Atypical serological profiles in hepatitis B virus infection

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Abstract During hepatitis B virus (HBV) infection, at least four antigen–antibody systems are observed: HBsAg and anti-HBs; preS antigen and anti-preS antibody; HBcAg and anti-HBc; and HBeAg and anti-HBe. Through the examination of these antigen–antibody systems, hepatitis B infection is diagnosed and the course of the disorder may be observed. Although the serologic findings that allow both the diagnosis of HBV infection as well as assessing of its clinical course are already well established, the dynamics of viral proteins expression and of the antibodies production may vary during the infection natural course. This causes the HBV infection to be occasionally associated with the presence of uncommon serological profiles, which could lead to doubts in the interpretation of results or suspicion of a serological result being incorrect. This paper is dedicated to the discussion of some of these profiles and their significance.

Introduction

The natural course of hepatitis B virus (HBV) infection is determined by the inter-relationship between viral replication via HBV protein production and the host’s immune response, and, therefore, clinical practice diagnosis of HBV infection is established by the serological detection of HBV protein products (antigens) as well as host-produced antibodies. During HBV infection, four structural antigen–antibody systems are observed: hepatitis B surface antigen (HBsAg) and its antibody (anti-HBs); the preS antigens associated with HBsAg particles and their antibodies; the particulate nucleocapsid antigen (HBcAg) and anti-HBc; and an antigen structurally related to HBcAg, namely, hepatitis B e antigen (HBeAg) and its antibody (anti-HBe). Through the examination of these antigen–antibody systems, hepatitis B infection is diagnosed and the course of the disorder may be observed.

Classically, 8–9 weeks following the infection, in the incubation period or 3–5 weeks before biochemical evidence of liver dysfunction and the appearance of clinical symptoms, it is possible to detect HBsAg in serum. HBsAg detection is used for the diagnosis of acute and chronic HBV and indicates potential infectiousness. In patients who subsequently recover from HBV infection, HBsAg usually becomes undetectable after 4–6 months [1]. If HBsAg persists for more than 6 months, spontaneous clearance is very improbable and the infected individual is considered to be a chronic HBV carrier [2].

However, during the acute phase of infection, following HBsAg detection, other viral markers can be easily detectable, including DNA polymerase and HBeAg. HBeAg appears shortly after the appearance of HBsAg and disappears within several weeks as acute hepatitis resolves. Its presence in the serum correlates with the presence of viral replication in the liver and HBsAg detection, while its disappearance, associated with the anti-HBe detection, is seen as a sign of the absence of viral replication and spontaneous resolution of acute infection.

Anti-HBc IgM antibodies are detectable at the outset of clinical disease, and, as the infection evolves, IgM anti-HBc levels gradually decline, often becoming undetectable
within 6 months and IgG class predominates, remaining for a long period of time (sometimes life-long) at detectable levels [2]. The presence of IgM anti-HBc usually indicates recent or continuous HBV replication, while the presence of IgG anti-HBc correlates with past infection [3].

As recovery and convalescence signal, antibodies to other viral proteins appear in the blood. Anti-HBe antibodies appear after HBeAg clearance, and may persist for many years after the resolution of acute HBV infection. In some cases, the phenomenon of “immunological window” of the HBe-anti-HBe system can be observed, with anti-HBe antibody detection about 2 months after the disappearance of its antigen precursor.

The antibody response to hepatitis B surface antigen (anti-HBs) becomes detectable in convalescence, from 6 weeks to 6 months after HBsAg clearance, although it is produced early in the course of an acute infection. Its detection indicates infection recovery. The antibodies to HBsAg are able to neutralize HBV infectivity and, therefore, clear circulating HBsAg and infectious HBV particles from peripheral blood. These neutralizing antibodies are especially important in the prevention of viral infection, since they could prevent viral attachment and entry into the cells by absorption of the viral particles [3]. Its presence is considered to be an indicator of immunity to HBV infection, although it could become undetectable in patients who have recovered fully from infection (for a review, see [4]). Table 1 shows the dynamics of the expression of HBV serological markers, the corresponding antibodies, and the interpretation of the profile, in accordance with the evolution in an typical serological profile, during the natural course of infection.

Currently, the most accurate tool available for the diagnosis of HBV infection is DNA detection, usually obtained by genomic amplification assays. However, anti-HBV-specific tests are used routinely: (i) to assess disease activity in persistent infection; (ii) in monitoring therapeutic regimens with antiviral agents, and, especially, (iii) in an attempt to diagnose the presence of infection and its development, according to the dynamics described above. Although the serologic findings that allow both the diagnosis of HBV infection as well as assessing its clinical course are already well established, the dynamics of viral proteins expression and of the antibodies production may vary during the infection natural course, due to factors related to the agent (infection by a new strain or viral serotype—antigenic variant) and/or to the host (immune tolerance, cellular immune response, and immunosuppression) [5–7]. This causes the HBV infection to be occasionally associated with the presence of uncommon serological profiles, which can lead to doubts in the interpretation of results or suspicion of an error in the serological diagnosis. The purpose of this paper is to discuss the atypical profiles most frequently found in HBV serology.

Profile 1

Isolated positivity for HBsAg and detectable DNA

\[ HBsAg [+] \; ; \; HBeAg [+/-] ; \; \text{anti-HBc} [-] ; \; \text{anti-HBe} [-] ; \; \text{anti-HBs} [-] ; \; \text{DNA/HBV} [+] \]

The isolated positivity for HBsAg comprises a finding often observed in HBV serology whose ample meaning was discussed recently [8]. Accordingly, this serological profile, associated with DNA detection in the bloodstream, suggests, initially, acute infection, which is a consequence of recent exposure to HBV, and this hypothesis, in most cases, can be confirmed after the subsequent expression of other markers detected by laboratory testing. However, the persistent isolated positivity for HBsAg and detectable DNA, in the absence of other markers, comprises an atypical serological profile, whose presence may indicate immune tolerance to HBV core antigen (an abnormality of the host’s immune system) or

<table>
<thead>
<tr>
<th>HBsAg</th>
<th>HBeAg</th>
<th>HBeAg IgM</th>
<th>HBcAg IgG</th>
<th>Anti-HBe</th>
<th>Anti-HBs</th>
<th>Interpretation</th>
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<td>NEG</td>
<td>NEG</td>
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<td>NEG</td>
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<td>POS</td>
<td>NEG</td>
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<td>Acute infection</td>
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<td>POS</td>
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<td>HBV infection (acute or chronic HBV infection)</td>
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<td>NEG/POS</td>
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<td>Chronic HBV infection or end of recent infection</td>
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<td>NEG</td>
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<td>Recent HBV infection. Beginning of the convalescence period</td>
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<td>POS</td>
<td>NEG</td>
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<td>Past HBV infection (immunological window)</td>
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<td>Immune, recent past infection</td>
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<td>Immune, past infection (old infection)</td>
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<td>NEG</td>
<td>POS</td>
<td>Immune, contact HBsAg, vaccine response</td>
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<td>NEG</td>
<td>NEG</td>
<td>NEG</td>
<td>Susceptible individual. Never had contact with HBV</td>
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</table>
suggest infection by an HBV mutant, factors that can compromise anti-HBc antibodies production.

The immune tolerance to HBeAg is known by the incapacity of the individual to produce anti-HBc or to produce it in undetectable levels. The immune tolerance can be mediated by a variety of mechanisms. For example, T-cell anergy is a tolerance mechanism in which the T cell is functionally inactivated following an initial antigen encounter, but remains alive in a hypoactivated state [9]. T-cell anergy has been proposed as a major mechanism for the maintenance of self-tolerance and regulation of the cellular immune response [10]. In addition, a small number of specific peripheral T lymphocytes, an inefficient antigen presentation, or lymphokine production by the antigen-presenting cell are, among others, possibilities associated with anti-HBc production [11]. Therefore, a selective immune system defect could lead the immune tolerance to the HBc and, consequently, the lack of production of corresponding antibody, justifying, therefore, the existence of this serological profile [12, 13].

The phenomenon of the immune tolerance to HBc antigen has also been associated with the vertical transmission of infection, and, consequently, the absence of an antibody response to HBc in infants born to HBe-positive carrier mothers [14]. It has been postulated that HBeAg may have an immunoregulatory function in promoting viral persistence [15]. The HBcAg and HBeAg are distinctly recognized by antibodies but, due to their extensive amino acid homology, are highly cross-reactive at the T-cell level [15]. Thus, HBe antigen, because of its small size, may traverse the placenta and elicit HBe/HBcAg-specific T-helper cell tolerance in utero [16]. Consequently, it may lead to fetal immunotolerance not only to HBeAg but also to HBcAg, and still inhibit anti-HBc antibody production when it is present in the serum.

As mentioned earlier, it is possible that agent-related factors can also justify the anti-HBc non-production by the host and contribute to the expression of this serological profile. Among them include infection by HBV which produces a non-responsive HBc [17, 18], therefore, unable to induce an immune response, or by HBV mutant with nucleotide sequence deletions in the core gene. These deletions may involve important epitopes to antigen recognition by immune response cells, and, therefore, compromise the HBc antigenicity, becoming a non-functional protein [19, 20].

The presence or absence of HBeAg in this profile may be inconsistent or even vary from case to case, since its detection (or not) will be associated with the mechanism that justifies the profile in question. For example, in the case of immune tolerance specific to c antigen or due to vertical transmission [12, 13], the HBeAg detection in the profile it seems likely, since, in these cases, there is no compromising of the viral antigens expression (the preC/C regions remain unchanged, with normal synthesis of viral antigen). On the other hand, following the occurrence of core gene deletions, depending on the region involved, the HBeAg expression may become compromised, since both antigens are encoded by the same gene.

Additionally, it is of great interest, and deserves highlight, the observation of this serological profile in the chronic infection context. Serologically, the chronic HBV infection is characterized by persistence of HBsAg in the bloodstream for more than 6 months, associated with the presence of anti-HBc antibodies and detectable DNA. During the natural course of chronic infection, the spontaneous seroclearance of HBsAg and even of DNA can occur after antiviral therapy, but anti-HBc antibodies remain detectable continually in peripheral blood [21]. However, in some circumstances, the presence of anti-HBc antibodies may not be observed in this clinical context, but only isolated HBsAg positivity and DNA detectable in the serological profile, comprising an unusual finding in medical practice.

In this case, the serological pattern is justified by immune complexes formation of anti-HBc antibodies with an excess of HBc antigen in the bloodstream, with anti-HBc becoming undetectable [22]. These immune complexes cannot be detected by available commercial tests, which are not able to detect antibodies in the presence of excess antigen in the serum [23]. Anti-HBc complexes could be detected only after immune complex dissociative treatment. Some methods are based on polyethylene glycol precipitation followed by ion chaotropic treatment [24] or based on sucrose gradient fractionation [25]. HBcAg is not a secreted protein and exists primarily in liver and in serum within HBV particles, not being directly accessible in the blood to the immune system. In exceptional cases, HBc epitopes may, however, be exposed to virions present in the serum [26] and also during liver necrosis, when nucleocapsids may be secreted from infected hepatocytes with a high level of HBV particles, explaining the presence of anti-HBc complexes in the bloodstream. This phenomenon has been observed in chronic HBV carriers during the phase of active replication in a state of immunosuppression, as seen in the context of transplantation (bone marrow, kidney, heart), during chemotherapeutical treatment for neoplasias [27], or an uncontrolled human immunodeficiency virus (HIV) infection, conditions which could also lead to an immune system defect. HBcAg is the most immunogenic HBV component and induces the production of high titers of anti-HBc antibodies in immunocompetent individuals who have been exposed to HBV [28]. However, the immunosuppression conditions can potentially induce decreased antibody production [3]. HBcAg-reactive material is released from HBV particles present at a high level in the serum and from hepatocytes undergoing lysis. Consequently, this could complex to low levels of anti-HBc produced in these patients and, therefore, lead to the non-detection of antibodies (Fig. 1).
Occult infection can be defined as the long-lasting persistence of viral genomes in the liver tissue (and, in some cases, also in the serum) of individuals negative for the HBV surface antigen [31]. In many instances, occult hepatitis B is associated with hepatitis B core antibody (anti-HBc) and/or anti-HBs [32]. In this context, the viral genome persistence under conditions not yet elucidated is characterized by a strong suppression of viral replication and expression of its genes, identified only by highly sensitive molecular biology techniques [33]. However, little is known about the molecular mechanism of viral persistence in these cases and the non-detection of HBsAg in this serological/molecular context.

The molecular basis of the occult infection seems to be strictly related to the peculiar life cycle of HBV. In particular, a fundamental step is the conversion of the relaxed circular genome into a covalently closed circular DNA (cccDNA). This comprises a long HBV replicative intermediate that persists in the cell nuclei (hepatocyte) as a stable episome and that serve as the template for mRNAs and RNA pregenomic transcription [34]. During the viral replication cycle, viral transcript is translated in capsid, together with polymerase proteins. After subsequent encapsidation, RNA pregenomic is retranscripted into new partial double-strand viral genomes. The new nuclear capsid containing DNA may be secreted as a complete virion after the attachment of coating protein or, then, may redirect the viral genome to the hepatocyte nucleus to pool the maintenance of cccDNA and to retain permanently infected cells [31, 35, 36]. Thus, considering the stability and long-term persistence of viral cccDNA molecules in the liver tissue, it seems plausible to affirm that, (i) HBV infection, once it has occurred, may possibly continue for life, and (ii) resolution of disease does not imply complete eradication of infection, but, more likely, reflects the capacity of the host’s immune response to restrain HBV and to keep persistent control of its replication [33, 35].

Accordingly, histopathological analysis of the liver by immunohistochemistry in individuals with occult infection has revealed the expression of HBsAg or HbcAg in the liver tissue comparable to those of chronic HBV carriers, despite the signals being lower [37, 38]. Additionally, the presence of extrachromosomal HBV/DNA and pregenomic RNA in the liver and peripheral blood mononuclear cells by molecular techniques [polymerase chain reaction (PCR) and in
situ hybridization] has been demonstrated in some patients, suggesting the maintenance of transcriptional activity, and, therefore, HBV activity [39]. Finally, in the chronic infection context, the persistence of replicative forms of the viral DNA in the hepatocytes nucleus, after HBsAg disappearance (spontaneously or due to a successful treatment), has also been detected by in situ hybridization, despite the inhibition of DNA synthesis by antiviral therapy [40]. These data illustrate that, (i) it is possible that some patients, even after the loss of serological markers of active infection, may still demonstrate a low degree of infection with liver disease development, despite the antibodies presence, and (ii) the persistent infection can occur without the production of viral particles. The frequency with which this occurs is unknown, although there are reports which have indicated that 3 out of every 16 monitored patients after acute infection remain viremic for a period of several years [41, 42].

Even considering the low levels of replication and intrahepatic HBV transcription maintained during the natural history of occult infection in these individuals, a question remains open. What reasons would justify the non-detection/expression of HBsAg in this situation? Two possibilities have been discussed: the antigen may be absent from the peripheral blood or may be present but not detectable. In the first situation, the absence of antigenemia could be due to mutations characterized by deletions in the preS region [43] or in promoter sequences of the HBV genome which would block HBsAg secretion, causing its accumulation inside the cell [38, 44]. In the second situation, the HBsAg may be present but, however, escaping, the recognition by enzyme immunoassays currently standardized, depending on the sensitivity of detection [45]. There is, however, an additional explanation for the non-detection of HBsAg: mutations in regions that code for protein S [46]. Specifically, mutations in the major hydrophilic region of HBsAg, the main target for antibodies used in diagnostic tests. These mutations could lead not only to an escape from recognition by the tests routinely used, but may also reduce the replicative capacity of the virus, reducing the levels of HBsAg, which could compromise its detection in the bloodstream. Bréchot et al. [47], however, have provided strong evidence that most cases of occult HBV is more related to low levels of HBV wild-type (low viral load) than the presence of HBV mutants that do not express surface proteins or that produce conformational structure changes.

Concerning the absence of anti-HBs antibodies in the serological profile, the failure to detect these antibodies in many patients in this context does not necessarily indicate the absence of humoral immune response, and may be justified by the formation of immune complex HBsAg anti-HBs. As a practical proof of this fact, it has been shown that anti-HBs can be detected in all the patients that evolve into chronic infection, when measured by an assay able to detect an antibody in the form of immune complexes [23]. Another explanation for failure in detecting anti-HBs could be the low sensitivity of commercial assays currently standardized, which are not adjusted to detect antibodies produced in low levels, since an increase in its sensitivity would also increase the risk of false-positive results [48, 49]. This observation suggests that the marker detection depends on the sensitivity of the test adopted and of the detection limit, which is arbitrarily determined by the manufacturer [49, 50].

Finally, the occurrence of residues substitutions in HBsAg, involving specific epitopes, could be able to prevent the protein recognition by T-cells CD4+ or minimize the ability of its stimulation, compromising, consequently, the anti-HBs production [51, 52] and its detection in the serological profile.

In conclusion, in this clinical setting, the detection or not of anti-HBs antibodies in the serological profile HBsAg [−]; anti-HBc [+]; anti-HBs [+/−]; DNA/HBV [+], does not seem to have great significance and implications, since these antibodies, in detectable levels, do not fulfill its function of neutralizing, whereas its presence below detection levels, despite characterizing the presence of immune response, is insufficient to prevent viral replication and establish infection control.

**Profile 3**

HBeAg positivity/negativity for HBsAg

\[ \text{HBsAg [−]: HBeAg [+]; anti-HBc [+]; DNA/HBV [+]} \]

HBeAg is a secretory protein that is processed from the precore protein of HBV. It is generally considered to be a marker of HBV replication and infectivity, although it is not required for infectivity or viral replication [53]. As a non-structural component of HBV, HBeAg is abundantly secreted into the bloodstream during the acute phase of infection, appearing shortly after the appearance of HBsAg. Classically, the HBeAg presence in the bloodstream correlates with high levels of viral replication in hepatic tissue and HBsAg detection in serum. Therefore, HBeAg positivity/HBsAg negativity in the serological profile comprises an atypical pattern that deserves to be addressed.

The ‘a’ determinant of HBV consists of a defined serological region, located between amino acid residues 124 and 147 of the HBsAg, which induces a protective immune response common to all HBV subtypes [54]. Variations in its primary structure have been demonstrated to markedly alter the antigenic conformation and antigenicity of HBsAg. For example, the loss of the proline at position 120 significantly minimizes the binding of antibodies mapped to that region, as does the insertions between residues 122 and 124.
A similar effect is the occurrence of substitutions at positions 122 and 123, which demonstrate that this region is critical in the HBsAg antigenicity [56]. In vitro studies have shown that the HBsAg antigenicity is also lost when cysteine residues at positions 124, 137, 139, 147, or 149 are replaced by serine, suggesting that this amino acid is of fundamental importance in maintaining the conformational structure of the molecule [57]. Thus, the maintenance of the tertiary molecular structure of HBsAg, especially of its immunodominant region, is of crucial importance to recognition by antibodies, since antibodies directed to this region protect against HBV infection, and HBV variants that are not recognized by them can escape its protective action [58].

On the other hand, the commercial tests usually detect this protein using a capture monoclonal antibody and a detection monoclonal antibody (or polyclonal) anti-HBs wild-type, such as those produced after vaccination [45, 46]. The antibodies bound to the antigen captured tend to be specific for epitopes present in the determinant ‘a’ of HBsAg and oriented to recognize its cyclic peptide sequence [45, 54]. Since the immune response to HBsAg is primarily directed against conformational epitopes of the ‘a’ determinant, and that its antigenicity has been employed for HBsAg detection in the serum [54, 59], changes in its tertiary structure could not only prevent the binding of neutralizing antibodies targeted to the wild-type virus, but, also, the antigenic detection by the assay used for diagnosis [58, 60, 61] (depending on the epitopes recognized by the test configuration [62]), leading to false-negative results, justifying, therefore, the serological profile HBeAg [+]/HBsAg [−] and detectable DNA.

Recent studies have shown conflicting results between the tests applied in the detection of HBsAg mutants [63, 64]. This has occurred, presumably, due to differences existing between the tests applied, related to the analytical sensitivity (> or <0.1 ng/ml) or associated with the system configuration of capture and detection. With regards to the capture and detection system, the sensitivity of the tests currently designed to detect HBV mutants can be variable, being established between 57 % for testing monoclonal/capture and 80.6 to 97.4 % for testing polyclonal/capture, which has been linked to the non-recognition of HBsAg epitopes by monoclonal anti-HBs antibody used in the test [65] and shows a significant advantage of polyclonal capture systems. However, it is known that the polyclonal tests do not guarantee full sensitivity in the HBsAg mutant detection, which makes it important to incorporate specific antibodies against mutant forms of HBV in commercial tests. Thus, it should be noted that the test configuration is not the only factor that predicts the capability to detect the mutant, but, rather, the ability of epitope recognition by the reagent used to immobilize or detect the antigen involved [64].

**Profile 4**

Simultaneous positivity for HBsAg and anti-HBs (and detectable DNA)

*HBsAg [+]; HBeAg [+/-]; anti-HBc [+]; anti-HBs [+]; DNA/HBV [+]*

Concurrent HBsAg and hepatitis B surface antibodies comprise a peculiar serological finding, since it contemplates components that indicate immunity and, at the same time, developing infection. However, despite the presence of neutralizing antibodies, this serological profile correlates with active viral replication and may be observed in acute or chronic hepatitis B infection, although the mechanisms that could lead to this serological pattern have not been well delineated and understood. Its existence has been justified mainly by the emergence of HBV mutants and the presence of non-protective anti-HBs antibodies, as was recently discussed [30].

In the acute infection context, the identification of this profile may be justified by the occurrence of reinfection with a new HBV strain. This new HBV strain could have an ‘a’ determinant different from the first HBV infection (mutant), whose circulating antibodies would not have a neutralizing action, and, therefore, were not protective. Obviously, in this case, the presence of anti-HBs does not indicate the removal of HBV and means that HBV carriers with concurrent anti-HBs still have active virus replication, suggesting that some of the infecting viruses are surface antigen mutants.

The emergence of HBV escape mutations has been a phenomenon associated with the replication mechanism (see below), and also as a result of a selection process due to a selective advantage of variants during the course of HBV infection in a patient (for a review, see [6, 7, 66]). Additionally, the primary prevention of HBV infection, such as vaccination in infants born to HBsAg-positive mothers and immunoprophylaxis in orthotopic liver transplantation recipients, due to exerting strong selective immune pressure on HBV, in some cases, can also induce escape mutations within the ‘a’ determinant region, affecting several amino acid positions, as previously mentioned [67–70]. HBV mutants can be extremely stable and maintain their ability to replicate at high titer in the presence of anti-HBs [67]. They can also be transmitted horizontally to other humans, and, therefore, justify cases of HBV reinfection/superinfection [71]. HBV reinfection comprises a common phenomenon in the liver transplantation context, reaching rates of more than 90 % if there is no treatment [72]. With the use of LMV and HBIG for prophylaxis, these rates can be reduced to almost 3.0 %, as demonstrated by Wang et al. [73].
Cases of HBV infection in previously vaccinated individuals may also occur [74, 75], and justify the co-existence of HBsAg/anti-HBs serological profile. Obviously, this would not be a case of HBV reinfection (since the anti-HBs present in the bloodstream was induced by vaccination), but demonstrates the possibility of replication of HBV (which produces an HBsAg mutant or not, but which is different from HBsAg vaccine), in the presence of preexisting anti-HBs antibodies (vaccine-induced), but which is unable to recognize the circulating virus (Fig. 2a).

In the chronic infection context, the observation of this profile can be justified by the detection of HBV mutants naturally selected due to existing anti-HBs endogenous response [76–79]. Consequently, some chronic carriers have HBsAg as anti-HBs; however, the antibody detected is being directed against HBsAg epitopes not shared by original HBsAg present in the bloodstream, and, therefore, not protective [80] (Fig. 2b). In this case, the presence of anti-HBs does not indicate viral elimination and suggests that HBV carriers with HBsAg and anti-HBs demonstrate active replication. Moreover, the presence of anti-HBs does not exclude the possibility of a new HBV infection or the reactivation of preexisting HBV infection.

These mutants have been characterized, presenting deletions [80, 81], point mutations [82], and genetic recombination [83] within the preS/S genes, which have been identified, frequently, in HBV DNA sequences obtained from the sera of inactive carriers.

HBV produces three envelope proteins which are all encoded in the preS/S open reading frame. The major hepatitis B surface (HBsAg) is coded by the S gene. The middle HBsAg is coded by the preS2 and S genes. The large HBsAg is coded by the preS1, preS2, and S genes. The preS sequences exhibit the highest heterogeneity of the HBV genome [84, 85]. Mutations in the preS region, such as deletions involving the preS2 translation initiation codon, could promote the synthesis of an anomalous HBsAg detectable in the bloodstream, but, however, not recognized by previously anti-HBs antibodies formed against the original HBsAg [76, 86]. These mutations can arise in the natural course of HBV infection and have been more frequently observed in patients with anti-HBs who did not receive hepatitis B immunoglobulin or vaccine than in those HBV carriers who do not have anti-HBs. These mutations are more frequently observed in asymptomatic carriers [86].

Deletion in the preS1 region has also been associated to the co-occurrence of HBsAg/anti-HBs, being related with persistent viremia. In a previous study, a deletion of nucleotide 31 of the HBs gene in a patient with anti-HBs seroconversion but who still remained an HBV carrier with a virus load of 10^4 DNA molecules/ml of serum was observed. This deletion led to frame-shift and introduced a stop codon after the 21 amino of HBs. The resulting HBsAg lacking the major epitopes recognized by specific antibodies could favor ongoing viral replication, despite the presence of anti-HBs [81].

Fig. 2  a Schematic representation of a infection with a new HBV strain in a vaccinated individual (immune), demonstrating non-recognition and non-neutralization of the circulating virus by anti-HBs antibodies induced by vaccination. b Schematic representation showing emergence of HBV mutants induced by immune pressure (anti-HBs endogenous). In the chronic infection context, the HBV/HBsAg exists in both forms, free and immunoglobulin-bound, whereas the anti-HBs antibodies only exist in the form of immune complex. Due to the immune pressure (anti-HBs induced), the emergence of HBV mutants with later HBV seroconversion (to wild-type virus) and consequent simultaneous detection of anti-HBs and HBsAg mutant in the bloodstream occurs.

**Table 1**

<table>
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<th>Seroconversion</th>
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<td>Anti-HBs free [anti-wild type]</td>
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<td>HBsAg Mutant</td>
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</tr>
<tr>
<td>HBV/HBsAg immunoglobulin-bound</td>
<td>HBV Mutant</td>
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**Fig. 2 a** Schematic representation of a infection with a new HBV strain in a vaccinated individual (immune), demonstrating non-recognition and non-neutralization of the circulating virus by anti-HBs antibodies induced by vaccination. **Fig. 2 b** Schematic representation showing emergence of HBV mutants induced by immune pressure (anti-HBs endogenous). In the chronic infection context, the HBV/HBsAg exists in both forms, free and immunoglobulin-bound, whereas the anti-HBs antibodies only exist in the form of immune complex. Due to the immune pressure (anti-HBs induced), the emergence of HBV mutants with later HBV seroconversion (to wild-type virus) and consequent simultaneous detection of anti-HBs and HBsAg mutant in the bloodstream occurs.
Besides deletions, and as aforementioned, variations in the primary structure of the ‘a’ determinant have also contributed to justifying the presence of this profile, since these variations may markedly alter the antigenic conformation and antigenicity of HBsAg. Therefore, the binding of both monoclonal and polyclonal anti-HBs antibodies can be affected by a single aa change, and antibody raised to the wild-type can be bound less well to the HBsAg variant. On the occurrence of these variations, there could be, then, the presence of HBsAg mutant and the concomitant presence of anti-HBs antibodies not bound to the mutated antigen.

It must be highlighted also that an additional cause to justify the mutants emergence during the natural course of HBV infection has been linked to the complex mechanism of viral replication, characterized by the synthesis of viral DNA through an RNA intermediary, by reverse transcription [87]. As result of this mechanism and due to biochemical processes of the host cell (besides the infidelity of the viral replication machinery), errors of bases incorporation into DNA strands may inevitably occur during viral replication. Considering the large number of viral particles generated in an infected person (10^{11} viral particles per day), and that the error rate (because of the polymerase reverse transcription) can be of the order of 1 error per 10^{10} bases, in active infection, 10^{10} base-pairing errors could be generated per day over the 3,200-bp genome in a given patient [88]. These errors lead to the generation of multiple variant transcripts from a single template, and, consequently, the formation of a quasispecies pool [88]. While these new sequences are viable, they effectively compete with the wild type and comprise a material source for the emergence of mutants in an environment with significant selective immune pressure during the natural course of HBV infection. In this context, in the proportion that any newly generated mutation confers selective advantages to the virus, it could allow the corresponding viral population to express more than wild-type virus, indirectly contributing to HBV seroconversion.

Thus, HBsAg/anti-HBs simultaneous detection could occur, suggesting that the acquisition of anti-HBs could be associated with the emergence of immune escape variants persisting for several years in association with wild-type strains. The presence of HBsAg variants would arise naturally in chronic carriers and should be a viral strategy to evade the immune system. Therefore, the detectable HBsAg is a mixture of the wild type and variants, whereas the detectable anti-HBs are targeted against the wild-type viruses.

The presence of heterologous subtype-specific antibodies could be another factor that could also lead to the co-occurrence of HBsAg and anti-HBs in the chronic HBV infection context. These antibodies could be directed against HBsAg subtypes different from the coexisting HBsAg, justifying the serological profile [81, 83, 89]. In these cases, HBV persistence in the presence of anti-HBs is not associated with the emergence of HBV escape mutants with changed HBsAg sequences, but due to natural sequence variations.

In such cases, it is possible that the major fraction of anti-HBs antibodies in patients with concurrent HBsAg/anti-HBs positivity may have the ‘wrong’ specificity and appear to be unable to bind to serum HBsAg in the same patient. Importantly, it must be highlighted that the HBsAg sequence in such cases demonstrates not the mutated HBsAg, but, rather, the wild-type sequence associated with anti-HBs antibodies that are heterotype specific. However, the mechanism by which subtype-specific anti-HBs can be induced in patients with chronic infection it is not clear.

Finally, the simultaneous detection of HBsAg and anti-HBs in the bloodstream could be associated with a case of viral reactivation (a phenomenon related to the presence of occult infection), since the products of viral reactivation could be a mutant, and the preexisting anti-HBs antibodies could be targeted to wild-type virus [30].

Regarding the HBeAg in this serological profile, in all situations addressed, its detection is likely to occur, since, in the mechanisms described, there is no compromising of the genomic sequence coding for the e protein. This means that the HBeAg detection (or non-detection) in this context would be more in dependence of the serological phase in which the sample collection/analysis occurred (before or after HBeAg clearance, in low- or high-replication phase), than probably due to the production (or non-production) of protein.

**Profile 5**

HBeAg negativity/positivity for anti-HBe and detectable DNA

\[ \text{HBsAg}^{+}; \text{HBeAg}^{-}; \text{anti-HBe}^{+}; \text{DNA/HBV}^{+} \]

It is universally acknowledged that HBeAg detection in the circulation correlates with viral replication in the liver, while its disappearance, associated with anti-HBe seroconversion, is seen as a sign of replication absence and spontaneous resolution of infection. However, this statement may not be absolute, since, in the cases of precore region HBV mutants, HBeAg expression may not occur, but there is maintenance of viral replication, whose existence cannot be measured by the HBe system [90]. In these cases, HBV/DNA and anti-HBe antibodies can be found in the bloodstream at the same time, justifying the occurrence of this atypical serological pattern.

The gene ‘C’ of HBV (nucleotide 1814 to nucleotide 2450) [82] is divided into the precore region and the core region by two in-frame initiating ATG codons. Translation
from the second initiation codon (nucleotide 1901) results in unprocessed core polypeptides (183-aa), which are assembled into HBeAg particles, and comprise the virion nucleocapsid. Initiation of translation at the first site (nucleotide 1814) produces a 312 amino acid polypeptide that has a signal peptide directing it to the endoplasmic reticulum, where the signal piece is removed by signal peptidases, cleaving the N-terminal 19 amino acid residues. With subsequent processing, the C-terminal 34 amino acid residues are removed, and the resultant polypeptide is secreted as HBeAg, a soluble protein, detectable in the bloodstream [85, 90]. The function of secretory HBeAg in the viral life cycles is unknown inasmuch as it is not required either for infection or replication. However, it has been suggested that the HBeAg may have a function in the immune response modulation during chronic HBV infection in adults and in promoting neonatal tolerance [15].

In the typical course of HBV infection or during antiviral therapy in chronic HBV infection, the seroconversion of HBeAg to anti-HBe is usually accompanied by a decrease in viral replication and hepatic disease remission [90]. Among some patients, for unknown reasons, the immune pressure associated with seroconversion contributes to the viral variants emergence, which are characterized by not expressing HBeAg or expressing at low levels, although these patients have ongoing viral replication associated with liver damage [90, 91].

During chronic HBV infection, two major types of HBV core gene variants have been frequently reported, which affect the HBeAg expression: the precore mutants and the basal core promoter (BCP) mutants.

The most prevalent precore region mutation, known as G1896A, is characterized by a guanine-to-adenine transition at nucleotide position 1896, which converts codon 28 from tryptophan (TGG) to a premature stop codon (TAG), and, by so doing, aborts the translation of HBeAg precursor made of 29 amino acids coded for by the precore region and the 183 amino acids encoded by the core region [92]. The precursor loses amino-terminal 19 amino acids and carboxy-terminal 34 amino acids to become HBeAg. This mutation occurs within the epsilon (ε) structure, a highly conserved stem-loop at the pregenomic RNA, essential for the initiation of encapsidation and replication of the HBV pregenome, during the viral replication cycle [92]. However, the stability of this structure depends on the strict conservation of base pairing in the stem region, since mutations that alter the combination of these bases may lead to less efficient viral replication or the production of non-viable viral particles [93].

Thus, in the ε structure, the base guanine, at the nucleotide 1896 (codon 28), on wild-type virus, forms a pair with the base thymidine or cytosine of the nucleotide 1858 (codon 15), depending on the genotype involved [85]. In genotypes A, F, and H, the presence of cytosine at nt 1858 (C1858) stabilizes the ε structure and makes the occurrence of a stop codon in the precore a rare event, maintaining the preferred pairing G-C base proposed by Watson-Crick [65, 94]. However, the substitution of guanine by adenine at position 1896 (A1896) would result in an unstable base pairing (A-C), destabilizing the ε structure’s loop and also reducing the efficiency of viral replication [65].

In contrast, the HBV genotypes B, D, and E, and some strains of genotype C, have a thymidine at nucleotide 1858 (T1858). Thus, a mutation for adenine at position 1896 (A1896) stabilizes the ε structure, forming a new base pair (A-T), in parallel, introducing a new stop codon [94] and increasing the viral replication efficiency [65]. As a result, viral strains which are HBeAg-negative are often found in infections by genotypes B–E. With regard to genotype G, the situation is peculiar, since all isolates are defined by the presence of adenine at nt 1896, which introduces a stop codon natural, making them incapable of expressing HBeAg [94]. However, despite two stop codons (at positions 2 and 28) in the precore region characteristic of HBV genotype G [92, 95], the HBeAg can still be detected in the serum of infected individuals by this genotype, but this marker is attributed to genotype A, with which the occurrence of co-infection is frequent [95].

In conclusion, patients infected with mutants in the precore region can then express high levels of DNA/HBV, despite the presence of anti-HBeAg in the serum. However, the cellular immune response modulation, induced by HBeAg against the peptides HBeAg/HBcAg related and expressed on the surface of infected hepatocytes, is lost [96], which can correlate with exacerbation of liver injury and a worse prognosis [97].

The second group of mutations affects the BCP (nt 1742 to nt 1849), with the double exchange of adenine by thymidine at nt 1762 (A1762T) and guanine by adenine at nt 1764 (G1764A) being the most common. These mutations are often present in patients with chronic hepatitis or fulminating hepatitis, and less frequently in asymptomatic carriers, in immunosuppressed patients, and in infected individuals devoid of any serological markers [98]. They arise before or during the HBeAg seroconversion to anti-HBe and result in suppression at the transcriptional level of mRNA coding for the core and precore. The consequence of these changes is the decrease in HBeAg expression to approximately one-third of the levels observed in the wild viral strain and, also, contribution to an improvement in the ability of viral genome replication [85, 90]. These observations are supported by transfection studies which show low levels of preC mRNA as well as of HBeAg secretion in the presence of 1762 T and 1764 A [98].

As previously mentioned, it has been postulated that HBeAg induces an immune tolerance against itself or to HBcAg, or even against both antigens. As the HBeAg
shares some epitopes with the core antigen, its absence or presence in low concentrations in the circulation causes the HBcAg to be targeted directly by the humoral and cellular immune system, leading to necrosis of hepatocytes and liver damage [98]. This suggests a close relationship between mutation in the BCP and liver disease advancement, which leads to increased risk of developing hepatocellular carcinoma [99].

Profile 6

Absence of serologic markers of infection and detectable HBV/DNA

HBsAg [−]; HBeAg [−]; anti-HBc [−]; DNA/HBV [+]

The isolated detection of HBV/DNA in the absence of serological markers is a typical serological/molecular profile and a relatively common laboratory finding, particularly in the blood transfusion context, when the research of this molecular marker is already established in the donors screening. At first, this finding does not seem curious or unusual, since, in most cases, it can be observed progressively the emergence of other serological markers of viral replication, which is indicative of infectious blood detection still in a recent phase of infection.

However, the situations of persistently detectable DNA, in the absence of other markers, can define an atypical serological profile in HBV infection and lead to the suspicion of mutant virus infection, especially the X-defective HBV mutant, whose characteristic is briefly described below.

The X-ORF (X-region) encodes for a 154aa protein called HBx (protein X). Protein X is known to transactivate transcriptional regulatory elements, which include the enhancer and core promoter elements. The core promoter, located at nt 1586 to nt 1849 [98, 100], regulates the transcription of the core/pregenome, and enhancer II, located at nt 1687 to nt 1805 [101], is implicated in the activation of the S-promoter [98, 101]. The sequence nt 1742 to nt 1849 is called the BCP. It is supposed to be sufficient for the correct transcription initiation of the pregenome and precore mRNA [82]. DR1 (direct repeated) and DR2, which are implicated in the origin of HBV DNA, are also located in the X-region.

The X-defective HBV mutant is commonly characterized by an 8-nt deletion between nt 1770 and 1777 and a point mutation of DR2 (T-to-C) in the X-ORF [102]. This deletion results in a C-terminally truncated X protein of 134aa (due a loss of 23aa and the addition of three normal aa), which causes a loss of its transactivating activity. The 8-nt deletion of the core promoter/enhancer II complex sequence may diminish the function of this transcriptional regulatory element [103], promoting the suppression of viral replication and the synthesis of HBcAg and HBsAg. Consequently, patients infected with X-defective HBV mutant are negative for HBsAg and anti-HBc, despite the presence of HBV replication and detectable DNA. X-defective HBV mutants have been isolated from patients with acute or chronic hepatitis [102].

Serological aspects of clinical and laboratorial significance

Acute infection and non-detection of HBsAg

Classically, 2–3 months after contact with HBV—so still in the incubation period, even before the biochemical evidence of hepatic dysfunction—the detection of HBsAg is possible [104]. Usually, it remains detectable in serum until about 2 months after the onset of clinical manifestations. However, this period may extend to over 3–6 months in individuals with acute infection [1].

During this period (i.e., during 2 months after the onset of clinical manifestations), the early clearance of HBsAg can occur before marker identification is made in the laboratory. This phenomenon is a result of the inhibition of HBV replication (and decrease of HBsAg), whose mechanisms are not well clarified, but are dependent on viral determinants (such as genotype, mutations) [105, 106], and also on the host’s immune response, involving the induction of a CD8(+) T-cell response (key cellular effectors mediating HBV clearance from the liver) by CD4(+) cells (the master regulators of the adaptive immune response to HBV), as was demonstrated by Yang et al. [107]. Therefore, the negativity of this marker does not exclude the diagnosis of acute HBV infection!

Detection of IgM anti-HBc antibodies: acute or chronic infection?

As already mentioned, IgM antibodies appear early, even during the incubation period. When the disease is clinically expressed, IgM and IgG antibodies are usually present. After 2–6 months, IgM disappears, leaving only IgG antibodies [4].

In contrast to most viral infections, patients with acute and chronic HBV often produce both IgM class and IgG class anti-HBc antibodies; therefore, the mere presence of IgM anti-HBc is not diagnostic of an acute infection. However, higher levels of IgM anti-HBc are generally produced during the acute phase as compared with chronic infection, and this quantitative difference can be the only serological means of differentiating an acute HBV infection from an
Correlation between anti-HBe and anti-HBc antibodies production

Although HBcAg and HBeAg are serologically distinct, they are structurally related, since these two proteins keep in its structure a very extensive homology in the amino acid residues sequence [53]. This has been observed from experiments that demonstrate the existence of cross-reactivity in recognition of these antigens at the Th-cell level for antibodies production [28, 53], although the immune responses to these antigens appear to be regulated independently.

It is known that anti-HBc antibody can be produced via T-cell-independent and T-cell-dependent pathways, whereas antibody production to the HBcAg is strictly T-cell-dependent [28]. Thus, the cross-reactivity of Th-cell recognition of HBcAg and HBeAg predicts a correlation between the T-cell-dependent pathway of anti-HBc antibody production and anti-HBe antibody production. Accordingly, the emergence of anti-HBe antibodies is associated with an increasing T-cell response to the core protein, and is accompanied by a slight increase in anti-HBc antibodies [11]. Therefore, low anti-HBc titers may be consistent with only T-cell-independent anti-HBc production, may reflect a deficit in HBc/HBcAg-specific Th-cell function, and are usually related to anti-HBe negativity in the serological profile. On the other hand, high titers of anti-HBc antibodies are associated with its production via T-cell-independent and T-cell-dependent pathways, and correlates with the presence of anti-HBe antibodies in the serological profile [23], supporting the theory that, if T-cells assist in the anti-HBc production, they would presumably also assist in the anti-HBe production (Fig. 3). These data demonstrate that, although the anti-HBc antibodies presence is observed in only 33 % of anti-HBc-positive individuals [108], its detection presupposes the existence of cellular immune mechanisms responsible for HBV clearance, suggests convalescence, and progresses toward recovery, whereas its absence in the profile may suggest the opposite. Obviously, the anti-HBe detection in the absence of anti-HBe antibodies suggests a profile initially incoherent and likely to be revised.

Seroconversion to anti-HBe and anti-HBs antibodies

The seroconversion to anti-HBe antibodies are frequently correlated with viral clearance and remission of liver disease during chronic infection. However, antibodies to HBeAg may not develop in all HBV-infected patients, or may appear at various times after the appearance of anti-HBc. The seroconversion to anti-HBs antibodies indicates viral elimination. However, these antibodies can become undetectable in patients who have recovered fully from infection. Although there is still limited knowledge about the kinetics and regulation of HBs/HBe-specific antibody response [109], the low HBs and HBe immunogenicity [110, 111] comprises one of several potential immunological mechanisms that could explain the non-development or non-detection of these antibodies in the clinical course of HBV infection. Regardless of this factor, it has been shown that antibody production to viral protein HBe and HBs is a T-cell-dependent process [28], and that the Th-cell response to HBcAg/HBeAg supports the production of HBc and HBe antibodies, as well as anti-envelope antibodies [112]. Considering that HBC/BeAg-specific Th cells in humans mediate anti-envelope antibodies production, and that there is a significant correlation between the activation of CD4+ T lymphocytes by HBcAg/HBeAg and the seroconversion phase to anti-HBe and anti-HBs in acutely infected patients [11], it is tempting to postulate that the seroconversion to anti-HBe antibodies predicts the seroconversion to anti-HBs antibodies. Nevertheless, it is possible that anti-HBe antibodies can be detected even in the absence of anti-HBs antibodies [113, 114], or that anti-HBs antibodies are present alone in the serological profile (or in combination with other markers, other than anti-HBe), without which these serological situations are necessarily characterized as atypical [114].

Prevalence of atypical serological profiles in HBV infection

Atypical serological profiles in HBV infection include serological situations that tend to occur most frequently in the chronic infection context in response to a long history of HBV infection, associated with virological characteristics of the agent (involving persistent viral replication, replication mechanism, natural variability, and wide capacity of mutation), but also due to an inadequate HBV-specific immune pressure of the host. And that is what the serological findings observed in studies conducted in regions of high prevalence of HBV infection (where the majority of infections are contracted perinatally or in early childhood) have suggested, demonstrating that, in chronic carriers, involving patients with chronic active hepatitis, or with chronic persistent hepatitis or asymptomatic carriers, the presence of atypical patterns is more incident [86, 115, 116]. However, these serological patterns can be observed in any clinical setting of HBV infection, since its occurrence may also be associated with the agent [117] and/or to the host [118], but not just due to the long history of HBV infection.
Despite these conjectures, there are no data on the prevalence of these patterns (or which patterns are more prevalent) in the context of acute or chronic HBV infection, since its occurrence, in most cases, have only been mentioned in the context of other investigations on HBV [116, 119–121] or identified primarily by routine laboratory tests in the context of HBV diagnosis [122] (or accidentally, as a result of screening protocols [123]), but not from serological studies correlating the incidence of atypical serological profiles in a population of HBV-infected individuals.

Atypical serological profile and HBV genotypes

So far, there has been no study carried out to establish any relationship between the presence of atypical patterns and genotype of the infecting HBV. However, it seems logical to assume that infection by certain genotypes, due to some specific peculiarities, may justify this finding and even favor its appearance. For example, with regard to infection by the G genotype (possibly also by B and E), the occurrence/presence of mutations in the precore region (or BCP) is expected and may comprise a frequent finding in some populations, especially in France and the USA, where infection by that genotype is incident [120]. Therefore, the association of infection by this genotype with the coexistence of anti-HBe and DNA in the circulation, may be wide. Another illustrative example is related to HBV/C genotype infection. HBV/C has been given some peculiarities, among which are the highest frequency of BCP mutations and preS/S deletions when compared with other genotypes [124]. Since deletions in preS/S has been a factor to justify the co-occurrence of HBsAg and anti-HBs in the bloodstream [79, 84], it seems plausible to associate a greater possibility of detection of atypical serological patterns in the occurrence of infection with this genotype [30].

However, it is observed that there is a poor relationship between atypical patterns and genotypes of HBV, and, even if there was such an association, the investigation of the genotype would not comprise a tool of immediate practical applicability to justify a particular atypical profile found, especially in the context of serological routine diagnosis. Even so, this correlation deserves further investigation.

Conclusion

The natural course of hepatitis B virus (HBV) infection is directly linked to immune tolerance, infection by viral variants, and viral clearance. The Immune tolerance, infection by viral variants, and viral clearance, in turn, comprise factors which are closely related to DNA levels produced by the agent, genetic expression of viral antigens, and also the patient’s immune response. The interrelationship of these factors, most of the time, may justify the identification of atypical serological patterns which are beyond those classically known and universally accepted, such as those described here. Thus, it seems possible that acute or chronic carriers of HBV are identified, showing serological patterns that do not characterize them as such.
Moreover, the advent of the next generation of immuno-assays, the improvement in the sensitivity and specificity of molecular assays, and better knowledge of the biology of HBV also tend to reveal other curious serological situations, such as HBsAg detection in the absence of HBV/DNA [125], and which will be added to a constellation of atypical profiles, which are yet to be revealed, in several clinical contexts of HBV infection. These profiles could be a problem in the medical clinic and laboratory diagnosis of hepatitis B, but, also, could be able to characterize different infection phases and even revise the current clinical concepts of HBV infection.

Conflict of interest The author has declared that they have no conflict of interest.

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