Novel Methods for Specific Detection and Quantification of Covalently Closed Circular DNA in Sera and Biopsies of Hepatitis B Patients

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Abstract

Over 350 million people worldwide suffer from chronic hepatitis B virus (HBV) infection, which leads to many cases of cirrhosis and hepatocellular carcinoma. HBV covalently closed circular DNA (cccDNA) is a critical intracellular replicative intermediate and cannot be eliminated during antiviral therapy. Current methods for cccDNA detection are limited by false positive detection due to the interference by HBV relaxed circular DNA (rcDNA). The tests also have limited sensitivity to detect cccDNA at low concentrations. Hence, we aimed to develop a highly sensitive and highly specific assay for cccDNA detection with wide linear range.

With this background, two new cccDNA assays were developed and optimized. Bowden's assay was used as a standard to evaluate the performance of new assays. The first new assay (modified Bowden's assay) involved the use of new primers and probes that targeted more conserved regions in the HBV genome. The second assay adopted the bisulfite conversion method, which introduced gene sequence changes into the HBV genome and thereby enhance the specificity of the assay. Capillary sequencing was performed to find mutations in primers and probe range of different assays.

The modified Bowden's assay had the highest intrahepatic cccDNA detection rate (60 positive results out of 61 cases). The detection rate of the modified

Bowden's assay is significantly higher than that of the Bowden's assay. On the other hand, the cccDNA detection rate in serum samples was low at 20-27% by all 3 assays. In 5 samples in which cccDNA was undetectable by the Bowden's assay but detectable by the other two assays, a point mutation in the HBV genome was found in the forward primer binding site of the Bowden's assay. This partly explained the false negative results.

The quantification result of cccDNA by the bisulfite conversion assay was significantly lower than that by the Bowden's assay assay (P=0.001) and the modified Bowden's assay (P=0.003). When the total HBV DNA was higher than 10^7 copies/ml, the serum cccDNA level detected by the bisulfite conversion assay was significantly lower than that detected by the Bowden's assay (P=0.008) and the modified Bowden's assay (P=0.046). When the total HBV DNA is less than 10^7 copies/ml, there were no significant differences. This suggests that the bisulfite conversion assay was less affected by rcDNA even in samples containing a high viral load.

In conclusion, two new methods of cccDNA quantitation were developed and validated. The two assays are complementary to each other and may be used in patients with extreme HBV DNA levels. These cccDNA assays should be further validated in larger studies and may become important tests for diagnostic, prognostic and treatment monitoring purposes.

摘 要

全球有超過三億五千萬人感染了乙型肝炎病毒，乙肝病毒感染導致了很多例肝硬化和肝癌。乙肝病毒共價閉合環狀去氧核糖核酸是乙肝病毒複製過程中的一個關鍵的中間媒介，而且不能被抗病毒藥物清除。當前應用的乙肝病毒共價閉合環狀去氧核糖核酸檢測方法都被由乙肝病毒鬆弛環狀去氧核糖核酸的干擾而產生的假陽性結果所限制。靈敏度的不足導致無法檢測過低含量的乙肝病毒共價閉合環狀去氧核糖核酸也是一個限制。本研究的目的就是提出一種新的檢測方法，能夠高效，高特異性地在一個很寬的濃度範圍內檢驗乙肝病毒共價閉合環狀去氧核糖核酸。

在這樣的背景之下，兩個新的檢測方法分別被開發和改良。Bowden 方法被用作評估新方法的標準。第一個檢測方法是對現有的 Bowden 方法的改良（modified Bowden 方法），包括了新設計的針對一個更保守的乙肝病毒序列區域的引物和探針。第二個是全新的基於重亞硫酸鹽轉化的檢測方法。重亞硫酸鹽轉化可以將序列的不同引入乙肝病毒基因組，從而增強檢驗的特異性。毛細管測序用來尋找各個方法的引物與探針區域的突變。

在肝組織樣本組中，modified Bowden 方法擁有最高的檢出率（61 個樣本中檢驗出 60 個陽性結果），明顯高於傳統的 Bowden 方法。然而在血清樣本組中，三個方法的檢出率都低至 20%到 27%。在 5 個可以被兩個新方法檢驗出乙肝病毒共價閉合環狀去氧核糖核酸卻不能被傳統的 Bowden 方法檢驗出的樣本中，發現了一個在 Bowden 方法引物區域的點突變。這個發現部分解釋了假陰性結果。

在肝組織樣本組中，重亞硫酸鹽轉化方法檢測出的乙肝病毒共價閉合環狀去氧核糖

核酸含量明顯低於 Bowden 方法（P=0.001）和 modified Bowden 方法（*P*=0.003）。在血清樣本組中，當血清裏的乙肝病毒去氧核糖核酸總濃度高於 10^7 拷貝每毫升時，重亞硫酸鹽轉化方法檢測出的乙肝病毒共價閉合環狀去氧核糖核酸含量也明顯低於 Bowden 方法（P=0.008）和 modified Bowden 方法（*P*=0.046）。當血清裏的乙肝病毒去氧核糖核酸總濃度低於 10^7 拷貝每毫升時，差異不明顯。這說明了即使在很高病毒載量的樣本中，重亞硫酸鹽轉化方法受到乙肝病毒鬆弛環狀去氧核糖核酸的干擾依然比較少。

總之，這個研究中提出並驗證了兩個新的檢測定量方法。這兩個新方法可以互補並可以用於極端乙肝病毒去氧核糖核酸濃度的樣本中。這兩個方法在更進一步的驗證之後，可能會成爲重要的用於診斷，預測和治療監控的測試。

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List of Abbreviations

A	Adenosine
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
Anti-HBe	Antibody against hepatitis B e
ATP	Adenosine triphosphate
C	Cytosine
cccDNA	Covalently closed circular DNA
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DR	Direct repeat
G	Guanine
HBcAg	Hepatitis B core antigen
HBeAg	Hepatitis B e antigen
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus
kb	Kilobase
MGB	Minor groove binder
mRNA	Messenger RNA

ORF	Open reading frame
PCR	Polymerase chain reaction
pgRNA	Pregenomic RNA
rcDNA	Relaxed circular DNA
RNA	Ribonucleic acid
siRNA	small interfering RNA
T	Thymidine
TMAC	*Tetramethylammonium chloride*
U	Uracil
WHO	World Health Organization

CHAPTER 1 Introduction

1.1 General introduction

Chronic hepatitis B is the leading cause of cirrhosis and hepatocellular carcinoma in Asia. [1] In chronic hepatitis B patients with advanced liver fibrosis or active hepatitis, antiviral therapy with oral nucleoside or nucleotide analogs or interferon may reduce the risk of disease progression and hepatocellular carcinoma development. [2, 3] While these antiviral drugs effectively suppress the viral load in the body, normalize serum alanine aminotransferase (ALT) level and lead to histological improvement, virological relapse is common after treatment cessation. [4]

The reason of frequent virological relapse is the persistence of covalently closed circular deoxyribonucleic acid (cccDNA) in the hepatocytes. cccDNA is the intracellular template for hepatitis B virus (HBV) replication. Oral nucleoside or nucleotide analogs inhibit the HBV polymerase and suppress viral replication. [5] However, they have little effect on cccDNA. When antiviral drugs are stopped, transcription of the cccDNA resumes, leading to virological relapse. In previous studies, intrahepatic cccDNA level could be used to predict long-term clinical response to antiviral therapy. [6, 7] Accurate quantitation of cccDNA level is important clinically.

The traditional method for cccDNA quantitation makes use of the structural difference between cccDNA and relaxed circular DNA (rcDNA), which is the

major viral DNA in hepatocytes. [8] Since rcDNA carries a gap in the gene sequence which is absent in cccDNA, primers across the gap region should theoretically only amplify cccDNA and not rcDNA. However, this method is not foolproof. In reality, the polymerase chain reaction (PCR) product of rcDNA will gradually extend across the gap region after several cycles of amplification. These products interfere with the quantitation of cccDNA.

In this project, we described the development and assessment of new cccDNA assay methods to improve the detection rate and specificity.

1.2 Chronic hepatitis B

1.2.1 Epidemiology of chronic hepatitis B

It is estimated that over 350 million people are chronically infected by HBV worldwide. [9] Three quarters of chronic hepatitis B patients are in Asia. Each year, over 1 million people die from hepatitis B-related end stage liver disease or hepatocellular carcinoma. In Asia, 60% to 80% of all hepatocellular carcinoma cases are due to chronic HBV infection.

The prevalence of HBV infection is 10% to 20% in endemic areas such as Southeast Asia and sub-Saharan Africa. [10] In Mediterranean countries, Japan, India and Singapore, the prevalence is moderate at around 3% to 5%. A low prevalence rate of 0.1% to 2% is observed in the United States, Western Europe, Australia and New Zealand. Within the United States, the prevalence is lowest among Caucasians and higher in Asians, African-Americans and Hispanics. [11] In China, nationwide survey in 1992 showed that the prevalence of hepatitis B surface antigen (HBsAg) was 9.75%.[12] It is estimated that about 10% of the Hong Kong population are carriers. The seroprevalence of HBsAg is generally lower in women than in men. Before the introduction of the HBV vaccine, the male-to-female ratio was 1.4:1 in China, 1.3:1 in Thailand, and 1.1:1 in Hong Kong.[13]

In Asia, vertical transmission is the most common route of transmission, accounting for up to 50% of chronic hepatitis B. Horizontal spread during early childhood is more common in Africa and the Middle East. In contrast, horizontal spread during adulthood by sexual exposure or sharing needles is observed in low endemic Western countries.

Universal vaccination against HBV was started in Taiwan in early 1980s and was gradually introduced to other countries. In 1991, the World Health Organization (WHO) recommended universal HBV vaccination for neonates. The adoption of the vaccination program effectively reduces the number of infected individuals. In Taiwan, the effect of HBV vaccination on the incidence of hepatocellular carcinoma has been demonstrated first in children and later also in adolescents. [14] By the end of 2006, universal HBV immunization programs for newborns, infants and/or adolescents had been implemented in 168 countries. [15]However, since the prevalence of chronic hepatitis B in the adult population remains high in endemic areas, it will take several more decades before the infection and its associated complications can be well controlled. In addition, the variability of vaccine coverage in developing countries, especially in rural areas, limits the full impact of the vaccine.

In the United States, the number of chronic hepatitis B patients listed for liver transplantation because of end stage liver disease peaked in 2000 and declined by 37% in 2006. [16] This was accompanied by a reduction in the age-adjusted

mortality rate among chronic hepatitis B patients.

1.2.2 Natural history of chronic hepatitis B

The natural course of perinatal-acquired chronic hepatitis B infection is commonly described as four phases: immune tolerance, immune clearance, low or non-replicative (also known as inactive carrier stage) and reactivation phases. Patients may progress from one phase to the next or reverse backwards. The phases represent the interaction between HBV and the host immune system. The degree of liver injury depends on the duration and severity of hepatic necroinflammation.

During the immune tolerance phase, the host has minimal immune response to HBV. As a result, there is active HBV replication and the hepatitis B e antigen (HBeAg) is positive. However, the patient is asymptomatic, the serum ALT level is normal, and liver histology shows minimal liver damage. [17]

During the second to fourth decades of life, the immune clearance phase occurs. During this phase, the host immune system is activated against HBV. As a result, the serum ALT level rises and HBV DNA drops. At this time, HBeAg seroconversion (disappearance of HBeAg and development of antibody against hepatitis B e [anti-HBe]) occurs at a rate of 10% to 20% per year. [18] The duration of the immune clearance phase is variable. The shorter the duration, the better the prognosis. On the other hand, if cirrhosis has developed through prolonged immune clearance, the risk of liver-related complications remains

substantial even though the virus may be under control.

After HBeAg seroconversion and successful viral suppression, the patient enters the inactive carrier state. Serum ALT level is normalized. Liver necroinflammation and fibrosis may also improve. Some patients may further develop hepatitis B surface antigen (HBsAg) seroclearance. The annual rate of HBsAg seroclearance is around 0.5% to 1% in patients below the age of 30 and 1.5% to 2% in those after the age of 40. [19] Most patients with HBsAg seroclearance have excellent prognosis unless cirrhosis has developed or seroclearance occurs after the age of 50. [20]

In 20% to 30% of patients with HBeAg seroconversion, reactivation occurs. HBeAg reversion may or may not develop. HBeAg-negative chronic hepatitis B is usually due to HBV mutants with defective HBeAg production.

Overall, up to 40% of men and 15% of women with perinatally-acquired chronic hepatitis B die from liver-related complications eventually. [21] The survival is markedly reduced once cirrhosis develops. In one study, the 5-year survival in patients with chronic active hepatitis was 86%. The survival dropped to 55% in those with cirrhosis. [22]

The duration of the replicative phases determines the long-term prognosis. In a

population study conducted in Taiwan, positive HBeAg at baseline was independently associated with future risk of developing hepatocellular carcinoma. [23] Subsequent studies in Taiwan and Hong Kong also confirmed that high HBV DNA level was associated with cancer risk. [24, 25]

1.2.3 Antiviral therapy for chronic hepatitis B

Antiviral therapy for chronic hepatitis B is divided into two main groups: oral nucleoside or nucleotide analogs and interferon (Table 1.1). Oral nucleoside or nucleotide analogs are administered orally and carry few side effects. They are inhibitors of the HBV polymerase and suppress viral replication directly. On the other hand, they have minimal effect on cccDNA. [26-28] Virological relapse is common after treatment cessation. [4] Thus, most patients require long-term treatment. However, long-term antiviral treatment is limited by the risk of developing drug resistance, which may lead to hepatitis flares and liver decompensation. [29] The use of newer nucleoside analogs such as entecavir reduces but not eliminates the risk of drug resistance. [30]

In patients with HBeAg-positive chronic hepatitis B, virological relapse upon cessation of oral nucleoside analog treatment occurs in 30% to 80% even after HBeAg seroconversion.[4, 31] Although younger age and longer consolidation therapy after HBeAg seroconversion are associated with better durability after treatment cessation, the prediction is imperfect. [32] On the other hand, the durability of oral nucleoside or nucleotide analogs in patients with HBeAg-negative chronic hepatitis B is extremely poor. After stopping lamivudine or adefovir dipivoxil treatment, less than 10% of patients have durable response. [33, 34] Since persistence of intrahepatic cccDNA is the main reason for virological relapse, it is theoretically possible to use this marker to predict whether a patient

can be taken off treatment. However, such data are scarce at present.

In comparison, interferon is an immunomodulatory agent to increase the potency of immune clearance. In patients with HBeAg-positive chronic hepatitis B, a course of peginterferon alfa leads to HBeAg seroconversion in 30% to 40% of cases at 6 months after treatment cessation. [35-37] Moreover, intrahepatic cccDNA is decreased in responders to peginterferon. [7] Therefore, the response to peginterferon is more durable. Among patients who develop HBeAg seroconversion after peginterferon treatment, around 80% continue to have HBeAg seroconversion 5 years later. [38] On the other hand, the drawback of interferon includes the need for parenteral administration and numerous side effects.

1.3 Virology of HBV

1.3.1 Genomic structure

HBV is the smallest DNA virus known till now. It is enveloped and carries a partially double-stranded DNA. The genome is approximately 3.2 kilobase (kb) in length. The viral DNA contains the plus and minus strands. The minus strand is 3.2 kb long and has a protein covalently linked to its 5' end. In contrast, the plus strand is shorter and has a capped oligo-ribonucleotide at its 5' end. There are two 11-bp direct repeat (DR) regions in the viral genome. DR1 is located at the 5' end of the minus strand, while DR2 is located at the beginning of the plus strand (Figure 1.1).

The HBV genome has four partially overlapping open reading frames (ORFs). The *C* region encodes for the hepatitis B core antigen (HBcAg) and HBeAg. The *P* region encodes for the polymerase protein. The *S* region encodes for HBsAg. There are three forms of surface proteins: small, middle and large surface proteins. Finally, the *X* region encodes for the hepatitis B X protein. The function of the X protein is not completely understood but is generally believed to be involved in hepatocarcinogenesis through transactivation. [39, 40] The *S* ORF is located within the *P* ORF. Both the *X* ORF and *C* ORF overlap partially with the *P* ORF.

HBV polymerase is involved in viral replication. Unlike many other DNA viruses, HBV replicates via reverse transcription. Reverse transcriptase is one domain of the HBV polymerase. Viral DNA is synthesized using either RNA or DNA templates. [41] The use of reverse transcription and the lack of proofreading activity explain the high rate of viral mutations. The mutation rate of HBV is around 10,000 times higher than that of other DNA viruses. [42, 43] As a result, a large number of HBV quasispecies exist in the host. This has major implication in the development of antiviral drug resistance. In addition, this may affect the performance of molecular assays such as cccDNA quantitation.

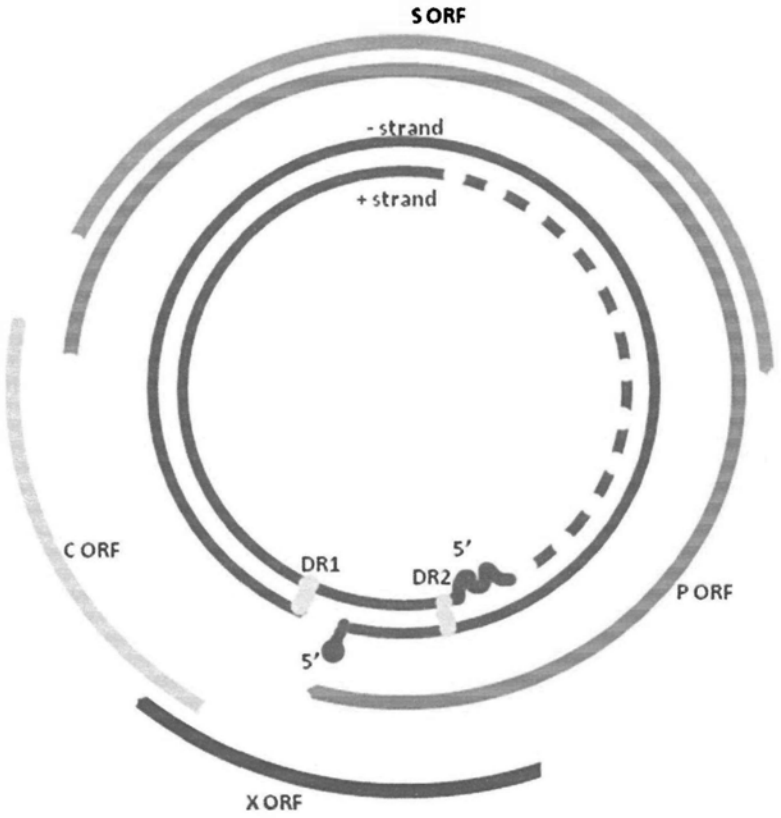

Figure 1.1. Organization of the HBV genome

The inner circle represents the HBV genome. HBV is a partially double-stranded DNA virus. The minus (-) strand is 3.2 kilobase in length. The plus (+) strand is shorter than the minus strand. The outer circle represents the four open reading frames: *C*, *S*, *P* and *X*.

1.3.2 Replication cycle

HBV is a hepatotropic virus. Replication takes place in hepatocytes. [44] Figure 1.2 depicts the life cycle of HBV. HBV is believed to enter hepatocytes by binding to receptors on the surface of the cell membrane. At present, the receptors for viral entry are yet to be discovered. As the virus enters the cytoplasm and releases its genome into the host cell nucleus, rcDNA is converted to cccDNA, which serves as the template for further viral replication by the host DNA polymerase. With the catalytic action of cellular ribonucleic acid (RNA) polymerase II, pregenomic RNA (pgRNA) and subgenomic messenger RNA (mRNA) are synthesized and translated into viral proteins in the cytoplasm.

Reverse transcription from pgRNA to rcDNA and formation of new virions take place in the cytoplasm. The pgRNA is encapsidated together with the viral polymerase. In newly formed nucleocapsids, viral polymerase interacts with encapsidation signal (known as 'epsilon' signal) on the pgRNA and initiates strand synthesis. As the minus-strand DNA is synthesized, pgRNA is gradually degraded by the ribonuclease H domain of the polymerase. Using the newly synthesized minus strand as the template, the plus-strand DNA is then synthesized by the viral polymerase. At this time, the mature viral genome with the capsid is assembled with the surface antigen and lipid envelop into a complete virion, which is then released into the circulation by infected cells.[45] Some mature capsids are not released from the cells but delivered back to the

nucleus for further replication. This process is partially regulated by the expression of the large surface protein. When the intracellular concentration of HBsAg is low, mature nucleocapsids are retained in hepatocytes for further viral replication. When the intracellular concentration of HBsAg has reached a certain threshold level, the infected hepatocytes would increase the release of virions in the circulation, leading to infection of new hepatocytes.

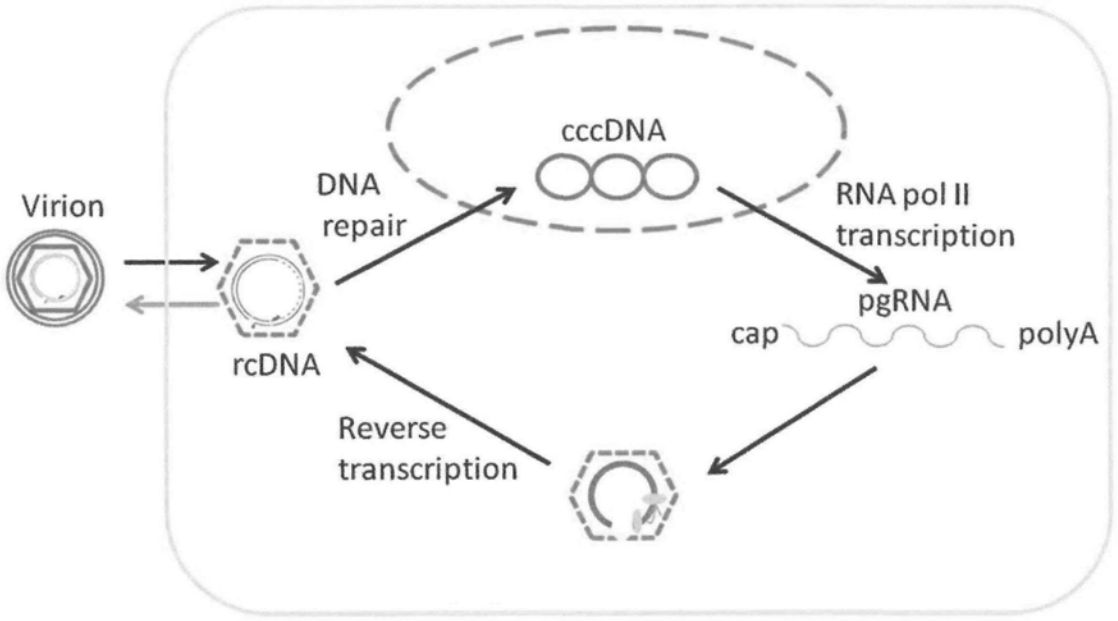

Figure 1.2. Replication cycle of HBV

HBV enters hepatocytes via an unknown receptor. The viral genome is transferred to the host nucleus, where cccDNA is formed and serves as the template for viral replication. HBV mRNA is transported to the host cytoplasm for translation and encapsidation. Mature virions are either transported back to the nucleus for further viral replication or released into the circulation.

1.3.3 HBV genotypes

Genotype is a viral variant with significant difference from other variants of the same virus to constitute a distinct phylogenetic group. For HBV, viruses are classified into eight genotypes, labeled from A to H (Table 1.2). Viruses of each genotype differ from other genotypes by at least 8% of the genetic sequences. [46] Moreover, viruses that differ in at least 4% of the genetic sequences are further classified into different subgenotypes. At present, at least 24 subgenotypes have been reported. [47]

HBV genotypes show distinctive geographical distribution (Table 1.2). In China and Japan, most patients are infected with genotypes B and C HBV. [48, 49] On the other hand, genotypes A and D are more common in Western countries. [50]

The knowledge of HBV genotypes is important for a number of reasons. First, genotypes affect the natural history of chronic HBV infection. Second, different genotypes may have different response to antiviral treatment. Third, from laboratory point of view, molecular assays for HBV have to be shown to have similar performance across different genotypes.

Compared to other genotypes, genotype C is associated with delayed HBeAg seroconversion. [51] In a study of 1158 chronic hepatitis B patients in Alaska, the

median age at HBeAg seroclearance was 48 years for genotype C, compared to 16 to 20 years for other genotypes. [52]

As a result of delayed HBeAg seroconversion, patients with genotype C HBV infection are subject to more prolonged immune clearance phase and ongoing liver injury. Thus, advanced liver fibrosis and cirrhosis are more commonly associated with genotype C virus.[53, 54] This also leads to increased risk of hepatocellular carcinoma. [55-57] Recently, subgenotype Ce was found to have the strongest association with hepatocellular carcinoma. [25] In addition, genotype C HBV is associated with higher recurrence rate after curative resection of hepatocellular carcinoma.[58]

In Caucasians, patients with genotypes A and B HBV infection have higher HBeAg seroconversion and HBsAg seroclearance rates than those with genotypes C and D after conventional interferon and peginterferon treatment.[59-61] However, studies in Asia failed to demonstrate significant differences between genotypes B and C. [38, 62]

In contrast, HBV genotypes do not appear to affect the response to oral nucleos(t)ide analogs. A small Italian study showed similar biochemical and virological response to lamivudine in patients with genotype A and genotype D virus, and studies in Hong Kong did not show any difference in virological response in patients with genotype B and genotype C virus. [63-65] Similarly,

according to pooled data of 694 patients in phase III studies of adefovir dipivoxil, the virological response was similar among patients infected with genotypes A, B, C and D HBV.[66]

Table 1.1. Geographical distribution of different HBV genotypes

Genotypes	Geographical distribution
A	North America, Northern and Middle Europe, South Africa
B	Asia
C	Asia
D	Mediterranean area, Middle East
E	Africa
F	South and Central America
G	Europe and North America
H	North and Central America

1.4 Covalently closed circular DNA

1.4.1 cccDNA in HBV replication

The HBV cccDNA is a critical intracellular replicative intermediate and is responsible for viral persistence in hepatocytes. It is approximately 3.2 kb in length, double-stranded and in circular form. Conversion of rcDNA to cccDNA in hepatocytes occurs within the first 24 hours after virus inoculation. cccDNA is the real transcription template for viral replication. The transcription products include viral pgRNA and mRNA coding for the multifunctional polymerase, core, surface and X proteins.

At least 5 steps are involved in the conversion of rcDNA to cccDNA. [67] First, the viral polymerase that is covalently attached to the 5' end of the minus strand of rcDNA is removed. Second, the short RNA oligomer is removed from the 5' end of the plus-strand rcDNA. The oligomer is responsible for the synthesis of the plus-strand. Third, one copy of the short terminal redundancy is removed from the minus-strand. Fourth, the plus-strand of rcDNA is completed by the cellular replicative machinery. Finally, the two viral DNA strands are ligated.

The mechanism governing the conversion of rcDNA to cccDNA is not completely understood. The conversion appears to go through an intermediate product, deproteinized rcDNA in the cytoplasm of hepatocytes, in which the

phosphodiester bond between the viral polymerase and the minus-strand of rcDNA is cleaved. [68] The deproteinized rcDNA is then attached to karyopherin-alpha and -beta, which leads to transportation of the whole complex to the nucleus of hepatocyte, where cccDNA is formed. [69]

All viral RNAs for protein production and viral replication are produced by the cellular transcriptional machinery. This is first done in the cytoplasm after reverse transcription of the pgRNA. [70] In duck models, the major source of cccDNA in infected hepatocytes comes from newly synthesized nucleocapsids. [71] This suggests that a stable pool of cccDNA does not require multiple rounds of HBV infection. In infected animals, around 1 to 50 cccDNA can be found in each cell. [72]

In quiescent hepatocytes, cccDNA is very stable. Reduction in cccDNA pool may occur through cytolytic and non-cytolytic mechanisms. During active cell death and liver regeneration, cccDNA concentration is reduced through dilutional effect. In the urokinase-type plasminogen activator / severe combined immunodeficiency mouse model, the cccDNA pool is diluted during liver regeneration. [73] Furthermore, the ratio of rcDNA to cccDNA also decreases by 75%, indicating a significant reduction in cccDNA transcription and viral productivity during liver regeneration. On the other hand, cccDNA clearance without hepatocyte damage may also occur in the presence of inflammatory cytokines. [74]

Epigenetic changes are involved in the control of cccDNA transcription and subsequent viral replication. In cell line models and human samples, the HBV replication status is associated with the acetylation status of histone proteins associated with cccDNA. [75] When Class I histone deacetylases inhibitors were added to a cell line HBV transfection model, cccDNA-bound acetylated histone protein 4 increased and HBV replication was enhanced. In addition, chronic hepatitis B patients with low viremia had histone hypoacetylation. Acetylation of cccDNA-bound histones is partially controlled by the recruitment of hepatitis B X protein on the cccDNA. [76] In another study of liver samples from 55 chronic hepatitis B patients, increased methylation of cccDNA was associated with reduced HBV replication, and was more commonly found in patients with negative HBeAg. [77]

Analysis of transcription products of cccDNA has shed light on the reason of different HBV replication activity in patients with HBeAg-positive chronic hepatitis B and inactive carrier state.[78, 79] In general, HBeAg-negative patients have lower intrahepatic cccDNA than patients with positive HBeAg. Furthermore, the ratio of pgRNA to cccDNA is reduced in HBeAg-negative patients, indicating that the reduced HBV replication is partly due to lowered efficiency of cccDNA transcription. On the other hand, HBsAg production is not affected in patients with negative HBeAg.

1.4.2 Clinical significance of cccDNA detection

The level of intrahepatic cccDNA reflects the amount of replicative templates in the liver, and has been used to monitor and predict treatment response. In a post-hoc analysis of a clinical trial comparing the combination use of peginterferon alfa-2b and lamivudine versus lamivudine monotherapy, intrahepatic cccDNA level at the end of treatment correlated well with sustained virological response at 6 months post-therapy. [7] At a cutoff value of -0.80 copies/genome equivalent, the sensitivity, specificity, positive predictive value and negative predictive value of log cccDNA at the end of treatment to predict sustained virological response were 73%, 78%, 56% and 86%, respectively. In another German study, 26 chronic hepatitis B patients were treated with peginterferon alfa-2b and adefovir dipivoxil for 48 weeks. [80] At week 48, the median intrahepatic cccDNA decreased by 2.4 logs. Among HBeAg-positive patients, those who achieved HBeAg loss had lower intrahepatic cccDNA both at baseline and week 48 of treatment. In the extension phase of the same study, the patients continued adefovir dipivoxil treatment till week 144, while peginterferon was stopped after week 48. [81] Although no further reduction in cccDNA level was observed from week 48 to week 144, the original cccDNA suppression was largely maintained with continuous adefovir dipivoxil therapy (median cccDNA reduction at week 144 compared to baseline was -2.2 log).

On the other hand, cccDNA reduction is modest in patients receiving oral

nucleoside or nucleotide analogs. In a study of 32 patients treated with adefovir dipivoxil monotherapy for 48 weeks, intrahepatic cccDNA only reduced by 0.8 log. [6] Similarly, a study of 40 chronic hepatitis B patients showed a reduction of intrahepatic cccDNA by 1 log after treatment with entecavir or lamivudine for 48 weeks. [82]

The role of cccDNA in hepatocellular carcinoma was also investigated. [83] The level of cccDNA in tumor tissue was higher than that in non-tumor liver tissue. In addition, the majority of HBV DNA in tumor tissue existed in the form of cccDNA. This suggests that HBV replication is ineffective in tumor cells.

In addition, cccDNA quantitation has been used in natural history studies. In a study of chronic hepatitis B patients with spontaneous HBsAg seroclearance, intrahepatic cccDNA was detected in 79% of patients. [20]

Although cccDNA can also be detected in serum and peripheral blood mononuclear cells, its clinical meaning remains unclear. [84-86] cccDNA is not released from hepatocytes. Therefore, cccDNA found in serum most likely represents leakage from damaged hepatocytes. In fact, serum cccDNA level is increased in patients with reactivation of chronic hepatitis B. [87] Furthermore, serum cccDNA declined by 2.1 logs after lamivudine treatment for 52 weeks in one study. [88] Since the reduction in serum cccDNA is more marked than the

reduction in intrahepatic cccDNA reported in other studies, the observation likely reflects both reduction in intrahepatic cccDNA and reduced leakage from hepatocytes to serum after necroinflammation is controlled.

Since intrahepatic cccDNA is the basis of viral persistence, novel treatments targeting or affecting cccDNA are being explored. In one study, therapeutic DNA vaccine was tested in a duck hepatitis B virus model. [89] Sixty-seven percent of ducks with surface antigen seroconversion also had cccDNA clearance, compared to only 4% of ducks without seroconversion. In another study using the duck hepatitis B virus model, zinc finger proteins were designed to target cccDNA and effectively reduce viral replication. [90] Similarly, small interfering RNAs (siRNAs) against cccDNA have been developed to inhibit cccDNA amplification. [91]

In summary, accurate quantitation of cccDNA can be used to predict treatment response, assess new therapy, and study the natural history of chronic hepatitis B. This is a useful tool in clinical research and potentially in clinical care in the future.

1.5 Detection of cccDNA

1.5.1 Overview of available methods for cccDNA detection and quantitation

The current methods for cccDNA detection include PCR-based assays and non-PCR-based assays. Total HBV DNA in the liver is a mixture of cccDNA and rcDNA. The major obstacle to cccDNA detection stems from the fact that the concentration of rcDNA is much higher than that of cccDNA, and rcDNA and cccDNA have identical gene sequence.

To distinguish between cccDNA and rcDNA, two methods are generally adopted. First, rcDNA can be removed from the sample. Since rcDNA is an incomplete circular double-stranded DNA, it can be digested by plasmid-safe deoxyribonuclease (DNase).

Second, the assays make use of the structural difference between rcDNA and cccDNA. While cccDNA is a complete circular DNA, there are gaps in the minus strand and plus strand of rcDNA (Figure 1.1). When elongation goes through the gap region, amplification efficiency is reduced by around 1000 fold. Therefore, primers spanning across the gap regions of rcDNA should theoretically amplify mostly cccDNA and not rcDNA.

In the following sessions, the principles of plasmid-safe DNase are first described. The more commonly used methods of cccDNA quantitation, including the Bowden's assay, Invader assay and chimeric assay, will then be described.

1.5.2 Plasmid-safe DNase

Plasmid-safe DNase (Epicenter Technology, Madison, WI) is a commercially available tool to selectively hydrolyze linear double-stranded DNA to deoxynucleotides at slightly alkaline pH. It also hydrolyzes closed circular single-stranded DNA at a lower efficiency. The reaction is adenosine triphosphate (ATP)-dependent and does not affect closed circular supercoiled or nicked circular double-stranded DNAs. This property is optimal for distinguishing between cccDNA and rcDNA. cccDNA is a closed circular double-stranded DNA which will not be affected by plasmid-safe DNase. In contrast, rcDNA is incompletely circular and can be hydrolyzed by plasmid-safe DNase (Figure 1.3). After the administration of plasmid-safe DNase, the amount of rcDNA is substantially reduced. Subsequent amplification product will more closely represent cccDNA. The effect of plasmid-safe DNase on rcDNA has been confirmed by Southern blot analysis on woodchuck liver samples. [6]

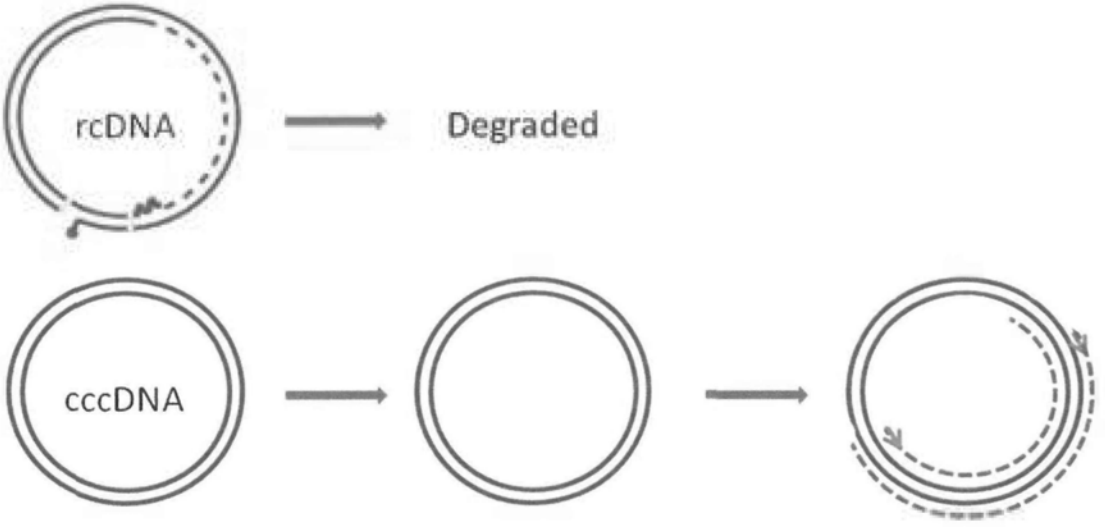

Figure 1.3. Plasmid-safe DNase treatment

Plasmid-safe DNase is an enzyme that hydrolyzes linear double-stranded DNA and closed circular single-stranded DNA. Its administration can be used to eliminate rcDNA from a pool of HBV DNA containing both rcDNA and cccDNA. cccDNA is a completely circular double-stranded DNA and is not affected by plasmid-safe DNase. In contrast, rcDNA is incompletely circular and is hydrolyzed by plasmid-safe DNase.

1.5.3 Bowden's assay

PCR-based assays, such as the Bowden's assay and its modification, are most commonly used in biomedical studies. [8] In the first step, the majority of rcDNA is removed from the sample by the addition of plasmid-safe DNase (see section 1.5.2). Afterwards, amplification of cccDNA is achieved by the use of two upstream primers and one downstream primer that target across the gap of the minus strand of rcDNA (Figure 1.4). The selective primers that target across the single-stranded gap region of rcDNA were designed using oligo5 software (MedProbe, Olso, Norway). Using this design, cccDNA would be predominantly amplified. Quantitation of cccDNA is done by real-time PCR using the LightCycler system and SYBR green chemistry. This method has been used to assess the treatment response of chronic hepatitis B patients to oral nucleoside or nucleotide analog and peginterferon. [6, 7]

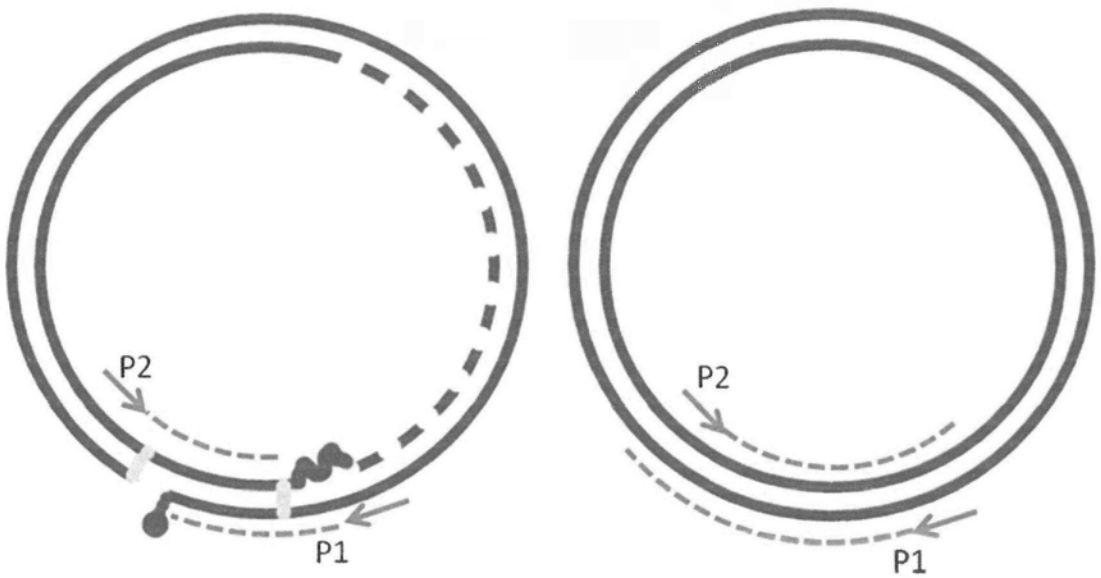

Figure 1.4. Bowden's cccDNA assay

The structure of rcDNA and cccDNA is shown. The primers target the single-stranded gap region of rcDNA and are less efficient in amplifying rcDNA. On the other hand, the strands of cccDNA are continuous structures and can be amplified normally.

1.5.4 Invader assay

The Invader technology is designed for the detection of nucleic acids. [92] High specificity is achieved by the use of hybridization and enzyme recognition. The assay has been used to detect mutations and single-nucleotide polymorphisms in humans.

This technique has been adopted for cccDNA quantitation (Figure 1.5). [93] First, the Invader probe and primary probe hybridize to the target sequence on cccDNA to form a partially overlapping structure. This is then cleaved by a Cleavase enzyme to generate an oligonucleotide called a 5'-flap. At a specific reaction temperature, the primary probe cycles on the target DNA and generates released 5'-flaps. Amplification of the released 5'-flaps is proportional to the concentration of the target DNA. Fluorescence resonance energy transfer cassettes react with the released flaps and generate a fluorescence signal that can be measured with real-time PCR.

As shown in Figure 1.6, the Invader assay also makes use of the gap of the plus-strand of rcDNA that is next to the DR2 region. Since the probe targets the gap region, 5'-flaps will not form on rcDNA. Cleavage products are only formed from cccDNA and can be measured by real-time PCR.

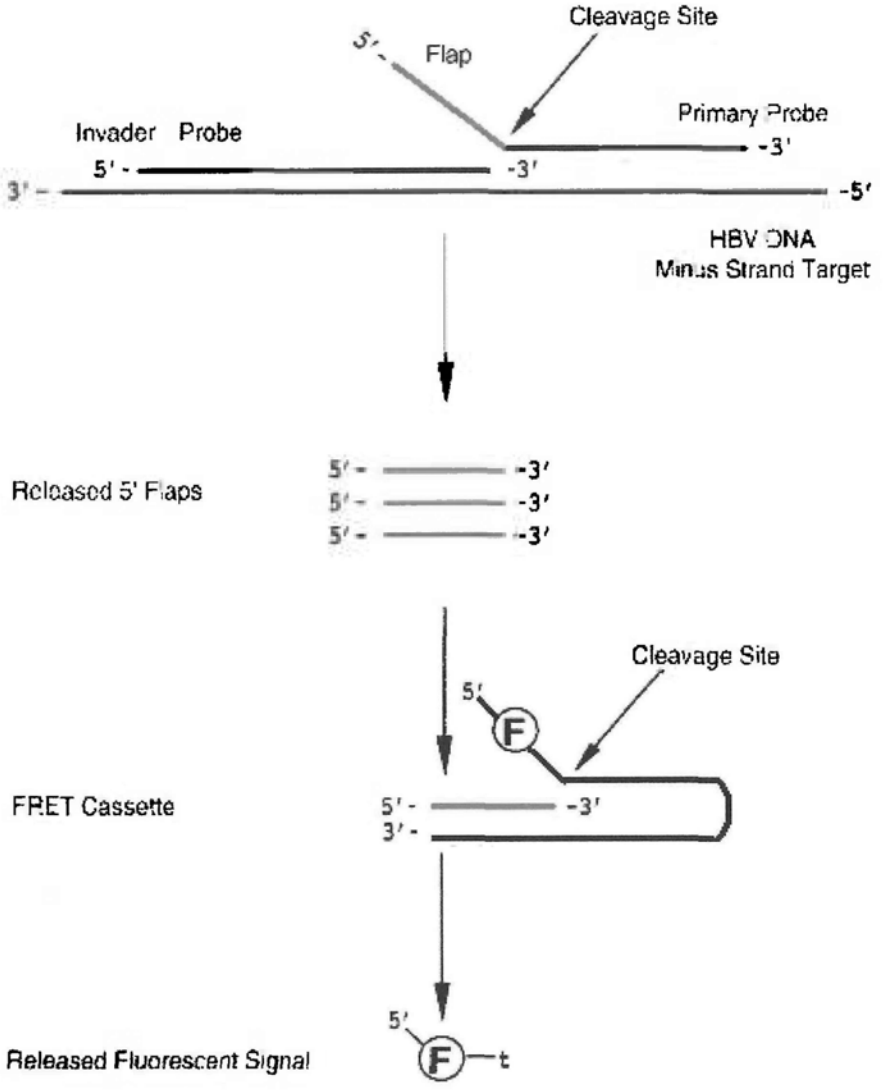

Figure 1.5. The Invader assay

The Invader assay is a method to quantitate the concentration of nucleic acids of specific sequence. As the Invader probe and primary probe are attached to the target sequence, the complex is recognized by the Cleavase enzyme and released as 5'-flaps. The cleavage product can be detected using fluorescence signal and reflects the concentration of the nucleic acids of interest.

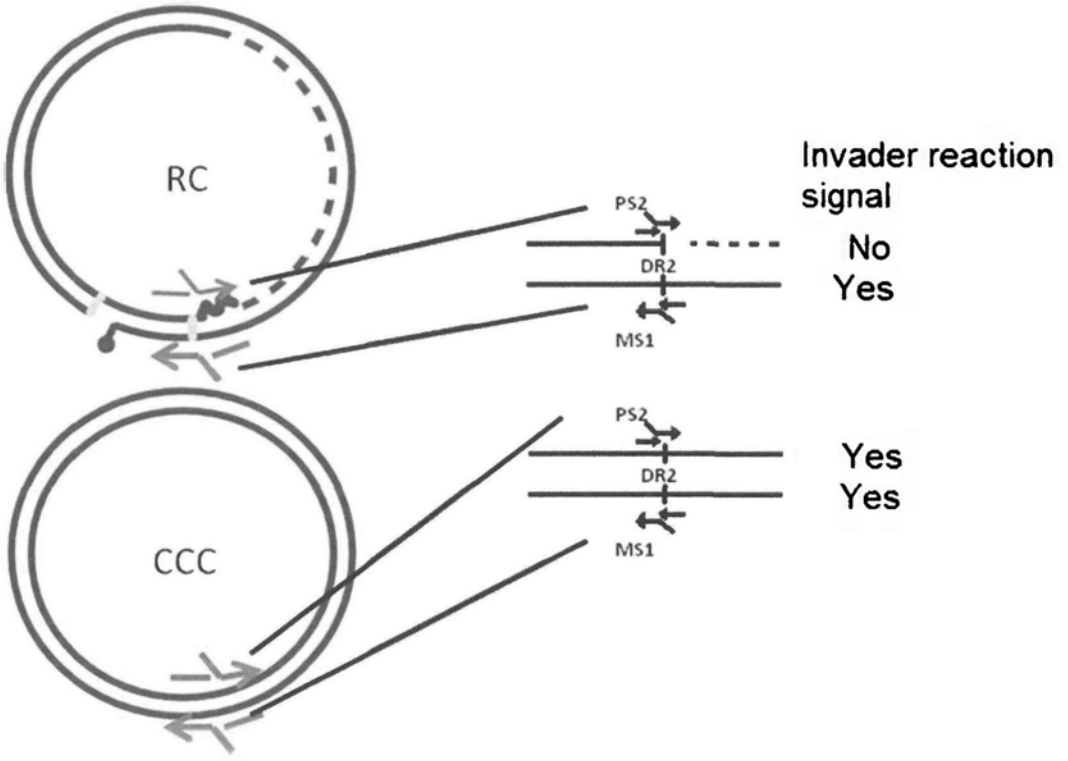

Figure 1.6. The Invader assay for cccDNA detection

Specific detection of cccDNA is achieved by using probes that target the gap region of the plus-strand. As such, the probes can only be attached to the plus-strand of cccDNA but not rcDNA.

1.5.5 Chimeric assay

Similar to the above assays, the chimeric assay also takes advantage of the gap region of the rcDNA (Figure 1.7). [94] The chimeric primer is composed of two segments. Segment A near the 3' end is complementary to the plus-strand of the HBV genome. Segment B near the 5' end is consensus to part of the human immunodeficiency virus genomic sequence but not the HBV genome. For cccDNA, since there is no gap at the DR2 site, the strand produced by primer extension will be relatively integrated. In contrast, nucleotide extension will be stopped at the DR2 gap of rcDNA. Elongated strands will become templates in the next round of PCR amplification, which contain one primer, identical to chimeric primer segment B (human immunodeficiency virus sequence), and another primer behind the DR2 gap. Thus, exponential amplification only occurs when the new single strand DNA is derived from cccDNA.

A fluorogenic probe is used for quantitation. As the specific PCR product from cccDNA accumulates, amplification of fluorescence occurs via Taq polymerase 5'-nuclease activity. The adoption of real-time quantitative PCR eliminates the variability of conventional PCR.

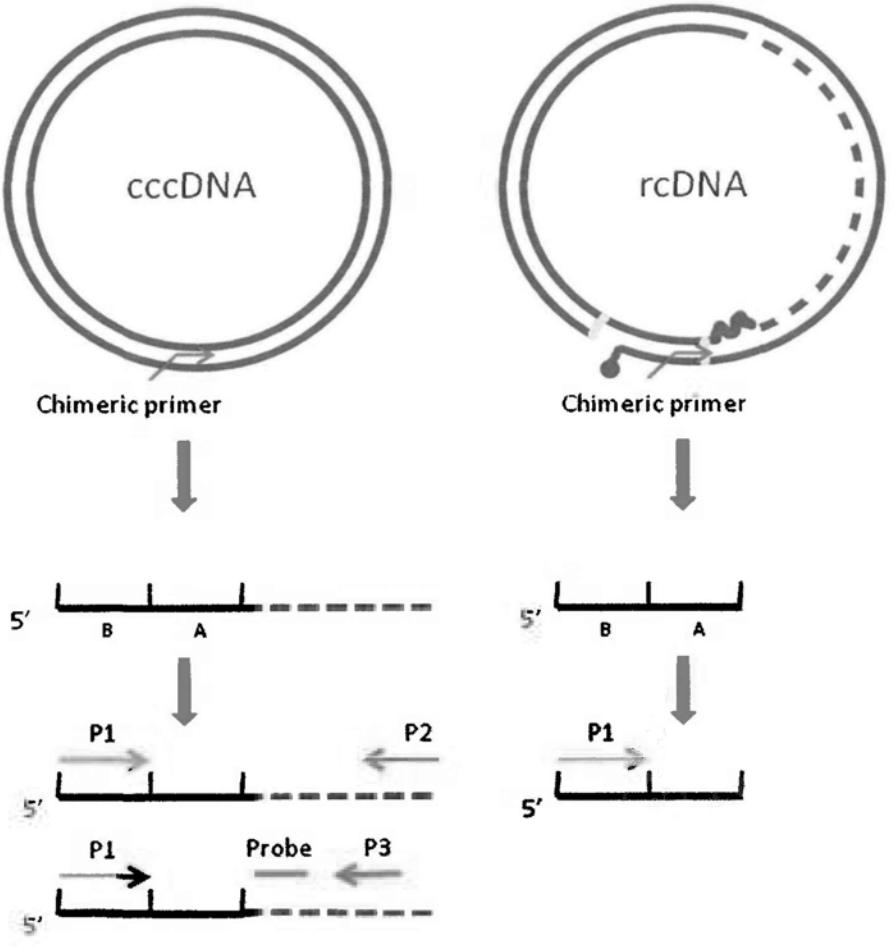

Figure 1.7. Chimeric assay

This assay utilizes a chimeric primer. Segment A of the primer is complementary to the HBV genome, while segment B is complementary to the gene sequence of human immunodeficiency virus but not HBV. In rcDNA, primer extension stops at the gap region. On the other hand, primer extension occurs smoothly on cccDNA. The elongated single-stranded DNA from cccDNA can then be amplified by a set of primers that target the HBV and human immunodeficiency virus gene sequences, respectively.

1.5.6 Limitations of current assays

An ideal assay should have high sensitivity and specificity. The major obstacle in quantitating cccDNA is the interference by rcDNA. Although plasmid-safe DNase can be used to eliminate rcDNA, the digestion is incomplete. Furthermore, though the above assays target the gap regions of rcDNA, amplification is not completely avoided. After several cycles of PCR, the amplification products will extend through the gap (Figure 1.8). The amplification products from the two primers are partially complementary. During the next cycle of PCR, the products can bind to each other and generate a full amplification product.

Although amplification across the gap region is much less efficient, this selection advantage is partially offset by the fact that the concentration of rcDNA is much higher than that of cccDNA. As a result, the cccDNA concentration may be overestimated, especially when the total HBV DNA concentration is high.

In addition, detection of cccDNA relies on complementary sequence between primers and cccDNA. HBV is a DNA virus with high rate of mutation due to the involvement of reverse transcription via RNA intermediate during viral replication and the lack of proof reading. It has been estimated that every nucleotide in the HBV genome is subject to mutation everyday. Therefore, although primers and probes used for cccDNA detection target at conserved regions, it is unclear if mutations at these sites may occur in patients in real life, leading to false-

negative results.

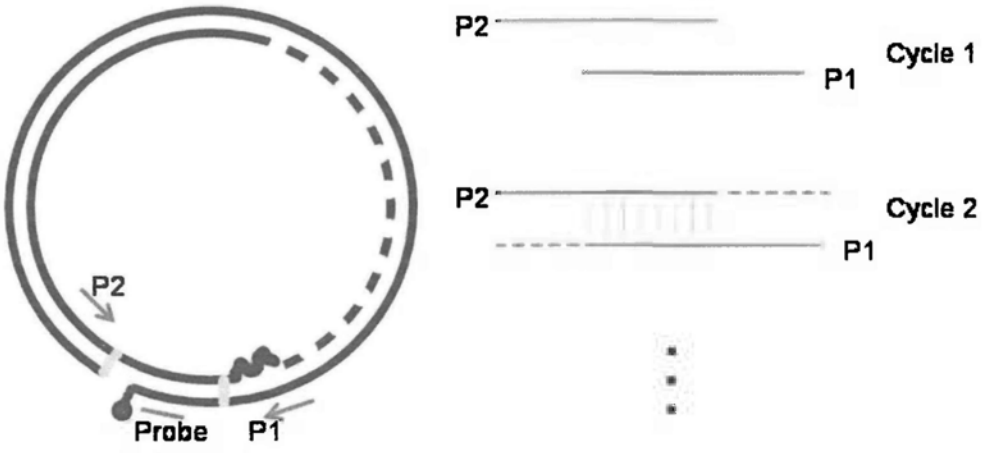

Figure 1.8. False amplification of rcDNA by cccDNA assay

Although primers for cccDNA should stop elongation at the gap region of rcDNA, in reality, amplification may still occur at a lower efficiency. At the first cycle, the primers extend to the edge of the gap region. These sequences are partially complementary and may bind to each other in the next round of PCR. This would finally result in a full amplification product.

1.6 DNA methylation and bisulfite conversion

1.6.1 DNA methylation

In this study, we proposed to use bisulfite conversion assay to overcome the technical difficulties in cccDNA detection. In this section, the concept of DNA methylation will be explained, followed by the explanation of why the bisulfite conversion method may be used for cccDNA detection.

Gene expression is under tight control by different mechanisms. One well-known mechanism governing gene expression is epigenetics. DNA methylation is one of the well-studied epigenetic mechanisms. This refers to the addition of methyl groups to the DNA, mostly at CpG sites, to convert cytosine to 5-methylcytosine. Some areas of the genome are more heavily methylated than others and are less transcriptionally active.

CpG sites represent around 5% to 10% of the human genome. The CpG notation refers to the phosphodiester bond between cytosine and guanine. The regions of DNA with high frequency of CpG sites are called CpG islands. CpG islands are usually 300 to 3000 bp in length with cytosine and guanine representing over 55% of all nucleotides and a higher than expected frequency of CpG sites. [95] CpG islands are typically located at or near the transcription start site of genes. [96] In over half of human genes, the transcription start sites overlap with CpG

islands. [97] In healthy human tissues, about 3.5% to 4% of cytosines are methylated. [98] Methylation of cytosine residues does not distribute evenly in the human genome.

The HBV genome also contains CpG islands, and methylation of HBV cccDNA has also been reported. [99] Higher rate of cccDNA methylation is observed in patients with HBeAg-negative chronic hepatitis B than those with positive HBeAg. [77] This supports the notion that cccDNA methylation is associated with the degree of cccDNA transcription and viral replication. In cell line experiments, transfection of methylated HBV DNA to hepatoma cell lines also reduces HBV mRNA levels.

1.6.2 Bisulfite conversion

The detection and quantitation of DNA methylation is useful in the study of cancer, genetic diseases, and control of gene expression. One common method for the study of DNA methylation is the bisulfite conversion technique. Treatment of DNA with bisulfite converts unmethylated cytosines into uracil. In contrast, methylated cytosines remain unchanged after bisulfite treatment. By comparing the DNA sequence before and after bisulfite treatment, the DNA methylation profile can be determined.

One result after bisulfite conversion is that the two DNA strands will no longer be complementary to each other (Figure 1.9). So the minus strand cannot serve as a template in a PCR targeting the plus strand after bisulfite conversion. In this study, this unique feature of bisulfite conversion is used for discriminating plus and minus strands of HBV DNA. Only cccDNA will be amplified when a PCR designed to amplify the bisulfite converted sequences before the DR2 region on the plus strand of cccDNA is performed, because rcDNA cannot be amplified by this PCR due to the loss of this region on the plus strand.

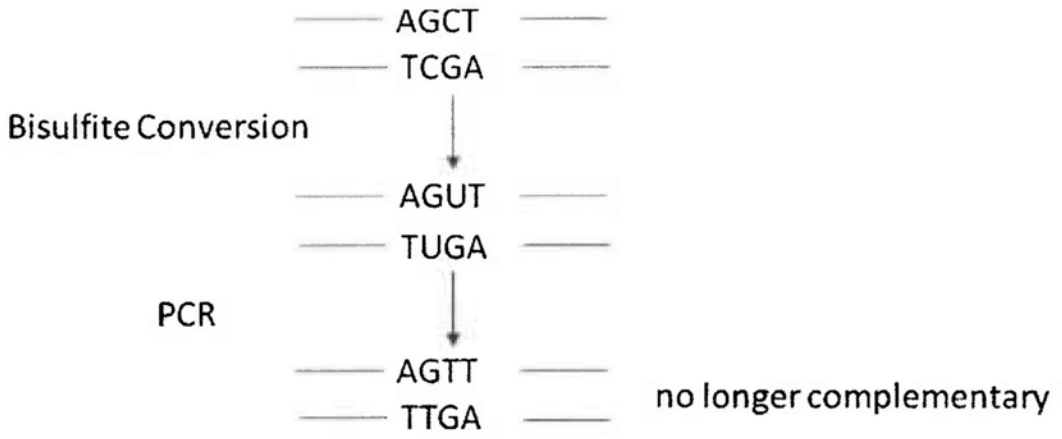

Figure 1.9. The effect of bisulfite conversion on the gene sequence

After bisulfite treatment, unmethylated cytosines (C) are converted to uracils (U). With further PCR, uracils become thymines (T). The newly formed thymines are no longer complementary to the guanines (G).

1.7 Hypothesis and aims of the study

We hypothesized that a PCR designed to amplify the bisulfite converted sequences before the DR2 region on the plus strand of cccDNA would mainly amplify cccDNA from a mixture of cccDNA and rcDNA and thus less affected by rcDNA.

The aim of the study was to develop cccDNA assays with improved detection rate and lower false amplification. The secondary aim of the study was to identify the cause of failed amplification in some cases.

CHAPTER 2 Materials and Methods

2.1 Study design overview

The study was divided into three parts. In part 1, the modified Bowden's assay and the bisulfite conversion assay were developed and optimized. In part 2, the detection rate and absolute concentration of cccDNA in human liver and serum samples were assessed. The two new assays were compared to the Bowden's assay as the reference method. In part 3, the reasons for undetectable cccDNA in some clinical samples were explored. The HBV genome was sequenced to test the hypothesis that some samples with undetectable cccDNA were due to gene mutations affecting the performance of the primers and probes.

2.2 Patients

Serum samples were obtained from 215 patients with chronic hepatitis B who attended the out-patient clinics of the Prince of Wales Hospital, Hong Kong SAR and Dong Hu Hospital, Shenzhen, China. In addition, 5 subjects without chronic hepatitis B from the Prince of Wales Hospital served as controls. Liver tissues from 61 chronic hepatitis B patients were collected from Dong Hu Hospital. These included 37 liver biopsy specimens from patients without hepatocellular carcinoma and 24 cancer biopsy specimens from patients with hepatocellular carcinoma. The subjects were aged 18 or above. All subjects provided informed written consents. The patients with chronic hepatitis B had positive HBsAg for at least 6 months. Subjects co-infected with hepatitis C virus or human immunodeficiency virus were excluded.

2.3 Clinical tests

For the group that provided serum samples, HBeAg, anti-HBe, HBV DNA and ALT level were checked at the time of blood taking. HBeAg and anti-HBe were measured by enzyme-linked immunosorbant assay (Sanofi Diagnostics, Pasteru, France). TaqMan real-time PCR assay was used to measure HBV DNA levels. [100] The detection range was 100 to 10^9 copies/ml with a correlation coefficient of the standard curve routinely above 0.990.

2.4 Viral DNA extraction

Viral DNA was extracted from serum using the QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA, US) according to the manufacturer's instructions. In brief, 20 µl of Qiagen Protease and 180 µl of buffer AL were added to 200 µl of serum samples. The mixture was incubated at 56°C for 10 minutes, followed by the addition of 200 µl of cold absolute ethanol. The mixture was transferred to the QIAamp Spin Column and centrifuged at 12,000 revolutions per minute for 1 minute. The extraction column was washed with 500 µl of buffers AW1 and AW2 at 12,000 revolutions per minute for 1 minute and 3 minutes, respectively. Finally, the DNA was eluted in 200 µl of sterile water at 12,000 revolutions per minute for 1 minute. The extracted DNA was stored at -20°C before the experiments.

As for the liver tissue samples, viral DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Twenty microlitres of Qiagen Protease K and 180 µl of buffer ATL were added to tissue samples. The mixture was incubated at 56°C for 2 to 4 hours until the liver tissue was completely lyzed. Afterwards, 200 µl of buffer AL was added and incubated at 70°C for 10 minutes. Then, 200 µl of cold absolute ethanol was added. The mixture was transferred to the QIAamp Spin Column and centrifuged at 6000 × *g* for 1 minute. The extraction column was washed with 500 µl of buffers AW1 and AW2 at 20,000 × *g* for 1 minute, and 3 minutes, respectively. The DNA was finally eluted in 200 µl of sterile water at 12,000 rotations per

minute for 1 minute. The extracted DNA was stored at -20°C before the experiments.

2.5 cccDNA detection

2.5.1 Primer and probe design

The online database of National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) was used to obtain HBV genome sequences. Based on the different HBV genome sequences provided, alignment was performed using Cluster X to determine conservative regions of the genome.

2.5.2 Plasmid-safe DNase treatment

Plasmid-safe DNase (Epicenter Technology, WI, US) was used to digest rcDNA before cccDNA amplification. The reaction mixture contained 1 × Plasmid-Safe Reaction Buffer, 1 mM adenosine triphosphate, 10 units of plasmid-safe DNase and 5 mM DNA. The total volume was 10 µl. The mixture was incuated at 37°C for 16 hours. Afterwards, the mixture was incubated at 70°C to inactivate plasmid-safe DNase.

2.5.3 Bowden's assay

After plasmid-safe DNase treatment, cccDNA quantitation was performed following the protocol described by Bowden et al. [8] Two upstream primers and one downstream primer were used (Table 2.1). The LightCycler™ system (Roche Diagnostics, Mannheim, Germany) and SYBR Green chemistry were used for real-time PCR quantitation. The reaction mixture had a volume of 20 µl and contained 0.5 µM of each primer, 0.2 µM of fluorescein-labeled probe, 0.4 µM of red-640-labeled probe, 3 mM of magnesium chloride, 2 µl of master hybridization probes kit (Roche Molecular Biochemicals, Canada) and 2 µl of DNA samples. The reaction capillaries were loaded, centrifuged, and placed in the carousels of the LightCycler™ instrument. The cycling conditions were as follows: incubation at 95°C for 7.5 minutes for Taq DNA polymerase activation, followed by 45 cycles of 95°C for 5 seconds for denaturation, 54°C for 15 seconds for annealing, and 72°C for 20 seconds for extension. Data were obtained during the annealing period in the single acquisition mode, with the channels set at F2/F1. Five serial dilutions of a single-copy HBV genome containing plasmids at concentration of 100 to 10^6 copies/ml were used as standard. The standards, negative controls and positive controls were added in each PCR run.

Table 2.1. Primers and probes for cccDNA detection

Bowden's assay	
Upstream primers	
CCC1	5'-GCGGWCTCCCCGTCTGTGCC
CCC2	5'-CTGTGCCTTCTCATCTGCCGGAC
Downstream primer	
CCC3	5'-GTCCATGCCCCAAAGCCACC
Modified Bowden's assay	
Forward primer	5'-CCGTCTGTGCCTTCTCATCTG
Reverse primer	5'-ACAGCTTGGAGGCTTGAACAGT
Probe	6FAM-CTGTAGGCATAAATTGGT
Bisulfite conversion assay	
Forward primer:	5'-TGTATTTTTTTTTATGTGGWTTTTTTGTTTG
Reverse primer:	5'-TTGTGTGTATTTTGTTTTATTTTTGTATGT
Probe:	6FAM-TGTTTTTTTATTTGTTGG

2.5.4 Modified Bowden's assay

To increase the detection rate of cccDNA, the Bowden's assay was modified by the use of primers and probes targeting more conserved regions of the HBV genome (Table 2.1). Similarly, the samples were first treated with plasmid-safe DNase. The amplification region corresponded to the HBV genome from nucleotides 1549 to 1880. The ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, US) was used for real-time PCR quantitation. The reaction mixture contained 1 × buffer A (TaqMan PCR reagent kit, Applied Biosystems), 3.5 mM of magnesium chloride, 200 μM of deoxyribonucleotide triphosphate mix, 100 nM of forward and reverse primers, 33 nM of TaqMan 3'-minor groove binder (MGB)-DNA probe, 0.01 units/μl of uracil-N-glycosylase, 1.25 units of Taqgold DNA polymerase and 5 μl of DNA samples. The total volume of the mixture was 50 μl. The PCR procedure started with an initial incubation at 50°C for 2 minutes, 95°C for 10 minutes, followed by 50 cycles of 95°C for 15 seconds, 60°C for 90 seconds, and 72°C for 30 seconds. Four serial dilutions of a single-copy HBV genome containing plasmids at concentration of 100 to 10^5 copies/ml were used as standard. The standards, negative controls and positive controls were added in each PCR run.

2.5.5 Bisulfite conversion assay

To increase the specificity of cccDNA detection, the bisulfite conversion assay was developed to introduce sequence differences between the plus-strand and minus-strand. The HBV DNA samples were modified by sodium bisulfite using the EZ DNA Methylation™ Kit according to the manufacturer's instructions (ZYMO Research, CA, US). Prior to the first use, the CT conversion reagent was prepared. Seven hundred and fifty microlitres of water and 210 µl of M-Dilution Buffer were added to a tube of the CT conversion reagent. The reagent was mixed at room temperature with frequent vortexing or shaking for 10 minutes. Afterwards, 5 µl of M-Dilution Buffer was added to 20 µl of DNA sample, and the total volume was adjusted to 50 µl with water. The mixture was incubated at 37°C for 15 minutes. Then, 100 µl of the prepared CT conversion reagent was added and the sample was incubated in the dark at 50°C for 12 to 16 hours. After this, the sample was incubated at 4°C for 10 minutes and loaded to a Zymo-Spin™IC Column with 400 µl of M-Binding Buffer. The mixture was centrifuged at 20,000 × *g* for 1 minute. The column was washed with 100 µl of M-Wash Buffer and centrifuged at 20,000 × *g* for another 30 seconds. Then 200 µl of M-Desulphonation Buffer was added to the column and incubated at room temperature for 20 minutes. The mixture was centrifuged at 20,000 × *g* for 30 seconds. The column was washed two times with 200 µl of M-Wash Buffer at 20,000 × *g* for 30 seconds. The converted DNA was finally eluted in 20 µl of sterile water at 20,000 × *g* for 1 minute. The extracted DNA was stored at -20°C

for future experiments.

The ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, US) was used in real-time PCR quantitation. The primers and TaqMan MGB probe were shown in Table 2.1. The reaction mixture contained 1 × buffer A (TaqMan PCR Reagent Kit, Applied Biosystems), 4 mM magnesium chloride, 200 μM deoxyribonucleotide triphosphate mix, 300 nM forward and reverse primers, 100 nM probe, 200 nM tetramethylammonium chloride, 1.25 units Taqgold DNA polymerase and 4 μl of converted DNA samples in a total volume of 50 μl. The PCR started with an initial incubation at 95°C for 10 minutes, followed by 45 cycles of 95°C for 15 seconds, 58°C for 90 seconds, and 72°C for 30 seconds. Bisulfite converted single-copy HBV genome-containing plasmids were used as standard. Negative controls and standards were added in each PCR run.

2.5.6 Direct sequencing for HBV mutations

We hypothesized that some cases of failed cccDNA detection were due to mutations at the target sequence of the primers and probes used in real-time PCR assay. To test this hypothesis, bi-directional sequencing of the PCR products was performed. Two overlapping fragments of the HBV genome were amplified for direct sequencing: fragment A (forward 5'-CCACTGGHTGGGGCTTGG and reverse 5'AAAAAGTTGCATGGTGCTGG) and fragment B (forward 5'-TTTTTCACCTCTGCCTAATCA and reverse 5'GGAGACTCTAAGGCCTCCCG). Fragments A and B covered position 1198 to 2035 of the HBV genome.

The reaction mixture contained 2 µl of viral DNA samples, 2.5 mM of magnesium chloride, 200 mM of deoxyribonucleotide triphosphate mix, 200 mM of each primer, 0.5 unit of TaqGold DNA polymerase (Applied Biosystems, Foster City, US), and 2.5 µl of 10 × buffer in a final volume of 25 µl. PCR started at 95°C for 10 minutes and went through 45 cycles with denaturation at 95°C for 40 seconds, annealing at 52°C for 40 seconds, elongation at 72°C for 90 seconds and a final extension at 72°C for 7 minutes. The PCR products were examined on a 2.0% agarose/ethidium bromide gel run in 1 × Tris/Borate/EDTA buffer.

Direct sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosytems, Foster City, US). The reaction mixture

comprised of 2 µl of BigDye-Terminator Ready Reaction Mix, 3.2 pmol specific primers and 1 µl of PCR products. The sequencing reaction included 25 cycles of 96°C for 30 seconds, 50°C for 15 seconds, and 60°C for 4 minutes. 10 µl of sodium acetate (5M, pH 5.2) and 50 µl of absolute ethanol were added to each reaction product. After storage at -80°C for 20 minutes, the mixture was centrifuged at 3,700 revolutions per minute for 30 minutes at 4°C. The supernatant was discarded and the pellets were washed once with 100 µl of 75% ethanol. Finally, the dried pellets were dissolved in 10 µl of Hi-Di™ Formamide (Applied Biosystems, Foster City, US). After denaturation at 95°C for 5 minutes, the sequencing solution was kept on ice for 2 minutes and then analyzed by the ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, US).

2.5.7 Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Science version 11.5. Continuous variables were presented as mean ± standard deviation and compared using paired *t* test or analysis of variance (ANOVA). Bonferroni correction was performed to adjust for multiple comparisons. Categorical variables were compared using the chi-squared test. Pearson test was performed to test the correlation between cccDNA and total HBV DNA levels and cccDNA levels detected by different assays. All statistical tests were two-sided. Statistical significance was taken as *P* value less than 0.05.

CHAPTER 3 Results

3.1 Primer and probe design

To design the optimal primers and probes for cccDNA detection, conserved regions of the HBV genome should be identified. The regions should have identical sequence in different HBV strains and genotypes. Using the database of National Center for Biotechnology Information, we obtained 20 sequences of HBV genomes that covered 6 genotypes. Sequencing alignment was performed by Cluster X. The alignment results for the primer and probe regions of the three cccDNA assays were listed in the Appendix and the primers and probes for different assays were shown in Table 2.1. The region chosen was before the DR2 region and extended through the gap on the minus strand (around position 1500 to 1900). This region was selected because it was incomplete in rcDNA but continuous in cccDNA. HBV genome mutations that may affect the primers and probes were allowed if the quasispecies constituted less than 5% of the entire viral population.

3.2 Patients

Liver tissue was obtained from 61 patients with chronic hepatitis B, including 24 from hepatocellular carcinoma and 37 from the liver parenchyma of non-cancerous patients (Table 3.1). This cohort was male-predominant and the viral load was 5.98 log copies/ml.

Serum samples were obtained from 215 patients, including 35 patients with cirrhosis and 180 patients without cirrhosis. Similarly, this cohort was male-predominant. The serum HBV DNA level was higher at 6.34 log copies/ml. The mean serum ALT was over 2 times the upper limit of normal. No patient in this cohort had hepatocellular carcinoma at the time of blood taking.

Table 3.1. Clinical characteristics of chronic hepatitis B patients in this study

	Group 1 (Liver tissue)	Group 2 (Serum)
N	61	215
Age (years)	34±8	36±10
Male gender, n (%)	50 (82)	176 (82)
HBV DNA (log10 copies/ml)	5.98±0.92	6.74±2.04
Positive HBeAg, n (%)	NA	168 (78)
Positive anti-HBe, n (%)	NA	144 (67)
Serum alanine aminotransferase (IU/l)	NA	114±122
Cirrhosis, n (%)	NA	40 (19)
Hepatocellular carcinoma, n (%)	24 (39)	0

3.3 Optimization of the bisulfite conversion assay

If the loss of DNA is too much during bisulfite conversion, the sensitivity of the assay would be affected. Thus, the recovery efficiency after bisulfite conversion was assessed. After comparing the quantitation of standard samples treated by bisulfite and a synthesized polynucleotide with the bisulfite converted sequences, the recovery efficiency after bisulfite conversion was determined to be above 80%.

After bisulfite conversion, unmethylated cytosine residues were converted to uracils. The content of thymidine and uracil residues in the viral genome was almost doubled. The melting temperature during PCR was largely reduced. Besides, the primers used in this assay were long and contained a lot of thymidine residues (Table 2.1). As a result, the risk of non-specific amplification was increased.

Therefore, we attempted a few methods to optimize the bisulfite conversion assay. First, the TaqMan MGB probe was used to increase the specificity of amplification and the annealing temperature. However, no specific amplification was obtained (Figure 3.1). Similar results were obtained despite adjustment of temperature and the use of alternative primers.

Afterwards, tetramethylammonium chloride (TMAC) was used to reduce non-specific priming events. A TMAC concentration of 40 mM was found to be optimal

for satisfactory amplification (Figure 3.2).

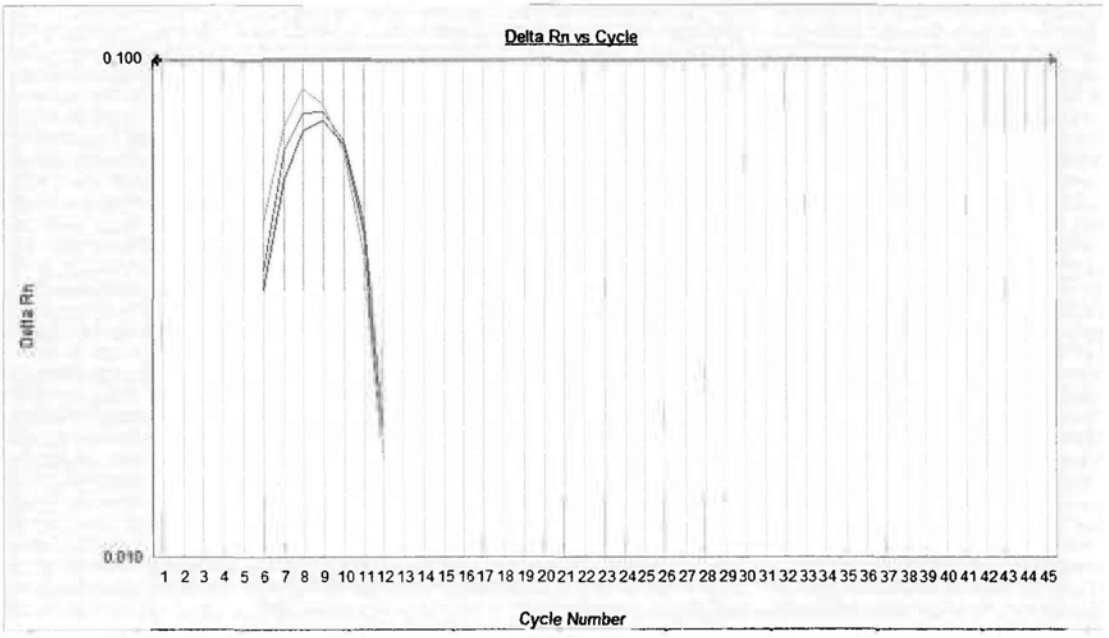

Figure 3.1. Failed amplification of cccDNA after bisulfite conversion.

The primers used in the bisulfite conversion assay were long and contained many thymidine residues. The figure represents an example of failed amplification of cccDNA before the assay was optimized.

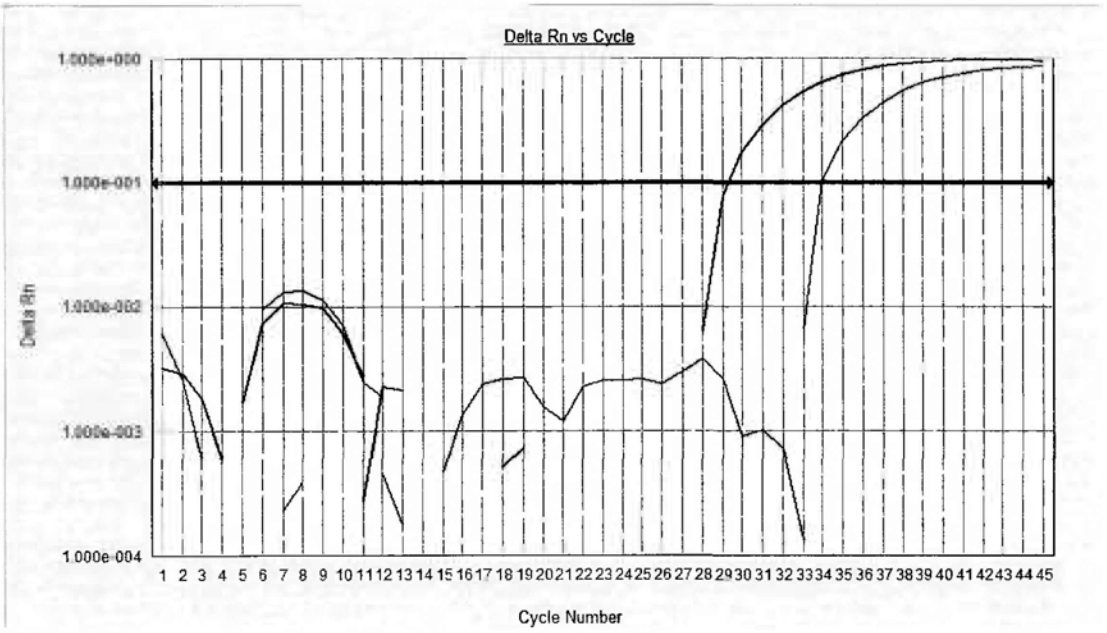

Figure 3.2. Optimization of the bisulfite conversion assay by the addition of tetramethylammonium chloride (TMAC).

TMAC is a chemical commonly used during PCR to reduce or eliminate non-specific priming events. In this example, specific and successful amplification of cccDNA was achieved after the addition of TMAC. The amplification curves were represented by the blue and grey curves at the right upper corner of the figure.

3.4 Detection rate by the three cccDNA assays

When the LightCycler software was used, some amplification curves that were low in amplitude were still reported as positive detection. However, this kind of weak and irregular amplification was commonly seen, non-specific and probably represented minor fluctuation of the baseline only. Therefore, all amplification curves in this study were manually checked to confirm genuine amplification. Amplification curves that were low in amplitude and had abnormal waveform were classified as negatives (Figure 3.3). Nine amplification curves, all generated by the Bowden's assay, were classified as negatives after manual checking.

Among 61 liver tissue samples, the cccDNA detection rate was lowest by the Bowden's assay, with only 42 (69%) cases tested positive. In comparison, the modified Bowden's assay had the highest detection rate, with 60 (98%) cases tested positive. Fifty (82%) cases had cccDNA detected by the bisulfite conversion assay (Figure 3.4).

In contrast, the cccDNA detection rate was substantially lower in serum samples using all three assays. Among 215 serum samples, cccDNA was detected in 58 (27%) cases using the Bowden's assay and in 54 (25%) cases using the bisulfite conversion assay (Figure 3.5). The detection rate was marginally higher using the modified Bowden's assay, with positive detections in 43 (20%) cases.

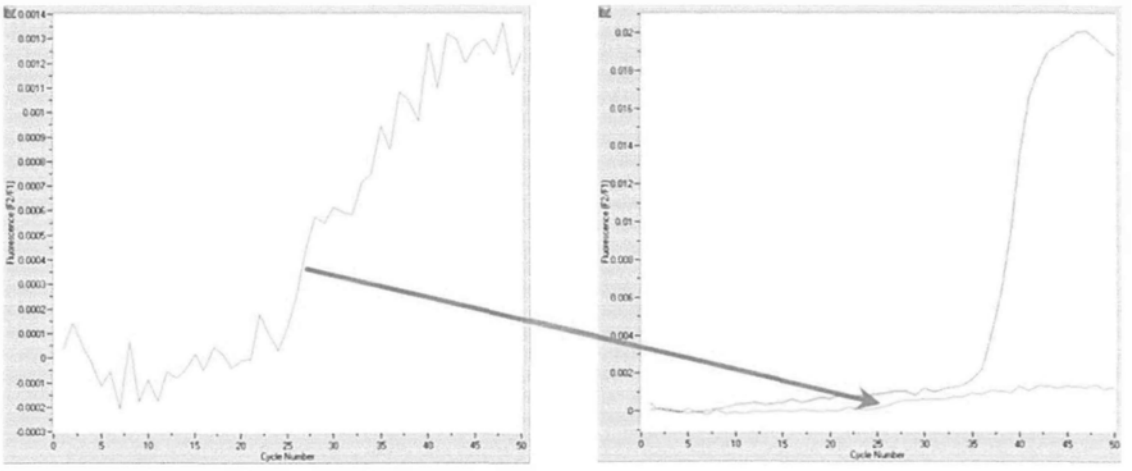

Figure 3.3. Non-specific amplification curves.

Although the LightCycler software reports amplification curves as positive when an upslope pattern is detected, they may be non-specific and represent minor fluctuations of the baseline only. An example was shown in this figure. When compared to a genuine amplification curve in red, the peak fluorescence level of green curve remained low even up to the 50^{th} cycle. To avoid false-positive results, every amplification curve in this study was checked manually.

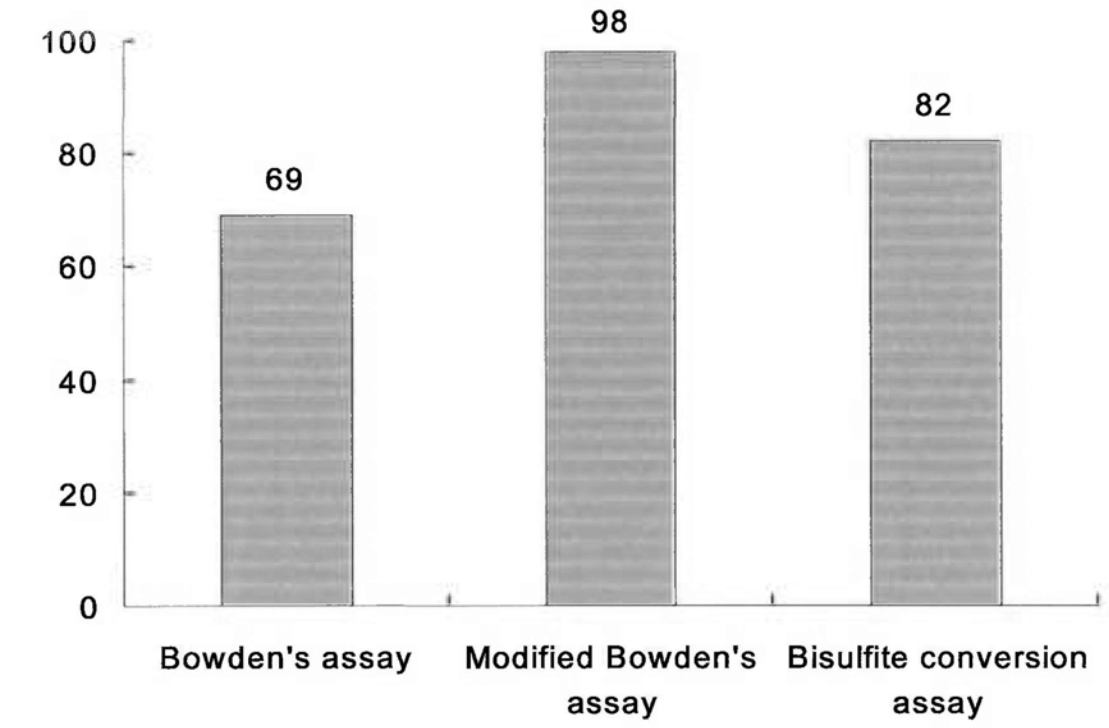

Figure 3.4. cccDNA detection rate of the Bowden's assay, modified Bowden's assay and bisulfite conversion assay in 61 liver samples.

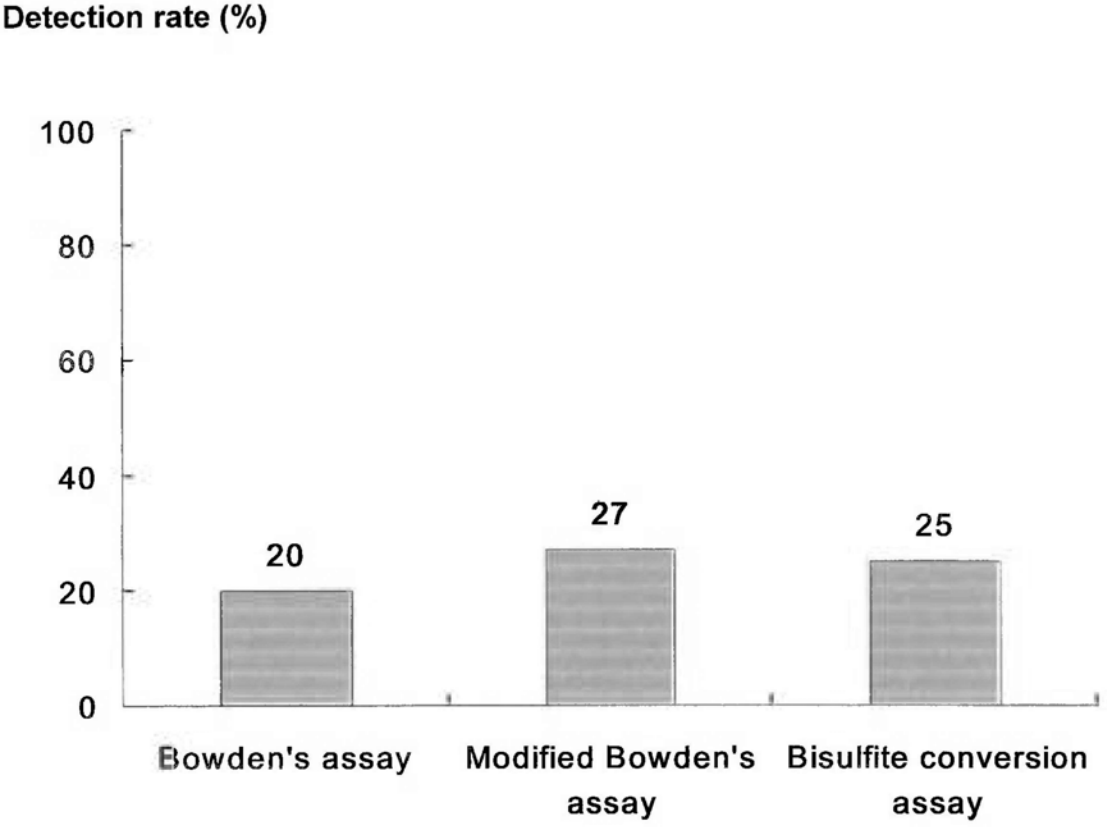

Figure 3.5. cccDNA detection rate of the Bowden's assay, modified Bowden's assay and bisulfite conversion assay in 185 serum samples.

3.5 cccDNA quantitation by the three assays

The standard curves of the cccDNA assays were linear over the range tested and had correlation coefficients routinely above 0.999 (Figure 3.6). Overall, cccDNA level had weak to moderate correlation with total HBV DNA level (Figure 3.7, 3.8, 3.9). The correlation of cccDNA quantitation by different assays was shown in Figure 3.10, 3.11, 3.12.

In liver tissue, the cccDNA quantitation was 5.00±0.94, 4.85±0.90 and 4.24± 0.87 (log10 copies/ml) using Bowden's assay, modified Bowden's assay and bisulfite conversion assay, respectively. The cccDNA quantitation by the bisulfite conversion assay was significantly lower than that by the Bowden's assay and modified Bowden's assay (Figure 3.13).

In serum samples, the cccDNA quantitation was 3.93±0.94, 3.65±1.30 and 3.92 ±0.55 (log10 copies/ml) using Bowden's assay, modified Bowden's assay and bisulfite conversion assay, respectively. The difference among the three assays was not significant in the whole serum group (Figure 3.14).

The bisulfite conversion assay was developed as a more specific test to quantitate cccDNA. In other words, this assay should be less interfered by rcDNA. In this regard, the cccDNA quantitation by the bisulfite conversion assay should be lower than that by the Bowden's assay when the total HBV DNA was high, and the difference should be less obvious when the total HBV DNA was low.

Indeed, among patients with total serum HBV DNA above 10^7 log copies/ml, the cccDNA quantitation by the bisulfite conversion assay was significantly lower than that by the Bowden's assay and modified Bowden's assay (Figure 3.15). On the other hand, among patients with total serum HBV DNA below 10^7 log copies/ml, the cccDNA quantitation by the bisulfite conversion assay was not significantly lower than the Bowden's assay (Figure 3.16). Due to the lower detection limit and better detection rate, more samples with lower cccDNA levels were detected by the modified Bowden's assay. The cccDNA quantitation by the modified Bowden's assay was significantly lower than that by the Bowden's assay.

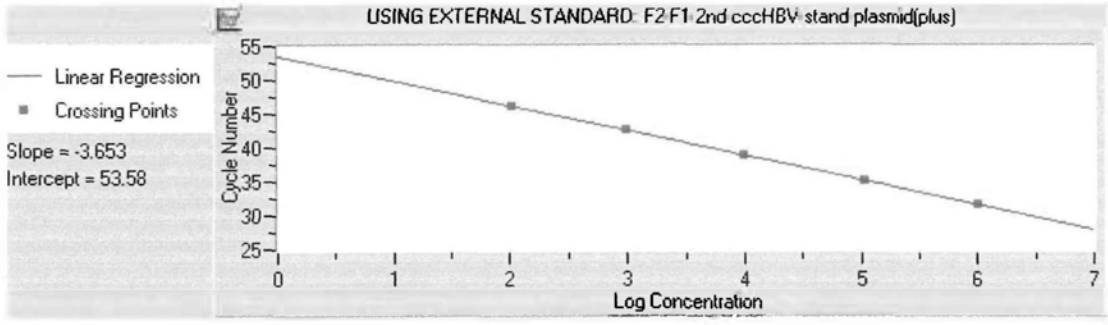

Figure 3.6. Standard curve of the cccDNA assays.

This example represents the Bowden's assay. Within the range tested, the standard curve was linear.

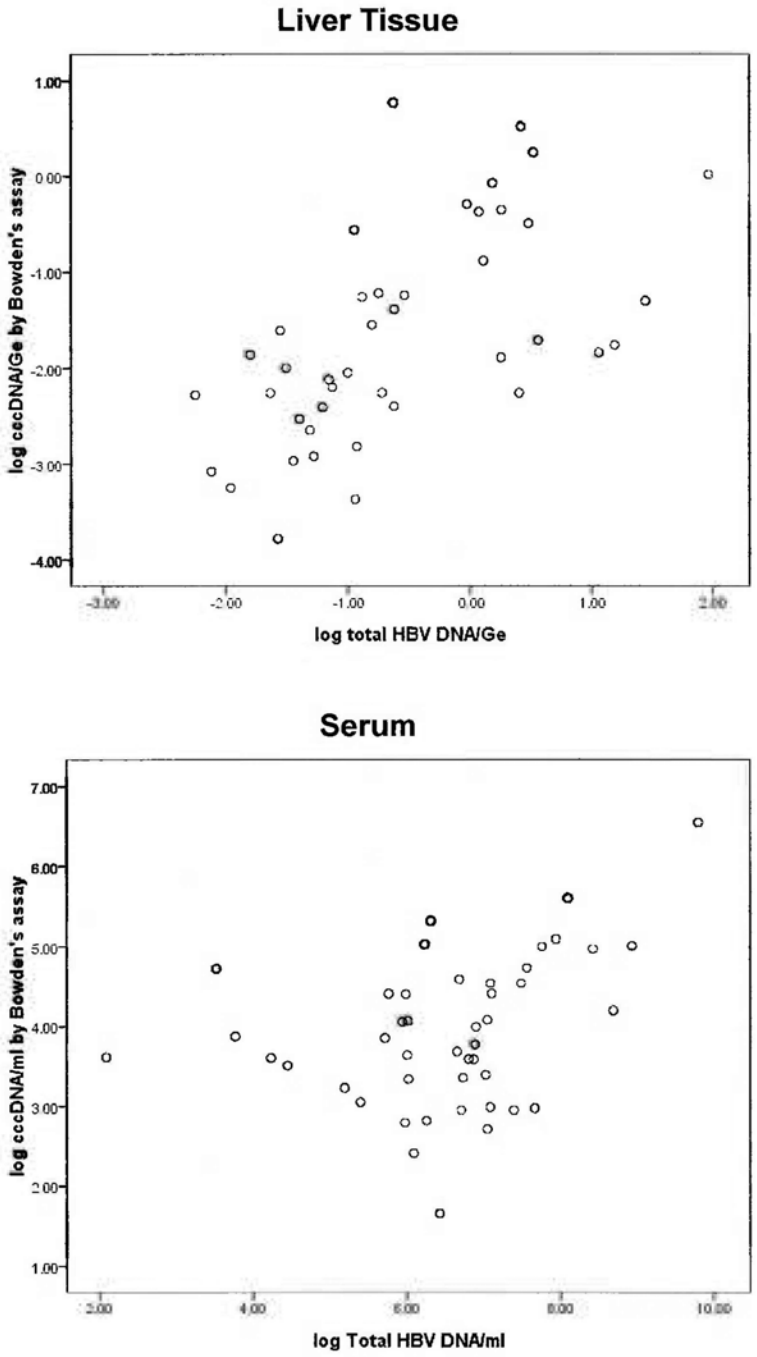

Figure 3.7. Correlation between cccDNA quantitation and total HBV DNA by Bowden's assay.

The Pearson R was 0.594 (P<0.001) and 0.360 (P=0.015) of liver tissue group and serum samples group, respectively.

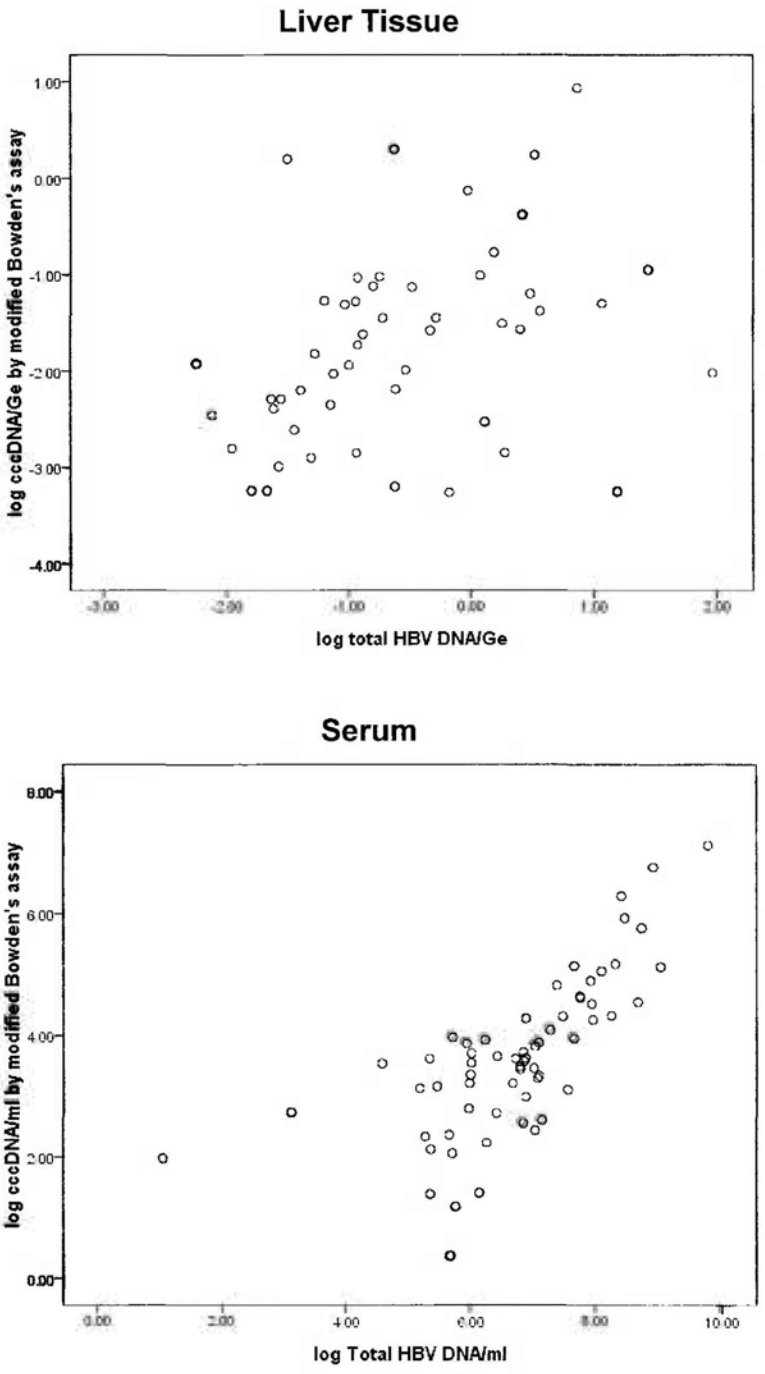

Figure 3.8. Correlation between cccDNA quantitation and total HBV DNA by the modified Bowden's assay.

The Pearson R was 0.353 (P=0.011) and 0.717(P<0.001) of liver tissue group and serum samples group, respectively.

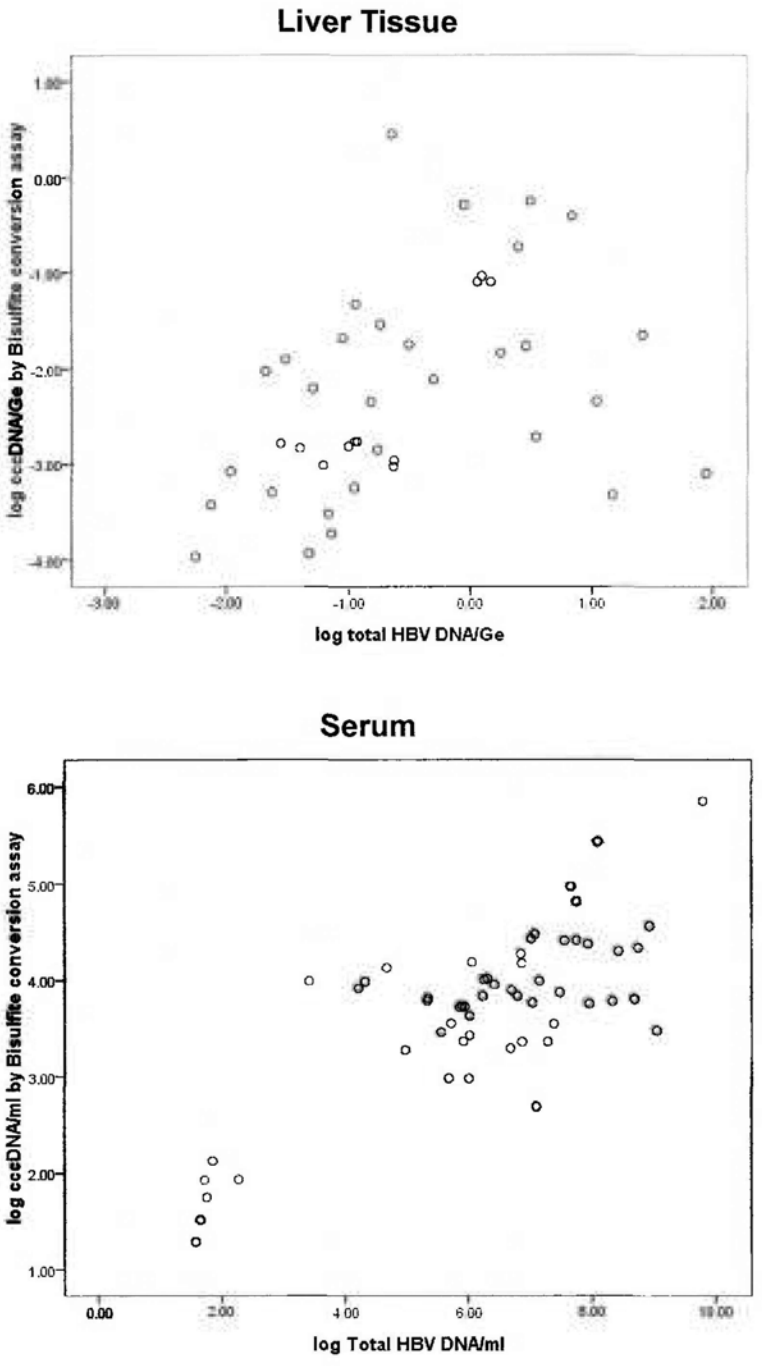

Figure 3.9. Correlation between cccDNA quantitation and total HBV DNA by the bisulfite conversion assay.

The Pearson R was 0.417 (P=0.007) and 0.773 (P<0.001) of liver tissue group and serum samples group, respectively.

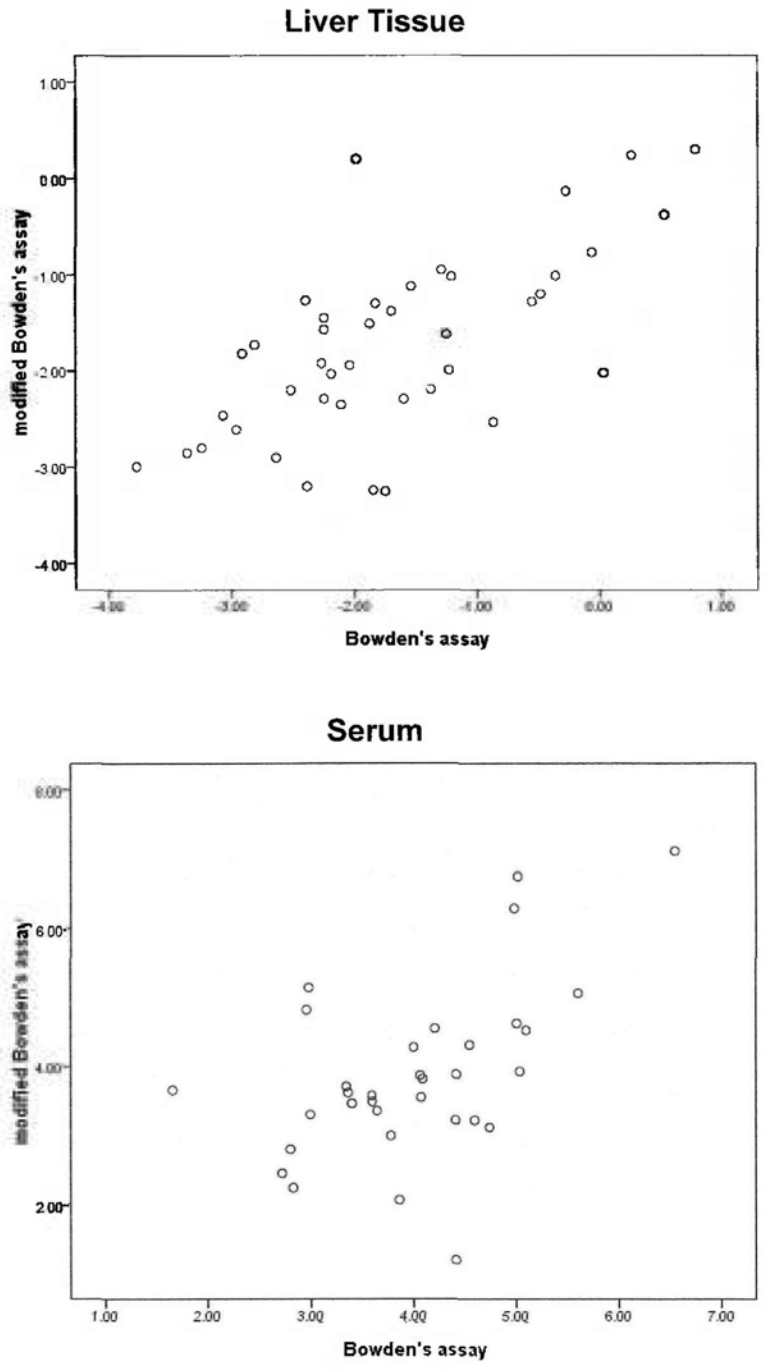

Figure 3.10. Correlation between cccDNA quantitation by Bowden's assay and the modified Bowden's assay.

The Pearson R was 0.667 (P<0.001) and 0.515 (P=0.002) of liver tissue group and serum samples group, respectively.

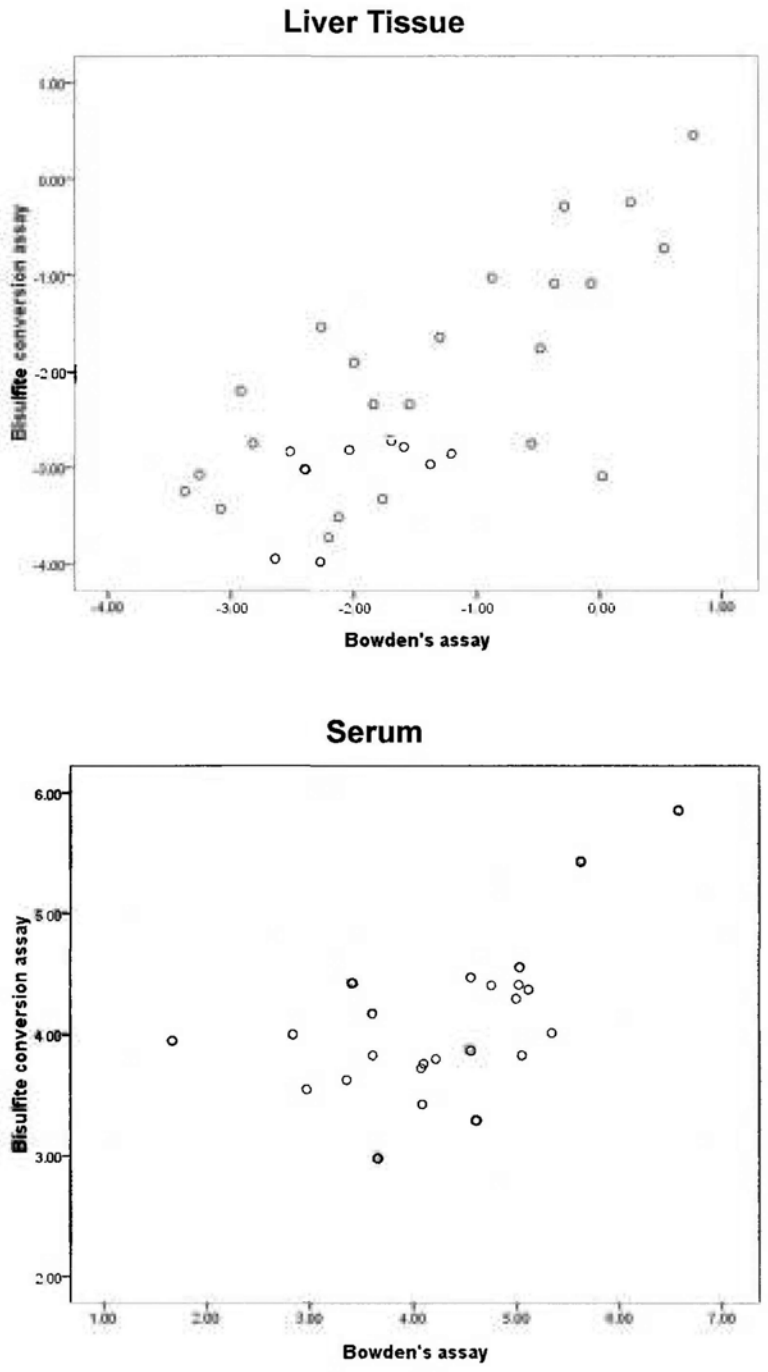

Figure 3.11. Correlation between cccDNA quantitation by Bowden's assay and the bisulfite conversion assay.

The Pearson R was 0.733 (P<0.001) and 0.587 (P=0.003) of liver tissue group and serum samples group, respectively.

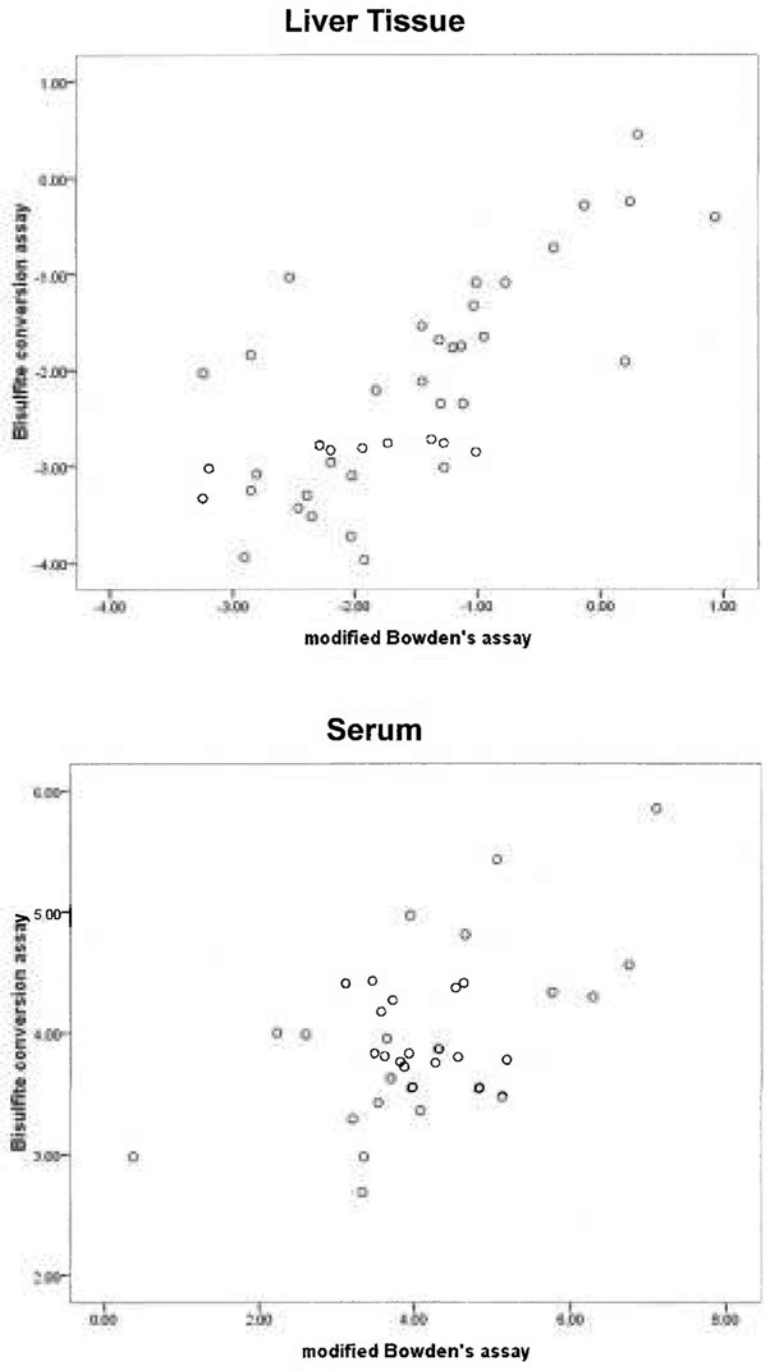

Figure 3.12. Correlation between cccDNA quantitation by the bisulfite conversion assay and the modified Bowden's assay.

The Pearson R was 0.722 (P<0.001) and 0.549 (P=0.001) of liver tissue group and serum samples group, respectively.

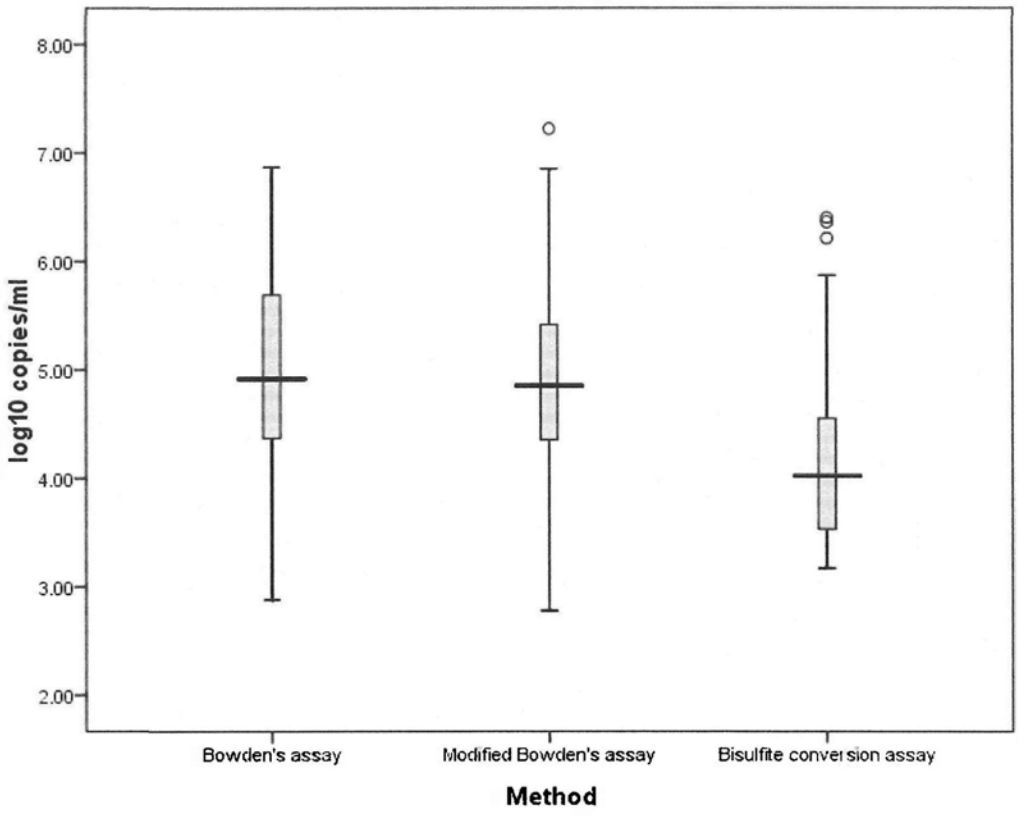

Figure 3.13. cccDNA quantitation in 61 liver tissues by the Bowden's assay, modified Bowden's assay and bisulfite conversion assay.

The cccDNA quantitation among the three assays was significantly different (P<0.001 by ANOVA). After Bonferroni correction, the intrahepatic cccDNA quantitation by the bisulfite conversion assay remained significantly lower than that by the Bowden's assay (P=0.001) and modified Bowden's assay (P=0.003).

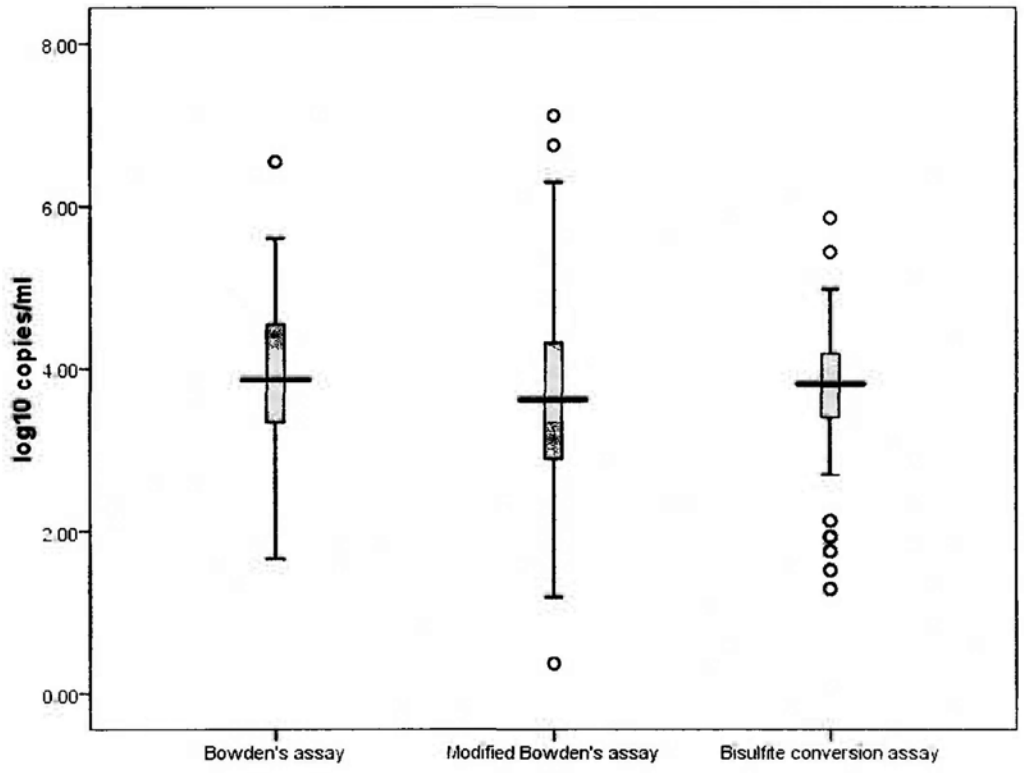

Figure 3.14. cccDNA quantitation in 215 serum samples by the Bowden's assay, modified Bowden's assay and bisulfite conversion assay.

The difference of cccDNA quantitation among the three assays was not significant (*P*=0.231 by ANOVA).

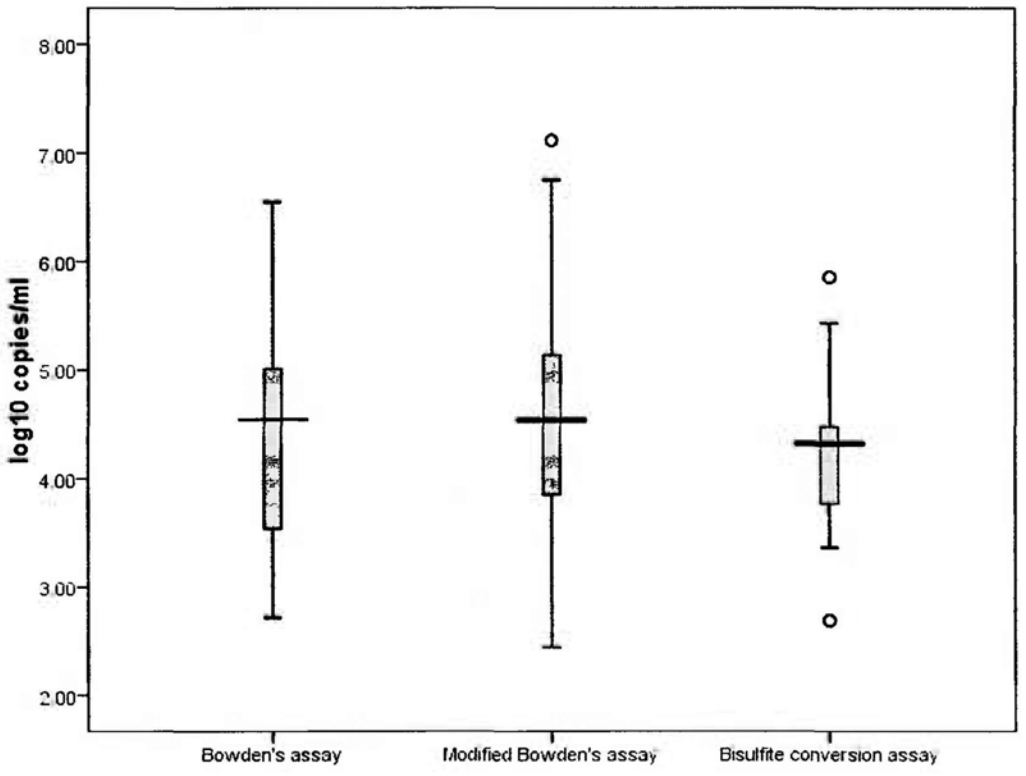

Figure 3.15. cccDNA quantitation in 29 serum samples in patients with total HBV DNA above 10^7 log copies/ml.

The cccDNA quantitation among the three assays was not significantly different (P=0.479 by ANOVA). When using paired-samples T-test, the serum cccDNA quantitation by the bisulfite conversion assay remained lower than that by the Bowden's assay (P=0.008) and the modified Bowden's assay (P=0.046).

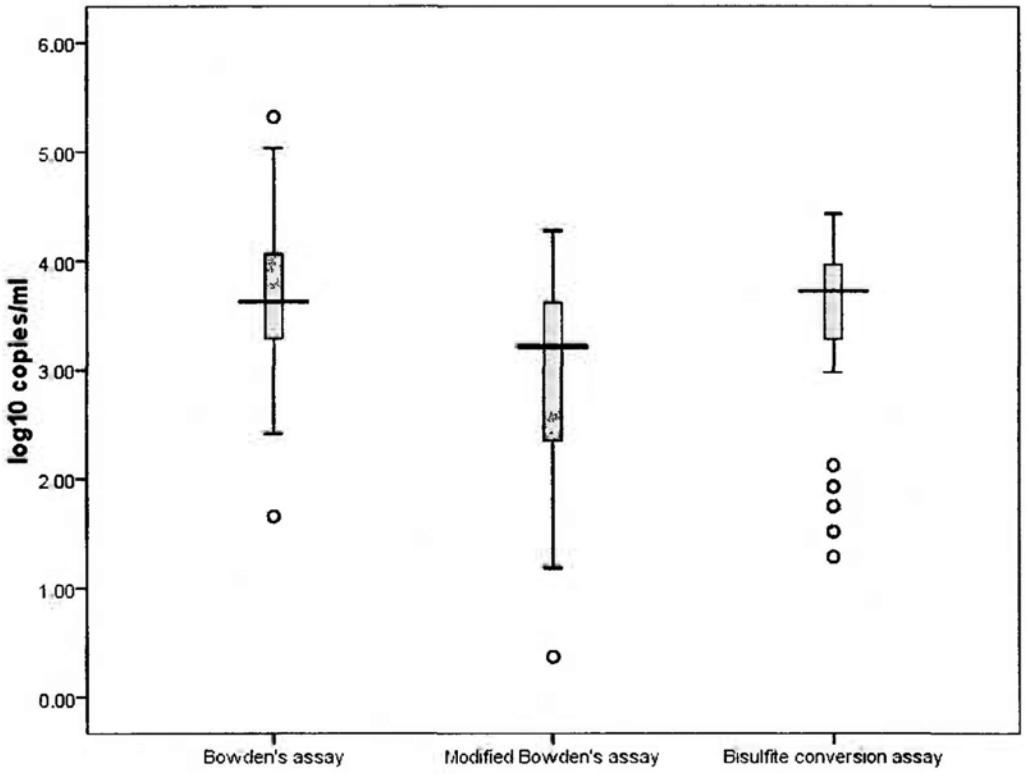

Figure 3.16. cccDNA quantitation in 186 serum samples in patients with total HBV DNA below 10^7 log copies/ml.

The cccDNA quantitation among the three assays was significantly different (*P*=0.004 by ANOVA). After Bonferroni correction, the serum cccDNA quantitation by the modified Bowden's assay was significantly lower than that by Bowden's assay (*P*=0.003).

3.6 Direct sequencing for HBV mutations

Negative cccDNA results could be explained by a few possibilities. First, the absolute amount of cccDNA might be small and below the detection limit of the assay. Second, HBV mutations might affect the binding and performance of the primers and probes. To test the latter possibility, the HBV DNA in 42 samples with negative cccDNA results was sequenced.

In 5 samples in which cccDNA was undetectable by the Bowden's assay but detectable by the modified Bowden's assay and the bisulfite conversion assay, the same C to A mutation was found in the forward primer binding site of the Bowden's assay (Figure 3.17). On the other hand, no HBV mutation was found in the binding sites of the backward primer and probe of the Bowden's assay, as well as the primer and probe binding sites of the modified Bowden's assay and the bisulfite conversion assay. This might partially explain the lower cccDNA detection rate when the Bowden's assay was used.

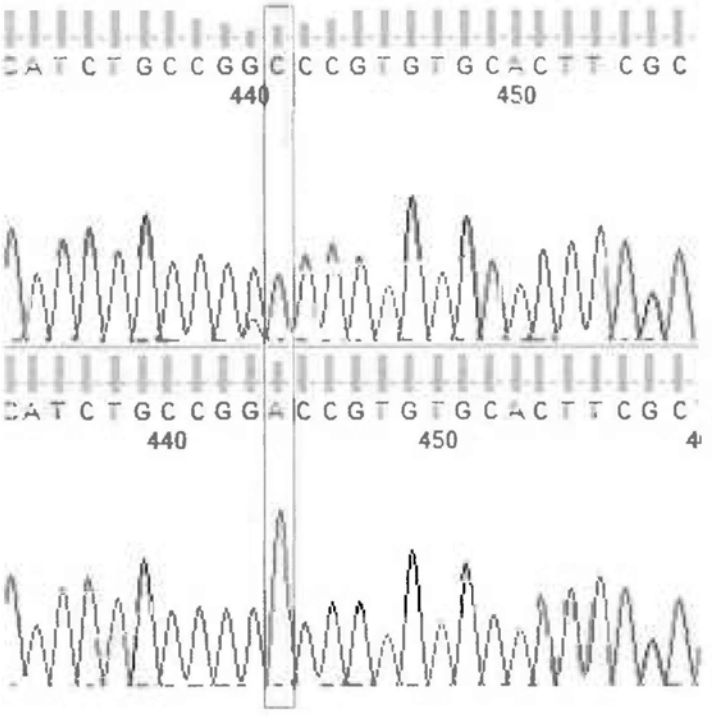

Figure 3.17. HBV mutation affecting the performance of the Bowden's assay.

In 5 samples, a C to A mutation in the HBV genome resulted in decreased binding of the forward primer of the Bowden's assay and undetectable cccDNA. No similar mutations were detected in the binding regions of the modified Bowden's assay and the bisulfite conversion assay.

CHAPTER 4 Discussion

4.1 Performance of the new cccDNA assays

We set out to develop new cccDNA assays with improved detection rates and specificity. This is to tackle the problem of non-specific amplification of rcDNA. Since cccDNA and rcDNA have identical gene sequences, traditional assays attempt to distinguish between the two simply by designing primers that run through the gap regions of rcDNA. Although we know that amplification efficiency is largely reduced through the gap regions, this distinction is partly offset by the fact that the concentration of rcDNA is much higher than that of cccDNA. Thus, current assays still cannot eliminate the interference of rcDNA.

By using the bisulfite conversion, unmethylated cytosines are converted into uracils. As a result, the two DNA strands are no longer complementary to each other. When this technique is applied to HBV DNA, the gene sequences of rcDNA and cccDNA become different. In other words, we not only make use of the structural difference between rcDNA and cccDNA, but also introduce gene sequence differences to distinguish between them. The latter would enhance the specificity in detecting cccDNA.

We anticipated that more specific cccDNA assay should be less interfered by rcDNA and would report a lower cccDNA concentration than the less specific assays, especially when the concentration of rcDNA is high. Indeed, we confirmed that the cccDNA concentration by the bisulfite conversion assay was

lower than that detected by the Bowden's assay or modified Bowden's assay in both liver tissues and serum. The difference was significant when the serum total HBV DNA level was high.

On the other hand, high sensitivity in cccDNA detection is expected when PCR-based assays are used. However, we found that up to 30% of patients had undetectable intrahepatic cccDNA using the Bowden's assay even though total HBV DNA was detectable. While some cases of undetectable cccDNA might be explained by the low level approaching the detection limit, we postulated that HBV mutations affecting the binding sites of the primers and probes used in the Bowden's assay might partly explain the phenomenon as well. Indeed, when a new set of primers and probe were designed based on more conserved regions of the HBV genome, intrahepatic cccDNA could be detected in 98% of cases by the modified Bowden's assay.

In contrast, the difference in detection rate of cccDNA was less marked in serum samples, although the modified Bowden's assay still had the highest detection rate. In the liver, cccDNA serves as the replication template. The same does not occur in the peripheral blood and cccDNA is not actively released from the hepatocytes to the circulation. Therefore, cccDNA detected in the serum is likely released from damaged hepatocytes. In patients with little hepatic necroinflammation, serum cccDNA concentration should be low. In a small study of 10 Chinese patients in Hong Kong, cccDNA was detected in the liver of all

patients but only in 60% of the serum samples despite that those patients had active viremia with serum total HBV DNA in the range of 5 to 10 log copies/ml. [87] The low absolute concentration of cccDNA in the serum of usual chronic hepatitis B patients explains the small difference in the detection rate among the three assays.

Full evaluation of cccDNA assays is limited by the lack of a gold standard. For example, when cccDNA is not detected by the Bowden's assay but detected by the modified Bowden's assay, this may represent a false negative result of the former test or a false positive result of the latter. Nevertheless, total HBV DNA was detectable in all liver samples of this study. In addition, in 5 of 42 cases of undetectable cccDNA by the Bowden's assay, the same HBV mutation over the binding site of the primer was demonstrated. These suggest that the modified Bowden's assay is indeed more sensitive due to the use of primers and probe over more conserved region of the HBV genome.

4.2 Development and optimization of the bisulfite conversion assay

All of the previous assays are targeting the structural differences between cccDNA and rcDNA, including the gap near the DR1 region and cessation of nucleotide strand next to the DR2 region. The main barrier of a PCR-based method is specificity. On the other hand, non-PCR-based assays are limited in sensitivity. The source of all these obstacles is the identical gene sequences of cccDNA and rcDNA. If one only focuses on the structural characteristics of rcDNA, these two opposing limitations cannot be resolved.

In this study, we artificially introduced sequence differences to cccDNA and rcDNA. After bisulfite conversion, the plus strand and minius strand of HBV DNA have changed to two totally different sequences. Then, the lost part on the plus strand of rcDNA became the breakthrough. This lost part is not only a structural difference but also a sequence difference now which can be used for a real-time PCR quantitation. This is a completely new sight of this question. By introducing the sequence differences, we developed this novel PCR-based method with improved sensitivity and specificity.

But the development of this method was not easy. We met serious problem when performing the amplification using the usual method of real-time PCR. The non-specific amplification was very strong and totally covered the real amplification curve.

After CT conversion, unmethylated cytosine residues are converted to uracils, leaving only 3 residues in converted DNA. The melting temperature and complexity of sequences are both largely reduced and more nucleotide sequences in human genome which are similar to that of the primers and probe will emerge. As a result, the chance of non-specific amplification increases a lot. To avoid this, a longer primer and MGB probe to raise the melting temperature is usually adopted. But in this assay, this was not enough. A lot of PCR additives were used to increase the primer affinity or to decrease the non specific amplification, such as PCR enhancer by Qiagen, dimethyl sulfoxide and TMAC. After many trials, TMAC was finally introduced to the reaction. After adjusting the concentration of TMAC, primers and probe and annealing temperature, we finally got very specific amplification curves.

4.3 Mutation analysis

HBV is a DNA virus, but the estimated nucleotide substitution rate is 10^4 times higher than normal DNA viruses (1.4×10^{-5} to 5×10^{-5} per site per cycle[42] [43, 101]), similar to that of RNA viruses [102, 103]. The high mutation rate is mainly due to the reverse transcription via an RNA intermediate during HBV replication cycle and the lack of proofreading. Spontaneous mutations exist even in highly conserved regions. The frequency of induced mutations is also high when patients are exposed to antiviral drugs. Mutations in the primer and probe regions will dramatically decrease the priming affinity and it may be an important factor resulting in failed detection.

After sequencing 42 samples which cccDNA was not detected in by one or more of these three assays, the mutation A/C@1574 was found in 5 samples by one or more of these three assays. It is a spontaneous mutation and located on the primer region of Bowden's assay. The same mutation does not affect the performance of the modified Bowden's assay and bisulfite conversion assay. This may partly explain the higher detection rate of these two assays.

4.4 Limitations

Our study had a few limitations. First, all clinical samples were from Chinese patients in Hong Kong and Shenzhen, China. As a result, all patients harbored genotypes B or C HBV. The performance of the novel assays in other HBV genotypes needs to be validated. However, the sample size of this study was relatively large and provided useful data on the performance of different cccDNA assays.

Second, there is yet a standard for cccDNA. Without a gold standard, the superiority of one cccDNA assay can only be inferred but not confirmed. However, we attempted to validate the methods from different angles. For example, the detection rate of cccDNA in liver samples was used to compare the sensitivity of different assays. In addition, the performance of different assays in patients with high and low total HBV DNA was compared to test the potential interference by rcDNA.

Third, mutations in the HBV genome only explained partially why the detection rate of cccDNA by the Bowden's assay was lower than that by the new assays. Further work is required to elucidate the cause of undetectable cccDNA by the Bowden's assay.

Fourth, from technical point of view, bisulfite conversion is not 100% efficient. Around 10% to 20% of DNA is lost during the procedure. This may affect the

lower limit of detection of the bisulfite conversion assay, especially in patients with low viral load. In these cases, the modified Bowden's assay is valuable.

Finally, most previous studies on cccDNA concentrated on liver samples. Although we developed novel cccDNA assays with good performance both in serum and liver samples, the clinical use of serum cccDNA assay is currently unclear. More studies using clinical endpoints such as the development of hepatocellular carcinoma and response to antiviral drugs are required to fully evaluate the utility of this biomarker in clinical practice.

4.5 Conclusions

In conclusion, the bisulfite conversion assay is a novel method to reduce the false detection of rcDNA and thus results in enhanced specificity in the quantitation of cccDNA in liver and serum. The modified Bowden's assay adopted probes and primers targeting more conserved regions of the HBV genome and has higher detection rate. The two assays are complementary to each other and may be used in patients with extreme HBV DNA levels. These cccDNA assays should be further validated in larger studies and may become important tests for diagnostic, prognostic and treatment monitoring purposes.

Appendix

Sequence alignment of cccDNA in different genotypes:

```
gi|56377730|dbj|AB109476.1|     CTGCCGTTTCGTCCGACCACGGGGCGCACCTCTCTTTACGCGGACTCCCC 1550
gi|56377768|dbj|AB110075.1|     CTGCCGTTTCGTCCGACCACGGGGCGCACCTCTCTTTACGCGGTCTCCCC 1550
gi|90991096|dbj|AB222713.1|     CTGCCGTTTCGTCCGACCACGGGGCGCACCTCTCTTTACGCGGACTCCCC 1550
gi|87295370|gb|DQ315778.1|      CTGCCGTTTCGACCGACCACGGGGCGCACCTCTCTTTACGCGGACTCCCC 1550
gi|87295361|gb|DQ315776.1|      CTGCCGTTCCGACCGACCACGGGGCGCACCTCTCTTTACGCGGACTCCCC 1550
gi|66267696|dbj|AB205189.1|     CTGCCGTTCCAGCCGACCACGGGGCGCACCTCTCTTTACGCGGTCTCCCC 1550
gi|78675492|dbj|AB219534.1|     CTGCCGTTCCGGCCGACCACGGGGCGCACCTCTCTTTACGCGGTCTCCCC 1520
gi|15425696|dbj|AB056515.1|     CTGCCGTTCCTGCCGACCACGGGGCGCACCTCTCTTTACGCGGTCTCCCC 1550
gi|29124907|gb|AY217365.1|      CTATTGTACCGACCGACCACGGGGCGCACCTCTCTTTACGCGGACTCCCC 1550
gi|29124911|gb|AY217366.1|      CTGTTCTACCGACCGACCACGGGGCGCACCTCTCTTTACGCGGACTCCCC 1549
gi|29124902|gb|AY217364.1|      CTGCTGTACCGACCGACCACGGGGCGCACCTCTCTTTACGCGGACTCCCC 1484
gi|6009775|dbj|AB026814.1|      CTGCCGTTCCGGCCGACCACGGGGCGCACCTCTCTTTACGCGGTCTCCCC 1548
gi|56342116|dbj|AB113876.1|     CTGCCGTTCCGGCCGACCACGGGGCGCACCTCTCTTTACGCGGTCTCCCC 1550
gi|56342126|dbj|AB113878.1|     CTGCCATTCCGACCGACCACGGGGCGCACCTCTCTTTACGCGGTCTCCCC 1550
gi|6063457|dbj|AB033557.1|      CTGCCGTTCCGGCCGACCACGGGGCGCACCTCTCTTTACGCGGTCTCCCC 1550
gi|39979292|dbj|AB112471.1|     CTGCCGTTCCGGCCGACCACGGGGCGCACCTCTCTTTACGCGGTCTCCCC 1550
gi|70609938|gb|DQ089765.1|      CTGCCGTTCCGGCCGACTACGGGGCGCACCTCTCTTTACGCGGTCTCCCC 1550
gi|11191883|dbj|AB036911.1|     CTGCCGTTCCAGCCGACGACGGGTCGCACCTCTCTTTACGCGGACTCCCC 1550
gi|11191955|dbj|AB036920.1|     CTGCCGTTCCAGCCGACGACGGGTCGCACCTCTCTTTACGCGGACTCCCC 1550
gi|18146691|dbj|AB064315.1|     CTGCCGTTCCGGCCAACGACGGGTCGCACCTCTCTTTACGCGGACTCCCC 1541
                                ** * * ** ** ***** ********************* ******

gi|56377730|dbj|AB109476.1|     GTCTGTGCCTTCTCATCTGCCGGACCGTGTGCACTTCGCTTCACCTCTGC 1600
gi|56377768|dbj|AB110075.1|     GTCTGTGCCTTCTCATCTGCCGGACCGTGTGCACTTCGCTTCACCTCTGC 1600
gi|90991096|dbj|AB222713.1|     GTCTGTGCCTTCTCATCTGCCGGACCGTGTGCACTTCGCTTCACCTCTGC 1600
gi|87295370|gb|DQ315778.1|      GTCTGTGCCTTCTCATCTGCCGGACCGTGTGCACTTCGCTTCACCTCTGC 1600
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gi|15425696|dbj|AB056515.1|     GTCTGTTCCTTCTCATCTGCCGGACCGTGTGCACTTCGCTTCACCTCTGC 1600
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gi|6063457|dbj|AB033557.1|      GTCTGTGCCTTCTCATCTGCCGGTCCGTGTGCACTTCGCTTCACCTCTGC 1600
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gi|11191955|dbj|AB036920.1|     GTCTGTTCCTTCTCATCTGCCGGACCGTGTGCACTTCGCTTCACCTCTGC 1600
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gi|18146691|dbj|AB064315.1|     TATATAAGAGGACTCTTGGACTTTCGCCCCGGTCAATGACCTGGATTGAG 1691
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gi|11191955|dbj|AB036920.1|     GAATACATCAAAGACTGTGTATTTAAGGACTGGGAGGAGCTGGGGGAGGA 1750
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gi|66267696|dbj|AB205189.1|     GCTTAGATTAAAGGTCTTTGTACTAGGAGGCTGTAGGCATAAATTGGTCT 1800
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gi|70609938|gb|DQ089765.1|      GATTAGATTAAAGGTCTTTGTACTGGGAGGCTGTAGGCATAAATTGGTCT 1800
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gi|90991096|dbj|AB222713.1|     GCGCACCAGCACCATGCAACTTTTTCACCTCTGCCTAATCATCTCTTGTT 1850
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gi|29124902|gb|AY217364.1|      CATGTCCTACTGTTCAAGCCTCCAAGCTGTGCCTTGGGTGGCTTTGGGGC 1834
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Primer and probe regions of Bowden's assay, modified Bowden's assay and bisulfite conversion assay are in red, blue and grey, respectively. The genotypes of these sequences are shown below:

Gynotype B: AY217365.1, AY217366.1, AY217364.1

Gynotype C: AB026814.1, AB113876.1, AB113878.1, AB033557.1, AB112471.1, DQ089765.1

Gynotype D: AB109476.1, AB110075.1, AB222713.1, DQ315778 D1, DQ315776.1 D3

Gynotype E: AB205189.1, AB219534.1,

Gynotype F: AB036911.1, AB036920.1,

Gynotype G : AB056515.1, AB064315.1

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