World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v20.i40.14615

World J Gastroenterol 2014 October 28; 20(40): 14615-14625
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (9): Hepatitis B virus

Recent advances in molecular diagnostics of hepatitis B virus

Sibnarayan Datta, Soumya Chatterjee, Vijay Veer

Sibnarayan Datta, Vijay Veer, Molecular Virology Laboratory, Defence Research Laboratory, Assam, PIN 784001, India
Soumya Chatterjee, Vijay Veer, Biodegradation Technology Laboratory, Defence Research Laboratory, Assam, PIN 784001, India
Author contributions: Datta S and Chatterjee S reviewed the available literature and wrote the manuscript; Veer V critically revised the draft with significant intellectual inputs.
Supported by Defence Research and Development Organization, Ministry of Defence, Government of India
Correspondence to: Dr. Sibnarayan Datta, Scientist, Molecular Virology Laboratory, Defence Research Laboratory, Post bag No. 2, Tezpur, Assam, PIN-784001, India. sndatta1978@gmail.com
Telephone: +91-3712-258508 Fax: +91-3712-258534
Received: November 27, 2013 Revised: January 26, 2014
Accepted: June 2, 2014
Published online: October 28, 2014

Abstract

Hepatitis B virus (HBV) is one of the important global health problems today. Infection with HBV can lead to a variety of clinical manifestations including severe hepatic complications like liver cirrhosis and hepatocellular carcinoma. Presently, routine HBV screening and diagnosis is primarily based on the immuno-detection of HBV surface antigen (HBsAg). However, identification of HBV DNA positive cases, who do not have detectable HBsAg has greatly encouraged the use of nucleic acid amplification based assays, that are highly sensitive, specific and are to some extent tolerant to sequence variation. In the last few years, the field of HBV molecular diagnostics has evolved rapidly with advancements in the molecular biology tools, such as polymerase chain reaction (PCR) and real-time PCR. Recently, apart of PCR based amplification methods, a number of isothermal amplification assays, such as loop mediated isothermal amplification, transcription mediated amplification, ligase chain reaction, and rolling circle amplification have been utilized for HBV diagnosis. These assays also offer options for real time detection and integration into biosensing devices. In this manuscript, we review the molecular technologies that are presently available for HBV diagnostics, with special emphasis on isothermal amplification based technologies. We have also included the recent trends in the development of biosensors and use of next generation sequencing technologies for HBV.

Key words: Hepatitis B virus; Polymerase chain reaction; Isothermal amplification; Real time polymerase chain reaction; Biosensors; Next generation sequencing

Core tip: In the past decades, introduction of molecular biology tools such as polymerase chain reaction (PCR) has entirely revolutionized the field of hepatitis B virus (HBV) diagnosis by allowing sensitive and specific detection of the virus in body fluids. In the recent years a number of isothermal nucleic acid amplification have also evolved, that provide certain advantages over PCR. These isothermal amplification techniques are comparatively sensitive, specific, require less instrumentation and automation. This manuscript reviews the advantages/disadvantages of prevailing isothermal technologies to serve as an assay selection guide for the readers, working in the field of HBV molecular diagnostics.

Datta S, Chatterjee S, Veer V. Recent advances in molecular diagnostics of hepatitis B virus. *World J Gastroenterol* 2014; 20(40): 14615-14625 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i40/14615.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i40.14615

INTRODUCTION

Hepatitis B virus (HBV) infection is amongst the most

important global health problems today. According to estimates, approximately 2 billion people worldwide have serologic evidence of past or present HBV infection, of which 350-400 million people are chronic carriers of HBV[1,2]. Although a highly immunogenic and effective vaccine against HBV is available, but its cost has precluded its use for mass vaccination in less developed nations where HBV is highly endemic. As a result, approximately, 10-30 million people get infected with HBV each year, and estimated 1 million people die annually due to HBV related complications[3]. Worldwide, chronic HBV infection is responsible for 80% of all primary hepatocellular carcinoma (HCC), which positions HBV infection as the 10th leading cause of death and HCC as the 5th most frequent cancer[1]. HBV is primarily transmitted through infective blood and other body fluids, and is almost 100 times more infective than the human immunodeficiency virus (HIV)[1].

DIAGNOSIS OF HBV

Specific diagnostic methods are required to discriminate HBV infection from other hepatitis causing pathogens; hence, clinical tests are essential. Diagnosis of HBV is primarily based on detection of HBV antigens [*e.g.*, HBV surface antigen (HBsAg), hepatitis B e antigen (HBeAg)], human antibodies against these antigens (anti-hepatitis B surface antigen, hepatitis B core antibody *etc.*) and also the presence of viral nucleic acids (HBV DNA), primarily in blood, followed by liver and in other extra hepatic sites, in certain circumstances. Based on the presence or absence of a combination of antigens/antibodies, acute/chronic, ongoing/past infections with HBV could be distinguished[4]. Among the different markers, laboratory diagnosis of HBV infection is, to a great extent, based on the immune-detection of the HBsAg, ever since the discovery of the virus[3]. However the issue of HBsAg non detection due to diagnostic-escape mutations in the epitopes or due to low antigen levels has greatly emphasized the importance of using robust molecular biology tools for efficient detection of HBV. Nevertheless, the advancement of molecular based assays has also greatly augmented the field of molecular diagnostics of HBV[5,6]. Molecular methods include thermal cycling based techniques for amplification of HBV DNA, such as polymerase chain reaction (PCR), qPCR or isothermal amplification based methods such as nucleic acid sequence based amplification (NASBA), transcription mediated amplification (TMA), loop mediated isothermal amplification (LAMP), rolling circle amplification (RCA) *etc.*

Every detection method has its own advantages and disadvantages, which promote or restrict their use in clinical settings. The main purpose of this review is to discuss the contemporary molecular methods or those having significant future potential in molecular diagnostics of HBV. Since a number of reviews have been published on sero-diagnostics based approaches, we have kept this review focused on nucleic acid detection based techniques, with emphasis on isothermal nucleic acid amplification based methods.

NUCLEIC ACID BASED DETECTION ASSAYS FOR HBV DNA

Since the last decade, with the increased findings of groups of serologically negative HBV infections [HBeAg negative chronic hepatitis B (CHB), occult HBV Infection *etc.*] and rapid emergence of diagnostic escape mutants, nucleic acid based HBV DNA assays have gained importance in clinical settings. With the technological advancement and reduction in cost, nucleic acid based methods have become supplementary or alternative to the sero-diagnostic assays. Moreover, nucleic acid based methods have allowed more accurate quantification of HBV DNA levels in the patients, crucial for assessment of replicative stage and efficacy of antiviral treatment[4].

Initially there was a dilemma regarding the levels of serum HBV DNA for recommending patients for antiviral therapy or to assess the efficacy of therapeutic response. To address this issue, an arbitrary value of 20000 IU/mL was recommended as a cut-off at the 2000 NIH conference as a diagnostic criterion for CHB[7]. However, later it was found that serum HBV DNA levels may fluctuate widely among the CHB patients and low HBV DNA is also a predictive factor of severe liver diseases such as LC and HCC[4,8]. It is now recognized that lower HBV DNA levels (approximately 3-5 $\log_{10}$ IU/mL) may also be associated with liver disease progression and necessitate treatment[4]. Hence, highly sensitive HBV DNA detection methods are essential and serial monitoring of HBV DNA levels is important than a single point arbitrary cut-off value for prognostic and diagnostic purposes[9]. To make HBV DNA assays highly sensitive, a large number of techniques have been developed using different molecular biology tools. Broadly, assays for detection of HBV nucleic acids can be divided into two categories: (1) Direct detection assays, utilizing probes to hybridize directly to the HBV DNA. These assays are relatively simple, requirement of sophisticated instruments is less but they lack sensitivity. The detection limit of direct assays is typically 10^3-10^5 templates. Sensitivity may be increased by adopting different methods of signal amplification; and (2) Indirect detection involves an *in vitro* amplification step to increase the amount of the target sequence, followed by detection of the amplified target. This format of detection is highly sensitive and can even detect as low as 1-10 templates in a reaction. However, amplification based assays need technical expertise and sophisticated instrumentation. A number of *in vitro* target nucleic acid amplification methods have evolved in the last three decades. Although, PCR based detection assays are the most widely practiced procedure, other techniques such as LAMP, NASBA, TMA, RCA *etc.* have also been employed for HBV DNA detection and quantification. A common problem with the amplification based techniques is the issue of contamination or false positive re-

sults. However, this may be avoided by adopting specific precautions and use of proper controls in the assays[10,11].

POLYMERASE CHAIN REACTION BASED STRATEGIES

Polymerase chain reaction (PCR), developed by Mullis *et al*[12] is the amplification technique that has entirely transformed the fields of biological research as well as clinical diagnostics[13]. Subsequent to the discovery of PCR, identification and availability of thermostable Taq polymerases and reverse transcriptase enzymes have enabled scientists and researchers to adapt and modify the basic PCR technology for automated amplification and detection of DNA and RNA. Furthermore, with the development of fluorescence based real-time amplification detection methods, simultaneous detection and quantification have become possible in clinical environments[14]. Theoretically, PCR reactions are capable of exponentially amplifying single copy target DNA, and are thus highly sensitive. A number of modifications have been done on the basic PCR to detect several nucleic acid sequences simultaneously in a multiplex PCR or by making the technique more sensitive and specific by second round of amplification with "nested" primers, or the amplification of divergent but related sequences using degenerate primers.

For HBV diagnosis through PCR, covalently closed circular DNA of HBV (cccDNA) level monitoring in the hepatocytes is the most precise way of assessing the number of infected hepatocytes, but it requires invasive procedures and thus are not part of routine diagnostics. However, quantitation of HBV DNA in the serum provides an alternative to cccDNA detection, through less invasive method. According to the recommendations of the Taormina Group[15], detection of very low levels of HBV DNA should be done with highly sensitive PCR using primers specific for highly conserved sequences (genotype independent) of different HBV genomic regions. It has been observed that sensitivity of the HBV DNA detection by PCR may vary across different genetic regions of the HBV genome[16,17]. Interestingly, it has been reported that the S gene PCR is most sensitive for HBV DNA detection in serum, while X gene PCR is more sensitive for HBV DNA in liver tissues[18].

Most PCR based methods of HBV DNA detection for clinical purposes have a sensitivity of 50-200 IU/mL with dynamic range of 4-5 $\log_{10}$ IU/mL[19]. In comparison, RTD PCR based assays have higher sensitivity (5-10 IU/mL) with a wider dynamic range 8-9 $\log_{10}$ IU/mL[20]. A large number of laboratories have developed highly sensitive "in-house" PCR and RTD PCR based methods, which exhibit remarkable sensitivity and reproducibility as well[21]. Previously there was a problem regarding the use of units for expression of HBV DNA levels (copies/genome equivalent/IU per mL) and standardization of quantitative assays. This issue has also been addressed by an international effort to establish a WHO international standard for HBV DNA (NAT) assays[22]. The standard comprises a dilution of the Eurohep reference *R1* (genotype A2, subgenotype Ae/A2, HBsAg subtype *adw2*) in anti-HIV-1; anti-HCV; HCV RNA, syphilis and HBV-negative pooled human plasma and has been calibrated in IU[23,24].

Apart from standard PCR and RTD PCR, a number of modified PCR methods have been widely employed in HBV diagnostics[25]. PCR-restriction fragment length polymorphism (RFLP) methods have been developed for HBV genotyping and mutation detection[26,27]. These PCR-RFLP techniques are simple, rapid and are suitable for large number of samples. However, due to single round PCR, these assays lack sensitivity required for typing of samples with low HBV DNA levels. To overcome this problem, nested PCR based genotyping methods were developed. Naito *et al*[28] developed a nested multiplex PCR method for the detection and genotyping of HBV genotypes A-F, using type specific primers in the second round of amplification. This method was further modified by Kirschberg *et al*[29], where genotypes A-F could be detected in a single PCR. Another multiplex PCR based assay was developed for HBV genotyping by a single PCR, that also allowed subgeno typing of genotypes B and C in an additional PCR reaction[30]. As compared to the RFLP methods, multiplex PCR is more sensitive, accurate and also capable of detecting minor population of mix genotypes[25]. However, developing an efficient multiplex PCR requires complex modeling and laborious standardization with respect to primer design, primer-template ratios, fragment lengths, PCR buffer composition and thermal cycling conditions[31].

In addition to increase in sensitivity, development of RTD PCR techniques have also solved the problem of contamination to large extent, by obviating the requirement for post-PCR manipulations, and also permitted very high throughput capabilities. The advancement in optical instrumentation and use of different fluorophore chemistries has further allowed multiplexing for detection of multiple targets simultaneously. RTD PCR is a kinetics-based quantitative PCR technique, where the quantity of synthesized DNA is calculated at each cycle throughout the PCR process[32]. RTD PCR has been extensively used in rcHBV DNA detection, cccDNA detection, quantitation, HBV genotyping as well as mutation screening[33-36]. The main disadvantage of RTD PCR genotyping method is that methods involving multiplex RTD PCR require extensive standardization, while methods involving non-specific DNA binding dyes (such as SYBR green) are not specific enough for exact distinction of the genotypes, based on melting temperatures of the amplicons.

LIGASE CHAIN REACTION

Ligase chain reaction (LCR) was originally developed by the Abbott Laboratories to amplify shorter DNA targets and is also known as ligase-mediated amplification

or oligonucleotide ligation assay[37,38]. LCR uses a DNA polymerase enzyme and a DNA ligase enzyme to drive the reaction. LCR uses the ligase to join two same-strand targeting oligonucleotides, which are designed to hybridize at adjacent positions of the template nucleic acid. When the target sequence is present in the reaction mix, oligonucleotides correctly hybridize to adjacent sequences of the target sequence and the gap between the oligonucleotides is ligated by a DNA ligase and a continuous fragment is generated. This ligation product then serves as a template for a PCR like reaction resulting in amplification of the target nucleic acid molecule[39]. A modified reaction, known as the gap-LCR was developed later, that allowed amplification of longer DNA stretches by inclusion of a polymerase extension[40]. Like other target amplification techniques, LCR can also be integrated into real time detection or can be used for multiplexing.

LCR has been utilized for detection of HBV S gene and PreC mutations[41-43]. Although the limit of detection for LCR alone was found to be very high for detection or precore mutations[43], LCR has been reported to be useful for semi-quantitative detection of HBV[44]. Like TMA, LCR has also been less utilized in the field of HBV diagnostics.

ISOTHERMAL AMPLIFICATION STRATEGIES

As opposed to the requirement of thermal cycling of different temperatures, isothermal amplification based methods are designed to take place at a uniform temperature. In these amplification strategies, strand displacing polymerases/specially designed primers/chemicals are used, that displace two strands of the dsDNA during primer extension. Till date a number of isothermal amplification strategies have been described in the literature, but these technologies have started gaining importance only recently due to their potential for application in field conditions, at point of care (POC) or in biosensing devices. Detailed mechanism of these techniques have already been reviewed elsewhere[45,46]. Here we will discuss only those isothermal amplification techniques that have been used for HBV DNA detection or have significant potential for application in this field.

LAMP

LAMP (developed at Eiken Chemical Co. Ltd, Japan) is a fast amplification method that amplifies DNA with high specificity, efficiency using a strand displacing Bst DNA polymerase and a set of four to six specially designed primers, two of which are designed to form loop for self priming[47,48]. This is a one-step amplification reaction, which takes place at 58-65 ℃ for 30-60 min and results in synthesis of a large amount of DNA (more than 500 μg/mL). This technique also allows simple detection either by agarose gel electrophoresis, visual inspection of turbidity or by visualizing fluorescence under ultraviolet light. Moreover, LAMP may also be used for RNA templates as well as it may also be integrated with real time turbidity detection for quantification[49]. In comparison to PCR, LAMP is reported to be a robust technique, being less vulnerable to inhibitors, generally present in clinical samples. Thus very less sample preparation is required for LAMP. Successful LAMP requires perfectly designed primers, which have been referred to as a reason for its less application in practice[45]. Nevertheless, a web-based software is available for designing LAMP primers (http://primerexplorer.jp/) and the method is now being increasingly adapted for detection of different pathogens.

Incidentally, the original paper on LAMP used HBV DNA extracted from HBV-positive serum, as template[47]. It was demonstrated that using cloned HBV DNA as a template, 600 and 6000 copies of DNA were detected at 13 and 11 min, respectively, reflecting a very high efficiency of the LAMP method. However, since then, only a few papers reported using this technique for HBV detection. Recently Cai *et al*[50] developed and evaluated a highly sensitive real-time fluorogenic (RtF-LAMP) protocol to quantitate HBV DNA. They documented a lower detection limit of 48 IU/mL, dynamic range of 8 orders of magnitude, low intra-assay and inter-assay variability (4.24%-12.11%) and excellent correlation between RtF-LAMP and real-time PCR ($r^2 = 0.96$). Similar highly sensitive LAMP assays were reported by other research groups also[51,52]. More recently LAMP has been employed in developing biosensors for on-site detection of the HBV[53], showing the immense potential of this technique for future diagnostic devices.

NASBA

NASBA was originally developed by Organon Teknika. Similar to LAMP, it is also an isothermal process and is particularly suited to the detection of RNA viruses. However this can be used for amplification of DNA templates with certain modifications in the basic method. The amplification process utilizes a modified primer including the T7 promoter sequence, which forms a modified cDNA after hybridizing with the template. This is subsequently amplified into ssRNA amplicons in a process catalyzed by *T7 RNA* polymerase (T7 RNA Pol)[45,54]. Since its development, NASBA has been broadly used in the detection of a variety of targets through quantitative real-time assays[45].

In the field of HBV diagnostics, NASBA has been used since 2001 by Yates *et al*[55]. They reported a wide detection range of 10^3 to 10^9 copies/mL of HBV DNA, with good reproducibility and precision when NASBA was used with real-time detection with molecular beacon technology. Recently, Deiman *et al*[56] reported the amplification of HBV DNA by NASBA and found it to be capable of detecting even 10 IU/mL with a dynamic detection range of 10^2 to 10^9 IU/mL. Like LAMP, incorporation of NASBA with molecular beacon detection onto lab-on-a-chip systems, pathogen capture devices and

microfluidic devices have been attempted, that show high sensitivity even in microliter and nanoliter reaction volumes[57,58]. This robust technology also has great potential for application in future detection devices.

ROLLING CIRCLE AMPLIFICATION

The rolling circle amplification (RCA) model of isothermal amplification (developed by Molecular Staging Inc.) imitates natural replication strategy of circular DNA molecules[59,60]. This powerful technique utilizes the strand displacement activity of the highly processive Phi29 bacteriophage DNA polymerase (ϕ29 DNA polymerase), acting on circular DNA molecules at low temperature (30-60 ℃). RCA reaction is initiated by annealing of primers to the circular ssDNA, followed by elongation of the new strand upto the point of initiation, displacing the strand and continuing again and again. This repeated elongation due to strand displacement, generates a continuous catenated ssDNA even upto 10^9 folds[46]. RCA has been integrated with various detection strategies and employed for pathogen detection[45]. Apart from the original RCA, a number of variants of RCA amplification have been developed that can amplify as low as 10 copies to a detectable amount within 30-90 min. A major advantage of RCA is that it is resistant to inhibitors present in clinical samples and requires little or no assay optimization. Moreover, RCA can amplify targets in solution or on solid support, offering opportunity for biosensor and microarray applications[61].

The requirement for a circular template for RCA makes it ideal for detection of HBV DNA especially the cccDNA of HBV in the hepatocytes. RCA has been used for amplification of rcDNA (with some enzymatic modification) as well as for direct amplification of cccDNA. Margeridon *et al*[62] used RCA for amplification of full genome of HBV DNA with low viral loads, from sera as well as from liver. They could amplify as low as 13 copies of cccDNA from liver biopsy samples. Martel *et al*[63] developed a RCA based method for complete genome amplification of HBV rcDNA from sera, with viral loads ranging from 10^3 IU/mL to 10^8 IU/mL. Recently, RCA has been used in combination with quartz crystal microbalance biosensor for direct detection of HBV DNA in clinical samples, which could detect as low as 10^4 copies/mL of HBV DNA[64].

TRANSCRIPTION MEDIATED AMPLIFICATION

Transcription mediated amplification (TMA reaction) (developed by GenProbe Inc.) is an isothermal amplification method that produces single-stranded RNA amplicons from RNA target molecules. TMA uses a reverse transcriptase that converts the template RNA into a cDNA, which in turn is converted by an RNA polymerase into numerous copies of RNA that are detected with fluorescent or chemiluminescent probes. TMA produces amplicon RNA exponentially since the newly produced RNAs repeatedly undergo TMA in the subsequent stage of amplification. The amplicons generated in this process may also be detected in real time, using fluorescent tagged probes. TMA produces 100-1000 copies per cycle (RNA) rather than the two copies per cycle produced by PCR (DNA)[65].

Although TMA has been used by different laboratories for sensitive detection and quantification of different pathogens, it has been used much less for HBV. Kamisango *et al*[66] developed a transcription-mediated amplification and hybridization protection assay, which could detect 5×10^3 to 5×10^8 genome equivalents (GE)/mL of HBV. Ide *et al*[67] used TMA for monitoring changes in the HBV DNA level during the treatment of CHB patients with lamivudine. Kubo *et al*[68] utilized this technique to measure HBV DNA among patients who underwent HCC resection. Overall TMA is a highly sensitive technology, with multiplexing possibilities and being isothermal reaction, it is well suited for routine clinical applications in laboratories with basic molecular biology facilities.

STRAND DISPLACEMENT AMPLIFICATION

Strand displacement amplification (SDA) (developed by Becton Dickinson and Company) is a form of isothermal nucleic acid amplification technique which was first described by Walker *et al*[69,70]. It utilizes restriction enzyme cleavage of the DNA template for strand displacement. SDA use two pairs of specially designed primers for template recognition. The inner pair is also designed to contain a restriction enzyme motif. Initially, template DNA is denatured by heating at 95 ℃, to which the primer pairs anneal and extend with the help of *Escherichia coli* DNA polymerase 1 (exo-Klenow), incorporating the restriction target into the new amplicons. The outer primers in turn displace the strands synthesized by the inner primers. Similarly, consecutive rounds of primer binding and extension generates DNA duplex incorporating restriction sites, upon which restriction enzyme acts and nicks one of the newly formed strands. This nick allows the DNA polymerase to displace the existing strand and extension of the nick to add in a new amplicon. This repetition of nick and run scheme results in exponential amplification.

The main advantage of SDA is its simplicity and requirement of user intervention only for initial heat denaturation and addition of enzymes (DNA polymerase and restriction enzyme). In a single reaction, SDA can produce 10^9 copies of target DNA in less than an hour. These features make SDA suitable for point-of care applications. However, since SDA is performed at low non-stringent temperatures, generation of high background signals with clinical samples (due to the abundance of human genetic material) restrict the use of SDA in diagnostic applications. Although, SDA has been used in a commercially available tuberculosis diagnostic assay[71], its

use in commercial or lab-brew HBV diagnostics is almost negligible.

THERMOPHILIC HELICASE-DEPENDENT ISOTHERMAL AMPLIFICATION

Thermophilic helicase dependent amplification (tHDA) (developed by Biohelix Corporation) is an isothermal nucleic acid amplification method that imitates the cellular replication fork mechanism. tHDA utilizes the DNA strand unwinding potential of thermophilic helicase in the presence of adenosine tri phosphate (ATP) to substitute the thermal denaturing[72]. In this reaction, helicase unwinds DNA strands and keeps them denatured with the help of single-strand binding proteins and accessory proteins. Subsequently, two sequence specific primers anneal to each of the single stranded DNA templates and are extended by the DNA polymerases (exonuclease deficient) to produce a double stranded DNA. The process is repeated at a single temperature to produce amplicons in exponential manner.

As compared to other isothermal amplification techniques, HDA has certain advantages. tHDA has enormous potential in diagnostics due to its extremely simple format, does not require initial high temperature for denaturing DNA strands, has high speed (approximately 100 bp/s) and high processivity (approximately 10 kb/binding)[73]. Although, these properties of tHDA offer prospective for the development of simple DNA diagnostic assays, but like SDA, this technology has been very less used in HBV diagnostics.

NEXT GENERATION SEQUENCING

Gene sequencing has always been a gold standard, especially in viral diagnostic setting. Sequencing and computational tools have allowed identification and confirmation of HBV mutants/variants, genotypes, subgenotypes etc, which has helped enormously in patient management, in drug resistance testing, and for the epidemiological analysis of disease outbreaks[33]. Initially, two DNA sequencing technologies were available (1) Sanger sequencing (DNA sequencing with chain terminating inhibitors); and (2) Maxam-Gilbert sequencing (chemical cleavage technique). Of them, the Sanger method for its simplicity became popular and remained a standard for more than three decades.

Nevertheless, in the past decade, the field of sequencing technology has been entirely revolutionized by the introduction and development of advanced chemistries for nucleotide sequencing, such as sequencing-by-synthesis chemistry coupled with enzymatic luminometric inorganic pyrophosphate detection assay[74,75] which ultimately led to the evolution of the pyrosequencing technology. Pyrosequencing was further developed into an array-based massively parallel microfluidic-sequencing platform for extremely high throughput sequencing[76]. Alongside the pyrosequencing technology, alternate technologies have also evolved, such as clonal bridge amplification and sequencing-by-ligation technologies, which have allowed simultaneous sequencing of millions of templates. These technologies are known as Next Generation sequencing technologies and the present parallel sequencing platforms can produce several Giga bases (Gb) of high quality sequence data in a single run. However, handling and analysis of such huge sequence data require very high level computational instrumentation and complex pipeline of softwares. These technologies have permitted whole genome sequencing, ultra-deep sequencing, amplification and identification of previously unknown microbes (no prior sequence information is required for sequencing of unknown genomes) and also the analyses of viral quasispecies populations within very short time.

Although these next generation sequencing technologies are still expensive to be used for clinical virology diagnostics, but they have already proven their power and significance in clinical research[77]. These are gradually replacing Sanger sequencing, by allowing direct sequencing of the mixed virus population and even quantifying the relative abundance of certain mutations with extremely high coverage within a short time frame. In a recent study, Margeridon-Thermet *et al*[78] performed ultra-deep pyrosequencing of hepatitis B virus quasispecies from nucleoside and nucleotide reverse-transcriptase inhibitor (NRTI)-treated patients and NRTI-naive patients and detected coinfection and recombination among two different HBV genotypes in some of the patients. In another recent study, ultradeep sequencing was used to examine the diversity between intrahepatic HBV strains and those circulating in the serum[79]. Interestingly drug-resistant HBV variants were identified in treatment naive patients, suggestive of natural existence of HBV variants in patients, a finding that clearly supports Darwinian theory of evolution in emergence of HBV drug resistance variants and has significant implications HBV management. Very recently, this powerful technique has been used for analysis of HBV reverse transcriptase quasispecies heterogeneity, to identify host genes that are frequent sites of HBV integration and to study the effects of HBV integration into the genomes of HCC patients[80-82]. Increasing number of studies, based on these technologies is being published rapidly; those are uncovering or may in future reveal more interesting facts about HBV.

BIOSENSORS, MICROARRAY AND MICROFLUIDIC DEVICES

In the recent years, development of biosensors has gained vast momentum due to their wide application in the fields of clinical diagnostics, public health and even for military purposes. Newman *et al*[83] defined biosensor as: "a compact analytical device incorporating a biological or biologically-derived sensing element either integrated within or intimately associated with a physicochemical transducer". Earlier biosensors especially integrated enzymes with transducers that convert the biological

Table 1 Comparison between different nucleic acid amplification technologies

Method	Template requirement	Sensitivity/ specificity	Enzyme(s) required	Temperature requirement	Primers/ primer design	Multiplex possibility	Rapid detection possibility	Time to detect in (min)	Sensitive to biological inhibitors
PCR	dsDNA[1], RNA	About 1-10 copies/ Very high	DNA polymerase	95 ℃ →55-60 ℃ → 68-72 ℃ (Cyclic)	≥ 2/ simple	Yes	Yes[2]	About 40-120	Yes
LCR	DNA[1]	About 1-10 copies/ Very high	DNA ligase and DNA polymerase	94 ℃ →65 ℃ (Cyclic)	4/ simple	Yes	Yes[2]	About 100-180	Yes
LAMP	ssDNA[1], RNA	About 5 copies/ Very high	Bst DNA polymerase	60-65 ℃ (Isothermal)	4-6/ complex	N/A	Yes[2,3,4]	About 60-90	Less
NASBA	RNA[1], DNA	About 1 copy/ Very high	Reverse transcriptase, T7 RNA polymerase, RNAse H	37-41 ℃ (Isothermal)	2/ simple	Yes	Yes[2,4]	About 60-120	Yes
RCA	Circular ssDNA[1]	About 10 copies/ high	ϕ29 DNA polymerase	30-65 ℃ (Isothermal)	1/ simple	N/A	Yes[2]	About 60-90	Less
TMA	RNA[1], DNA	About 1-10 copies/ high	Reverse transcriptase, RNA polymerase	50-60 ℃ (Isothermal)	2/ simple	Yes	Yes[2]	About 120-140	Yes
SDA	ssDNA[1], RNA,	About 10 copies/ low	Bst DNA polymerase or exo$^-$ Klenow Fragment	95 ℃ →37 ℃ (Isothermal)	4/ complex	Yes	Yes[2,4]	About 90-120	Yes
tHDA	dsDNA[1]	About 1-10 copies/ Very high	Helicase, DNA polymerase	60-65 ℃ (Isothermal)	2/ simple	Yes	Yes[2,4]	About 75-90	Less

[1]Preferred template; [2]Real time detection of fluorescence; [3]Real time detection of turbidity; [4]Nucleic acid lateral flow. PCR: Polymerase chain reaction; LCR: Ligase chain reaction; LAMP: Loop mediated isothermal amplification; NASBA: Nucleic acid sequence based amplification; RCA: Rolling circle amplification; TMA: Transcription mediated amplification; SDA: Strand displacement amplification; tHDA: Thermophilic helicase dependent amplification.

reaction into a measurable electrical or electronic signal. Later, biosensors utilized other biological materials, such as antibodies, receptors and more recently nucleic acids. Presently, most of the biosensors being developed utilize affinity interaction (antigen-antibody or receptor-ligand interaction) or nucleic acid hybridization based interaction for generating biological responses. Although antigen-antibody or receptor-ligand based biosensors are faster, biosensors that utilize nucleic acid or signal amplification based technologies are more specific and sensitive[84,85]. Currently available biosensors technologies and applications have been reviewed in details elsewhere[45,86]. As with other viruses, a number of biosensors based on different principles have been developed recently for the detection of HBV DNA, or its antigens or anti-HBV human antibodies[87-89]. Assembly of numerous (up to a few thousand) DNA biosensors onto the same detection platform results in DNA microarrays (or DNA chips), devices that are increasingly used for large-scale transcriptional profiling and single-nucleotide polymorphism discovery. Microarray based diagnostic systems have been developed for HBV detection, genotyping and detection of mutants[90,91]. However such systems are time consuming and require sensitive instrumentation for detection, limiting their application for clinical diagnostics.

Apart from biosensors and microarrays, microfluidic devices are being developed since 1990, which are also known as "lab-on-chip" capable of sample and reagent processing as rapid micro total analysis system[92]. Revolutionary advancement of micro-electro-mechanical systems has made fabrication of microchambers/microchannels possible, leading to miniaturization of reaction chambers, significantly decreasing assay time, reagent volume and sample use[93,94]. High-speed PCR performed on microfluidic devices have been shown to be extremely rapid, capable of amplifying upto 500 bp fragments in just 1.7 min and 997 bp in only 3.2 min[95]. NASBA based pathogen detection microfluidic devices have also been developed[96]. Recently, microfluidic devices have been used for study of HBV detection, replication and genotyping[97-99].

CONCLUSION

Molecular diagnostic methods have evolved dramatically over the past decades. Among these, nucleic acid based methods have better sensitivity, and specificity; however, these are comparatively expensive and are mostly confined to research laboratories. Nevertheless, there is a huge potential for their application in clinical diagnostics, military and other settings where faster and accurate detection is required at the POC. Though many sensitive nucleic acid amplification based technologies have been developed in the last few decades, but none of them have become so popular, as PCR. Despite being highly sensitive, isothermal amplification techniques have remained less-utilized for developing detection assays, especially HBV. Although some kits based on isothermal amplification methods (*e.g.*, TMA, LAMP, NASBA, SDA *etc.*) are available for other viruses (HSV, HPV, HIV *etc.*) but most of the commercially available kits for HBV detection and quantitation are primarily based on PCR.

As evident from the ongoing discussion, a number of isothermal amplification methods have prospective to be used for HBV diagnostics. A comparison of these important techniques has been put into Table 1, for ready ref-

erence. Among these techniques, LAMP, NASBA, RCA and tHDA are good candidates for the purpose, although with certain advantages and disadvantages. NASBA is a very sensitive technique, but the use of three different enzymes may be economically prohibitive for certain laboratories. Similarly, the requirement of circular DNA as a template restricts the use of RCA mostly for amplification of cccDNA. LAMP and tHDA are reported to be tolerant to biological inhibitors, which are most often present in clinical preparation. This compatibility with clinical samples obviates the need of highly pure template preparations for these methods and is definitely an advantage for off-lab situations. Earlier primers designing for LAMP were complex, but at present softwares are available, that can easily work out primers. In comparison, among the isothermal amplification techniques, tHDA is a fast and simple reaction, require simple primer sets, highly sensitive assay that also supports multiplex and real time detection formats. In addition, most of the isothermal amplification techniques require simple thermal conditions for amplification, support different rapid detection formats and thus could be easily integrated for biosensor applications. Taken together, there is a range of amplification techniques available, which could be selected and further evaluated for their wide application in the field of HBV molecular diagnostics.

Ironically, even after decades of discovery of HBV and availability of a highly effective vaccine, HBV is transmitted to 10-30 million naive people, of which more than 1 million die worldwide each year. Notably, majority of the HBV transmissions and deaths related to it occur in less developed or poor countries, where HBV is highly endemic. Thus most important advancement required urgently in HBV diagnostics is to make available low cost sensitive assays in highly endemic countries, to contain the "man-hunt" of this "silent killer".

REFERENCES

1 **Global Alert and Response (GAR)**. Hepatitis B. Available from: URL: http://www.who.int/csr/disease/hepatitis/whocdscsrlyo20022/en/index3.html

2 **Datta S**, Chatterjee S, Veer V, Chakravarty R. Molecular Biology of the Hepatitis B Virus for Clinicians. *J Clin Exp Hepatol* 2012; **2**: 53-365 [DOI: 10.1016/j.jceh.2012.10.003]

3 **Blumberg BS**. Hepatitis B virus, the vaccine, and the control of primary cancer of the liver. *Proc Natl Acad Sci USA* 1997; **94**: 7121-7125 [PMID: 9207053]

4 **Lok AS**, McMahon BJ. Chronic hepatitis B. *Hepatology* 2007; **45**: 507-539 [PMID: 17256718 DOI: 10.1002/hep.21513]

5 **Weber B**. Recent developments in the diagnosis and monitoring of HBV infection and role of the genetic variability of the S gene. *Expert Rev Mol Diagn* 2005; **5**: 75-91 [PMID: 15723594 DOI: 10.1586/14737159.5.1.75]

6 **Gerlich WH**. Medical virology of hepatitis B: how it began and where we are now. *Virol J* 2013; **10**: 239 [PMID: 23870415 DOI: 10.1186/1743-422X-10-239]

7 **Lok AS**, Heathcote EJ, Hoofnagle JH. Management of hepatitis B: 2000--summary of a workshop. *Gastroenterology* 2001; **120**: 1828-1853 [PMID: 11375963]

8 **Yuen MF**, Yuan HJ, Wong DK, Yuen JC, Wong WM, Chan AO, Wong BC, Lai KC, Lai CL. Prognostic determinants for chronic hepatitis B in Asians: therapeutic implications. *Gut* 2005; **54**: 1610-1614 [PMID: 15871997 DOI: 10.1136/gut.2005.065136]

9 **Chu CJ**, Hussain M, Lok AS. Quantitative serum HBV DNA levels during different stages of chronic hepatitis B infection. *Hepatology* 2002; **36**: 1408-1415 [PMID: 12447866]

10 **Kwok S**, Higuchi R. Avoiding false positives with PCR. *Nature* 1989; **339**: 237-238 [PMID: 2716852 DOI: 10.1038/339237a0]

11 **Victor T**, Jordaan A, du Toit R, Van Helden PD. Laboratory experience and guidelines for avoiding false positive polymerase chain reaction results. *Eur J Clin Chem Clin Biochem* 1993; **31**: 531-535 [PMID: 8218586]

12 **Mullis KB**, Faloona FA. Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. *Methods Enzymol* 1987; **155**: 335-350 [PMID: 3431465 DOI: 10.1016/0076-6879(87)55023-6]

13 **Yang S**, Rothman RE. PCR-based diagnostics for infectious diseases: uses, limitations, and future applications in acute-care settings. *Lancet Infect Dis* 2004; **4**: 337-348 [PMID: 15172342 DOI: 10.1016/S1473-3099(04)01044-8]

14 **O'Connor L**, Glynn B. Recent advances in the development of nucleic acid diagnostics. *Expert Rev Med Devices* 2010; **7**: 529-539 [PMID: 20583889 DOI: 10.1586/erd.10.22]

15 **Raimondo G**, Allain JP, Brunetto MR, Buendia MA, Chen DS, Colombo M, Craxì A, Donato F, Ferrari C, Gaeta GB, Gerlich WH, Levrero M, Locarnini S, Michalak T, Mondelli MU, Pawlotsky JM, Pollicino T, Prati D, Puoti M, Samuel D, Shouval D, Smedile A, Squadrito G, Trépo C, Villa E, Will H, Zanetti AR, Zoulim F. Statements from the Taormina expert meeting on occult hepatitis B virus infection. *J Hepatol* 2008; **49**: 652-657 [PMID: 18715666 DOI: 10.1016/j.jhep.2008.07.014]

16 **Datta S**, Banerjee A, Chandra PK, Biswas A, Panigrahi R, Mahapatra PK, Panda CK, Chakrabarti S, Bhattacharya SK, Chakravarty R. Analysis of hepatitis B virus X gene phylogeny, genetic variability and its impact on pathogenesis: implications in Eastern Indian HBV carriers. *Virology* 2008; **382**: 190-198 [PMID: 18952249 DOI: 10.1016/j.virol.2008.09.007]

17 **Datta S**, Banerjee A, Chandra PK, Chakravarty R. Selecting a genetic region for molecular analysis of hepatitis B virus transmission. *J Clin Microbiol* 2007; **45**: 687; author reply 688 [PMID: 17277176 DOI: 10.1128/JCM.02046-06]

18 **Torbenson M**, Thomas DL. Occult hepatitis B. *Lancet Infect Dis* 2002; **2**: 479-486 [PMID: 12150847 DOI: 10.1016/S1473-3099(02)00345-6]

19 **Pawlotsky JM**. Molecular diagnosis of viral hepatitis. *Gastroenterology* 2002; **122**: 1554-1568 [PMID: 12016423 DOI: 10.1053/gast.2002.33428]

20 **Weiss J**, Wu H, Farrenkopf B, Schultz T, Song G, Shah S, Siegel J. Real time TaqMan PCR detection and quantitation of HBV genotypes A-G with the use of an internal quantitation standard. *J Clin Virol* 2004; **30**: 86-93 [PMID: 15072760 DOI: 10.1016/j.jcv.2003.08.015]

21 **Paraskevis D**, Haida C, Tassopoulos N, Raptopoulou M, Tsantoulas D, Papachristou H, Sypsa V, Hatzakis A. Development and assessment of a novel real-time PCR assay for quantitation of HBV DNA. *J Virol Methods* 2002; **103**: 201-212 [PMID: 12008014 DOI: 10.1016/S0166-0934(02)00033-2]

22 **Saldanha J**, Gerlich W, Lelie N, Dawson P, Heermann K, Heath A. An international collaborative study to establish a World Health Organization international standard for hepatitis B virus DNA nucleic acid amplification techniques. *Vox Sang* 2001; **80**: 63-71 [PMID: 11339072 DOI: 10.1046/j.1423-0410.2001.00003.x]

23 **Heermann KH**, Gerlich WH, Chudy M, Schaefer S, Thomssen R. Quantitative detection of hepatitis B virus DNA in two international reference plasma preparations. Eurohep Pathobiology Group. *J Clin Microbiol* 1999; **37**: 68-73 [PMID: 9854066]

24 **Fryer JF**, Heath AB, Wilkinson DE, Minor PD; the Collaborative Study Group. Collaborative study to evaluate the pro-

posed 3rd WHO International Standard for hepatitis B virus (HBV) for nucleic acid amplification technology (NAT)-based assays. WHO ECBS Report 2011; WHO/BS/2011.2170. Available from: URL: http://www.who.int/biologicals/expert_committee/BS2011.2170HBV_NAT.pdf

25 **Guirgis BS**, Abbas RO, Azzazy HM. Hepatitis B virus genotyping: current methods and clinical implications. *Int J Infect Dis* 2010; **14**: e941-e953 [PMID: 20674432 DOI: 10.1016/j.ijid.2010.03.020]

26 **Mizokami M**, Nakano T, Orito E, Tanaka Y, Sakugawa H, Mukaide M, Robertson BH. Hepatitis B virus genotype assignment using restriction fragment length polymorphism patterns. *FEBS Lett* 1999; **450**: 66-71 [PMID: 10350059 DOI: 10.1016/S0014-5793(99)00471-8]

27 **Lindh M**, Andersson AS, Gusdal A. Genotypes, nt 1858 variants, and geographic origin of hepatitis B virus--large-scale analysis using a new genotyping method. *J Infect Dis* 1997; **175**: 1285-1293 [PMID: 9180165 DOI: 10.1086/516458]

28 **Naito H**, Hayashi S, Abe K. Rapid and specific genotyping system for hepatitis B virus corresponding to six major genotypes by PCR using type-specific primers. *J Clin Microbiol* 2001; **39**: 362-364 [PMID: 11136801 DOI: 10.1128/JCM.39.1.362-364.2001]

29 **Kirschberg O**, Schüttler C, Repp R, Schaefer S. A multiplex-PCR to identify hepatitis B virus--enotypes A-F. *J Clin Virol* 2004; **29**: 39-43 [PMID: 14675868 DOI: 10.1016/S1386-6532(03)00084-2]

30 **Chen J**, Yin J, Tan X, Zhang H, Zhang H, Chen B, Chang W, Schaefer S, Cao G. Improved multiplex-PCR to identify hepatitis B virus genotypes A-F and subgenotypes B1, B2, C1 and C2. *J Clin Virol* 2007; **38**: 238-243 [PMID: 17224304 DOI: 10.1016/j.jcv.2006.12.007]

31 **Markoulatos P**, Siafakas N, Moncany M. Multiplex polymerase chain reaction: a practical approach. *J Clin Lab Anal* 2002; **16**: 47-51 [PMID: 11835531 DOI: 10.1002/jcla.2058]

32 **Kubista M**, Andrade JM, Bengtsson M, Forootan A, Jonák J, Lind K, Sindelka R, Sjöback R, Sjögreen B, Strömbom L, Ståhlberg A, Zoric N. The real-time polymerase chain reaction. *Mol Aspects Med* 2006; **27**: 95-125 [PMID: 16460794 DOI: 10.1016/j.mam.2005.12.007]

33 **Ratcliff RM**, Chang G, Kok T, Sloots TP. Molecular diagnosis of medical viruses. *Curr Issues Mol Biol* 2007; **9**: 87-102 [PMID: 17489437]

34 **Malmström S**, Berglin-Enquist I, Lindh M. Novel method for genotyping hepatitis B virus on the basis of TaqMan real-time PCR. *J Clin Microbiol* 2010; **48**: 1105-1111 [PMID: 20107090 DOI: 10.1128/JCM.01442-09]

35 **Liu Y**, Hussain M, Wong S, Fung SK, Yim HJ, Lok AS. A genotype-independent real-time PCR assay for quantification of hepatitis B virus DNA. *J Clin Microbiol* 2007; **45**: 553-558 [PMID: 17182753 DOI: 10.1128/JCM.00709-06]

36 **Takkenberg RB**, Zaaijer HL, Molenkamp R, Menting S, Terpstra V, Weegink CJ, Dijkgraaf MG, Jansen PL, Reesink HW, Beld MG. Validation of a sensitive and specific real-time PCR for detection and quantitation of hepatitis B virus covalently closed circular DNA in plasma of chronic hepatitis B patients. *J Med Virol* 2009; **81**: 988-995 [PMID: 19382261 DOI: 10.1002/jmv.21477]

37 **Landegren U**, Kaiser R, Sanders J, Hood L. A ligase-mediated gene detection technique. *Science* 1988; **241**: 1077-1080 [PMID: 3413476 DOI: 10.1126/science.3413476]

38 **Landegren U**. Ligation-based DNA diagnostics. *Bioessays* 1993; **15**: 761-765 [PMID: 8292008]

39 **Lisby G**. Application of nucleic acid amplification in clinical microbiology. *Mol Biotechnol* 1999; **12**: 75-99 [PMID: 10554773]

40 **Abravaya K**, Carrino JJ, Muldoon S, Lee HH. Detection of point mutations with a modified ligase chain reaction (Gap-LCR). *Nucleic Acids Res* 1995; **23**: 675-682 [PMID: 7534908]

41 **Osiowy C**. Sensitive detection of HBsAg mutants by a gap ligase chain reaction assay. *J Clin Microbiol* 2002; **40**: 2566-2571 [PMID: 12089278 DOI: 10.1128/JCM.40.7.2566-2571.2002]

42 **Karthigesu VD**, Mendy M, Fortuin M, Whittle HC, Howard CR, Allison LM. The ligase chain reaction distinguishes hepatitis B virus S-gene variants. *FEMS Microbiol Lett* 1995; **131**: 127-132 [PMID: 7557320]

43 **Minamitani S**, Nishiguchi S, Kuroki T, Otani S, Monna T. Detection by ligase chain reaction of precore mutant of hepatitis B virus. *Hepatology* 1997; **25**: 216-222 [PMID: 8985293 DOI: 10.S0270-9139(97)00046-3]

44 **Trippler M**, Hampl H, Goergen B, Spies U, Knolle P, Grimm B, Meyer zum Büschenfelde KH, Gerken G. Ligase chain reaction (LCR) assay for semi-quantitative detection of HBV DNA in mononuclear leukocytes of patients with chronic hepatitis B. *J Viral Hepat* 1996; **3**: 267-272 [PMID: 8914008]

45 **Craw P**, Balachandran W. Isothermal nucleic acid amplification technologies for point-of-care diagnostics: a critical review. *Lab Chip* 2012; **12**: 2469-2486 [PMID: 22592150 DOI: 10.1039/C2LC40100B]

46 **Gill P**, Ghaemi A. Nucleic acid isothermal amplification technologies: a review. *Nucleosides Nucleotides Nucleic Acids* 2008; **27**: 224-243 [PMID: 18260008 DOI: 10.1080/15257770701845204]

47 **Notomi T**, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, Hase T. Loop-mediated isothermal amplification of DNA. *Nucleic Acids Res* 2000; **28**: E63 [PMID: 10871386 DOI: 10.1093/nar/28.12.e63]

48 **Tomita N**, Mori Y, Kanda H, Notomi T. Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products. *Nat Protoc* 2008; **3**: 877-882 [PMID: 18451795 DOI: 10.1038/nprot.2008.57]

49 **Parida M**, Sannarangaiah S, Dash PK, Rao PV, Morita K. Loop mediated isothermal amplification (LAMP): a new generation of innovative gene amplification technique; perspectives in clinical diagnosis of infectious diseases. *Rev Med Virol* 2008; **18**: 407-421 [PMID: 18716992 DOI: 10.1002/rmv.593]

50 **Cai T**, Lou G, Yang J, Xu D, Meng Z. Development and evaluation of real-time loop-mediated isothermal amplification for hepatitis B virus DNA quantification: a new tool for HBV management. *J Clin Virol* 2008; **41**: 270-276 [PMID: 18296109 DOI: 10.1016/j.jcv.2007.11.025]

51 **Nagamine K**, Watanabe K, Ohtsuka K, Hase T, Notomi T. Loop-mediated isothermal amplification reaction using a nondenatured template. *Clin Chem* 2001; **47**: 1742-1743 [PMID: 11514425]

52 **Moslemi E**, Shahhosseiny MH, Javadi G, Praivar K, Sattari TN, Amini HK. Loop mediated isothermal amplification (LAMP) for rapid detection of HBV in Iran. *Afr J Microbiol Res* 2009; **3**: 439-445

53 **Chuang TL**, Wei SC, Lee SY, Lin CW. A polycarbonate based surface plasmon resonance sensing cartridge for high sensitivity HBV loop-mediated isothermal amplification. *Biosens Bioelectron* 2012; **32**: 89-95 [PMID: 22209071 DOI: 10.1016/j.bios.2011.11.037]

54 **Compton J**. Nucleic acid sequence-based amplification. *Nature* 1991; **350**: 91-92 [PMID: 1706072 DOI: 10.1038/350091a0]

55 **Yates S**, Penning M, Goudsmit J, Frantzen I, van de Weijer B, van Strijp D, van Gemen B. Quantitative detection of hepatitis B virus DNA by real-time nucleic acid sequence-based amplification with molecular beacon detection. *J Clin Microbiol* 2001; **39**: 3656-3665 [PMID: 11574587 DOI: 10.1128/JCM.39.10.3656-3665.2001]

56 **Deiman B**, Jay C, Zintilini C, Vermeer S, van Strijp D, Venema F, van de Wiel P. Efficient amplification with NASBA of hepatitis B virus, herpes simplex virus and methicillin resistant Staphylococcus aureus DNA. *J Virol Methods* 2008; **151**: 283-293 [PMID: 18514336 DOI: 10.1016/j.jviromet.2008.04.009]

57 **Gulliksen A**, Solli L, Karlsen F, Rogne H, Hovig E, Nord-

strøm T, Sirevåg R. Real-time nucleic acid sequence-based amplification in nanoliter volumes. *Anal Chem* 2004; **76**: 9-14 [PMID: 14697026 DOI: 10.1021/ac034779h]
58 **Dimov IK**, Garcia-Cordero JL, O'Grady J, Poulsen CR, Viguier C, Kent L, Daly P, Lincoln B, Maher M, O'Kennedy R, Smith TJ, Ricco AJ, Lee LP. Integrated microfluidic tmRNA purification and real-time NASBA device for molecular diagnostics. *Lab Chip* 2008; **8**: 2071-2078 [PMID: 19023470 DOI: 10.1039/b812515e]
59 **Fire A**, Xu SQ. Rolling replication of short DNA circles. *Proc Natl Acad Sci USA* 1995; **92**: 4641-4645 [PMID: 7753856]
60 **Liu D**, Daubendiek SL, Zillman MA, Ryan K, Kool ET. Rolling Circle DNA Synthesis: Small Circular Oligonucleotides as Efficient Templates for DNA Polymerases. *J Am Chem Soc* 1996; **118**: 1587-1594 [PMID: 20830216]
61 **Fakruddin M**, Mannan KS, Chowdhury A, Mazumdar RM, Hossain MN, Islam S, Chowdhury MA. Nucleic acid amplification: Alternative methods of polymerase chain reaction. *J Pharm Bioall Sci* 2013; **5**: 245-252 [DOI: 10.4103/0975-7406.120066]
62 **Margeridon S**, Carrouée-Durantel S, Chemin I, Barraud L, Zoulim F, Trépo C, Kay A. Rolling circle amplification, a powerful tool for genetic and functional studies of complete hepatitis B virus genomes from low-level infections and for directly probing covalently closed circular DNA. *Antimicrob Agents Chemother* 2008; **52**: 3068-3073 [PMID: 18606836 DOI: 10.1128/AAC.01318-07]
63 **Martel N**, Gomes SA, Chemin I, Trépo C, Kay A. Improved rolling circle amplification (RCA) of hepatitis B virus (HBV) relaxed-circular serum DNA (RC-DNA). *J Virol Methods* 2013; **193**: 653-659 [PMID: 23928222 DOI: 10.1016/j.jviromet.2013.07.045]
64 **Yao C**, Xiang Y, Deng K, Xia H, Fu W. Sensitive and specific HBV genomic DNA detection using RCA-based QCM biosensor. *Sens Actuators B-Chem* 2013; **181**: 382-387 [DOI: 10.1016/j.snb.2013.01.063]
65 **Chevaliez S**, Rodriguez C, Pawlotsky JM. New virologic tools for management of chronic hepatitis B and C. *Gastroenterology* 2012; **142**: 1303-1313.e1 [PMID: 22537437 DOI: 10.1053/j.gastro.2012.02.027]
66 **Kamisango K**, Kamogawa C, Sumi M, Goto S, Hirao A, Gonzales F, Yasuda K, Iino S. Quantitative detection of hepatitis B virus by transcription-mediated amplification and hybridization protection assay. *J Clin Microbiol* 1999; **37**: 310-314 [PMID: 9889209]
67 **Ide T**, Kumashiro R, Hino T, Murashima S, Ogata K, Koga Y, Sata M. Transcription-mediated amplification is more useful in the follow-up of patients with chronic hepatitis B treated with lamivudine. *Hepatol Res* 2001; **21**: 76-84 [PMID: 11470630]
68 **Kubo S**, Hirohashi K, Tanaka H, Shuto T, Takemura S, Yamamoto T, Uenishi T, Kinoshita H, Nishiguchi S. Usefulness of viral concentration measurement by transcription-mediated amplification and hybridization protection as a prognostic factor for recurrence after resection of hepatitis B virus-related hepatocellular carcinoma. *Hepatol Res* 2003; **25**: 71-77 [PMID: 12644041]
69 **Walker GT**, Fraiser MS, Schram JL, Little MC, Nadeau JG, Malinowski DP. Strand displacement amplification--an isothermal, in vitro DNA amplification technique. *Nucleic Acids Res* 1992; **20**: 1691-1696 [PMID: 1579461 DOI: 10.1093/nar/20.7.1691]
70 **Walker GT**, Little MC, Nadeau JG, Shank DD. Isothermal in vitro amplification of DNA by a restriction enzyme/DNA polymerase system. *Proc Natl Acad Sci USA* 1992; **89**: 392-396 [PMID: 1309614]
71 **McHugh TD**, Pope CF, Ling CL, Patel S, Billington OJ, Gosling RD, Lipman MC, Gillespie SH. Prospective evaluation of BDProbeTec strand displacement amplification (SDA) system for diagnosis of tuberculosis in non-respiratory and respiratory samples. *J Med Microbiol* 2004; **53**: 1215-1219 [PMID: 15585500 DOI: 10.1099/jmm.0.45780-0]
72 **Vincent M**, Xu Y, Kong H. Helicase-dependent isothermal DNA amplification. *EMBO Rep* 2004; **5**: 795-800 [PMID: 15247927 DOI: 10.1038/sj.embor.7400200]
73 **Jeong YJ**, Park K, Kim DE. Isothermal DNA amplification in vitro: the helicase-dependent amplification system. *Cell Mol Life Sci* 2009; **66**: 3325-3336 [PMID: 19629390]
74 **Melamede RJ**. Automatable process for sequencing nucleotide. US Patent 4,863,849 (1989). Available from: URL: http://www.google.com/patents/US4863849
75 **Nyrén P**. Enzymatic method for continuous monitoring of DNA polymerase activity. *Anal Biochem* 1987; **167**: 235-238 [PMID: 2831754 DOI: 10.1016/0003-2697(87)90158-8]
76 **Margulies M**, Egholm M, Altman WE, Attiya S, Bader JS, Bemben LA, Berka J, Braverman MS, Chen YJ, Chen Z, Dewell SB, Du L, Fierro JM, Gomes XV, Godwin BC, He W, Helgesen S, Ho CH, Irzyk GP, Jando SC, Alenquer ML, Jarvie TP, Jirage KB, Kim JB, Knight JR, Lanza JR, Leamon JH, Lefkowitz SM, Lei M, Li J, Lohman KL, Lu H, Makhijani VB, McDade KE, McKenna MP, Myers EW, Nickerson E, Nobile JR, Plant R, Puc BP, Ronan MT, Roth GT, Sarkis GJ, Simons JF, Simpson JW, Srinivasan M, Tartaro KR, Tomasz A, Vogt KA, Volkmer GA, Wang SH, Wang Y, Weiner MP, Yu P, Begley RF, Rothberg JM. Genome sequencing in microfabricated high-density picolitre reactors. *Nature* 2005; **437**: 376-380 [PMID: 16056220 DOI: 10.1038/nature03959]
77 **Radford AD**, Chapman D, Dixon L, Chantrey J, Darby AC, Hall N. Application of next-generation sequencing technologies in virology. *J Gen Virol* 2012; **93**: 1853-1868 [PMID: 22647373 DOI: 10.1099/vir.0.043182-0]
78 **Margeridon-Thermet S**, Shulman NS, Ahmed A, Shahriar R, Liu T, Wang C, Holmes SP, Babrzadeh F, Gharizadeh B, Hanczaruk B, Simen BB, Egholm M, Shafer RW. Ultra-deep pyrosequencing of hepatitis B virus quasispecies from nucleoside and nucleotide reverse-transcriptase inhibitor (NRTI)-treated patients and NRTI-naive patients. *J Infect Dis* 2009; **199**: 1275-1285 [PMID: 19301976 DOI: 10.1086/597808]
79 **Nishijima N**, Marusawa H, Ueda Y, Takahashi K, Nasu A, Osaki Y, Kou T, Yazumi S, Fujiwara T, Tsuchiya S, Shimizu K, Uemoto S, Chiba T. Dynamics of hepatitis B virus quasispecies in association with nucleos(t)ide analogue treatment determined by ultra-deep sequencing. *PLoS One* 2012; **7**: e35052 [PMID: 22523569 DOI: 10.1371/journal.pone.0035052]
80 **Gong L**, Han Y, Chen L, Liu F, Hao P, Sheng J, Li XH, Yu DM, Gong QM, Tian F, Guo XK, Zhang XX. Comparison of next-generation sequencing and clone-based sequencing in analysis of hepatitis B virus reverse transcriptase quasispecies heterogeneity. *J Clin Microbiol* 2013; **51**: 4087-4094 [PMID: 24088859 DOI: 10.1128/JCM.01723-13]
81 **Ding D**, Lou X, Hua D, Yu W, Li L, Wang J, Gao F, Zhao N, Ren G, Li L, Lin B. Recurrent targeted genes of hepatitis B virus in the liver cancer genomes identified by a next-generation sequencing-based approach. *PLoS Genet* 2012; **8**: e1003065 [PMID: 23236287 DOI: 10.1371/journal.pgen.1003065]
82 **Jiang Z**, Jhunjhunwala S, Liu J, Haverty PM, Kennemer MI, Guan Y, Lee W, Carnevali P, Stinson J, Johnson S, Diao J, Yeung S, Jubb A, Ye W, Wu TD, Kapadia SB, de Sauvage FJ, Gentleman RC, Stern HM, Seshagiri S, Pant KP, Modrusan Z, Ballinger DG, Zhang Z. The effects of hepatitis B virus integration into the genomes of hepatocellular carcinoma patients. *Genome Res* 2012; **22**: 593-601 [PMID: 22267523 DOI: 10.1101/gr.133926]
83 **Newman JD**, Tigwell LJ, Turner APF, Warner PJ. Biosensors " A clearer view. Proceedings of the 8th World Congress on Biosensors; 2004 May 24-26; Granada, Spain. UK: Cranfield University Publication, 2004: 17-20
84 **Yao C**, Zhu T, Tang J, Wu R, Chen Q, Chen M, Zhang B, Huang J, Fu W. Hybridization assay of hepatitis B virus by

QCM peptide nucleic acid biosensor. *Biosens Bioelectron* 2008; **23**: 879-885 [PMID: 17951045 DOI: 10.1016/j.bios.2007.09.003]

85 **Iqbal SS**, Mayo MW, Bruno JG, Bronk BV, Batt CA, Chambers JP. A review of molecular recognition technologies for detection of biological threat agents. *Biosens Bioelectron* 2000; **15**: 549-578 [PMID: 11213217]

86 **Mascini M**, Tombelli S. Biosensors for biomarkers in medical diagnostics. *Biomarkers* 2008; **13**: 637-657 [PMID: 19061054 DOI: 10.1080/13547500802645905]

87 **Ding C**, Zhao F, Zhang M, Zhang S. Hybridization biosensor using 2,9-dimethyl-1,10-phenantroline cobalt as electrochemical indicator for detection of hepatitis B virus DNA. *Bioelectrochemistry* 2008; **72**: 28-33 [PMID: 18096445 DOI: 10.1016/j.bioelechem.2007.11.001]

88 **Qi C**, Zhu W, Niu Y, Zhang HG, Zhu GY, Meng YH, Chen S, Jin G. Detection of hepatitis B virus markers using a biosensor based on imaging ellipsometry. *J Viral Hepat* 2009; **16**: 822-832 [PMID: 19486471 DOI: 10.1111/j.1365-2893.2009.01123.x]

89 **Perdikaris A**, Alexandropoulos N, Kintzios S. Development of a Novel, Ultra-rapid Biosensor for the Qualitative Detection of Hepatitis B Virus-associated Antigens and Anti-HBV, Based on "Membrane-engineered" Fibroblast Cells with Virus-Specific Antibodies and Antigens. *Sensors (Basel)* 2009; **9**: 2176-2186 [PMID: 22574007 DOI: 10.3390/s90302176]

90 **Song Y**, Dai E, Wang J, Liu H, Zhai J, Chen C, Du Z, Guo Z, Yang R. Genotyping of hepatitis B virus (HBV) by oligonucleotides microarray. *Mol Cell Probes* 2006; **20**: 121-127 [PMID: 16431078 DOI: 10.1016/j.mcp.2005.11.004]

91 **Wang YZ**, Wu GX, Luo LB, Chen M, Ruan LH. Oligonucleotide chip, real-time PCR and sequencing for genotyping of hepatitis B virus. *World J Gastroenterol* 2007; **13**: 4260-4263 [PMID: 17696258]

92 **Manz A**, Graber N, Widmer JM. Miniaturized total chemical analysis systems: a novel concept for chemical sensing. *Sens Actuat B-Chem* 1990; **1**: 244-248 [DOI: 10.1016/0925-4005(90)80209-i]

93 **Zhang C**, Xing D. Miniaturized PCR chips for nucleic acid amplification and analysis: latest advances and future trends. *Nucleic Acids Res* 2007; **35**: 4223-4237 [PMID: 17576684 DOI: 10.1093/nar/gkm389]

94 **Yager P**, Edwards T, Fu E, Helton K, Nelson K, Tam MR, Weigl BH. Microfluidic diagnostic technologies for global public health. *Nature* 2006; **442**: 412-418 [PMID: 16871209 DOI: 10.1038/nature05064]

95 **Hashimoto M**, Chen PC, Mitchell MW, Nikitopoulos DE, Soper SA, Murphy MC. Rapid PCR in a continuous flow device. *Lab Chip* 2004; **4**: 638-645 [PMID: 15570378 DOI: 10.1039/B406860B]

96 **Zaytseva NV**, Goral VN, Montagna RA, Baeumner AJ. Development of a microfluidic biosensor module for pathogen detection. *Lab Chip* 2005; **5**: 805-811 [PMID: 16027930 DOI: 10.1039/B503856A]

97 **Sodunke TR**, Bouchard MJ, Noh HM. Microfluidic platform for hepatitis B viral replication study. *Biomed Microdevices* 2008; **10**: 393-402 [PMID: 18165913 DOI: 10.1007/s10544-007-9148-1]

98 **Liu D**, Shi M, Huang H, Long Z, Zhou X, Qin J, Lin B. Isotachophoresis preconcentration integrated microfluidic chip for highly sensitive genotyping of the hepatitis B virus. *J Chromatogr B Analyt Technol Biomed Life Sci* 2006; **844**: 32-38 [PMID: 16899416 DOI: 10.1016/j.jchromb.2006.06.041]

99 **Zhi X**, Deng M, Yang H, Gao G, Wang K, Fu H, Zhang Y, Chen D, Cui D. A novel HBV genotypes detecting system combined with microfluidic chip, loop-mediated isothermal amplification and GMR sensors. *Biosens Bioelectron* 2014; **54**: 372-377 [PMID: 24292142 DOI: 10.1016/j.bios.2013.11.025]

P- Reviewer: Al-Gayyar MMH, Fisher RA, Shi Z, Wang K
S- Editor: Gou SX **L- Editor**: A **E- Editor**: Zhang DN

Published by **Baishideng Publishing Group Inc**
8226 Regency Drive, Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
http://www.wjgnet.com

ISSN 1007-9327
9 771007 932045 40>