

## ADVANCES IN MOLECULAR APPROACHES IN THE DIAGNOSIS OF HEPATITIS-B VIRUS (HBV)

<sup>1</sup>Harindi J. Salgado, <sup>2</sup>Fahad M. Rismy

*School of Science, Bms, Colombo*

*<sup>1</sup>hjs\_jay@hotmail.com, <sup>2</sup>fahad@bms.edu.lk*

### ABSTRACT

Hepatitis B virus (HBV) is a globally spread, public health concern with increasing rates of morbidity and fatality. The infection could progress into acute or chronic phases, eventually causing life-threatening complications. Virological diagnosis depends on immunoassays and molecular approaches, which analyze peripheral blood to detect HBV-specific antigens and HBV-DNA respectively. With the arising need for early diagnosis, a rapid evolution is observed in the utilization of advanced molecular tools like polymerase chain reaction (PCR) and isothermal amplification methods. This review emphasizes on the advantages and limitations of contemporary PCR and isothermal assays, their advancements and their diagnostic role in future applications. Different variants of conventional PCR including nested and real-time PCR, and isothermal assays such as rolling circle amplification (RCA) and loop-mediated isothermal amplification (LAMP) have been compared and evaluated. Accordingly, conventional PCR is the most extensively adopted technique as its variants support automation and multiplexing. Of the multiple variants available, real-time PCR has been commonly exploited for HBV detection, due to its augmented specificity and sensitivity, broad detection capability and reproducibility. This is crucial in therapeutic decision-

making, in monitoring drug responses and in point-of-care treatment. Among the isothermal amplification techniques, LAMP attested to be more efficient in HBV detection due to its tolerance to biological inhibitors, which becomes convenient during off-lab situations. Yet, one of the highest advancements achieved so far in this regard is the integration of these assays to portable, miniaturized biosensing devices, which represent a pragmatic alternative for early diagnosis and prompt management of the infection.

Keywords: Molecular tools, hepatitis-B virus, loop-mediated isothermal amplification, rolling circle amplification, biosensor

### INTRODUCTION

Hepatitis-B virus (HBV), which belongs to family Hepadnaviridae; causes the life-threatening HBV infection. It possesses a 3200 base-pairs long circular-genome, constituting partially double-stranded DNA (Figure 1) (Riaz et al., 2016).

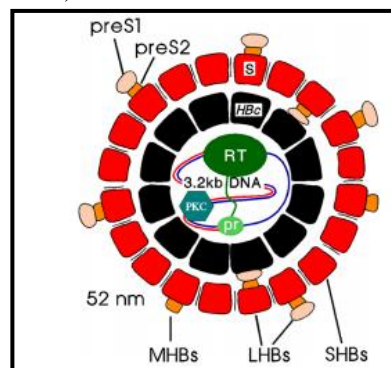


Figure 1 - Structural components of a Hepatitis-B virion (Gerlich, 2013)

Exposure of percutaneous or mucosal surfaces to infected body fluids, parenteral contact, sexual intercourse and transmission via mother to neonate are the primary modes of HBV propagation (Ott et al., 2012). It directly affects the liver and often results in short, transient and asymptomatic acute infections. Majority with weakened immune systems undergo chronic infections causing substantial liver damage; eventually leading to cirrhosis and hepatocellular carcinoma (Mu et al., 2015). HBV affects over 2 billion people totally and 350 million of them are carriers of chronic-HBV (cHBV). HBV and its complications account for 780,000 deaths annually. Accordingly, it is positioned as the 10th leading cause of mortality (Klumpp et al., 2015). In quantitative HBV diagnosis as well as in population surveillance of HBV prevalence (Figure 2) and endemicity (Table 1), seromarkers are extensively used. The predominantly used HBV-specific seromarker is the Hepatitis-B surface antigen (HBsAg) (Ott et al., 2012).



Figure 2 - Global HBV prevalence (MacLachlan & Cowie, 2015).

Table 1 - Global prevalence and endemicity of HBV infection (MacLachlan & Cowie, 2015; Trepo et al., 2014).

Rate and percentage of prevalence/ endemicity	Geographical distribution	Percentage of HBV-infected population	Predominantly affected individuals
High; $\geq 8\%$	Asia-Pacific region, Sub-Saharan African region and Amazon basin	45%	Women of peak child-bearing age, infants and children
Intermediate; 2 - 7%	North Africa, Middle East, parts of Eastern and Southern Europe, parts of Latin America, South West and South Central Asia	40-43%	Infants, children and adults
Rate and percentage of prevalence/ endemicity	Geographical distribution	Percentage of HBV-infected population	Predominantly affected individuals
Low; $< 2\%$	North America, some regions in South America, Northern and Western Europe, Australia, Japan and Asia	12%	Adolescents and adults

Whole-genome sequencing studies has proven the existence of 10 widely distributed HBV genotypes; A-J, and their respective subgenotypes (Table 2) (MacLachlan & Cowie, 2015).

Table 2 - Geographical distribution of the HBV genotypes and subgenotypes (Lin & Kao, 2015).

Genotypes	Serotypes	Subtypes	Geographical location
A	adw	A1 A2 A3	Sub-Saharan Africa and India Northern Europe and India Western Africa
B	adw, ayw	B1 B2 - 5 B6	Japan East Asia, Taiwan, China, Indonesia, Vietnam and Philippines Alaska, Northern Canada and Greenland
C	adw, ayr, adr	C1 - 3 C4 C5 C6 - 11	Taiwan, China, Korea and Southeast Asia Australia Philippines and Vietnam Indonesia
D	ayw	D1 - 6	Africa, Europe, Mediterranean countries, India and Indonesia
E	ayw		Restricted to West Africa
F	adw	F1 - 4	Central and South America
G	adw		France, Germany and United States
H	adw		Central America
I	adw		Vietnam and Laos
J			Japan

Due to high mortality and morbidity rate, early detection of HBV is crucial. Virological diagnosis is based on immunoassays and nucleic acid detection assays (Figure 3). Immunoassays mainly detect the HBsAg, whereas molecular assays target genomic material of HBV (Portillo et al., 2015). However, low antigen levels and diagnostic-escape mutations cause non-detection of HBsAg, thus emphasizing the prominence of molecular tools for efficient HBV diagnosis. In molecular assays, HBV-DNA is quantitatively detected in peripheral blood, hepatocytes and in other extra-hepatic sites as it reliably indicates active HBV replication (Kania et al., 2014). Relative to immunoassays,

molecular techniques assist infection diagnosis, the evaluation of therapeutic responses to antiviral therapy, therapeutic decision-making and in monitoring viral replication kinetics. Currently, thermal cycling-based amplification techniques such as polymerase chain reaction (PCR), quantitative real-time PCR (qPCR), nested-PCR (nPCR) and multiplex-PCR have been implemented for HBV diagnosis. Their advantages include the availability of validated standard operating procedures, convenient access to reagents and equipment, higher sensitivity, specificity, simplicity and reproducibility, and broader detection capability (Wu et al., 2017; Zauli et al., 2016). Nevertheless, high equipment cost, risk of contaminations and inhibitions, requirement of post-amplification optimizations and technical expertise, lengthy, time-consuming procedures and the need to regulate temperature (Bandhavkar, 2016) has led to the development of alternative, thermal cyler-independent isothermal techniques. They function under constant temperature, acquiring a higher efficiency within a shorter amplification time. These include loop-mediated isothermal amplification (LAMP), rolling-circle amplification (RCA) and transcription-mediated amplification (TMA). Further, they can be applied in biosensing devices (Chuang et al., 2012). In this review, few of the contemporary PCR-based techniques and isothermal assays mentioned above and their advancements are compared, emphasizing on their potential to be adopted for HBV diagnosis in the future by those who are attentive on the molecular diagnostic field.

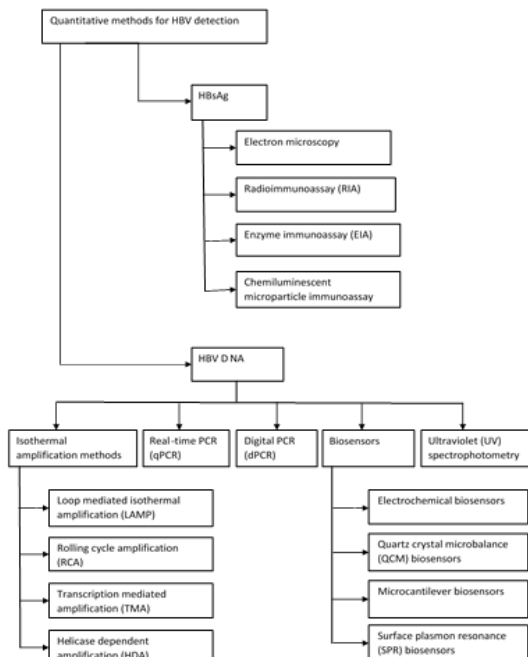


Figure 3 - Quantitative methods for HBV detection (Liu & Yao, 2015).

### Polymerase Chain Reaction based detection assays for HBV DNA

PCR-based techniques were first implemented by Mullis and Faloona (1987), to amplify nucleic acid sequences exponentially. They are important in HBV diagnosis. Covalently-closed circular DNA (cccDNA) found in infected liver cells, is the most precise marker to monitor disease progression, to assess anti-HBV therapy efficacy and for approximation of treatment end-points (Liang et al., 2016). As acquisition of cccDNA samples require invasive procedures (Li et al., 2017), HBV DNA are routinely quantitated for diagnostic purposes. Discovery of thermostable Taq polymerases and reverse transcriptase has resulted in modification and automation of the conventional PCR technology (Maasoumy et al., 2017).

Real-time Polymerase Chain Reaction (qPCR) Development of in-house real-time assays based on fluorescence emission allow qualitative and quantitative analysis of HBV simultaneously; with a higher sensitivity, reproducibility, broader linearity and minimal risk of carry-over contaminations (Sitnik et al., 2010). Zauli et al. (2016) conducted a novel in-house qPCR assay based on TaqMan-MGB system. Assessment of 49 samples revealed a 90% positive rate. The limit of detection (LOD) was  $1.9 \times 10^2$  IU/mL. It allowed determination of all HBV genotypes and enabled simultaneous quantification of both low and high viral loads in a large number of specimens. It detected a viral load ranging from  $5.1 \times 10^9$ - $5.1 \times 10^2$  IU/mL. The application of TaqMan minor groove-binding fluorescent probe technology has increased the quantification accuracy and facilitated detection of drug-resistant mutants. Such in-house methods were replaced by the development of commercial versions of COBAS® AmpliPrep/COBAS® TaqMan® (CAP/CTM) assays (Goedel et al., 2009). First generation CAP/CTM assay was limited to plasma specimen analysis; whereas the second generation assay allowed HBV DNA quantification in plasma as well as in serum with a 99% specificity and a LOD of 20 IU/mL. In CAP/CTM v2.0 assay, the use of capture and extender-probes at several loci along the length of the HBV genome allowed precise DNA quantification regardless of the genotype. Thus, it was used for large-scale HBV analysis on a routine basis (Chevaliez et al., 2010). Similarly, Pyne et al. (2012) concurrently related the analytical activity of fully-automated CAP/CTM v2.0 assay, manual CAP/CTM High Pure (HP) system and semi-automated Total Nucleic Acid Isolation (TNAI) assay using 208 clinical specimens. The findings suggested a

slightly higher sensitivity in CAP/CTM v2.0 assay when compared to HP and TNAI methods. The CAP/CTM, HP and TNAI assays achieved 100% sensitivity at a LOD of 10.2 IU/mL,  $\geq 10$  IU/mL and  $\geq 5$  IU/mL respectively. In cases where higher sample volumes were tested, both HP and TNAI assays presented substantial drawbacks in terms of training and operation. Such limitations were overcome in CAP/CTM assay by automating sample and master-mix preparation, thereby reducing hands-on time and enhancing throughput. According to a few studies, Abbott Real-time HBV assay has a slightly wider dynamic range than CAP/CTM v2.0. Therefore it is crucial for therapeutic-monitoring of HBV. Analysis of 220 HBV-infected serum samples showed a 98.2% sensitivity and a 100% specificity for Abbott real-time assay. An excellent reproducibility and correlation was observed between the two assays (Table 3) (Yeh et al., 2014; Caliendo et al., 2011).

Table 3 - Comparison of Abbott Real-time and CAP/CTM v2.0 assays (Yeh et al., 2014).

Parameter	Abbott Real-time assay	CAP/CTM v2.0 assay
Dynamic range	10 IU/mL – $1 \times 10^8$ IU/mL	20 IU/mL – $1.7 \times 10^8$ IU/mL
Intra-assay coefficient of variation (CV)	0.00% - 11.25%	1.22% - 8.22%

Table 4 - Comparison of different analytical parameters between LNA probe-based and TaqMan probe-based qPCR assays (Wang et al., 2012).

Probe used	Sensitivity (IU/mL)	Stability (Fluorescence decrease)	Optimal probe concentration	C <sub>t</sub> (threshold cycle) values of sample	Amplification efficiency
LNA	40	1%	75	26.56	95.9
TaqMan	80	36%	200	28.67	88.2

Table 5 - Comparison of the positive rates between LNA probe-based and TaqMan probe-based qPCR assays (Wang et al., 2012).

Assay	Number of samples evaluated	Number of HBV-positive samples	Positive rate
TaqMan probe-based real-time PCR	39	35	76.9%
LNA-probe based real-time PCR	39	39	100%

The assay developed by Wang et al. (2012) exploited locked nucleic acid (LNA) qPCR probes in HBV detection. It chemically modifies nucleotides, thereby increasing the stability and DNA affinity

of PCR. Relative to TaqMan probes used in CAP/CTM, LNA probes exhibited a higher sensitivity, wider linear detection range, improved amplification efficacy and a higher precision (Table 4). Both assays indicated a positive correlation in detecting HBV-positive DNA (Table 5). Moreover, the upgraded signal-to-noise ratio and the simple design of LNA assay permitted single-base mismatch identification in HBV DNA. The droplet-digital PCR (ddPCR) system employed by Mu et al. (2015) was capable of detecting trace amounts of target HBV DNA from blood and liver biopsies. The use of cccDNA-specific primers and water-in-emulsion droplets enabled ddPCR to achieve a LOD at single copy level and to measure exceptionally lower concentrations of DNA sequences. Also rare somatic mutations can be identified using ddPCR, with a greater reliability and precision than conventional qPCR-assays. Overcoming the limitation of low sensitivity, the conventional qPCR assays were replaced by ultrasensitive-qPCR. It is coupled with an ultrafiltration (UF)-based DNA preparation and extraction methodology to save time and curtail the high cost needed for sophisticated equipment. This ensures ultrafiltration of samples with low DNA content, leading to ultrasensitive detection of HBV DNA. Ability to handle both large and small batches of sample, lower sample/reagent consumption, lower running cost and shorter analysis time are the advantages of UF-qPCR over CAP/CTM-v2.0 assay (Table 6) (Wu et al., 2017).

Table 6 - Comparison of the characteristics of UF-qPCR assay and CAP-CTM V2.0 assay (Wu et al., 2017).

Characteristics	UF-qPCR	CAP-CTM V2.0
Serum volume input (μL)	250	500
DNA concentrate / total reaction volume (μL)	10 / 30	50 / 100
Dynamic range (IU/mL)	12 – 1.0 × 10 <sup>8</sup>	20 – 1.7 × 10 <sup>8</sup>
LOD	12	20
Additional equipment needed/ equipment cost (RMB, Yuan)	No / None	Yes / ~2 million
Running cost (RMB, Yuan)	50 - 60	300 - 350
Time-consuming	3 – 3.5 h (24 samples) Extraction: ~ 2 h Amplification: 1.5 h (40 cycles)	~5 h (24 – 72 samples a time) Extraction: ~2.5 h Amplification: 2.5 h (60 cycles)

According to Braun et al. (2017), the recently developed, fully-automated VERIS-HBV assay demonstrated an overall higher performance compared to other currently available qPCR assays discussed above.

Nested polymerase chain reaction (nPCR)

Nie et al. (2012) introduced a modification of conventional PCR by developing a type-specific nPCR assay; for HBV genotyping and subgenotyping. It assisted in investigating the HBV distribution in China (Table 7). The use of primers specific for each genotype and subgenotype contributed to the increased specificity. At a LOD ≥102.3 IU/mL, 100% of the samples were genotyped; thus contributing to improved sensitivity. However, the inability to detect low HBV viral loads (<10<sup>3</sup>) by this conventional assay, prevented detection of antiviral-resistant mutations.

Table 7 – Genotypes and subgenotypes detected using the type-specific nPCR assay (Nie et al., 2012).

Number of samples assessed	Detected Genotypes		Detected Subgenotypes	
	Genotype	Percentage	Subgenotype	Percentage
642	B	11.2%	B2	87.5%
	C	68.2%	C2	92.9%
	D	7.2%	Not detected	-
	Mixed genotypes	13.1%	-	-

As a solution, Wang et al. (2017) integrated a 2-stage nPCR with a high-throughput sequencing (HTS) assay for HBV whole-genome sequencing and for identification of mutations, virulence factors, drug-resistant genes and single-nucleotide variations. Use of 2 pairs of conserved, overlapping nested-primers enhanced the speed, sensitivity, specificity and accuracy of this approach. Compared to similar PCR techniques that use multiple primers (Chook et al., 2015), nPCR-HTS was convenient and cost-effective.

### Multiplex Polymerase Chain Reaction

To detect and genotype HBV genotypes A-F, a nested multiplex PCR assay was implemented by Naito et al. (2001). In this genotyping system, 55 HBV DNA-positive samples, which were acquired from entities of six different countries were used. The speciality in this particular assay is that in the second round of amplification, type-specific primers were exploited. On occasions where large scale epidemiological, pathological or transmission studies of HBV are conducted, this assay becomes crucial due to its rapidness and sensitivity in genotyping. Unlike the previously described method, where two different rounds of amplification were used, the assay developed by Kirschberg et al. (2004) uses a modified protocol which needs only a single round of amplification for the identification of genotypes A-F. The assay revealed a sensitivity which was within the range of 104 genome equivalents. When compared to the previous assay, total time and hands-on time required for this assay is relatively less, which is an improvement. However, both the assays failed to detect genotypes G and H, which were introduced recently. Similarly, Chen et al. (2007) implemented a multiplex PCR based assay which was also capable of specific differentiation of HBV genotypes A-F using a single round of amplification. However, this assay was superior to both the above described assays in the aspect that it could sub-genotype genotypes B and C using a subsequent PCR reaction. Of the 79 samples genotyped, 18 were infected with HBV genotype B, whereas 50 were infected with genotype C. Eleven were identified as mixed infections. Further analysis of the 68 samples infected with genotypes B and C using subgenotype-specific multiplex PCR revealed 18 samples infected with subgenotype B2, 2 samples infected with C1 and 48 samples infected with C2. This

assay is advantageous over the other two multiplex assays due to the comparatively higher sensitivity, simultaneous detection of four subgenotypes (B1, B2, C1, C2) and lower cost. Also it allows surveying of large-scale epidemiological areas and clinical examinations in areas where there is a high prevalence of the subgenotypes B1/B2 and C1/C2. Moreover, this assay permits detection of dual HBV infections with diverse genotypes.

### Isothermal based amplification strategies for detection of HBV

#### Loop-mediated isothermal amplification (LAMP)

Notomi et al. (2000) initially introduced the LAMP methodology. Later, Cai et al. (2008) implemented a real-time fluorescence-based LAMP (RtF-LAMP) technique. A sensitivity of 210 copies/mL was achieved by assessing 402 samples, of which 295 were positive. It replaced the conventional hybridized dye-based fluorescent emission method by adopting a 6-fold exponential amplification as well as a triple-amplification synchronization technique. Hence, its efficiency and sensitivity were relatively higher than that of qPCR assays. Cai et al. (2011) presented a genotype-specific LAMP assay to genotype and quantify HBV genotypes B and C synchronously. The use of single nucleotide polymorphisms as targets enabled differentiation of genotypes with a higher degree of specificity. Unlike qPCR-based genotyping, LAMP permitted genotype detection in a mixed infection, thereby indicating the relative proportions of the distinguished genotypes. Hence, this assay was successfully used in point-of-care treatment (POCT). Chang et al. (2015) proposed a LAMP-based lab-on-disk system which uses a low-cost, disposable microfluidic disk (Figure 4). Turbidity assessment allowed on-sight HBV detection on the disk. Automation of

steps reduced processing time, thereby making this method more advantageous than traditional LAMP assays

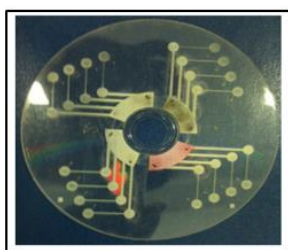


Figure 4 - The microfluidic disk used in the turbidity-based centrifugal microfluidics LAMP assay (Chang et al., 2015).

The turbidity-based detection posed limitations like irregular spatial distribution, non-homogenous particle sizes and reduced reactant amounts. These resulted in reduced sensitivity. Overcoming such challenges, an inexpensive surface-sensing method which uses a disposable surface plasmon resonant (SPR)-LAMP cartridge (Figure 5) was introduced. It provided a higher DNA yield with an enhanced sensitivity and a rapid detection rate (Chuang et al., 2012).

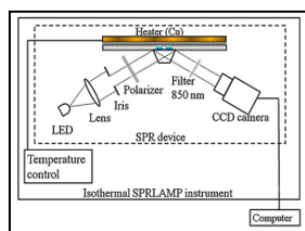


Figure 5 - The isothermal SPR-based LAMP system (Chuang et al., 2012).

Nyan et al. (2014) adopted a robust HBV-LAMP assay to detect all the important HBV genotypes in peripheral blood. By carrying out this assay, a 92% sensitivity (69 / 75 HBV- positive human plasma) and 100% specificity (107 / 107 healthy or HBV- negative human plasma) was achieved. Contrary to qPCR, the assay was executed on a non-complex digital heat-block in less than 60 minutes. Specialized equipment, expensive reagents or high technical expertise were not required. Use of multiple primers specific for the conserved regions of S-gene allowed detection of the genotypes without being subjected to cross-contaminations. Further, thermostable reaction buffer and Bst-DNA polymerase

allowed HBV DNA amplification even in heat-treated plasma samples in highly endemic populations, without compromising sensitivity. Nyan and Swinson (2015) has modified the previous assay, so that it can detect pathogens rapidly and simultaneously in a resource-limited setting. This multiplex fluorogenic-LAMP assay can detect 50 copies/reaction with a 97% sensitivity and 100% specificity. It is a one-step, single-tube procedure which uses a portable digital heating source to shorten the amplification time to 30-60 minutes. Similarly, it has used fluorogenic-oligonucleotides to supplant post-amplification addition of non-specific intercalating dyes and metal indicators. It can be used to investigate epidemiological trends of HBV and in point-of-care (POC) diagnostics. However, this assay has a relatively high cost.

### Rolling Circle Amplification (RCA)

Margeridon et al. (2008) applied RCA assay (Figure 6) to amplify the entire genome of HBV extracted from cccDNA and relaxed-circular DNA (RC-DNA). Its dependence specifically on a circular template avoided amplification of integrated HBV sequences. It was capable of amplifying very low viral loads present in patients with inactive or occult HBV infection. It can amplify viral loads ranging from 13 to 109 copies. In contrast to PCR, RCA produces a greater yield and product length, better fidelity and less amplification bias. Hence, this assay can be used in phenotype-testing of HBV-variants and for direct-probing of cccDNA pool. Martel et al. (2013) introduced an improved RCA technique, overcoming the limitations posed by the previous assay. Hence, the reduction of polymerase activity with time and mutant-induced activity impairment were resolved. This technique combined the principles of plus-strand RC-DNA ligation, RCA and genomic-PCR to



amplify full-length genomes extracted from RC-DNA; especially in low-titer sera. It was capable of amplifying HBV viral loads ranging from 10<sup>3</sup>-10<sup>8</sup> IU/mL. This method can be applied in genome cloning and in identification of HBV-specific genotypes and subgenotypes in sera and DNA extracts. Zhong et al. (2011) adopted a combined assay involving plasmid-safe ATP-dependent DNase digestion (PSAD), RCA and qPCR to quantitate cccDNA. Four RCA primer sets specific for multiple binding sites were used. In this way, it was able to achieve an increased LOD (raised by 2 logs), a greater sensitivity and specificity, a higher amplification efficacy and a wider dynamic range (Table 8) than conventional qPCR.

Table 8 - Positive rate, dynamic range and the detection limit of the combined assay of PSAD, RCA and qPCR (Zhong et al., 2011).

Number of liver biopsy samples analyzed	Number of HBV-cccDNA positive samples	Positive rate	Dynamic range	LOD
130	119	91.5%	10 <sup>2</sup> copies/mL - 10 <sup>10</sup> copies/mL	0.01 copies/cell

Similarly, Zhong et al. (2014) developed an assay combining in-situ PCR (IS-PCR) with RCA. Of the 26 samples tested, 73.07% were positive for HBV-cccDNA. This reflected an increment in LOD, despite the low copy number. Regardless of the lower sensitivity and specificity of IS-PCR, it enabled localization and distribution of HBV-cccDNA in hepatocytes, which cannot be achieved by performing RCA and qPCR alone. Also this combined assay allowed analysis of the link between the expression levels of cccDNA and pathological features of hepatocytes.

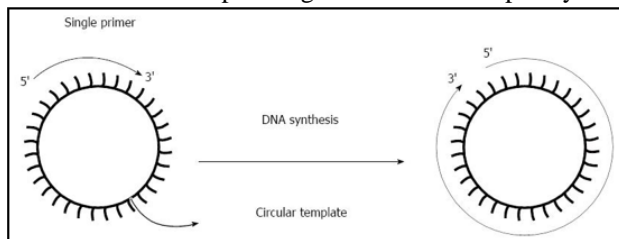


Figure 6 - Schematic diagram of the rolling circle amplification (Liu & Yao, 2015).

## Biosensors

As isothermal amplification techniques support many rapid detection setups under minimal thermal conditions, they can be conveniently integrated into biosensors (Yang et al. 2014). Zhang et al. (2010) devised a microfluidic device combining microbead-arrays and DNA probes labeled with quantum dots. It was used to identify HBV genotypes B and C with a sensitivity of 1x10<sup>3</sup> copies/mL (Figure 7). Its advantages include minimal consumption of reagents, reduced assay time and analogous detection.

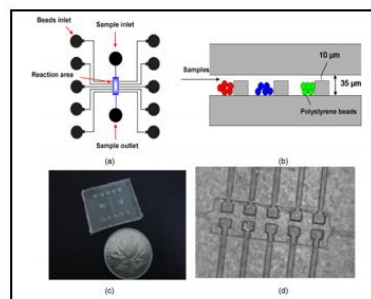


Figure 7 - Microfluidic chip incorporated with microbead array for analysis of HBV DNA. (a) A schematic diagram of the microfluidic design, (b) A cross-sectional view of the reaction area and detection area, (c) The microchip (d) The reaction area (Zhang et al., 2010).

Similarly, Zhi et al. (2014) incorporated LAMP and line probe assay (LiPA) on a microfluidic-biochip along with an independent giant magnetoresistive (GMR) sensor for HBV genotyping. According to results, genotypes B and C were identified. An LOD ranging from 10-10<sup>9</sup> copies/mL was accomplished within 1 hour. In contrast to the previous approach, this method demonstrated a higher sensitivity and broader detection range using only one set of LAMP primers. Also, it shortened reaction time and reduced both manufacturing cost and patients' test cost.

Yao et al. (2013) developed a RCA-based quartz crystal microbalance (QCM) biosensor to detect HBV genomic-DNA



directly. It has merged the powerful amplification capacity of RCA with the high sensitivity of QCM-biosensor. It can detect a viral load of 104 copies/mL. This method showed a relatively higher sensitivity when compared to the silica nanoparticle-enhanced microcantilever sensor employed by Cha et al. (2009), which could detect a viral load of 1,300,000 copies/mL at a DNA concentration of 2.3fM. In contrast, it showed a lesser sensitivity when compared to the LAMP assay proposed by Cai et al. (2011), which reached a detection limit of 210 copies/mL.

### **Future perspectives**

Integrating previously developed assays into novel platforms like biosensors is still an emerging concept in HBV diagnostics. Although chip-based isothermal systems are developed at present, acquiring a higher degree of portability in these systems with a proper quality control and reduced power consumption is challenging. Hence, implementation of portable, disposable, credit-card sized micro-systems incorporated with on-chip sample treatment modes is to be anticipated in the near future. Using the same concept, label-free home-care diagnostic devices will be developed (Yao et al., 2013). Also, ongoing research focuses on devising SPR-LAMP sensing cartridges for field-diagnosis of HBV in complex real-world samples; preferably in developing countries, with enhanced precision and reduced cost-per-test (Chuang et al., 2012). Moreover, the combined microfluidic-chip, LAMP and GMR-sensor system shows the potential to be utilized in multiple target molecule synchronous detection in the future (Zhi et al., 2014). These technological advancements aim on decentralizing the laboratory-based HBV testing system, so that POC and point-of-patient diagnostics

can be implemented in the future (Liu & Yao, 2015).

### **CONCLUSION**

Over the past decade, a dramatic advancement in molecular approaches has been observed in the field of HBV diagnosis. Of the many nucleic acid-detection assays available, conventional PCR is the most extensively adopted technique as its variants support automation and multiplexing. So far, real-time PCR has been the most commonly exploited PCR variant for HBV detection, due to its augmented specificity and sensitivity, broad detection capability and reproducibility. This is crucial in therapeutic decision-making, in monitoring response to anti-HBV drugs, in POCT and in assessing the risk factors that lead to fatality. However, the affordability of the assay is in question. According to the reviewed data, it is also evident that these real-time methods are confined to well-equipped, research laboratories.

Outperforming these drawbacks, isothermal amplification techniques showed the prospective to be used in HBV diagnosis. Despite the obvious advantage of increased sensitivity, literary research suggest that techniques like RCA are less useful in HBV diagnosis as it depend on circular-DNA templates for amplification. However, LAMP technique attested to be more preferable in this regard, as it can tolerate biological inhibitors. This high compatibility with clinical samples might become expedient in off-lab situations where clinical samples are more exposed to contaminants. Development of portable, miniaturized HBV diagnostic devices integrated with isothermal amplification methods are required in order to shift the laboratory-centered testing to confined family-units. In this way, the elderly and incapacitated

patients can self-monitor their disease condition.

The highest HBV transmission and mortality rate is reported in underdeveloped countries. Therefore, the most pragmatic advancement that can be recommended in the field of HBV diagnostics is the implementation of low-cost, sensitive and simple molecular assays in highly-endemic, resource-limited countries. In this way, early detection and hence, prompt management of the infection can be assured.

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