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Hepatitis B virus infection in post-vaccination South Africa: Occult HBV infection and circulating surface gene variants

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Abstract

Background and objective—The aim of this study was to investigate the prevalence of occult hepatitis B virus (HBV) infection and the HBV surface (S) gene variants circulating in the South African population after nearly two decades of universal hepatitis B vaccination.

Study design—From a previous serosurvey, 201 serum samples with serological evidence of exposure to HBV were identified and these were stratified into post- and pre-vaccine introduction populations. For all samples, HBV DNA was screened and quantified using a real-time PCR assay and results analysed together with HBV serological markers. Where HIV results were available, subset analysis was performed. The HBV S gene was PCR-amplified and sequences analysed for a total of 37 isolates.

Results—The prevalence of occult HBV infection reduced from 70.4% in the pre-vaccine introduction era to 66.0% post-vaccine introduction. There was an association between HIV infection and an increase in prevalence of occult HBV infection within the post-vaccine introduction population, although this was not statistically significant. Furthermore, sequence analysis revealed the following HBV subgenotypes; A1 ($n = 34$), A2 ($n = 2$) and a rare D4 isolate. HBV S gene variants, including diagnostic escape mutants were isolated.

Conclusion—There was a decline in the prevalence of occult HBV infection in post-vaccination South Africa, although the disease burden remains significant in the HIV co-infected population.

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Competing interests

None declared.

Ethical approval

Ethical clearance to conduct this study was approved by the Medunsa Research and Ethics Committee (MREC) (MREC/P/22/2012: PG).

After nearly two decades of a universal hepatitis B vaccination programme, no positive selection of vaccine escape mutants were observed.

Keywords

Hepatitis B vaccine; Occult HBV infection; Diagnostic escape mutants; Subgenotype D4; HIV infection

1. Background

South Africa is hyper-endemic for hepatitis B virus (HBV) infection which is associated with fatal chronic sequelae such as liver cirrhosis, end-stage liver disease and hepatocellular carcinoma (HCC) [21,27]. Prior to hepatitis B vaccine introduction, the burden of hepatitis B in the country was chiefly driven by the high prevalence (>10%) of childhood (<5 years) infections accounting for ~3 million potentially fatal chronic HBV infections [19,44].

In keeping with the World Health Assembly's 1992 recommendation for controlling the hepatitis B burden in hyper-endemic regions, the hepatitis B vaccine was introduced into the South African Expanded Programme on Immunisation (EPI-SA) in April 1995 [37,47]. Subsequent studies have reported a decline in the prevalence of chronic HBV infection since the vaccine's introduction [2,35,36]. What threatens the benefits of vaccination is the emergence of HBV S gene variants due to immune selection pressure from the HBV vaccine [50]. These variants, which may exhibit immune, diagnostic and vaccine escape, can also be transmitted [6,9,49]. While selection of these variants is a very rare event, vaccine escape mutants have been isolated in several countries with longstanding hepatitis B vaccination programmes, such as Taiwan [18,7] and The Gambia [12,46].

It is reasonable to expect the remarkable impact the hepatitis B vaccine has had on the prevalence of chronic HBV infection to be similar on occult HBV infection (OBI) which is also prevalent in South Africa, especially in the human immunodeficiency virus (HIV) infected population [28,25]. Clinically, OBI may be benign but is associated with increased risk of reactivation of HBV infection during immune compromise (for example during HIV infection) and can hasten progression of liver disease and hepatocarcinogenesis [38]. The public health concern is in the risk of transmission of HBV from asymptomatic and misdiagnosed occult HBV infected individuals to susceptible persons [30,38].

OBI is defined as the presence of HBV DNA in the hepatocytes or serum of individuals who test negative for the HBV surface antigen (HBsAg) by current available assays [33]. Various molecular mechanisms leading to the development of OBI have been described, this including mutations within the HBV S gene which may affect HBsAg detectability and result in an occult status [32,34].

2. Objective

The aim of this study was to examine the prevalence of OBI and investigate the HBV S gene variants circulating in a South African population post-vaccine introduction.

3. Study design

From a previous health facility-based hepatitis B serosurvey [2], 201 serum samples which tested positive for serological markers of exposure to HBV; HBsAg and/or antibody to HBV core antigen (anti-HBc), were identified out of a total sample size of 1206. These serologically exposed samples were the focus of this study. The serum samples were stratified by age based on the year (1995) the hepatitis B vaccine was introduced into EPI-SA, into a post-vaccine introduction population (POVP, 1–16 year olds) and a pre-vaccine introduction population (PRVP, 17–25 year olds). Where HIV results were available, a subset analysis of results was performed. The hepatitis B vaccination status of patients was not collected for the overarching study so as to mimic population-based nationwide hepatitis B serosurveys.

3.1. HBV DNA testing

Viral nucleic acid was extracted from 150 µl of each of the 201 serum samples using the Nucleospin® Viral Nucleic Acid Isolation kit (Macherey-Nagel, Düren, Germany). These extracts were screened for HBV DNA using an in-house quantitative real-time PCR (qPCR) assay (5X HOT FIREPol® EvaGreen® qPCR Mix Plus, Solis BioDyne, Estonia) performed on the LightCycler® 2.0 Real-Time PCR System (Roche, Germany). Primers designed to target the HBV basal core promoter (BCP) region (BcP1:5'-ACCACCAAATGCCCCCTAT-3' and BcP2:5'-TTCTGCGACGCGGCCGA-3') [17] were employed. A conversion factor of 2.8 copies/ml to IU/ml was used for all viral loads determined [31]. To avoid cross-contamination, physically separated rooms were used for HBV DNA extraction, qPCR master mix preparation and loading of the template. The possibility of false positives was excluded by performing conventional nested PCR on selected qPCR positive samples with varying viral loads (between 10² and 10⁴ copies/ml) and analysing the sequenced amplicons.

4. HBV S gene amplification and sequencing

For amplification of HBV DNA in the S region, a conventional nested PCR assay was performed using two sets of primer pairs (first round: S1 = 5'-CCTGCTGGTGGCTCCAGTTC-3', S2Na = 5'-CCACCATTCK(G/T)TTGACATACTTTCCA-3' and second round: S6Bs = 5'-GATCCGAGGACTGGGGAC-3', S7Ps = 5'-GGTTAGGGTTTAAATGTATAC-3') in a two-step reaction for all HBV DNA positive samples with viral loads >35.7 IU/ml (detection limit by this PCR assay). The cycling conditions used for amplification of the S gene were as outlined previously by Mphahlele et al. [28]. Confirmation of the desired amplicon (~681 bp) was performed by gel electrophoresis on a 2% agarose gel. Direct sequencing was then carried out with the ABI3500XL Genetic Analyzer (Applied Biosystems, CA, USA), using primers S6Bs and S7Ps.

4.1. Phylogenetic analysis of HBV S gene sequences

All sequences were edited and aligned using ChromasPro 1.49Beta (Technelysium Pty. Ltd.) and BioEdit Sequence Alignment Editor [15] respectively, and then analysed for both

nucleotide and amino acid variations. For phylogenetic analysis, representative sequences from different HBV subgenotypes were retrieved from the GenBank database for comparison with study sequences. All alignments were performed using the neighbour-joining method implemented in Clustal X [41]. The statistical robustness and reliability of the branching order was confirmed by bootstrap analysis using 1000 replicates [10]. Phylogenetic inference was also performed using a Bayesian Markov Chain Monte Carlo (MCMC) approach as implemented in the BEAST v1.8.0 program [8].

5. Results

5.1. Sample population

Serum samples ($N = 201$) were collected from various health facilities from five of the nine provinces in South Africa; Gauteng (49.8% [$n = 100$]), North West (40.3% [$n = 81$]), Mpumalanga (6.0% [$n = 12$]), Limpopo (3.5% [$n = 7$]), and the Northern Cape (0.5% [$n = 1$]). Of the 201 serum samples, 62 (30.8%) were classified by age into the POVP and 139 (69.2%) into the PRVP based on national introduction of the vaccine in 1995 (Table 1). The demographic background of the sample population has been described previously [2].

5.2. Prevalence of OBI

While 17.3% (24/139) and 14.5% (9/62) of the PRVP and POVP respectively were HBsAg positive, the majority of both populations were HBsAg-negative (Table 1). This proportion of HBsAg negative samples served as the population for evaluation of the prevalence of OBI. HBV DNA was detectable in 70.4% (81/115) and 66.0% (35/53) of the HBsAg negative PRVP and POVP respectively, with mean viral loads of 336.42 IU/ml and 356.07 IU/ml. Of the populations with OBI, 37.0% (30/81) of the PRVP and 40.0% (14/35) of the POVP had the isolated anti-HBc serological profile (Table 1).

Overall, HIV results were available for 34/139 (24.5%) and 23/62 (37.1%) of the PRVP and POVP respectively, with 70.6% (24/34) and 56.5% (13/23) being HIV positive. There was an association between HIV infection and an increase in the prevalence of OBI within the POVP (91.7% [11/12] in the HIV positive versus 70.0% [7/10] in the HIV negative subsets) although this was not statistically significant (Table 2).

5.3. Molecular analysis of the HBV S gene

Of the total 201 serum samples screened by qPCR for HBV DNA, 149 had detectable levels of HBV DNA. From the 149 HBV DNA positive samples, 47 (30 PRVP and 17 POVP samples) were selected for HBV S gene amplification based on viral loads >35.7 IU/ml (detection limit by the PCR assay). The HBV S gene was successfully amplified and sequenced for 37/47 isolates; 28 in the PRVP (24 HBsAg positive and 4 HBsAg negative) and 9 in the POVP (8 HBsAg positive and 1 HBsAg negative). Phylogenetic analysis showed the following subgenotypes; A1 (34), A2 (2) and a rare D4 isolate (Fig. 1).

A total of 20 different amino acid variations were observed; most notably M103I (PRVP-1082, POVP-0225, POVP-4028 and POVP-4288). The following variations were unique to HBV isolates from OBI cases with viral loads (ranging from 807.14 to 1.09×10^4

IU/ml) comparable to that in overt HBV infections: S45P (PRVP-3106), P70H (POVP-4162) and V168A + P217L (PRVP-5603). Two nonsense mutations were also observed towards the end of the S protein of isolates which remained HBsAg positive: Y200* (isolate POVP-5162) and L206* (PRVP-1082), caused by a stop codon (TAA) inserted by T600A and T647A nucleotide substitutions respectively. The antigenic region of the HBsAg – the Major Hydrophilic Region (MHR, aa110-aa165) containing the viral “α” determinant (aa124-aa147) – was also investigated for amino acid variations. Table 3 shows the variations observed.

6. Discussion

The distinction between the prevalence of OBI in the South African population nearly two decades post-introduction of universal hepatitis B vaccination has not been explored. A previous study conducted in children (aged 1–2 years) from the Eastern Cape province of South Africa determined that HBV DNA detection by a nested PCR assay in HBsAg negative serum samples significantly reduced from 6.5% in the unvaccinated cohort to 0.3% in those with documented proof of vaccination [16]. In the current study, we investigated the prevalence of OBI in a health facility-based population from five of the nine provinces in South Africa, born after the introduction of the hepatitis B vaccine into EPI-SA in 1995. It was found that universal hepatitis B vaccination had a positive impact on the prevalence of OBI, with a decline from 70.4% in the pre-vaccine introduction era to 66.0% in the POVP, although this was not a statistically significant reduction ($p = 0.57$). While the declining OBI trend was in support of results from the Hino et al. [16] study, the high number of OBI cases in the POVP could be explained by the difference in HBV DNA screening procedures, with qPCR assays, as used in this study, generally regarded to be highly sensitive (detection of <10 copies per reaction) compared to conventional nested PCR assays [32]. An alternative explanation could be drawn from a previous study conducted in Germany which proposed that although hepatitis B vaccination positively impacts the burden of overt HBV infection, it may instead support OBI [13]. It cannot be conclusively determined what proportion of the 66.0% POVP with OBI was fully vaccinated as vaccination records were not collected for the purpose of the overarching study, although some inference could be drawn from the current hepatitis B vaccination coverage rate (88%) in the country [48].

The majority of South African studies on OBI have been conducted in HIV infected populations in whom the prevalence of OBI has been shown to range from 22.1% to 88.4% [3–5,11,25,28]. With OBI prevalence rates of 63.6% and 91.7% in the HIV positive subset of the PRVP and POVP respectively, this study reiterates the fact that HIV positive individuals are at high risk for OBI and OBI reactivation in South Africa, even after the introduction of universal hepatitis B vaccination.

The HBV S gene was successfully sequenced for 28 PRVP and 9 POVP isolates. The following unique and previously identified variations could be associated with diagnostic escape properties: S45P (PRVP-3106), P70H (POVP-4162) and V168A + P217L (PRVP-5603). The S45P variation for example, is located within a cytotoxic T lymphocyte epitope at 28aa–51aa of the HBsAg. Variations within this region are associated with HBsAg undetectability [1,40]. The P70H variation found in an isolate from a HBsAg

negative, anti-HBs positive study patient in the POVP has been previously identified in vaccinated infants with a similar serological profile, born to HBsAg positive mothers in China [39]. While the V168A variation has not been shown to alter the antigenicity of HBsAg, the P217L variation is located within a T helper cell epitope at 215aa–223aa of the protein, and has also been linked with diagnostic escape [23,45].

Within the MHR, the majority of variations observed (K122R, N131T and T143S) were neutral substitutions (Table 3). The A159P (POVP-1474) variation has been identified previously in HBV sequences from neighbouring Zimbabwe but its significance to the protein is unknown [14]. The E164D (PRVP-7532) variation has been linked with reduced binding of HBsAg to anti-HBs and is selected for by a Lamivudine resistance-associated mutation (V173L) located within the overlapping polymerase open reading frame[42,43].

Finally, Kramvis and Paraskevis [22] recently used a large dataset of full length HBV reference sequences – those included in this study – to show clear monophyletic groups for subgeno-types A1 and A2, and we noted the same (data not shown). When the reference dataset was trimmed to the size (~681 bp) of the HBV S gene fragment amplified in this study, the ability to distinguish subgenotype A1 from A2 was not as obvious (Fig. 1). Nonetheless, phylogenetic analysis of all 37 HBV S gene study sequences demonstrated the predominant HBV subgenotypes to be A1 (34 isolates) and A2 (2 isolates), in line with South African literature [20]. The HBV genotype D is second to genotype A in terms of predominance in Southern Africa, with the dominant D subgenotype reported to be the D3 [20,29]. However, a recent South African study isolated the subgenotype D4 [26] as was the case in the current study, showing potential importation from other countries like Kenya [24] and Somalia [29] where the D4 subgenotype has been reported.

In conclusion, this study reports a decline in the prevalence of OBI in post-vaccination South Africa. There is a need for further investigations with similar study designs in larger community-based populations in order to further assess the impact of universal hepatitis B vaccination on OBI. While HBV S gene variants including diagnostic escape mutants were identified, selection of vaccine escape mutants following 19 years of a universal hepatitis B vaccination programme remains uncommon to South Africa.

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Abbreviations

HBsAg	hepatitis B surface antigen
Anti-HBs	antibody to hepatitis B surface antigen
Anti-HBc	antibody to hepatitis B core antigen
HBV	hepatitis B virus

HIV

human immunodeficiency virus

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Table 1

Patient demographics and OBI in the HBV serologically exposed PRVP versus POVP.

	PRVP (<i>n</i> = 139)	POVP (<i>n</i> = 62)	<i>p</i> -Value
Mean age (range)	22.7 years (17–25 years)	9.2 year (1–16 years)	–
Gender			
Female	108 (77.7%)	32 (51.6%)	0.0002
Male	30 (21.6%)	29 (46.8%)	0.0002
Unknown	1 (0.7%)	1 (1.6%)	0.5556
HBsAg +ve	24 (17.3%)	9 (14.5%)	0.6269
HBV DNA +ve	24/24 (100.0%)	9/9 (100.0%)	–
Mean viral load (IU/ml)	4.96×10^{34}	1.01×10^{10}	–
HBsAg –ve	115 (82.7%)	53 (85.5%)	0.6269
OBI ^a	81/115 (70.4%)	35/53 (66.0%)	0.5667
Mean viral load (IU/ml)	336.42	356.07	–
Isolated anti-HBc profile	30/81 (37.0%)	14/35 (40.0%)	0.7627

^aOBI indicates HBsAg negative with detectable serum HBV DNA levels.

Table 2

Prevalence of OBI in the HBV-exposed HIV positive versus HIV negative subsets.

	PRVP (n = 34)		p-Value	POVP (n = 23)		p-Value
	HIV +ve (n = 24)	HIV -ve (n = 10)		HIV +ve (n = 13)	HIV -ve (n = 10)	
HBsAg +ve	2 (8.3%)	1 (10.0%)	0.8759	1 (7.7%)	0 (0.0%)	0.3698
HBV DNA +ve	2/2 (100.0%)	1/1 (100.0%)	–	1/1 (100.0%)	–	–
Mean viral load (IU/ml)	5.96×10^{36}	2.07×10^7	–	18.25	–	–
HBsAg -ve	22 (91.7%)	9 (90.0%)	0.8759	12 (92.3%)	10 (100.0%)	0.3698
OBI ^a	14/22 (63.6%)	8/9 (88.9%)	0.1597	11/12 (91.7%)	7/10 (70.0%)	0.1895
Mean viral load (IU/ml)	3.13	1.75	–	67.86	782.14	–
Isolated anti-HBc profile	7/14 (50.0%)	2/8 (25.0%)	0.2513	6/11 (54.5%)	2/7 (28.6%)	0.1421

^a OBI indicates HBsAg negative with detectable serum HBV DNA levels.

Table 3

Amino acid variations identified within the major hydrophilic region (MHR; 110aa–165aa) of the HBsAg.

HBsAg +ve isolates	HBV subgenotype	Wild type amino acids	Amino acid position	Amino acids in study isolates
PRVP-4065	A1	Lysine/Arginine (K/R)	122	Arginine (R)
PRVP-6964	D4	Asparagine/Threonine (N/T)	131	Threonine (T)
PRVP-1177	A1	Threonine/Serine (T/S)	143	Serine (S)
POVP-1474	A1	Alanine/Glycine (A/G)	159	Proline (P)
PRVP-7532	A1	Glutamic acid (E)	164	Aspartic acid (D)