HBc is the first sign of immune response mounted by the infected host. This anti-HBc is usually followed by HBsAg and anti-HBs. However, it has been clearly shown in the past that between 10 and 40% of individuals with this serological pattern are chronic carriers of HBV[23,70-73]. They usually show very low virus concentration in their sera, but nevertheless can transmit the infection.

Advances in molecular biology and biotechnology are creating possibilities for DNA diagnostic. Amplification techniques such as PCR is an extremely sensitive method for detecting nucleic acid of HBV DNA in serum. The advantage of PCR technique in diagnosis of HBV is its ability to detect the presence of nucleic acid of the infecting microorganisms that may not be identified by conventional methods. PCR can also be modified to use for the diagnosis of low level of virus concentration in the sera of anti-HBc as the only serological marker of HBV infection. Nested PCR

increases the sensitivity and specificity of the test through two independent rounds of amplification using two discrete primer sets.

In this study, we have developed an ultrasensitive nested PCR technique for detecting the HBV DNA from serum of anti-HBc as only serological marker. The sensitivity of conventional PCR as determined by amplification of serially diluted known amount of HBV is approximately 100 copies/ml. The sensitivity of ultrasensitive PCR is approximately 25 copies/ml. The sensitivity of ultrasensitive nested PCR could be increased approximately 4 fold through a relatively simple modification of the specimen processing procedure. An ultracentrifugation step was performed prior to DNA extraction to concentrate the HBV particles. To increase sensitivity, the volume of specimen was also increased up to five fold, from 200 μl to 1 ml of plasma. The only disadvantage of this modification is it's time consuming during specimen preparation.

From our experiment, all samples that were undetectable of HBV DNA by conventional nested PCR were retested with ultrasensitive PCR. Four more samples were positive for HBV DNA. Thus, our results indicates that the ultrasensitive assay will provide more useful information than the conventional PCR. The technique can be used to detect extremely low level of HBV in patients plasma that fallen below the detection limit of conventional nested PCR. This finding demonstrates that all of anti-HBc positive and PCR positive samples usually show very low virus concentrations in their sera. The low level of viremia has been confirmed by using the Cobas Amplicor HBV Monitor^{TM} Test, which is an in vitro nucleic acid amplification test for the quantitation of HBV DNA in plasma. The lower limit of quantitation of the Cobas Amplicor HBV Monitor^{TM} Test is 200 copies/ml of plasma[74]. The Cobas Amplicor HBV Monitor^{TM} Test failed to detect HBV in all 8 samples due to the concentration is lower than the limit of detection of the test.

The serological diagnosis of hepatitis B virus infection is based on tests for HBsAg, anti-HBs, anti-HBc, HBeAg and anti-HBe. The analysis allows the differentiation between acute, chronic or past hepatitis B infection because different stages of infection are characterized by specific patterns of these markers. It has been

known for sometime that patients who have anti-HBc can be viremic and even infectious [75,76] for example after blood transfusion. In other study, it was shown that some samples had no markers of HBV infection yet were PCR positive. This discordances had led to a search for HBV variants that can be used to explain the HBsAg negative status.

In other situation such as discordance in reactivity between two HBsAg assays. There are two possibilities to explain discrepancies in commercial assays. First, there may be very low level of HBsAg which can only be detected by the most sensitive assay. Second, there are variant sequences which are not recognized by the antibodies employed in the assays. A further explanation for the lack of any detectable serological markers are variants in other parts of the genome (such as X gene) that may downregulate the production of antigens and, consequently, antibodies. This discordance have led to a search for variants that may be able to explain HBsAg negativity. There are some examples of variants that are not recognized by some commercial HBsAg assay. All of 158 samples in our study are HBsAg negative by Elecsys HBsAg(Roche Diagnostic, USA). In order to study any discordance in reactivity between two HBsAg assays, the 158 samples were retested by second commercial assay, AxSYM HBsAg[V2] (Abbott Diagnostics, Germany) which has been claimed that it can detect variant of HBsAg [52-54]. In our study, we could not detect the discordance in reactivity between the two HBsAg assays. This finding demonstrates that there is no variant sequence in S gene or it may be because of the too low concentration of HBsAg to be detected by both serological assays. The latter explanation may be true according to HBV DNA results. The additional search for variants was done by sequencing analysis of the S gene.

The hepatitis B virus has three envelope proteins. The major envelope protein is the S protein that consists of 226 amino acid. A hydrophilic region in the S protein between amino acid 98-156 (other report showed amino acid 124-147) is designated as the “a” determinant which is an antigenic determinant common to all HBV subtype. The “a” determinant is a conformational epitope which is made up of a hydrophilic two-loop structure linked by the disulfide bond between Cys124 and Cys137, Cys139 and Cys147, respectively[39]. The HBV DNA template is transcribed by cellular RNA polymerase to pregenomic RNA, which in turn is reverse

transcribed to DNA by virus polymerase. This unique way of HBV replication means a significant tendency to mutation. This structure projects out from the surface of the HBV particle. So, the antigenicity of this “a” determinant has been employed to detect HBsAg in the serum. It is also important for the induction of anti-HBs production.

The presence of HBsAg variable mutations within the “a” determinant has been reported in both the vaccinated and non-vaccinated populations. These mutation were observed in the non-vaccinated group. Therefore, HBsAg was undetected by assays that could not recognize these mutations. Most studies on occult HBV infection that the clinical significance of HBV mutants have been reported. Yamamoto reported point mutations converting Gly145 to Arg145 [48,52,53,54,56,59], or Ile(Thr)126 to Asn126 [59]. Some reported presence point mutations within the S gene at positions 129 (Glutamine to Asparagine) and at position 145 (Glycine to Alanine) [39,52] Pro120 to Thr120 [48]. Some individuals with anti-HBc alone are carrier of HBV. The reason for this unusual serological pattern is not clear. One possibility may be the mutations in the HBsAg rendering the molecule undetectable by conventional serological assays. In order to test this hypothesis, we sequenced the “a” determinant of the S gene and compared them to the S gene sequence of 4 isolates from randomly chosen HBsAg positive HBV carrier. The “a” determinant of the S gene of isolates from 8 HBV carriers who were positive for anti-HBc only were sequenced. All individuals had low level HBV containing less than 200 copies of the viral genome per ml in their serum as determined by quantitative PCR. Five of these isolates had mutated nucleotide leading to amino acid change as followed M103I, P105R, L109I, T/I126M and F134I/V, S143L and D144E (Table9). None of the mutations was observed in three different isolates. Isolates from HBsAg carrier showed no variation or amino acid change. In contrast, isolates from individuals with anti-HBc alone showed higher rate of amino acid exchange (5 in 8 isolate). Thus the amino acid exchanges or mutation in the “a” determinant differs significantly between the two groups. Therefore, one cannot neglect the possibility that structure alternations caused by changes of amino acid in the surface determinants might prevent recognition of HBsAg by the serological assays routinely used for diagnosis of hepatitis B infections. Additional information can be deduced from sequence analysis. The nucleotide sequences of all eight isolates were compared

with published genotype. Four of these isolates are phylogenetically related to genotype C and other four are genotype B.

Recently, the clinical significance of HCV infection that may interfere with HBsAg synthesis has been reported. In 1991, Y.F.Liaw reported hepatitis C virus superinfection followed by spontaneous hepatitis B e antigen seroconversion and hepatitis B surface antigen clearance in a patient with chronic type B hepatitis. The observations suggest that HCV may exert a suppressive effect on hepatitis B virus [38]. Although anti-HCV seropositivity may be merely a confounding factor, the significant association between HCV infection and delayed serum HBsAg clerance further suggests that HCV superinfection might exert a viral interference effect that suppresses or terminates the HBsAg carrier state [35,36]. HCV infection was presented in 65.4% of anti-HBc only reactive individuals [21]. Our study found anti-HCV+ in 10 samples (6.33%). There was no coinfection between HCV and HBV in samples of anti-HBc only positive individuals. Whereas, other paper showed high prevalence of HCV infection in sera with only anti-HBc positive, these results may occur from those were high risk group of HCV (high HCV seroprevalence), i.e., haemodialysis patients, organ transplant recipients, intravenous drug addicts, HIV-infected and one third of the patients were treated or under follow-up at the gastroenterology outpatient clinic for chronic hepatitis C virus infection [21]. Our study shows no association between anti-HCV positive and the presence of HBV DNA in serum of anti-HBc positive/HBsAg negative patients.

According to our finding, the most probable hypothesis that account for the presence of anti-HBc alone is the virus presence in a very low titer and below the detection limit of the serological assays. Interestingly, mutants usually cannot be detected by serology alone. Molecular technique will help to interprete the HBV infection in sera of those individuals with only anti-HBc as serologic marker. The clinical application of this study may be beneficial to use for detection of this acute phase marker in blood donors and donor of organ transplantation in order to discard possible donations from patients during the window period of infection or to detect mutant forms which may help to increase the diagnostic safety.

REFERENCES

1. Mahoney FJ. Update on diagnosis, management, and prevention of hepatitis B virus infection. Clin Microbiol Rev 1999;12(2):351-66.
2. Seeger C, Mason WS. Hepatitis B virus biology. Microbiol Mol Biol Rev 2000;64(1):51-68.
3. Weinberger KM, Kreuzpaintner EA, Hottentrager B, Neifer S, Jilg W. Mutation in the S-gene of hepatitis B isolates from chronic carriers with anti-HBc as the only serological marker of HBV infection. J Hepatol 1996;2:138-43.
4. Carman WF, Mimm LT. Pre-S/S gene variants of hepatitis B virus. J Hepatol 1996;2:108-15.
5. Norder H, Courouce AM, Coursaget P, Echevarria JM, Lee SD, Mushahwar IK, et al. Genetic diversity of hepatitis B virus strains derived worldwide: genotypes, subgenotypes, and HBsAg subtypes. Intervirology 2004;47(6):289-309.
6. Stuyver L, De Gendt S, Van Geyt C, Zoulim F, Fried M, Schinazi RF, et al. A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. J Gen Virol 2000;81(Pt 1):67-74.
7. Theamboonlers A, Tangkijvanich P, Pramoolsinsap C, Poovorawan Y. Genotypes and subtypes of hepatitis B virus in Thailand. Southeast Asian J Trop Med Public Health 1998;29(4):786-91.
8. Theamboonlers A, Jantaradsamee P, Kaew-In N, Tangkijvanich P, Hirsch P, Poovorawan Y. The predominant genotypes of hepatitis B virus in Thailand. Ann Trop Med Parasitol 1999;93(7):737-43.
9. Gutierrez C, Leon G, Loureiro CL, Uzcategui N, Liprandi F, Pujol FH. Hepatitis B virus DNA in blood samples positive for antibodies to core antigen and negative for surface antigen. Clin Diagn Lab Immunol 1999;6(5):768-70.
10. Hess G, Arnold W. The clinical relevance of the antibody to hepatitis B core antigen (anti-HBc): a review. J Virol Methods 1980;2(1-2):107-17.
11. Prieto M, Gomez MD, Berenguer M, Cordoba J, Rayon JM, Pastor M, et al. De novo hepatitis B after liver transplantation from hepatitis B core antibody-

positive donors in an area with high prevalence of anti-HBc positivity in the donor population. Liver Transpl 2001;7(1):51-8.

12. Dickson RC, Everhart JE, Lake JR, Wei Y, Seaberg EC, Wiesner RH, et al. Transmission of hepatitis B by transplantation of livers from donors positive for antibody to hepatitis B core antigen. The National Institute of Diabetes and Digestive and Kidney Diseases Liver Transplantation Database. Gastroenterology 1997;113(5):1668-74.
13. Uemoto S, Sugiyama K, Marusawa H, Inomata Y, Asonuma K, Egawa H, et al. Transmission of hepatitis B virus from hepatitis B core antibody-positive donors in living related liver transplants. Transplantation 1998;65(4):494-9.
14. Yotsuyanagi H, Yasuda K, Moriya K, Shintani Y, Fujie H, Tsutsumi T, et al. Frequent presence of HBV in the sera of HBsAg-negative, anti-HBc-positive blood donors. Transfusion 2001;41(9):1093-9.
15. Yotsuyanagi H, Yasuda K, Iino S, Moriya K, Shintani Y, Fujimie H, et al. HBV DNA in serum of HBsAg-negative, anti-HBc-positive blood donors. Transfusion 2002;42(12):1616-7.
16. Arraes LC, Ximenes R, Andrieu JM, Lu W, Barreto S, Pereira LM, et al. The biological meaning of anti-HBC positive result in blood donors: relation to HBV-DNA and to other serological markers. Rev Inst Med Trop Sao Paulo 2003;45(3):137-40.
17. Almeida Neto C, Strauss E, Sabino EC, Sucupira MC, Chamone DA. Significance of isolated hepatitis B core antibody in blood donors from Sao Paulo. Rev Inst Med Trop Sao Paulo 2001;43(4):203-8.
18. Katsurada A, Marusawa H, Uemoto S, Kaburagi A, Tanaka K, Chiba T. Circulating antibody to hepatitis B core antigen does NOT always reflect the latent hepatitis B virus infection in the liver tissue. Hepatol Res 2003;25(2):105-14.
19. Chaudhuri V, Nanu A, Panda SK, Chand P. Evaluation of serologic screening of blood donors in India reveals a lack of correlation between anti-HBc titer and PCR-amplified HBV DNA. Transfusion 2003;43(10):1442-8.
20. Zekri AR, Awlia AA, El Mahalawi H, Ismail EF, Mabrouk GM. Evaluation of blood units with isolated anti HBC for the presence of HBV DNA. Dis Markers 2002;18(3):107-10.

21. Weber B, Melchior W, Gehrke R, Doerr HW, Berger A, Rabenau H. Hepatitis B virus markers in anti-HBc only positive individuals. J Med Virol 2001;64(3):312-9.

22. Joller-Jemelka HI, Wicki AN, Grob PJ. Detection of HBs antigen in "anti-HBc alone" positive sera. J Hepatol 1994;21(2):269-72.

23. Brechot C, Degos F, Lugassy C, Thiers V, Zafrani S, Franco D, et al. Hepatitis B virus DNA in patients with chronic liver disease and negative tests for hepatitis B surface antigen. N Engl J Med 1985;312(5):270-6.

24. Marusawa H, Uemoto S, Hijikata M, Ueda Y, Tanaka K, Shimotohno K, et al. Latent hepatitis B virus infection in healthy individuals with antibodies to hepatitis B core antigen. Hepatology 2000;31(2):488-95.

25. Mason AL, Xu L, Guo L, Kuhns M, Perrillo RP. Molecular basis for persistent hepatitis B virus infection in the liver after clearance of serum hepatitis B surface antigen. Hepatology 1998;27(6):1736-42.

26. Larsen J, Hetland G, Skaug K. Posttransfusion hepatitis B transmitted by blood from a hepatitis B surface antigen-negative hepatitis B virus carrier. Transfusion 1990;30(5):431-2.

27. Cabrerizo M, Bartolom inverted question marke J, Caramelo C, Barril G, Carreno V. Molecular analysis of hepatitis B virus DNA in serum and peripheral blood mononuclear cells from hepatitis B surface antigen-negative cases. Hepatology 2000;32(1):116-23.

28. Oon CJ, Chen WN, Goh KT, Mesenas S, Ng HS, Chiang G, et al. Molecular characterization of hepatitis B virus surface antigen mutants in Singapore patients with hepatocellular carcinoma and hepatitis B virus carriers negative for HBsAg but positive for anti-HBs and anti-HBc. J Gastroenterol Hepatol 2002;17 Suppl:S491-6.

29. Bodhiphala, P., et al. Detection of HBV genome by gene amplification method in HBsAg negative blood donors. J Med Assoc Thai 1999;82(5):491–5.

30. Brenda GL, Kristin RF, Angela MC, Frederick SN. Development and verification of an automated sample processing protocol for quantitation of human immunodeficiency virus type1 RNA in plasma. J Clin Microbiol 2003;41(5):2062-7.

31. Rita S, Joanne KU, Harsha J, Jo CK, Donald B, Steven H, Maurice R, Joanne

S. Ultrasensitive reverse transcription-PCR assay for quantitation of human immunodeficiency virus type1 RNA in plasma. J Clin Microbiol 1998;36(10):2964-9.

32. Dreier J, Kroger M, Diekmann J, Gotting C, Kleesiek K. Low-level viremia of hepatitis B virus in an anti-HBc- and anti-HBs-positive blood donor. Transfus Med 2004;14(2):97-103.
33. Piwowar-Manning EM, Henderson TA, Brisbin L, Jackson JB. A modified ultrasensitive assay to detect quantified HIV-1 RNA of fewer than 50 copies per milliliter. Am J Clin Pathol 2003;120(2):268-70.
34. Shih CM, Lo SJ, Miyamura T, Chen SY, Lee YH. Suppression of hepatitis B virus expression and replication by hepatitis C virus core protein in HuH-7 cells. J Virol 1993;67(10):5823-32.
35. Sheen IS, Liaw YF, Chu CM, Pao CC. Role of hepatitis C virus infection in spontaneous hepatitis B surface antigen clearance during chronic hepatitis B virus infection. J Infect Dis 1992;165(5):831-4.
36. Drosten C, Nippraschk T, Manegold C, Meisel H, Brixner V, Roth WK, et al. Prevalence of hepatitis B virus DNA in anti-HBc-positive/HBsAg-negative sera correlates with HCV but not HIV serostatus. J Clin Virol 2004;29(1):59-68.
37. Tsai JF, Margolis HS, Jeng JE, Ho MS, Chang WY, Hsieh MY, et al. Immunoglobulin- and hepatitis B surface antigen-specific circulating immune complexes in chronic hepatitis B virus infection. Clin Immunol Immunopathol 1998;86(3):246-51.
38. Liaw YF, Lin SM, Sheen IS, Chu CM. Acute hepatitis C virus superinfection followed by spontaneous HBeAg seroconversion and HBsAg elimination. Infection 1991;19(4):250-1.
39. Koyanagi T, Nakamuta M, Sakai H, Sugimoto R, Enjoji M, Koto K, et al. Analysis of HBs antigen negative variant of hepatitis B virus: unique substitutions, Glu129 to Asp and Gly145 to Ala in the surface antigen gene. Med Sci Monit 2000;6(6):1165-9.
40. Mesenas SJ, Chow WC, Zhao Y, Lim GK, Oon CJ, Ng HS. Wild-type and 'a' epitope variants in chronic hepatitis B virus carriers positive for hepatitis B surface antigen and antibody. J Gastroenterol Hepatol 2002;17(2):148-52.

41. Chen WN, Oon CJ. Hepatitis B virus mutants: an overview. J Gastroenterol Hepatol 2002;17 Suppl:S497-9.

42. Locarnini S, McMillan J, Bartholomeusz A. The hepatitis B virus and common mutants. Semin Liver Dis 2003;23(1):5-20.

43. Grethe S, Monazahian M, Bohme I, Thomssen R. Characterization of unusual escape variants of hepatitis B virus isolated from a hepatitis B surface antigen-negative subject. J Virol 1998;72(9):7692-6.

44. Chen WN, Oon CJ. Human hepatitis B virus mutants: significance of molecular changes. FEBS Lett 1999;453(3):237-42.

45. Zuckerman AJ, Harrison TJ, Oon CJ. Mutations in S region of hepatitis B virus. Lancet 1994;343(8899):737-8.

46. Kao JH, Chen PJ, Lai MY, Chen DS. Sequence analysis of pre-S/surface and pre-core/core promoter genes of hepatitis B virus in chronic hepatitis C patients with occult HBV infection. J Med Virol 2002;68(2):216-20.

47. Alhababi F, Sallam TA, Tong CY. The significance of 'anti-HBc only' in the clinical virology laboratory. J Clin Virol 2003;27(2):162-9.

48. Basuni AA, Butterworth L, Cooksley G, Locarnini S, Carman WF. Prevalence of HBsAg mutants and impact of hepatitis B infant immunisation in four Pacific Island countries. Vaccine 2004;22(21-22):2791-9.

49. Theamboonlers A, Chongsrisawat V, Jantaradsamee P, Poovorawan Y. Variants within the "a" determinant of HBs gene in children and adolescents with and without hepatitis B vaccination as part of Thailand's Expanded Program on Immunization (EPI). Tohoku J Exp Med 2001;193(3):197-205.

50. Kajiya Y, Hamasaki K, Nakata K, Nakagawa Y, Miyazoe S, Takeda Y, et al. Full-length sequence and functional analysis of hepatitis B virus genome in a virus carrier: a case report suggesting the impact of pre-S and core promoter mutations on the progression of the disease. J Viral Hepat 2002;9(2):149-56.

51. Laskus T, Radkowski M, Wang LF, Nowicki M, Rakela J. Detection and sequence analysis of hepatitis B virus integration in peripheral blood mononuclear cells. J Virol 1999;73(2):1235-8.

52. Louisirirotchanakul S, Kanoksinsombat C, Theamboonlert A, Puthavatana P, Wasi C, Poovorawan Y. Mutation of the "a" determinant of HBsAg with discordant HBsAg diagnostic kits. Viral Immunol 2004;17(3):440-4.

53. Moerman, B., et al. Evaluation of sensitivity for wild type and mutant forms of hepatitis B surface antigen by four commercial HBsAg assays. Clin Lab 2004; 50(3-4):159-62.

54. Coleman PF, Chen YC, Mushahwar IK. Immunoassay detection of hepatitis B surface antigen mutants. J Med Virol 1999;59(1):19-24.

55. Chen WN, Oon CJ. Pre-S deletion mutants co-exist with wild-type virus at low viral replication stage in Singapore chronic hepatitis B virus carriers. Curr Microbiol 2002;44(2):145-7.

56. Chakravarty R, Neogi M, Roychowdhury S, Panda CK. Presence of hepatitis B surface antigen mutant G145R DNA in the peripheral blood leukocytes of the family members of an asymptomatic carrier and evidence of its horizontal transmission. Virus Res 2002;90(1-2):133-41.

57. Ma CL, Fang DX, Chen HB, Li FQ, Jin HY, Li SQ, et al. A mutation specific polymerase chain reaction for detecting hepatitis B virus genome mutations at nt551. World J Gastroenterol 2003;9(3):509-12.

58. Chen HB, Fang DX, Li FQ, Jing HY, Tan WG, Li SQ. A novel hepatitis B virus mutant with A-to-G at nt551 in the surface antigen gene. World J Gastroenterol 2003;9(2):304-8.

59. Yamamoto K, Horikita M, Tsuda F, Itoh K, Akahane Y, Yotsumoto S, et al. Naturally occurring escape mutants of hepatitis B virus with various mutations in the S gene in carriers seropositive for antibody to hepatitis B surface antigen. J Virol 1994;68(4):2671-6.

60. Carman, W. F., A. R. Zanetti, P. Karayiannis, J. Waters, G. Manzillo, E.Tanzi, A. J. Zuckerman, and H. C. Thomas. Vaccine-induced escape mutant of hepatitis B virus. Lancet 1990;336:325–9.

61. Harrison, T. J., E. A. Hopes, C. J. Oon, A. R. Zanetti, and A. J. Zuckerman. Independent emergence of a vaccine-induced escape mutant of hepatitis B virus. J Hepatol 1991;13 Suppl:S105-7.

62. Howard, C. R. The structure of hepatitis B envelope and molecular variants of hepatitis B virus. J Viral Hepatitis 1995;2:165–70.

63. McMahon, G., P. H. Ehrlich, Z. A. Moustafa, L. A. McCarthy, D. Dottavio, M. D. Tolpin, P. I. Nadler, and L. Ostberg. Genetic alterations in the gene encoding the major HBsAg: DNA and immunological analysis of recurrent HBsAg

derived from monoclonal antibody-treated liver transplant patients. Hepatology 1992; 15:757–66.

64. Nainan, O. V., C. E. Stevens, and H. S. Margolis. Hepatitis B virus (HBV) antibody resistant mutants: frequency and significance. In Ninth Triennial International Symposium on Viral Hepatitis and Liver Disease 1996: 29.

65. Zanetti, A. R., E. Tanzi, G. Manzillo, G. Maio, C. Sbreglia, N. Caporaso, H. Thomas, and A. J. Zuckerman. Hepatitis B variant in Europe. Lancet ii 1988 :1132–3.

66. Zuckerman, A. J., T. J. Harrison, and C. J. Oon. Mutation in S region of hepatitis B virus. Lancet 1994; 343:737–8.

67. Ogata, N., R. H. Miller, K. G. Ishak, A. R. Zanetti, and R. H. Purcell. Genetic and biologic characterization of two hepatitis B virus variants: a precore mutant implicated in fulminant hepatitis and a surface mutant resistant to immunoprophylaxis. Springer Verlag 1993.

68. Oon, C. J. Studies on the transmissability of HBV vaccine escape 145 Gly to Arg mutants in immune and hepatitis B carriers. In Ninth Triennial International Symposium on Viral Hepatitis and Liver Disease 1996: 265.

69. Merican, I., R.Guan, D. Amarapuka, et al. Chronic hepatitis B virus infection in Asian countries. J. Gastroenterol. Hepatol 2000;15: 1356-61.

70. Shin L, Sheu J, Wang J, Huang G, Yang P, Lee H, Sung J, Wang T, Chen D. Serum hepatitis B virus DNA in healthy HBsAg-negative chinese adults evaluated by polymerase chain reaction. J Med Virol 1990;32:257-60.

71. Brechot C, Kremsdorf D, Paterlini P, Thiers V. Hepatitis B virus DNA in HBsAg-negative patients – Molecular characterization and clinical implications. J Hepatol 1991; 13(suppl.4): 49-55.

72. Wang JT, Wang TH, Sheu JC, Shin LN, Lin JT, Chen DS. Detection of hepatitis B virus DNA by polymerase chain reaction in plasma of volunteer blood donors negative for hepatitis B surface antigen. J Infect Dis 1991;163:397-9.

73. Jilg W, Sieger E, Zachoval R, Schatzl H. Individuals with antibodies against hepatitis B core antigen as the only serological marker for hepatitis B infection: high percentage of carriers of hepatitis B and C virus. J Hepatol 1995;23:14-20.

74. Cobas Amplicor HBV Monitor^{TM} Test(Roche Molecular Systems, Inc.). Revision 2.0 2001;6:1-33.

75. Hoofnagle JH, Seeff LB, Bales ZB, Zimmerman HJ. Type B hepatitis after transfusion with blood containing antibody to hepatitis B core antigen. N Eng J Med 1978;298:1379-83.

76. Rakela J, Mosley JW, Aach RD, Gitnick GL,Hollinger FB, Stevens CE, Szmuness W. Viral hepatitis after transfusion with blood containing antibody to hepatitis B core antigen. Gastroenterology 1980;78:1318.

APPENDICES

APPENDIX A

REAGENTS AND EQUIPMENTS

1. Equipments

ABI PRISM Sequencer (ABI 310 Genetic Analyzer, Applied Biosystem, USA)
Automatic adjustable micropipette (Gilson Medical Electronic, France)
AxSYM System (Abbott Diagnostics, Germany)
Centrifuge 5417R (Eppendorf, Germany)
Cobas Core II (Roche Diagnostics, USA)
Chemi Doc (BIORAD, USA)
DNA speed VAC (Savent, USA)
Gelmate 2000 electrophoresis(Toyobo, Japan)
Mini Gel Migration Tank (National Labnet, USA)
Mixer-Vertex2 Genie (Scientific industries,USA)
Optima ^{TM} TLX Ultrasentrifuge (Beckman, USA)
PCR machine Gene Amp PCR System 2400 (Perkin Elmer, USA)
Polycarbonate centrifuge tube (Beckman, USA)
Rotor model TLA-120.2 (Beckman, USA)
Spectrophotometer (BIORAD, USA)
Thermomixer (Eppendorf, USA)
UV transilluminator (FotoPrepI, Fotodyne USA)

2. Reagents

100 bp molecular marker (Promega corporation,USA)
AxSYM® HBsAg[V2] (Abbott Diagnostic, Germany)
Absolute ethanol (Merck, Germany)
Agarose (GIBCO, Grand Island,N.Y. USA)
BigDye ™ Terminator Cycle Sequencing reaction kit (Perkin Elmer, USA)
Bromphenol blue (Sigma, Mo.,USA)
Cobas Amplicor HBV Monitor ™ Test (Roche diagnostic, USA)
Cobas core Anti-HCV EIA II (Roche Diagnostics, USA)
Disodium ethylenediamine tetraacetic acid : EDTA (Sigma, Mo.,USA)
Ethidium bromide (Sigma, Mo.,USA)
Glacial acetic acid (Merck, Germany)
QIAamp DNA Blood Kit (Qiagen, Hilden, Germany)
QIAquick PCR Purification Kit (Qiagen, Hilden, Germany)
Sodium acetate (Merck, Germany)
Tris base (sigma, USA)

3. Laboratory supplies

Pipette tip for P10, P20, P200, P1000 (Treff Lab, Switzerland)
Glassware (Pyrex, USA)
Parafilm (American National Can, USA)

4. Enzymes

Taq DNA polymerase (Promega Corporation, USA)

5. Reagents of PCR

10X PCR buffer : 500 mM KCl, 200 mM Tris-HCl pH8.4, 25 mM $MgCl_2$ (Promega Corporation, USA)

Deoxynucleotide triphosphates (dNTPs) (Promega Corporation, USA)

Oligonucleotide primers (S.M.Chemical supplies)

6. Reagent of DNA Sequencing

ABI PRISM ™ Dye Terminator Cycle Sequencing Ready Reaction kit with Amplitaq® DNA polymerase FS, (Perkin-Elmer, USA)

APPENDIX B

REAGENTS PREPARATION

1. Reagent for DNA Extraction

1.1 Protease K

Reconstituted of protease K (lyophilized) with 1.25 ml protease solvent , stored at –20^{o}C

1.2 Buffer AL (Ready to used)

1.3 Buffer AW1

Buffer AW1 is supplied as a concentrate. Before using for the first time, add the 25 ml of ethanol (96-100%) to buffer AW1 concentrate as indicated on the bottle.

1.4 Buffer AW2

Buffer AW2 is supplied as a concentrate. Before using for the first time, add the 30 ml of ethanol (96-100%) to buffer AW2 concentrate as indicated on the bottle.

1.5 Buffer AE (Ready to used)

2. Reagents for agarose gel electrophoresis

2.1 0.5 M EDTA, pH8.0

Disodiun ethylene diamine tetraacetate.$2H_2O$	186.1 g
Distilled water	800 ml
Adjust pH to 8.0 with sodium hydroxide pellet	
then adjust volume to	1,000 ml

2.2 50X Tris-acetate buffer (TAE)

Tris base	242.0 g
Glacial acetic acid	57.1 ml
0.5 M EDTA pH 8.0	100 ml

Adjust the volume to 1 liter with deionized distilled water and sterilize by autoclaving at 121 ^{o}C for 15 min.

2.3 10 mg/ml Ethidium bromide (Stock)

Ethidium bromide	1 g
Distilled water	100 ml

Stir on a magnetic stirrer for several hours to ensure that dye has dissolved. Wrap the container in aluminum foil or transfer to a dark bottle and stores at 4^{o}C

2.4 2% Agarose gel

Agarose ultrapure	0.4 g
1X TAE	40 ml

3. Reagent for PCR product purification

3.1 Buffer PB (Ready to used)

3.2 Buffer PE

Buffer PE is supplied as a concentrate. Before using for the first time, add the 55 ml of ethanol (96-100%) to buffer PE concentrate as indicated on the bottle.

4. Reagent for Sequencing

4.1 3 M Sodium acetate, pH 8.0

Sodium acetate, 3 H_2O	408.1 g
Distilled water	800 ml
Adjust pH to 8.0 with glacial acetic acid, Adjust volume to	1,000 ml

APPENDIX C

1. 64 codon on DNA

First Position	Second Position				Third Position
	T	C	A	G	
T	F	S	Y	C	T
	F	S	Y	C	C
	L	S	STOP CODON	STOP CODON	A
	L	S	STOP CODON	W	G
C	L	P	H	R	T
	L	P	H	R	C
	L	P	Q	R	A
	L	P	Q	R	G
A	I	T	N	S	T
	I	T	N	S	C
	I	T	K	R	A
	M	T	K	R	G
G	V	A	D	G	T
	V	A	D	G	C
	V	A	E	G	A
	V	A	E	G	G

Abbreviation: A,alanine; C,cysteine; D,aspartic acid; E,glutamic acid; F,phenylalanine; G,glycine; H,histidine; I,isoleucine; K,lysine; L,leucine; M,methionine; N,asparagine; P,proline; Q,glutamine; R,arginine; S,serine; T,threonine; V,valine; W,trytophan; Y,tyrosine, NCCLS

2. The results of HBsAg by AxSYM HBsAg[V2], Anti-HCV by Cobas Core Anti-HCV EIA II and HBV DNA by Nested and Ultrasensitive PCR from serum of anti HBc only marker.

Code No.	Sex	HBsAg by AxSYM		Anti-HCV (EIA)		HBV PCR (core region)	
		S/N*	Result	Absorbance**	Result	Nested PCR	Ultrasensitive PCR
H001	M	0.80	-	0.030	-	-	-
H002	F	0.74	-	0.032	-	+	ND
H003	M	0.91	-	0.021	-	-	-
H004	M	0.85	-	0.029	-	-	-
H005	F	0.78	-	0.020	-	-	-
H006	M	0.68	-	0.028	-	-	-
H007	M	0.64	-	0.019	-	-	-
H008	F	0.77	-	0.066	-	-	-
H009	F	0.84	-	0.019	-	-	-
H010	M	0.77	-	0.023	-	-	-
H011	M	0.78	-	3.500	+	-	-
H012	M	0.66	-	0.027	-	-	-
H013	F	0.68	-	0.019	-	-	-
H014	M	0.73	-	0.026	-	-	-
H015	M	0.83	-	0.024	-	-	-
H016	M	0.85	-	0.024	-	-	-
H017	M	0.75	-	0.029	-	-	-
H018	M	0.83	-	0.021	-	-	+
H019	M	0.73	-	0.021	-	-	-
H020	M	0.72	-	0.025	-	-	-
H021	M	0.75	-	0.020	-	-	-
H022	F	0.75	-	0.020	-	-	-
H023	M	0.81	-	0.026	-	-	-
H024	F	0.74	-	0.024	-	-	-
H025	M	0.72	-	0.023	-	-	-
H026	F	0.69	-	0.025	-	-	-

Code No.	Sex	HBsAg by AxSYM		Anti-HCV (EIA)		HBV PCR (core region)	
		S/N*	Result	Absorbance**	Result	Nested PCR	Ultrasensitive PCR
H027	F	0.93	-	0.026	-	-	-
H028	M	0.82	-	0.020	-	-	-
H029	F	0.84	-	0.026	-	-	-
H030	F	0.68	-	0.043	-	-	-
H031	M	0.75	-	0.022	-	-	-
H032	M	0.74	-	0.023	-	-	-
H033	M	0.78	-	0.025	-	-	-
H034	F	0.65	-	0.023	-	-	-
H035	M	0.65	-	0.031	-	-	-
H036	M	0.64	-	0.032	-	-	-
H037	F	0.60	-	0.035	-	-	-
H038	F	0.77	-	0.027	-	-	-
H039	M	0.70	-	0.035	-	-	-
H040	M	0.75	-	0.031	-	-	-
H041	M	0.86	-	0.025	-	+	**ND**
H042	F	0.65	-	0.021	-	-	-
H043	M	0.72	-	0.026	-	-	-
H044	M	0.84	-	0.028	-	-	-
H045	F	0.74	-	0.034	-	-	-
H046	M	0.83	-	0.023	-	-	-
H047	M	0.78	-	0.020	-	-	-
H048	M	0.86	-	3.500	+	-	-
H049	F	0.87	-	0.025	-	-	-
H050	M	0.87	-	0.023	-	-	-
H051	M	0.66	-	0.021	-	-	-
H052	M	0.95	-	0.032	-	-	-
H053	M	0.76	-	0.021	-	-	-
H054	F	0.75	-	0.025	-	-	-

Code No.	Sex	HBsAg by AxSYM		Anti-HCV (EIA)		HBV PCR (core region)	
		S/N*	Result	Absorbance**	Result	Nested PCR	Ultrasensitive PCR
H055	F	0.75	-	0.026	-	-	-
H056	M	0.88	-	3.500	+	-	-
H057	F	0.76	-	0.029	-	-	-
H058	F	0.71	-	0.031	-	-	-
H059	M	0.74	-	0.035	-	-	-
H060	F	0.73	-	0.018	-	-	-
H061	F	0.79	-	0.019	-	-	-
H062	M	0.75	-	0.026	-	-	-
H063	M	0.91	-	0.032	-	-	-
H064	M	1.14	-	0.025	-	-	-
H065	F	0.77	-	0.014	-	-	-
H066	F	0.95	-	0.026	-	-	-
H067	F	0.72	-	0.018	-	-	-
H068	M	0.65	-	0.029	-	-	-
H069	M	0.93	-	0.031	-	+	**ND**
H070	F	0.72	-	0.025	-	-	-
H071	M	0.78	-	0.026	-	-	-
H072	F	1.05	-	0.023	-	-	-
H073	F	0.88	-	3.500	+	-	-
H074	F	0.99	-	0.017	-	-	-
H075	F	0.85	-	0.025	-	-	-
H076	M	0.92	-	0.021	-	-	-
H077	M	0.82	-	0.026	-	-	-
H078	M	0.86	-	0.019	-	-	-
H079	M	0.85	-	3.500	+	-	-
H080	M	1.10	-	0.021	-	-	-
H081	M	0.93	-	0.023	-	-	-
H082	F	0.85	-	0.018	-	-	-

Code No.	Sex	HBsAg by AxSYM		Anti-HCV (EIA)		HBV PCR (core region)	
		S/N*	Result	Absorbance**	Result	Nested PCR	Ultrasensitive PCR
H083	M	0.76	-	0.035	-	-	-
H084	M	0.93	-	0.031	-	-	-
H085	F	0.82	-	0.021	-	-	-
H086	M	0.79	-	0.025	-	-	-
H087	M	0.77	-	0.024	-	-	+
H088	M	0.77	-	0.018	-	-	-
H089	M	0.78	-	0.019	-	-	-
H090	M	0.68	-	0.021	-	-	-
H091	M	0.64	-	0.026	-	-	-
H092	M	0.91	-	0.023	-	-	-
H093	F	0.77	-	0.028	-	-	-
H094	F	0.84	-	0.029	-	-	-
H095	M	0.75	-	0.031	-	-	-
H096	F	0.73	-	0.035	-	-	-
H097	F	0.85	-	0.021	-	-	-
H098	M	0.66	-	0.015	-	-	-
H099	M	0.75	-	3.500	+	-	-
H100	M	0.72	-	0.021	-	-	-
H101	F	0.84	-	0.026	-	-	-
H102	M	0.82	-	0.028	-	-	-
H103	M	0.77	-	0.018	-	+	**ND**
H104	F	0.78	-	0.013	-	-	-
H105	F	0.75	-	0.029	-	-	-
H106	F	0.84	-	0.032	-	-	-
H107	M	0.76	-	0.021	-	-	-
H108	M	0.77	-	0.023	-	-	-
H109	F	0.75	-	0.026	-	-	-
H110	F	0.74	-	0.025	-	-	-

Code No.	Sex	HBsAg by AxSYM		Anti-HCV (EIA)		HBV PCR (core region)	
		S/N*	Result	Absorbance**	Result	Nested PCR	Ultrasensitive PCR
H111	M	0.81	-	0.026	-	-	-
H112	F	0.82	-	0.018	-	-	-
H113	M	0.91	-	0.026	-	-	-
H114	F	0.85	-	3.500	+	-	-
H115	M	0.76	-	0.021	-	-	-
H116	F	0.77	-	0.032	-	-	-
H117	F	0.85	-	0.035	-	-	-
H118	F	0.83	-	0.029	-	-	-
H119	M	0.76	-	0.026	-	-	-
H120	M	0.78	-	0.019	-	-	-
H121	F	0.74	-	0.026	-	-	-
H122	M	0.82	-	0.035	-	-	-
H123	F	0.86	-	0.029	-	-	-
H124	F	0.84	-	3.500	+	-	-
H125	M	0.76	-	0.029	-	-	-
H126	M	0.75	-	0.028	-	-	-
H127	M	0.77	-	0.019	-	-	-
H128	M	0.78	-	0.019	-	-	+
H129	M	0.84	-	0.023	-	-	-
H130	M	0.86	-	0.025	-	-	-
H131	F	0.78	-	0.018	-	-	-
H132	M	0.82	-	0.013	-	-	-
H133	F	0.78	-	0.031	-	-	-
H134	M	0.85	-	0.031	-	-	-
H135	M	0.86	-	0.025	-	-	-
H136	M	0.77	-	0.026	-	-	-
H137	M	0.71	-	0.018	-	-	-
H138	M	0.65	-	3.500	+	-	-

Code No.	Sex	HBsAg by AxSYM		Anti-HCV (EIA)		HBV PCR (core region)	
		S/N*	Result	Absorbance**	Result	Nested PCR	Ultrasensitive PCR
H139	M	0.68	-	0.017	-	-	-
H140	F	0.75	-	0.023	-	-	-
H141	M	0.78	-	0.021	-	-	-
H142	M	0.86	-	0.035	-	-	-
H143	M	0.88	-	0.016	-	-	-
H144	M	0.78	-	0.018	-	-	-
H145	F	0.76	-	0.026	-	-	-
H146	F	0.79	-	0.021	-	-	-
H147	M	0.84	-	0.023	-	-	-
H148	F	0.81	-	0.028	-	-	+
H149	M	0.75	-	0.023	-	-	-
H150	M	0.77	-	3.500	+	-	-
H151	F	0.74	-	0.021	-	-	-
H152	M	0.85	-	0.015	-	-	-
H153	M	0.86	-	0.027	-	-	-
H154	F	0.88	-	0.036	-	-	-
H155	F	0.73	-	0.032	-	-	-
H156	F	0.71	-	0.024	-	-	-
H157	M	0.85	-	0.016	-	-	-
H158	F	0.86	-	0.032	-	-	-

*Cut off (S/N) of HBsAg >2.00 = Positive; ** Cut off anti-HCV >0.273 = Positive

Abbreviations : F= Female; M= Male; - Negative; + Positive; ND= Not done

BIOGRAPHY

Miss. Niramol Thammacharoenrach was born on October 15, 1975 in Prachubkirikan, Thailand. She graduated with the Bachelor degree of Biochemistry from the Faculty of Science, Chulalongkorn University in 1997. She is currently a member of Immunology Unit, Department of Microbiology, King Chulalongkorn Memorial Hosipital and was given the opportunity to pursue her MS degree in the Inter-Department of Medical Microbiology, Faculty of Graduate School, Chulalongkorn University since 2002.