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REVIEW

Primer and probe conservation issue in the quantification of hepatitis B virus DNA

Chye Phing Teh[1,2] | Jack Bee Chook[2] | Yun Fong Ngeow[3] | Tommy Yuh Koon Tong[1] | Kok Keng Tee[4] | Jan Jin Bong[5] | Rosmawati Mohamed[6]

[1]Department of Biological Sciences, School of Science and Technology, Sunway University, Petaling Jaya, Selangor, Malaysia

[2]Department of Medical Sciences, School of Healthcare and Medical Sciences, Sunway University, Petaling Jaya, Selangor, Malaysia

[3]Department of Pre-Clinical Sciences, Faculty of Medicine and Health Sciences, Universiti Tunku Abdul Rahman, Kajang, Malaysia

[4]Department of Medical Microbiology, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia

[5]Sunway Medical Centre, Petaling Jaya, Selangor, Malaysia

[6]Department of Medicine, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia

Correspondence
Jack Bee Chook, Department of Medical Sciences, School of Healthcare and Medical Sciences, Sunway University, 5, Jalan Universiti, Bandar Sunway, Petaling Jaya, Selangor 47500, Malaysia.
Email: jackbeec@sunway.edu.my

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Summary

Current treatment strategies for chronic hepatitis B virus (HBV) infection aim at long-term suppression of the viral replication since a cure remains elusive. Its clinical management therefore relies greatly on routine monitoring of serum HBV DNA levels using quantitative polymerase chain reaction (qPCR) assays. Designing a highly conserved oligonucleotide set for the qPCR assay can be challenging due to the high genetic heterogeneity of the virus. The ever-increasing number of HBV genomes deposited in the GenBank nucleotide database warrants a revisit to previous primer and probe designs. We examined primer and probe sets from 53 qPCR assays published in the past 2 decades for their coverage in 9864 complete HBV genomes retrieved from GenBank. Of all the 53 qPCR assays, only 17% achieved ≥80% coverage. About 40% of the 53 assays covered less than 20% of the 9864 genomes. *In silico* DNA thermodynamics analysis demonstrated reduced primer/probe binding affinity, which further increases the risk of viral load misdetection and underestimation for certain HBV variants. Taken together, there is a pressing need for improving available qPCR designs for the quantification of HBV DNA based on the updated genome data.

KEYWORDS
HBV DNA quantification, nucleic acid testing, qPCR assay, real-time PCR, viral load

1 | INTRODUCTION

Approximately 2 billion of the world population have been infected with hepatitis B virus (HBV), and over 250 million are living with chronic HBV infection.[1,2] HBV caused 890,000 deaths (66% of all viral hepatitis deaths) in 2015, mostly due to the complications of chronic infection associated with liver cirrhosis or hepatocellular carcinoma.

Chronic HBV infection is defined as hepatitis B surface antigen (HBsAg) positivity for more than six months. Although there is an effective vaccine to prevent hepatitis B since the 1980s, a cure remains elusive. Current treatment strategies aim at long-term suppression of viral replication to prevent disease progression which can cause severe and irreversible damage to the liver.[3,4] The clinical management of chronic HBV infection therefore relies to a great

Abbreviations: BioEdit, BioEdit Sequence Alignment Editor software; cccDNA, covalently closed circular DNA; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; MAFFT, Multiple Alignment using Fast Fourier Transform; NCBI, National Center for Biotechnology Information; OBI, occult HBV infection; qPCR, quantitative polymerase chain reaction; Ta, annealing temperature; Tm, melting temperature.

degree on routine monitoring of the viral load, which is done by measuring the circulating HBV DNA levels in patient samples using a quantitative polymerase chain reaction (qPCR) assay.

In addition, HBV infection cannot be excluded in all cases tested negative for HBsAg. It was reported that more than 20% of HBV infected individuals were found positive for HBV DNA even though they were seronegative for all other biomarkers indicating a history of HBV infection.[5] Such cases are classified as occult HBV infection (OBI), which is defined by HBsAg-negativity with very low amount (typically <200 IU/ml) of HBV DNA in the serum.[6] The prevalence of OBI varies in different regions of the world, ranging from 1% to as high as 87%.[7]

Accurate determination of circulating HBV DNA level is no doubt essential for excluding false-negative detection of HBV infection (particularly for detection during the seronegative window period, and identification of OBI),[8,9] assessing the phase of the infection and response to treatment, detecting antiviral resistance and viral reactivation, guiding treatment decisions and proper management of HBV-infected patients. Designing a highly conserved oligonucleotide set for qPCR assays can be challenging due to the high genetic heterogeneity of the virus. The ever-increasing number of HBV genomes deposited in the GenBank nucleotide database from 187 in 1999 to 11,033 in 2019 warrants a revisit to previous primer and probe designs.

While the impact of escape mutants on the detection of HBsAg by commercial assays is a diagnostic issue that is not infrequently discussed,[10–13] data on primer and probe sequence conservation are lacking in most qPCR assays used for HBV DNA quantification. The analytical sensitivity and accuracy of a qPCR assay is highly questionable if there were polymorphisms occurring at the primer and/or probe binding sites in the viral genomes. With tremendous increase in the number of HBV variants over the years, there is a need to re-examine the conservation of existing primer and probe sequences.

In this study, we investigated the coverage of existing qPCR primer and probe sequences based on the up-to-date 9864 HBV genomes retrieved from the GenBank nucleotide database. We also assessed the potential impact of sequence variability on the performance of the assays by analysing the binding affinity of those primer and probe sequences in silico.

2 | MATERIALS AND METHODS

2.1 | Retrieval and alignment of HBV genomic sequences

HBV genomes were searched for in the GenBank nucleotide database of the National Center for Biotechnology Information (NCBI) using the keywords '(HBV [Organism]) AND 2800:3400 [Sequence Length]' with the 'release date' filter set to 31 December 2018. The search result revealed 10,696 hits. The number of new sequences released each year over the past 2 decades were investigated. Patented genome sequences, incomplete sequences, cloned sequences, sequences determined from non-human hosts, sequences having one or more fragments of 5 unknown nucleotides or above, as well as other unrelated hits were further removed, leaving the final search result of 9864 entries. These full-length HBV genomes were aligned with the Multiple Alignment using Fast Fourier Transform alignment software version 7,[14] inspected and edited with the BioEdit Sequence Alignment Editor software (BioEdit) version 7.2.5 (Ibis Therapeutics).[15] The aligned sequence database in FASTA format will be provided upon request.

2.2 | Compilation of primers and probes from existing qPCR assays

To collect primer and probe sets used for existing qPCR assays, we used the keywords 'HBV DNA quantification' in the PubMed database, and 'Title contains ("Hepatitis B" OR "HBV") AND ("DNA") AND ("real-time" OR "PCR" OR "qPCR" OR "quantification" OR "quantitation")' in OneSearch of the Lancaster University library database. We included HBV DNA quantification assays using probe-based methods, digital PCR-based methods, SYBR Green, or EvaGreen methods, from journal articles published in the last 20 years (1999–2019). Assays using molecular beacon analysis, qPCR of which the oligo sequences were not disclosed, or any of the primer/probe sequences not specified, assays targeting the HBV covalently closed circular DNA (cccDNA), assays designed for other purposes such as detecting mutation, and duplicate assays by the same author(s), were excluded from our study.

2.3 | Calculation of qPCR coverage

We investigated the number of HBV genome sequences having perfect match with the qPCR oligo sets (primer and probe sequences) in their respective target regions, that is, the coverage of qPCR assays. A total of 9864 updated HBV genome sequences were used for this analysis. The coverage was calculated in percentage by comparing the oligo sequences of the primers and probes with the 9864 aligned sequences. First, the nucleotide position of each primer and probe oligo sequence was identified with reference to a 3215 bp (base pair) HBV genome (GenBank accession number D00329.1),[16] using the BioEdit software. The oligo sequences (either a primer or a probe individually, or the combination of both), were then compared in accordance with their respective regions in the 9864 aligned sequences and the results were recorded in Microsoft Excel 2016. Each matched result was coded as '1', and unmatched as '0'. The matched results were then summed up and the percent values were computed (Data S1).

2.4 | In silico analysis of primer/probe binding affinity

We analysed the binding affinity of nine primer and probe sets that covered at least 80% of the 9864 complete HBV genome sequences.

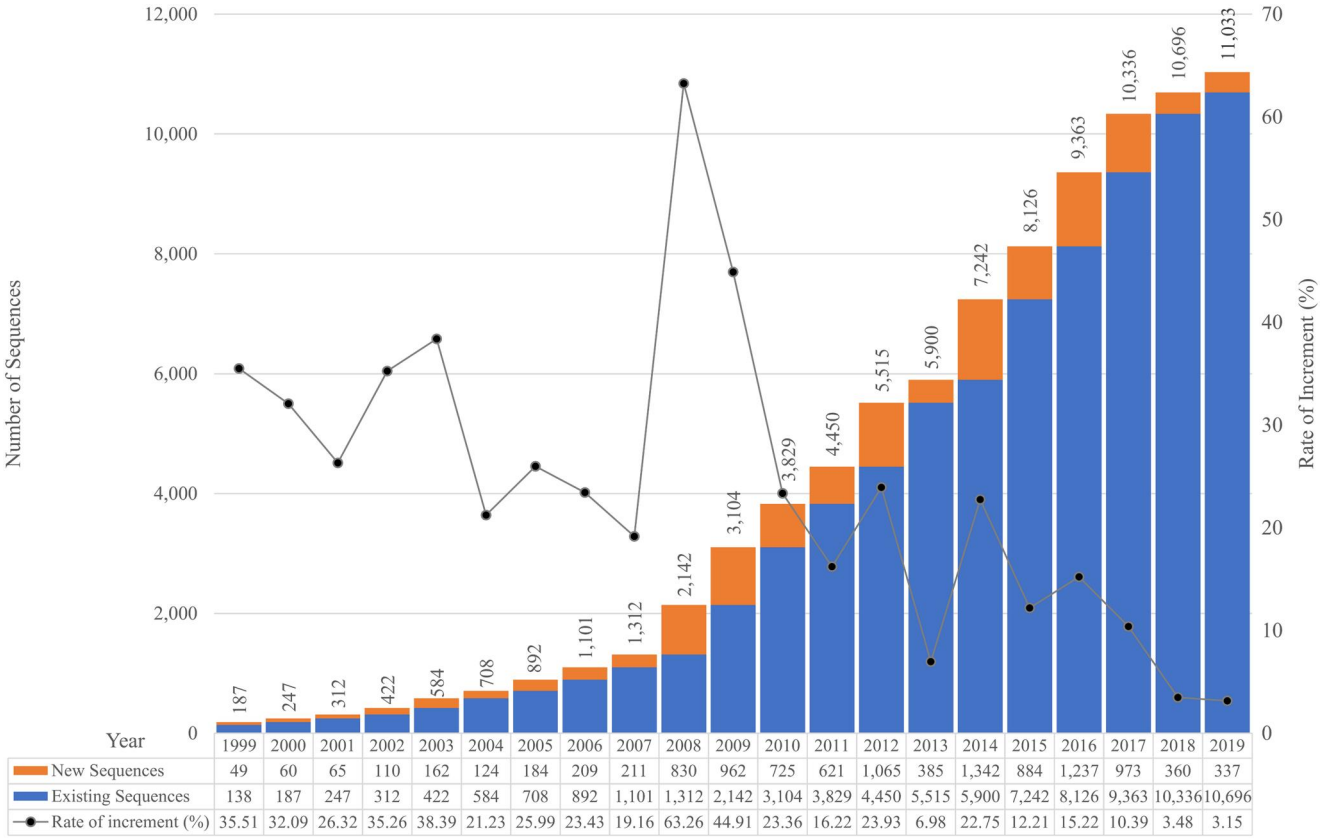

FIGURE 1 The number of HBV sequences released in the GenBank nucleotide database over the past 20 years (1999–2019). HBV, hepatitis B virus

The nucleotide position was identified based on the reference position in a 3215 bp HBV genome (GenBank accession number D00329.1).[16] The haplotype sequences of the quasispecies variants corresponding to the primer/probe target regions were identified. The primer/probe binding affinity at the assay annealing temperature (Ta) was calculated by pairing the oligo sequences with the complement counterparts of the haplotype variants occurring at the frequency of 0.1% and above. Reverse primers were converted into their reverse complements that were in concordance with the genome sequence before pairing.

This analysis was performed using the TM Mismatch function of the OligoAnalyzer software version 3.1 (Integrated DNA Technologies). The qPCR parameters were set at default values, that is, oligo concentration 0.2 μM, Na+ concentration 50 mM, Mg++ concentration 3 mM, and dNTPs concentration 0.8 mM. The total percentage without mismatch for primer/probe with ambiguous base(s) was calculated by summation of the binding affinity with their corresponding haplotype variants at the assay Ta. Since the TM Mismatch function only examines the thermodynamic effects of single base mismatches, the binding affinity of oligo sequences with multiple mismatched bases was determined through subtracting the total percentage decrease (total reduction in binding affinity of all single base mismatches) from the percent bound with exact match. For assays having more than one Ta in their cycling conditions, the lower Ta was used for the analysis as that allows stronger binding of a primer/probe to the target sequence (Data S2).

3 | RESULTS

3.1 | HBV sequences released in the GenBank nucleotide database over the past 20 years

The number of full-length HBV genome sequences has increased from 187 in 1999 to 11,033 in 2019, with an average raise of 24% each year (Figure 1). The number of new sequences released peaked in 2014, reaching a total of 1342 new HBV genome sequences deposited in the GenBank within a year.

3.2 | Coverage of existing qPCR assays

Our search for existing qPCR assays in the PubMed and Lancaster University library database revealed 486 and 365 hits respectively. The search results were collated and scanned through for identifying studies related to HBV DNA quantification assays following our inclusion criteria. Fifty-three qPCR assays from 47 journal articles published in the last 20 years (1999–2019) were included

TABLE 1 Number of qPCR assays analysed

Year of publication	Number of articles included	Number of qPCR assays analysed[a]
1999	2	4
2000	4	4
2001	3	4
2002	4	4
2003	0	0
2004	2	2
2005	3	3
2006	5	7
2007	3	3
2008	3	3
2009	1	1
2010	1	1
2011	2	2
2012	0	0
2013	0	0
2014	4	5
2015	2	2
2016	3	3
2017	2	2
2018	3	3
2019	0	0
TOTAL	47	53

Abbreviation: qPCR, quantitative polymerase chain reaction.
[a]51 simplex, and 2 duplex qPCR assays.

and analysed (Table 1). The TaqMan probe qPCR was the most used method, accounting for 71.70% (38/53) of the qPCR assays. This was followed by droplet digital PCR (ddPCR; 5/53, 9.43%), hybridization probes (3/53, 5.66%), SYBR Green (3/53, 5.66%), EvaGreen (1/53, 1.89%), and some that were not specified (3/53, 5.66%).

Figure 2 shows the overview of qPCR coverage based on 9864 HBV genome sequences retrieved from the GenBank nucleotide database released up to 31 December 2018. Of all the 53 qPCR assays analysed in our study, only 9 (17%) have successfully matched ≥80% of the 9864 complete HBV genome sequences, among which two were using dual probes. All these nine assays targeted the HBV S gene. Seven out of nine used TaqMan probes, while the other two used the SYBR Green method. About 40% of the assays covered less than 20% of the sequences. The mean coverage was 40.5% (3991/9864), and the median was 33% (3243/9864).

The profiles of various qPCR assays analysed are shown in Table 2. A total of 51 simplex (one oligo set per assay) and two duplex qPCR assays (two oligo sets per assay) were identified for their target regions in the HBV genome. The most targeted region was the HBV S gene (49%, 26/53 inclusive of one duplex assay), followed by the PreC/C gene (30%, 16/53) and the X gene (19%, 10/53). One duplex assay targeted both the S and C genes (2%).

3.3 | Primer and probe binding affinity of existing qPCR assays

Tables 3–5 illustrate the binding affinity of primers/probes from the nine qPCR assays that achieved at least 80% coverage in the 9864 complete HBV genome sequences. Four of the assays[33,39,59,60] had the same binding site, targeting nucleotide position 379–550 in the S region. The forward primer, probe, and reverse primer showing highest coverage were from Wang et al.[56] (96.98%, Mean [*M*] = 93.24, standard deviation [SD] = 1.99), Pas et al.[29] (98.01%, *M* = 94.09, SD = 4.15), and Taranta et al.[55] (97.97%, *M* = 94.94, SD = 2.26), respectively.

DNA thermodynamics analysis showed that the predicted binding affinity of oligo sequences having perfect match with the genome sequences ranged from 86.5% to 99.7% (*M* = 94.85, SD = 4.77) at the forward primer target sites, 1.30%–100% (*M* = 85.81, SD = 37.26) at the probe target sites, and 88.7%–99.85% (*M* = 95.61, SD = 4.27) at

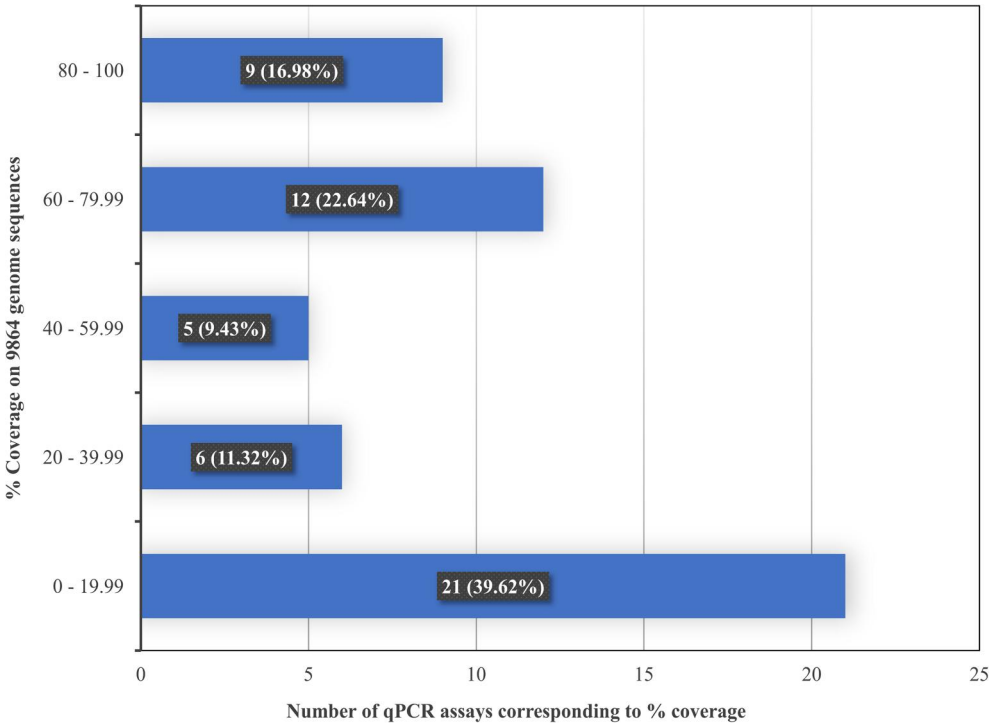

FIGURE 2 Overview of qPCR coverage based on 9864 genome sequences retrieved from the GenBank nucleotide database released up to 31 December 2018. Total qPCR assays analysed = 53 (51 simplex and 2 duplex qPCR). qPCR, quantitative polymerase chain reaction

the reverse primer target sites. As for other haplotype variants having mismatches with the oligo sequences, the predicted binding affinity was 37.35%–92.38% ($M = 63.02$, SD = 22.44) at the forward primer target sites, 0.66%–100% ($M = 83.01$, SD = 36.65) at the probe target sites, and 40.16%–90.27% ($M = 67.56$, SD = 19.79) at the reverse primer target sites.

The forward primer from Mendy et al.[39] and Prakash et al.[60] had the highest binding affinity with a hybridization efficiency of 99.7% ($M = 94.85$, SD = 4.77) with exact match and 92.38% ($M = 63.02$, SD = 22.44) with mismatch. Both of these assays were targeting the same primer region and were using the same Ta at 55°C. The probe and reverse primer from Prakash et al.[60] demonstrated the highest binding affinity among the nine assays analysed, with 100% (exact match $M = 85.81$, SD = 37.26; mismatch $M = 83.13$, SD = 36.65) efficiency for all sequence variants at the probe binding site, 99.85% ($M = 95.61$, SD = 4.27) and 90.05% ($M = 67.56$, SD = 19.79) efficiency with exact match and mismatch, respectively, at the reverse primer binding site.

4 | DISCUSSION

With an average of 24% increment in new HBV genome sequences released per year, the design of qPCR assays for HBV DNA quantification should keep pace with the latest HBV genome information for better coverage in order to improve the accuracy of detection and measurement of viral load. Based on 9864 complete HBV genomes deposited in the GenBank nucleotide database, our analysis indicated that the coverage of HBV viral load assays available today can be further improved. Strikingly, none of the assays included in this study has achieved more than 90% coverage. Only 17% (9/53) of the qPCR assays achieved ≥80% coverage, whereas most assays covered less than 20% of the HBV genomes (Figure 2). Those qPCR assays covering less than 20% of the published HBV genomes are likely to underestimate viral load in patient samples. Nevertheless, GenBank entries often do not reflect the actual genotype distribution across various parts of the world. That is, certain countries may sequence more genomes than others owing to better access to financial and scientific resources. Although some assays may appear as having low coverage from our analysis, they could still be efficient in some regions/countries in which only a small number of genotypes are predominant.

Almost all published qPCR assays showed reduction in hybridization efficiency, particularly for HBV minor variants and with the presence of base mismatches. This, together with inadequate coverage in the updated genome sequences, poses a risk of viral load misdetection and underestimation. Moreover, computational prediction of the binding affinity and effects of primer- and probe-template mismatches on PCRs is but a conservative estimate. Experimental evidence has shown that mismatches could result in up to 63% or more than seven cycles of quantification error in qPCRs.[68,69] An earlier study[70] reported certain mismatches reduced overall PCR product yield as much as 20- to 100-fold. Thus, it is essential to design oligonucleotides within the conserved region of the target sequence to avoid amplification inefficiency. Our study indicated that all the nine assays having higher coverage (≥80%) in

TABLE 2 Profiles of qPCR assays and their primer/probe coverage

No.	Source	Year	No. of sequences used for oligo design	qPCR method	Target region	Amplicon length (bp)[a]	Coverage with 1200% identity (%) in 9864 genomes	Remarks
1	Abe et al.[17]	1999		TaqMan	S	174	0 (0)	
					S	241	1239 (12.56)	
					X	331	319 (3.23)	
2	Mercier et al.[18]	1999	22	TaqMan	C	113	6886 (69.81)	
3	Drosten et al.[19]	2000	36	TaqMan	S	92	3203 (32.47)	Multiple primers
4	Guillou et al.[20]	2000		Not specified (commercial assay—Roche Amplicor)	PreC/C	104	345 (3.50)	ELISA compared with commercial assays (Murex & Roche)
5	Pas et al.[21]	2000		TaqMan	S	90	0 (0)	Compared with Digene & Roche
6	Weinberger et al.[22]	2000		TaqMan	S	81	1175 (11.91)	
7	Chen et al.[23]	2001	25	TaqMan	C	119	6362 (64.50)	Comparison between 2 qPCR sets
					C	228	1111 (11.26)	
8	Jardi et al.[24]	2001	44	Hybridization probes	C	138	0 (0)	
9	Zanella et al.[25]	2001	79	TaqMan	S	123	6117 (62.01)	
10	Hennig et al.[26]	2002	65	TaqMan	PreC/C	132	1893 (19.19)	
11	Mitsunaga et al.[27]	2002		TaqMan	C	120	6910 (70.05)	Multiple primers and probes
12	Paraskevis et al.[28]	2002		Hybridization probes	S	153	2413 (24.46)	
13	Pas et al.[29]	2002		TaqMan	S	90	8061 (81.72)	Dual probes. Primers from Pas et al. (2000),[21] probes modified.
14	Aliyu et al.[30]	2004		Hybridization probes	S	259	1 (0.01)	Modified from Yokosuka et al. (1993)[31]
15	Aytay et al.[32]	2004		TaqMan	C	148	2183 (22.13)	
16	Garson et al.[33]	2005	126	TaqMan	S	98	8202 (83.15)	
17	Qian et al.[34]	2005	150	TaqMan	C	80	1185 (12.01)	

TABLE 2 (Continued)

No.	Source	Year	No. of sequences used for oligo design	qPCR method	Target region	Amplicon length (bp)[a]	Coverage with 1200% identity (%) in 9864 genomes	Remarks
18	Zhao et al.[35]	2005		TaqMan	X	331	4809 (48.75)	
19	Lole & Arankalle[36]	2006	60	TaqMan	PreC	109	0 (0)	Dual probes
20	Lu et al.[37]	2006		TaqMan	S	74	1011 (10.25)	S Region showed highest copy number and Cq value
					C	156	984 (9.98)	
					X	93	82 (0.83)	
21	Mazet-Wagner et al.[38]	2006		TaqMan	PreC/C	132	1893 (19.19)	Taken from Hennig et al. (2002)[26]
22	Mendy et al.[39]	2006		SYBR Green	S	98	8519 (86.36)	Primers same as in Garson et al. (2005).[33] Also used in Mendy et al. (2010)[40]
23	Welzel et al.[41]	2006	340	TaqMan	X	84	5421 (54.96)	
24	Cheng at al.[42]	2007		TaqMan	S	184	8424 (85.40)	
25	Liu et al.[43]	2007	27	TaqMan	S	144	3243 (32.88)	
26	Olioso et al.[44]	2007	20	SYBR Green	C	447	6936 (70.32)	Primers same as in Rodrigues et al. (2001)[45]
27	Bayram et al.[46]	2008		TaqMan	S	376	4364 (44.24)	
28	Compston et al.[47]	2008		TaqMan	S	81	1 (0.01)	
29	Pan et al.[48]	2008		TaqMan	X	124	6630 (67.21)	
30	Daniel et al.[49]	2009		TaqMan	S	98	6545 (66.35)	Target same as in Garson et al. (2005),[33] without ambiguous bases
31	Sitnik et al.[50]	2010	24	TaqMan	S	116	8386 (85.02)	
32	Sun et al.[51]	2011	44	Not specified (duplex)	S	81	5780 (57.87)	
					S	117	3220 (32.64)	
							Total 6523 (66.13)	
33	Zhong et al.[52]	2011		TaqMan	S	151	488 (4.95)	Forward primer & probe from Weinberger et al. (2000)[22]
34	Kania et al.[53]	2014	12	TaqMan	X	84	5421 (54.96)	Adapted from Welzel et al. (2006)[41]
					S	98	6545 (66.35)	Adapted from Daniel et al. (2009)[49]

(Continues)

TABLE 2 (Continued)

No.	Source	Year	No. of sequences used for oligo design	qPCR method	Target region	Amplicon length (bp)[a]	Coverage with 1200% identity (%) in 9864 genomes	Remarks
35	dos Santos et al.[54]	2014		TaqMan	PreC	108	7831 (79.39)	
36	Taranta et al.[55]	2014		TaqMan	S	175	8530 (86.48)	
37	Wang et al.[56]	2014	891	TaqMan	S	182	1900 (19.26)	Dual probes (genotype B & non-B)
							6158 (62.43)	
							Total 8058 (81.69)	
38	Huang et al.[57]	2015		ddPCR	X	161	2419 (24.52)	
39	Mu et al.[58]	2015		ddPCR	X	162	75 (0.76)	
40	Aguiar et al.[59]	2016		SYBR Green	S	97	8520 (86.37)	Primers same as in Garson et al. (2005)[33] & Mendy et al. (2006)[39], except for the reverse primer is one nucleotide shorter
41	Prakash et al.[60]	2016	8	TaqMan	S	98	8273 (83.87)	Target same as in Garson et al. (2005)[33] & Daniel et al. (2009),[49] with more ambiguous bases
42	Tang et al.[61]	2016		ddPCR	X	161	2419 (24.52)	Same as in Huang et al. (2015)[57]
43	Liu et al.[62]	2017	1000	EvaGreen (duplex)	S	92	2551 (25.86)	Probe region same as in Garson et al. (2005)[33] with a shorter sequence
					C	120	4979 (50.48)	
							Total 7145 (72.44)	
44	Liu et al.[63]	2017		ddPCR	X	124	6630 (67.21)	
45	Larsson et al.[64]	2018		Not specified	C	90	5755 (58.34)	
46	Portilho et al.[65]	2018		TaqMan	S	78	784 (7.95)	
47	Yang et al.[66]	2018		ddPCR	C	93	1253 (12.70)	

Abbreviations: bp, base pairs; C, core; Cq, quantification cycle, previously known as the threshold cycle (Ct); ddPCR, droplet digital PCR; preC, precore; ELISA, enzyme-linked immunosorbent assay; S, surface.[67]

[a]Calculated based on reference position in a 3215 bp HBV genome (GenBank accession number D00329.1).[16]

TABLE 3 Binding affinity of forward primer from qPCR assays with high coverage

No.	Source	Nucleotide position[a]	Coverage in 9864 genomes (%)	Assay Ta (°C)	Predicted % bound at assay Ta	
					Exact match	Mismatch mean (SD)
1	Pas et al.[29]	182–201	89.69	60	98.20	77.75 (19.32)
2	Garson et al.[33]	379–398	93.14	60	90.10	37.56 (23.96)
3	Mendy et al.[39]	379–398	93.14	55	99.70	92.38 (4.59)
4	Cheng at al.[42]	252–273	94.17	60	97.70	74.78 (31.34)
5	Sitnik et al.[50]	362–382	91.49	60	92.95	43.18 (19.27)
6	Taranta et al.[55]	254–273	94.31	60	86.50	47.33 (42.65)
7	Wang et al.[56]	248–269	96.98	60	91.30	37.35 (23.75)
8	Aguiar et al.[59]	379–398	93.14	58	97.50	64.49 (17.20)
9	Prakash et al.[60]	379–398	93.14	55	99.70	92.38 (4.59)

Abbreviations: bp, base pairs; HBV, hepatitis B virus; SD, standard deviation; Ta, annealing temperature.
[a]Based on a 3215 bp HBV genome, GenBank accession no. D00329.1.[16]

TABLE 4 Binding affinity of probe from qPCR assays with high coverage

No.	Source	Nucleotide position[a]	Coverage in 9864 genomes (%)	Assay Ta (°C)	Predicted % bound at assay Ta	
					Exact match	Mismatch mean (SD)
1	Pas et al.[29]	316–245	98.01	60	99.99	98.78 (1.51)
2	Garson et al.[33]	403–430	95.87	60	100	99.88 (0.16)
3	Mendy et al.[39,b]	-	-	55	-	-
4	Cheng at al.[42]	374–399	92.06	60	100	98.80 (0.63)
5	Sitnik et al.[50]	419–435	97.60	60	1.30	0.66 (0.57)
6	Taranta et al.[55]	374–395	92.35	60	99.40	85.91 (5.92)
7	Wang et al.[56]	311–336	86.35	60	99.95	97.06 (3.17)
8	Aguiar et al.[59,b]	-	-	58	-	-
9	Prakash et al.[60]	403–430	96.37	55	100	100 (0)

Abbreviations: bp, base pairs; HBV, hepatitis B virus; qPCR, quantitative polymerase chain reaction; SD, standard deviation; Ta, annealing temperature.
[a]Based on a 3215 bp HBV genome, GenBank accession no. D00329.1.[16]
[b]SYBR Green assay without a probe.

TABLE 5 Binding affinity of reverse primer from qPCR assays with high coverage

No.	Source	Nucleotide position[a]	Coverage in 9864 genomes (%)	Assay Ta (°C)	Predicted % bound at assay Ta	
					Exact match	Mismatch Mean (SD)
1	Pas et al.[29]	247–271	96.48	60	99.40	86.93 (12.23)
2	Garson et al.[33]	456–476	92.57	60	91.10	40.16 (24.45)
3	Mendy et al.[39]	456–476	92.57	55	99.80	90.27 (14.14)
4	Cheng at al.[42]	414–435	96.88	60	95.30	72.01 (21.05)
5	Sitnik et al.[50]	456–477	94.75	60	98.88	75.25 (15.35)
6	Taranta et al.[55]	410–428	97.97	60	88.70	44.03 (23.50)
7	Wang et al.[56]	410–429	97.36	60	91.50	57.82 (29.15)
8	Aguiar et al.[59]	456–475	92.58	58	96.00	51.48 (24.83)
9	Prakash et al.[60]	456–476	93.30	55	99.85	90.05 (15.19)

Abbreviations: bp, base pairs; HBV, hepatitis B virus; SD, standard deviation; Ta, annealing temperature.
[a]Based on a 3215 bp HBV genome, GenBank accession no. D00329.1.[16]

the updated HBV genome sequences targeted the HBV S gene, suggesting the potential of this region contributing to a robust qPCR diagnostic tool is an area worth investigating. The development of a robust assay will not only ensure efficiency on contemporary isolates but also on emerging novel variants, at least in the near future.

Our analyses showed that the primer and probe set from Taranta et al.[55] is the most conserved, that is, having the highest HBV sequence coverage among all qPCR assays published to date. This is followed by the assays reported by Aguiar et al.[59] and Mendy et al.,[39] which are both using the SYBR Green method. In terms of hybridization efficiency, the oligonucleotide set from Prakash et al.[60] showed the strongest binding affinity with a fairly high coverage. This assay targeted the same region as in Garson et al.[33] and Daniel et al.[49] but with more ambiguous bases, particularly in the probe. Although the predicted binding efficiency of the assay designed by Taranta et al.[55] was relatively lower, its performance may still be improved through further experimental optimisation.

Apart from the qPCR sets included in our study, it is also notable that some commercial qPCR assays were reported to have underestimated viral load in blood samples from patients infected with HBV.[62,71] Attaining accurate HBV viral load measurement is no doubt challenging and is an issue that needs to be seriously addressed. It is crucial in the clinical management of chronic HBV infection for serial monitoring of HBV DNA levels, since consensus clinical practice guidelines advise the commencement or continuation of antiviral treatment for patients whose viral loads have reached certain thresholds. On top of that, effective antiviral treatment is defined as undetectable HBV DNA by a sensitive qPCR assay with a detection limit of 10–12 IU/ml.[3,4,72] At this low detection limit, poorly designed qPCR assays not based on a well conserved region in the HBV genome, or not covering most HBV genotypes and variants could result in false estimations of the viral load, thus leading to misguided treatment decisions, which might compromise proper medical care for individuals suffering from an active chronic HBV infection. Furthermore, HBV nucleic acid testing by qPCR has also become a routine screening for blood donors to prevent post-transfusion infection. Recent studies suggested using more than one primer or probe set to achieve better accuracy and reliability in HBV DNA quantification.[62,73] However, such an approach will inevitably increase the cost of nucleic acid tests and hence, is unlikely to be a practical solution.

It has been proposed that the sequence data of primers and probes in commercial qPCR assays should be made accessible to the public for the investigation of incoherent quantification results.[71] Our analysis raised a similar concern in regard to the accuracy and reliability of routine clinical diagnostic assays that are widely used for estimating viral load and monitoring therapeutic response. Granted that revealing the sequence data may not be feasible, such assays should be upgraded alongside emerging HBV strains over time and validated in respect of its efficiency, at least in silico. If this were not possible as well, production companies should at the minimum evaluate their primer and probe coverage based on the latest HBV genome data deposited in the GenBank nucleotide database.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Conceptualization: Jack Bee Chook; Methodology: Jack Bee Chook and Chye Phing Teh; Investigation: Chye Phing Teh and Jack Bee Chook; Analysis: Chye Phing Teh; Validation: Jack Bee Chook; Writing—original draft preparation: Chye Phing Teh and Jack Bee Chook; Writing—review and editing: Yun Fong Ngeow, Tommy Yuh Koon Tong, Kok Keng Tee, Jan Jin Bong and Rosmawati Mohamed; Visualization: Chye Phing Teh; Supervision: Jack Bee Chook and Rosmawati Mohamed; Project administration: Jack Bee Chook; Funding acquisition: Jack Bee Chook and Jan Jin Bong. All authors approved the final manuscript.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article. The aligned sequence database containing 9864 updated HBV genome sequences in FASTA format is available from the corresponding author upon request.

ORCID

Chye Phing Teh https://orcid.org/0000-0003-1434-6021

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