Use of PCR in resolving diagnostic difficulties potentially caused by genetic variation of hepatitis B virus

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Abstract

***Aims*—To assess the relevance of genetic variants of hepatitis B virus (HBV) and to demonstrate the usefulness of the polymerase chain reaction (PCR) in cases of HBV diagnostic difficulty.**

***Methods*—Five serum samples from patients that presented diagnostic difficulty in routine laboratories were sent to a research laboratory for PCR, and if appropriate, S gene sequencing, in vitro expression, and antigenic analysis.**

***Results*—The demonstration of HBV in serum by PCR allowed a definitive diagnosis of current infection. One serum sample with poor reactivity in a diagnostic assay had a minor hepatitis B surface antigen (HBsAg) variant and another with very poor reactivity had multiple variants of HBsAg. Transient HBsAg reactivity was observed in a recently vaccinated patient. A hepatitis Be antigen (HBeAg) false positive reaction was noted in a patient from a well defined risk group for HBV. One patient who was strongly HBsAg/HBeAg positive, but anti-hepatitis B core antibody negative, was viraemic.**

***Conclusions*—PCR may become the gold standard for the diagnosis of current HBV infection. HBV variants are responsible for a proportion of diagnostically difficult cases. Modification of commercial assays is necessary to increase the sensitivity of detection of such variants.**

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Hepatitis B virus (HBV) infection is a cause of significant morbidity and mortality worldwide. In the UK, much effort is expended on screening populations such as pregnant women and health care workers and developing policies to protect at risk groups. Although hepatitis B surface antigen (HBsAg) is used as the primary marker of current infection and thus infectivity, it is well known that only individuals with anti-hepatitis B core antibodies (HBc) can be infectious (because they are viraemic, as measured by HBV DNA). A proportion of these people are infected with variants of HBsAg that are not detected in one or more commercial assays. As both variants and standard viruses can be detected using nucleic acid methods, such as the polymerase chain reaction (PCR), HBsAg detection alone can no longer be considered the gold standard for measurement of current infection.

Variation in the properties of viruses results from evolutionary changes within both populations and individuals. The latter give rise to quasispecies that coexist in an individual. Two sequential factors, semi-random errors in polymerase mediated nucleotide incorporation and selection of the "fittest" of the resulting strains, account for this process. Strains that replicate poorly, or are in some other way defective, fail to survive, whereas strains with enhanced survival have a distinct advantage over the other strains in the viral population.[1] One of the best documented selection forces is the immune system. Escape mutants are strains that can survive in the presence of an effective immune response; in such cases, mutations are usually seen in epitopes associated with virus neutralisation. Variation occurs throughout the HBV genome (reviewed recently).[2 3] Some variants have been associated with a particular clinical outcome—for example, the recent findings of strains linked with fulminant hepatitis.[4 5]

Variants of the surface gene, which encodes HBsAg, can be classified as: natural (class 1) and medically induced (class 2). The latter tends to cluster within neutralising epitopes because of the nature of the selection pressure extended by medical intervention—either immunisation[6] or treatment with antibodies against HBsAg after liver transplantation.[7] Both class 1 and class 2 variants occur in the cluster of epitopes recognised by B cells within the major hydrophilic region of HBsAg (between amino acids 100 and 160).

We are at an early stage in our understanding of the frequency and antigenic importance of HBsAg variants. In immunised infants born to HBeAg positive mothers, a substitution from glycine to arginine at amino acid 145 of HBsAg (Gly145Arg) within the major hydrophilic region is the most common variant seen in those who become infected despite adequate levels of anti-HBsAg antibodies.[6 8–10] It is also the most common variant seen in liver graft recipients whose grafts become infected despite treatment with anti-HBsAg antibodies.[7 11] Yet, it is also a naturally occurring variant[12 13] that has been observed worldwide. As position 145 is within the neutralising epitope cluster, these observations are not surprising. Such genetic variation is relevant to the development of the next generation of commercial

Table 1 Hepatitis B virus serology

HBV marker	Method or name of assay (manufacturer)	Patient 1	2	3	4	5
HBsAg	Uniform II (Organon, Turnhout, Belgium)	< 1.0	ND	ND	ND	ND
	Amerlite HBsAg II (Kodak, Amersham, UK)	ND	11.9	ND	ND	845
	MicroTrak II (Syva, Maidenhead, UK)	ND	< 1.0	ND	ND	ND
	BioELISA (Biokit, Longfield, Kent, UK)	ND	1.8	ND	ND	34.8
	Auszyme (Abbott, Delkenheim, Germany)	ND	ND	2.7*	1.26	ND
	Wellcozyme (Wellcome Diagnostics, Dartford, Kent, UK)	ND	ND	< 1.0	ND	ND
	HBsAg-3 (Ortho, Raritin, New Jersey, USA)	ND	ND	ND	1.13	ND
	HBsAg GE14 (Murex, Dartford, Kent, UK)	ND	ND	ND	< 1.0	ND
	Radioimmunoassay	ND	ND	+	ND	ND
	Reverse passive haemagglutination assay	ND	ND	+	ND	+
anti-HBs	Amerlite	71 mIU/ml	ND	ND	ND	ND
HBeAg	Enzyme immunoassay	1.58†	< 1.0	7.9	< 1.0	21.7
anti-HBe	Enzyme immunoassay	1.58†	10.7	< 1.0	ND	< 1.0
anti-HBc total	Amerlite	2.2‡	ND	ND	ND	ND
	Biokit	ND	ND	ND	ND	< 1.0
	Murex	ND	1.5	ND	< 1.0	< 1.0
	Corzyme (Abbott)	ND	ND	+	ND	ND
anti-HBc IgM	Enzyme immunoassay	< 1.0	< 1.0	ND	ND	< 1.0

Methods are enzyme immunoassays unless otherwise stated. Amerlite HBsAg II, Auszyme, Wellcozyme, and HBsAg-3 HBsAg assays use monoclonal antibodies in both capture and detection stages; BioELISA, radioimmunoassay, and reverse passive haemagglutination assay use polyclonal antibodies only; UniformII and HBsAgGE14 use both polyclonal and monoclonal antibodies in their assay design.
Values are signal to cut off where values greater than 1.0 are positive.
*Sample strongly reactive in a modified version of this assay.
†In patient 1 values < 0.45 were positive for anti-HBe and values > 1.3 were positive for HBeAg (Amerlite assay).
‡Cut off < 18, so patient 1 strongly positive for anti-HBc.
+, indicates positive result.
ND, test not performed on this sample.

diagnostic assays for the detection of HBsAgs. As these depend on the interaction of HBsAgs with anti-HBsAg antibodies, the antibodies chosen by each manufacturer dictate the ability of that assay to detect antigenic variants. Both the absolute binding between these two ligands and the ability to bind to low concentrations of variant HBsAgs are important. The sensitivity of many assays for Gly145Arg is poor.[3 14] Other variants, such as amino acid insertions in the major hydrophilic region,[12 13 15] are even more antigenically diverse. These considerations have potentially major implications for diagnostic laboratories, for blood transfusion services, and for defining infectivity status in occupational situations.

Here, we describe five cases of potential or confirmed HBV infection, that gave difficulties in interpretation in routine diagnostic laboratories in the UK. Evaluation in a research laboratory, using PCR, sequencing, and antigenic analysis of the in vitro expressed HBsAgs, demonstrated that PCR should be more widely available to routine laboratories and that there were varied explanations for the diagnostic difficulties.

Materials and methods

CASE HISTORIES

Patient 1 was a 35 year old homosexual man who was a previous drug user. In two separate blood samples, his serum was negative for HBsAg, positive for anti-HBsAg antibodies, and weakly positive for both anti-HBc antibodies and HBeAg (table 1). He was positive for antihepatitis C virus (HCV) antibodies, HCV RNA positive, and human immunodefiency virus (HIV) negative.

Patient 2 was a 24 year old man from Hong Kong whose serum had been reported positive for HBsAg by the Hong Kong Blood Transfusion Service. He did not have clinical features of liver disease nor any history of exposure to drugs or sexual intercourse that would make him at risk of HBV infection. He was strongly positive for HBsAg in a monoclonal antibody based assay, but weakly positive in a polyclonal antibody based assay, and negative in a third assay; his serum was also negative for HBeAg, positive for anti-HBe antibodies, positive for total anti-HBc antibodies, and negative for anti-HBc IgM (table 1).

Patient 3 was a 42 year old man with chronic HBV infection who had received a renal transplant. The route and timing of infection were unknown. Detection of HBsAg was discrepant using two monoclonal antibody based enzyme immunoassays (EIA) but was positive in a polyclonal antibody based reverse passive haemagglutination assay (RPHA) (table 1). HBeAg was present in the serum. HBV virions and HBsAg spheres were seen with electron microscopy.

Patient 4 was a male blood donor who presented with a repeatably weakly reactive HBsAg screen results with two monoclonal assays, confirmed by a specific neutralisation test, but negative in a third, polyclonal capture, and monoclonal detection based assay. However, anti-HBc antibodies and HBeAg were negative (table 1). He had been given a dose of HBV vaccine five days previous to the donation.

Patient 5 was a 28 year old man from Botswana who was positive for anti-HIV antibodies. He was clinically well apart from recent multidermatomal shingles from which he recovered uneventfully. On routine testing, he was strongly reactive for HBsAg and HBeAg but negative for anti-HBc antibodies by two assays (table 1). HBsAg was neutralised, but only after serum dilution, as the HBsAg titre was high.

POLYMERASE CHAIN REACTION AND SEQUENCING

A standard PCR was performed to amplify the surface gene of HBV. The amplified DNA was sequenced using the Sequenase Version 2.0 DNA sequencing kit (USB, Ohio, USA; method and primer sequences available on request).

CLONING AND EXPRESSION OF HBsAg

The PCR products from patients 2 and 3 were cloned into the plasmid pRK5. This plasmid, with or without the HBV surface gene, was introduced by transfection into mammalian cells (COS 7); plasmid pRK5 containing the standard HBV DNA S gene (adr subtype) was used as a control for transfection and antigenic analysis. Forty eight hours after transfection,

Table 2 Diagnostic assay reactivity with expressed HBsAg

Cloned sequence	*Concentration*	*Auszyme (Abbott)*	*Ausria (Abbott)*	*Axsym (Abbott)*	*HBV 3.0 (Ortho)*	*HBV GE14 (Murex)*
adr control	1×	> 69.0	96.0	49.8	> 41.7	29.3
	5×	> 69.0	136.7	75.2	> 41.7	> 29.4
Patient 2	1×	20.6	3.8	4.0	14.4	6.8
	5×	64.9	14.9	13.4	> 41.7	27.1
Patient 3	1×	> 69.0	8.7	31.4	< 1.0	< 1.0
	5×	> 69.0	20.2	43.6	< 1.0	1.9
Capture antibody		Mouse mAb	Guinea pig pAb	Mouse mAb	Mouse mAb	Goat pAb
Detection antibody		Mouse mAb	Human pAb	Goat pAb	Mouse mAb	Mouse mAb

Values are signal to cut off where a value > 1.0 is positive.
> indicates that this was the highest value recordable in this assay.
mAb, monoclonal antibody; pAb, polyclonal antibody.

the culture media were harvested and coverslips collected for immunofluorescence analysis.

ANTIGENIC ANALYSIS OF EXPRESSED HBSAG

Immunofluorescence

COS 7 cells on the coverslips were fixed with methanol, washed and incubated first with goat anti-HBsAg polyclonal antibody, (Dako Ltd, High Wycombe, Bucks, UK) or mouse anti-HBsAg monoclonal antibodies H166, H57, H35, H95, H10, H53, and H12 for 60 minutes at room temperature. After washing, the cells were incubated with secondary antibody, either antigoat or antimouse FITC labelled immunoglobulin (IgG or IgM), for 30 minutes at room temperature. Monoclonal antibodies were a gift from Abbott Laboratories (North Chicago, Illinois, USA); their characteristics have been described previously by Peterson *et al.*[16]

Diagnostic assay reactivity

An aliquot of the collected supernatant was concentrated using Vivaspin membranes (Vivascience Ltd, Lincoln, UK) according to the manufacturer's instructions. The neat (1×) and concentrated (5×) samples were assayed using various commercially available diagnostic kits, according to manufacturers' instructions. A variety of monoclonal and polyclonal reagents are used in the kits (table 2).

Results

PCR AND SEQUENCING

Patients 2, 3, and 5 were HBV DNA positive; HBV DNA could not be amplified in the sera from patients 1 and 4. Amino acid sequences of the S region of HBV DNA were generated from patients 2 and 3. There was no suspicion of HBsAg variant sequence in patient 5 as HBsAg was strongly positive. A previously undescribed single amino acid substitution was observed at amino acid 156 (Trp156Leu) in patient 2. Patient 3 had a highly variant sequence with 11 amino acid substitutions: Tyr100Ser, Thr118Val, Lys133Ile, Phe134Asn, Pro142Ser, Thr143Leu, Gly145Lys, Leu175Ser, Pro178Leu, Gly185Asp, and Leu186Arg. Seven of these substitutions were located within the major hydrophilic region.

IMMUNOFLUORESCENCE

Results of the binding of anti-HBsAg antibodies to transfected COS 7 cells expressing HBsAgs from patients 2 and 3 are given in table 3. Monoclonal antibody H166,[17] which recognises a small loop (Cys121–Cys124) in the major hydrophilic region, reacted with both expressed HBsAgs. In contrast, a "y" determinant subtype specific anti-HBsAg monoclonal antibody, H10,[16] did not react with any expressed HBsAgs, indicating the presence of the "d" determinant containing subtype. Cells transfected with a cloned sequence from patient 2 expressed an HBsAg that reacted with all anti-HBsAg monoclonal antibodies except for H10, but reactivity was less than that of the control for all of the polyclonal anti-HBsAgs and monoclonal antibody H35. Expressed HBsAg from patient 3 could not be detected with anti-HBsAg monoclonal antibodies H57, H35, H10, and H12. With the exception of H166, antibody recognition of HBsAgs from patient 3 was weaker than that of the control HBsAgs.

DIAGNOSTIC ASSAY REACTIVITY

The reactivity of neat HBsAg containing supernatant from patient 2, although positive, was lower than that of the control for all assays (table 2). However, by concentrating the supernatant, reactivity was increased in all tests, indicating that the sensitivity of the assays was lower for this variant than for the control HBsAg. Note that the concentrated supernatant saturated the binding capacity of the Auszyme, Ortho, and Murex kits, but remained low in Axsym and Ausria assays.

Two assays (Ortho HBV 3.0 and Murex HBV EIA) did not react with the expressed HBsAg from patient 3. Reactivity in two assays (Ausria and Axsym) increased on concentrating the samples (table 2), whereas reactivity in the Auszyme was saturated in the neat sample.

Table 3 Binding of anti-HBs to transfected cells expressing HBsAg from patients 2 and 3 measured by immunofluorescence

		Cloned sequence	
Primary antibody	*adr control*	*Patient 2*	*Patient 3*
pAb anti-HBs	+++	++	+
mAb H166	+++	+++	+++
mAb H57	+	+/++	–
mAb H35	++	+	–
mAb H95	++	++	+
mAb H10	–	–	–
mAb H53	++	++	+
mAb H12	++	+/++	–

The degree of binding—that is, the avidity of anti-HBs for expressed HBsAg, was classified as high (+++), moderate (++), low (+), or negative (–), according to the intensity of fluorescence within a number of transfected cells.
mAb, monoclonal antibody; pAb, polyclonal antibody.

Discussion

This study demonstrates the value of PCR as a confirmatory test for current HBV infection. Such assays are no longer research tools, yet their widespread introduction continues to come up against this psychological barrier. The cost is often considered an impediment, but it is clear that the unit cost reduces dramatically with increased use. Second, although these five cases presented diagnostic problems that were potentially a result of variants within different regions of the HBV genome, they had, in fact, diverse explanations. The clinical importance of HBsAg variants is increasingly recognised, such that most, if not all, diagnostic kit manufacturers are actively assessing the choice of monoclonal antibody pools and the sensitivity of their kits, with a view to changing the format if required. With the increasingly stringent demands on the blood transfusion services and the mounting campaign to eradicate HBV, this issue will increase in importance.

In the first case, serum was negative for HBV DNA by PCR; consequently, the sample showed false seropositivity for HBeAg. The titre of HBeAg was very low and, in reference laboratories, the HBeAg would have been neutralised to confirm the specificity of the result. However, at the time, this case gave cause for concern in a Regional Virus Laboratory (which does not offer a neutralisation test of HBeAg) and therefore further investigations were required to confirm that HBeAg was truly negative.

For patient 2, we believe that the serological diagnostic difficulty resulted from the insensitivity of an assay for a minor HBsAg variant. A single, previously undescribed, substitution at amino acid 156 was found in patient 2 within an exposed loop of the major hydrophilic region[17]; as we demonstrated in vitro, this did affect antigenicity. Expression of the HBsAg protein in an in vitro cell system was performed to investigate whether low level HBsAg production or poor antigenicity of a minor variant was the problem in this patient. HBsAg produced in vitro from patient 2 bound to the same range of anti-HBsAg antibodies as HBsAg from control HBV DNA (by immunofluorescence), but there was decreased intensity of staining with polyclonal anti-HBsAg reagents and monoclonal antibody H35. The signal to cut off values of expressed HBsAg from patient 2 were increased at least threefold in all diagnostic assays by concentrating the culture supernatant. The corresponding values from the control sequence plasmid were not signficantly increased by concentration. As it seems unlikely that the efficiency of production of HBsAg from these otherwise identical plasmids should be any different, the rise in reactivity seen after concentration probably reflected initial poor binding between the two ligands. Reactivity in three assays became comparable with that of control HBsAg after concentration. The low reactivity in the other two assays was increased, although still low, after concentration. This remaining differential lends support to our conclusion that the serum from case 2 not only had a variant that was poorly detected, but also had a low absolute amount of HBsAg. This pattern of reactivity illustrates that it is not only absolute binding between anti-HBsAg reagents in the kit and variant HBsAgs from the patient's serum that detemines the sensitivity of an assay, but also the ability to detect low levels of variant antigen. Only recently has this concept been appreciated fully. In a recent study,[18] 5% of individuals positive for HBsAg by a polyclonal antibody based assay in Papua New Guinea were negative by a widely used monoclonal antibody based kit. Some of these samples had variants in the exposed loop of the major hydrophilic region from amino acid 154–157, but others were explained only by low HBsAg concentrations.

Samples may contain major HBsAg variants that fail to bind to anti-HBsAg antibodies in multiple assays; in these circumstances, absolute antigen concentrations probably have little effect on sensitivity. Patient 3 had 11 amino acid substitutions in the S gene, of which seven were located within the major hydrophilic region; clearly, these are sufficient to alter the three dimensional structure of the HBsAg and to affect its recognition properties by several antibodies. In vitro expressed HBsAg protein from patient 3 only reacted well with monoclonal antibody H166: H166 recognises the amino acid 121–124 loop of HBsAg as a continuous epitope.[17] The H166 epitope does not appear to be affected by conformational changes of the molecule and no amino acid substitution was found within this epitope. Diagnostic assay reactivity also corresponded with sequence variability. Two assays were non-reactive and a third (the polyclonal test) was very poorly reactive. Reactivity was still very low or not evident after concentration, suggesting that variable reactivity was not related to low amounts of HBsAg in the serum. It was surprising to find that the polyclonal Ausria assay result was weaker in sample 3 than some of the assays using monoclonal antibody technology, suggesting that the polyclonal antibody based assays are not always better for the detection of HBsAg variants. In addition, the different specificities of the monoclonal antibodies included in test kits is important, because we observed a difference in performance between the monoclonal antibody based tests.

There was a dicrepancy between HBsAg produced in vitro, which reacted strongly with one monoclonal assay (Auszyme), and HBsAg in patient 3's serum, which was only weakly reactive initially, but saturated the assay when the assay was modified to include an extra wash step. It may be that an inhibitory factor was present in the serum (but not in the cell culture derived HBsAg preparation) and that this factor affected binding; in addition, the antibodies used in this test may have changed since the serum sample was first tested 10 years previously.

Recently, lamivudine treated patients have been shown to select variants in the polymerase gene.[19] As this gene overlaps with HBsAg

coding sequences, the resulting HBsAg variants are a potential source of diagnostic problems.

The HBsAg result from patient 4 was genuine as it was confirmed positive in the Auszyme assay by specific neutralisation; there are three possible scenarios that could explain these results: (1) the donor was at a very early stage of an acute HBV episode; (2) the donor sample had been contaminated, which is unlikely as no other HBsAg positive samples had been detected during the same test run; or (3) the donor had been vaccinated recently with HBsAg. As it transpired, the third possibility was correct. This circumstance, although rare, has been described before[20] and it reflects the very high sensitivity of current assays for standard HBsAg sequences.

Case 5 highlights one of the problems of using anti-HBc antibody detection as a first line screen to detect current HBV infection. Because the anti-HBc assay was negative, this case would have been missed (without further testing) even though the patient had an exceptionally high concentration of HBsAg. There have been other reports of anti-HBc antibody negativity,[21] but no satisfactory explanation has been provided. It is possible that alterations occur within the sequence of the core protein containing the B and T cell epitopes which stimulate production of anti-HBc antibodies. However, not all gene sequences generated in such cases support this hypothesis.[21] The X gene (which helps to control transcription/replication of the virus) may be defective, but quite how this would lead to high concentrations of virus (and supposedly HBcAg) yet no anti-HBc antibody is not clear. Alternative explanations include immunodeficiency (this case was HIV positive but with good immune function) or a congenital inability to mount a humoral response to HBcAg, perhaps mediated by the human leucocyte antigen (HLA) system.

A further serological profile that has been linked to HBV variants is isolated anti-HBc (that is, HBsAg and anti-HBsAg antibody negative) reactivity. At least 10% of such individuals are PCR positive. In some cases, this profile is due to HBsAg antigenic variants,[18] but in others it appears that virus replication is very low, resulting in HBsAg concentrations below the sensitivity of current assays. An association between this pattern of productivity and variability in hepatitis B X protein and the enhancer/promoter regions of the genome (which may dampen down replication efficiency) has also been described.[22]

Further molecular investigations of the diagnostic dilemmas presented by these five cases illustrate graphically the complex phenomena that may underline patterns of reactivity to HBV antigens. Genetic variants of HBV may be significant in a small percentage of cases and it is wise to be aware of these. Although current commercial assays are very good, there is clearly room for improvement and the value of PCR as a confirmatory assay has been demonstrated. However, current PCR protocols and their performances are not standardised for general use, so diagnostic dilemmas should still be referred to specialist units.

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