The Isolated Anti-HBc Reactivity: New Developments

Die Bedeutung des isoliert anti-HBc positiven Befundes: neue Aspekte

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Summary: The presence of isolated antibody to hepatitis B virus (HBV) core antigen (anti-HBc) is the most frequent so-called unusual seroconstellation observed in HBV serology. Its clinical significance is still relatively unclear. In the last decade, its diagnostic significance has been intensively investigated. Actually, false positive results can be excluded through adequate confirmatory testing. Isolated anti-HBc reactivity is generally observed after resolved HBV infection (loss of anti-HBs or low-level anti-HBs). This serological pattern is also very frequently associated with HBsAg negative chronic low-level HBV DNA carriage. The reasons for the absence of HBsAg detection are inhibition of expression by HCV co-infection, and/or low-level HBsAg synthesis under the limit of detection of screening assays, presence of immune complexes and probably also in a minority of cases, HBsAg mutants. While anti-HBc screening of blood donations might further reduce the HBV related residual risk, it is not used worldwide because of its relatively poor cost-effectiveness and the burden of exclusion of donors through non-specific test results. Combined anti-HBc/HBsAg testing with a highly sensitive and specific assay is probably technically achievable and would permit to detect the rare cases of HBV DNA negative potentially infectious donations at a reasonable financial burden.

Keywords: HBV DNA; HBsAg; ALT; immune complexes; HBV mutants; HCV; HIV.

Zusammenfassung: Der Befund „isoliert Antikörper gegen Hepatitis B Virus (HBV) Core Antigen (anti-HBc) positiv“ ist eine ungewöhnliche, aber dennoch relativ häufige Serokonstellation. Während die klinische Bedeutung noch relativ unklar ist, wurde im letzten Jahrzehnt der isoliert anti-HBc positive Befund diagnostisch ausführlich abgeklärt. Heutzutage werden falsch positive Ergebnisse zuverlässig durch ausführliche „Bestätigungstests“ ausgeklammert. Isolierte anti-HBc-positive Ergebnisse werden im allgemeinen nach einer abgelaufenen Hepatitis B (Verschwinden von anti-HBs oder „low-level“ anti-HBs) beobachtet. Allerdings ist diese Serokonstellation auch sehr häufig mit einem HBsAg negativem chronischem „low level“ HBV DNA Trägerstatus assoziiert. Zu den Ursachen zählen eine Inhibition der HBsAg Expression durch eine HCV-Koinfektion und/oder eine eingeschränkte HBsAg-Synthese unter der Nachweisgrenze von Suchtests, Präsenz von Immunkomplexen und möglicherweise in einer Minorität von Fällen, HBsAg-Mutanten. Während ein anti-HBc-Screening von Blutspenden möglicherweise das HBV-assoziierte Restrisiko reduzieren würde, stehen die relativ schlechte Kosteneffizienz sowie die Gefahr eines zu hohen Ausschlusses von Spenden aufgrund unspezifischer Ergebnisse einer weltweiten Anwendung im Blutspendewesen im Wege. Kombinierte, hoch sensitive und spezifische anti-HBc/HBsAg Tests dürften keine allzu große technische Herausforderung darstellen und könnten mit einem relativ geringem Kostenaufwand seltene aber potentiell infektiöse HBV DNA negative Spenden erfassen.

Schlüsselwörter: HBV DNA; HBsAg; ALT; Immunkomplexe; HBV Mutanten; HCV; HIV.

Detection of antibody against hepatitis B virus core antigen (anti-HBc) by enzyme immunoassay (EIA) is used solely or in combination with HBV surface antigen (HBsAg) screening for diagnosis of hepatitis B. Anti-HBc detection is used as a screening method prior to hepatitis B vaccination in high prevalence groups and for blood donor testing in certain European countries and the USA. Anti-HCV screening has superceded blood donor screening for anti-HBc, which has been implemented in some countries as a surrogate marker for non-A non-B hepatitis.

The presence of anti-HBc without detectable HBsAg or anti-HBs antibody (isolated anti-HBc), as the only marker of hepatitis B is a relatively frequent finding, which needs further diagnostic resolution. The clinical significance of this serological pattern is still a challenge.

There is some confusion about the terminology of “isolated anti-HBc positive”, since most frequently the designation “isolated anti-HBc positive” is used regardless of the anti-HBe status, HBeAg is, however, absent.

Table 1 gives an overview on the potential causes for this “unusual” seroconstellation. The first step in the

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Table 1 Causes of isolated anti-HBc reactivity

	Relative frequency
False positive result	Relatively low with specific assays (< 1 %)
Previous infection	
– Low-level anti-HBs	High
– Loss of anti-HBs	High
Acute HBV infection	Low
Chronic infection	
– Low level HBsAg	Low
– Immune complexes	Low
– HCV co-infection	High
– HBsAg mutation	Low
Passive immunisation	
– Maternal-neonatal transmission	Low
– Blood derivative therapy	Low
– Immune globulin administration	Low

resolution of anti-HBc reactivity alone is to verify the specificity of the test result (Table 2).

Verification of the specificity of an isolated anti-HBc positive result

The detection of anti-HBc with routine EIAs in a competitive test format was subject to rather frequent non-specific reactions causing false-positive results [1–5]. Isolated false-positive anti-HBc reactivity (presence of anti-HBc; HBsAg and anti-HBs negative) has been attributed to cross-reactive antibodies or interfering substances in serum; this effect was also observed for anti-HBs [2, 3]. One of the major reasons for false-positive reactivity is the non-specific activation of premature B-lymphocytes resulting in the production of IgA- or IgM-related molecules without previous exposure to HBV [6, 7]. Pre-treatment of serum samples with reducing agents, i. e. dithiothreitol (DTT) or potassium bisulphite (MBS) significantly improves the specificity of the anti-HBc determination [8, 9]. The specificity of the anti-HBc assays with sample pre-treatment varies between 99.8 and 99.9 %.

Although newer anti-HBc assays with reducing agents are specific, the validity of a result can be enhanced by retesting the initially reactive sample and by distinguishing between strong- and weak-positive samples. The latter are frequently non-specific and give mostly a negative result in an alternative assay

Table 2 Recommended diagnostic "confirmatory" and "supplementary" testing for isolated anti-HBc reactivity

"Confirmatory" testing	Consider only high positive results as true positive
	Repeat measurement with an alternative assay
	Follow-up with a second sample weeks to months later
"Supplementary" testing	Anti-HBe detection
	HBV DNA detection
	Anti-HBs detection with a second assay
	Anti-HBc-IgM detection
	HBcAg neutralisation
	Immune complex dissociation
Diagnostic "vaccination"	

[9]. For these reasons, only samples presenting inhibition values ≥ 90 % or a very low index value (signal sample/signal cut-off) in a competitive assay should be considered as probably positive. Up to 3 % of isolated anti-HBc positive results are non-specific, if the cut-off is set at an inhibition value > 90 % [10]. With a cut-off at 60 % inhibition value, 20.5 % of the results would be potentially non-specific. Confirmation should be sought by another anti-HBc test with a different format, results of testing for HBV DNA measured by an amplifying method, diagnostic "vaccination", neutralisation or blocking of the sample with HBcAg, HBsAg immune complex dissociation and control testing after weeks to months [11, 12]. Generally, strong signals are reproducible over years, whereas weak reactivities are not.

Alternative data from selected populations indicate that samples with very low levels of reductant-stable anti-HBc activity are associated with anti-HBs and are presumably true anti-HBc-IgG positive [7, 13]. This suggests that the sensitivity of anti-HBc assays should be increased in order to detect true low-level reactive samples. On the basis of the results from Weare *et al.* [8], the required sensitivity of 0.3 to 0.4 PEIU/ml could be achieved by lowering the cut-off of anti-HBc assays. This approach however may have negative effects on the specificity of current assays.

Up to 5 % of highly anti-HBc positive samples may give discordant results if retested with another assay [1, 14]. Variations in pre-treatment, incubation and washing protocols even between assays of the same manufacturer may be responsible for differences in frequency of isolated anti-HBc reactivity [10].

Alternatively, false positive results (primary response) or prior infection by HBV (anamnestic response) can be detected by anti-HBs response after HBV vaccination [15]. A single injection of HBV vaccine usually leads to a secondary anti-HBs response with a high titer within 1–2 weeks [16]. In a study from Ural *et al.* [15], 20 subjects in an isolated anti-HBc group (41.6 %) but none of the subjects from an HBV negative control group responded with a titer of > 50 IU/l, 30 days after immunization, which suggested an anamnestic response due to prior infection and immunity. Furthermore, 23 subjects in the isolated anti-HBc group (47.9 %) finally responded after three doses of vaccination (anti-HBs titer > 10 IU/l), thus excluding chronic infection and suggesting initial false positive results.

The presence of anti-HBe helps validate the anti-HBc reactivity [12]; a negative anti-HBe result however does not exclude a true positive anti-HBc reactivity.

Differentiation of recent, previous and chronic HBV infection and passive immunisation

The next step after exclusion of a non-specific result is the determination of the stage of HBV infection. Isolated anti-HBc can be observed during recent (window phase), chronic and resolved HBV infection (late immunity).

A small proportion of the individuals with "anti-HBc alone" are assumed to be in the so-called window phase of a resolving acute HBV infection when HBsAg disappears followed by anti-HBs a few weeks later. The presence of a strongly positive anti-HBc-IgM result is definitive evidence for a recent infection, in which the sample was collected in the early convalescent stage of the illness when HBsAg has declined to undetectable levels [12]. These sera are likely to contain anti-HBe, and it is assumed that these patients are still infectious. Anti-HBs seroconversion is observed after 2 to 8 weeks.

In the absence of anti-HBs and high-titered IgM-specific anti-HBc, a serologic distinction between a chronic infection with undetectable HBsAg or past infection in which the anti-HBs level has declined to below the range of detectability needs to be performed.

HDV DNA detection and/or a challenge with HBsAg vaccine permit a differentiation between past and chronic infection in the great majority of the cases. Fewer than 3 % of HBV-susceptible patients (see also exclusion of false positive results) and chronic carriers will generate anti-HBs levels > 10 IU/l. HBV DNA detection should be performed with a highly sensitive amplification method with a threshold of 10–50 copies/ml in order to permit a reliable diagnosis of low level HBV DNA carriers [11]. Individuals without detectable HBV DNA and normal ALT level should be checked routinely every 5 years.

Alternatively, individuals with active infection may be detected by using highly sensitive anti-HBc-IgM assays which detect anti-HBc-IgM levels under 100 Paul Ehrlich Units (PEI)/ml [17, 18]. Low levels of specific IgM antibodies may be present in up to 8.5 % of patients with confirmed isolated anti-HBc reactivity. Anti-HBc-IgM may be observed in any form of liver disease associated with HBV infection, independently of the duration of infection and level of virus replication. A positive anti-HBc-IgM result in a solely anti-HBc positive patient correlates with active viral replication, even if the viral burden is under the detection limit of HBV DNA hybridisation (for review see [18]). Anti-HBc-IgM determination may be useful in unusual serologic constellations, such as isolated anti-HBc reactivity, in order to confirm the result and to provide information of potential HBV related liver disease of the patient, especially in those cases where HBV DNA detection is negative.

The reasons for isolated anti-HBc reactivity in past HBV infection are a progressive loss of anti-HBs antibody, most often decades after resolution of infection or a "false negative" result for anti-HBs determination. Comparative studies using anti-HBs tests from different manufacturers clearly indicated that anti-HBs concentrations differ considerably from assay to assay [19]. A high proportion of individuals with past HBV infection present low anti-HBs titers under 100 IU/l. In the concentration range between 10 and 100 UI/l, the discrepancies between commercial immunoassays are particularly high. Similar to HBsAg tests, anti-HBs assays detect a variety of antibodies to HBsAg, including antibodies to the *a* and subtype specific determinants of HBsAg. In unselected blood donors, 3.4 % showed discrepant results among 3 anti-HBs tests. One test gave a positive result up to 400 IU/ml, while two other tests scored the same sample negative. In a population of selected anti-HBc positive individuals, 14.4 % showed discrepant results between two anti-HBs assays [14].

Individuals with "anti-HBc alone" as a sign of late immunity probably do not differ from those showing also anti-HBs in terms of susceptibility to HBV re-infection. Despite the lack of the later marker, they seem to be protected against re-infection by cellular immune mechanisms and a marked immunological memory [11].

Passive immunisation can be excluded by follow-up testing, 6 to 8 weeks later.

Chronic HBV infection

Between 3 and 40 % of individuals with the serological pattern anti-HBc alone are chronic carriers of HBV (for a review see [20]). The reason for the lack of HBsAg in isolated anti-HBc positive individuals is not clear, but several explanations have been suggested. HBsAg may be hidden in circulating immune complexes [21, 22]. Variations in the pre-S region, or mutations in the surface antigen itself and especially in the *a* determinant which is recognized by anti-HBs, may render HBsAg undetectable by conventional assays [23–25]. A certain proportion of isolated anti-HBc positive individuals may be low-level HBsAg carriers, with antigen concentrations below the detection limit of serological tests [26].

With a new highly sensitive HBsAg assay, 2.7 % of isolated anti-HBc reactive samples were tested positive; only one of these samples was PCR positive [27]. Of 2000 antenatal clinic attendees in Papua New Guinea, 5 % of HBsAg positive subjects were negative in a widely used monoclonal assay but PCR positive. The monoclonal assay had a sensitivity of 0.5–1 ng/ml. These samples were reactive in another assay with a sensitivity of 0.1 ng/ml. Over 50 % of these discordant samples had rare or unique variants of the major hydrophilic region of HBsAg [26]. These data also demonstrate that the sensitivity of current screening HBsAg assays should be increased arguing for implementation of detection of HBV DNA in blood donors. Another reason for the possible failure of commercial assays in detecting surface antigen in isolated anti-HBc reactive HBV carriers is that the *a* determinant is more variable and shows a larger variability of the whole protein than HBsAg positive controls [28]. Polyclonal-antibody-based assays do not guarantee full sensitivity [29]. Modification of commercial assays is necessary to increase the sensitivity of detection of S gene variants. By using monoclonal antibodies directed against different S gene mutants in a prototype assay, it was possible to achieve a higher sensitivity in dilution series of HBsAg variants than with classical serological assays [14]. A further explanation for the lack of HBsAg is that there are variants in other parts of the genome that down-regulate the production of antigens [30, 31]. Weinberger *et al.* [28] observed that the exchange rate per amino acid in the "a" determinant in isolates from isolated anti-HBc positive individuals was significantly higher than in the residual parts of the molecule and even higher than in the same region of the HBsAg positive controls. On the other hand, sequences were found from solely anti-HBc positive individuals which were previously published to stem from "normal" HBsAg positive HBV carriers. Besides, no single position seems to be predominantly mutated. Cacciola *et al.* [32] did not observe changes in sequenced HBV genomes of HBsAg negative infections that are known to interfere with viral activity and gene expression. It is possible that structural alterations of HBsAg prevent the recognition by serological assays in anti-HBc alone positive individuals. Therefore, immunological properties of recombinant antigens should be examined.

HBsAg circulating immune complexes are a common feature of acute and chronic hepatitis B and correlate with HBV replication [11, 33]. Joller-Jemelka *et al.* [22] demonstrated that complexed HBsAg was present in more than 30 % of anti-HBc alone positive sera, highest rates were observed in individuals with hepatopathies (up to 80 %), in i.v. drug users (up to 63 %) and in haemodialysis patients (39 %). HBV DNA was detected by nested PCR in 39 % of the patients. Other authors [14] did not detect immune complexes among isolated anti-HBc positive samples. The absence of immune complexes in a more recent study from Weber *et al.* [14] may be attributed to different test methods. While in most of the previous studies [21, 33], immune complexes were removed by passage through C1q affinity column, Immune Complex Dissociation (ICD) by acid pre-treatment of serum samples is used. This technique proved to dissociate immune complexes even in the presence of a large excess of anti-HBs [34]. Another possible explanation is the fact that a relatively low percentage of selected samples (14.4 %) were viraemic and a high proportion were probably "healthy" carriers.

Prevalence of "anti-HBc alone" and follow-up

Within a given geographic region or country, the prevalence of isolated anti-HBc reactivity varies greatly. A recent study that included a representative portion (n = 5377) of the German population revealed that individuals with this pattern (1.4 %) are found even more frequently than HBsAg carriers (0.6 %) [35]. There was a trend to higher rates of this pattern in males than in females; a significantly higher percentage of persons with anti-HBc only was found individuals below 31 years than in older individuals. Five participants with anti-HBc only (7.7 %, or about 0.1 % of the whole population) showed HBV-DNA despite the absence of HBsAg.

Among healthy blood donors, 0.2 to 1.0 % are solely anti-HBc reactive [11, 36–38]. In 9006 pregnant women from all parts of Switzerland, the prevalence was 1.2 % [39]. During January 1994 to February 1996, 28,596 samples from single individuals were screened for the presence of anti-HBc, HBsAg and anti-HBs at the routine serologic department of the Institute of Virology, University Frankfurt. These subjects included organ transplant recipients, dialysis patients, HIV positive individuals, haemophiliacs, patients from different clinics, i. e. dermatology, gynaecology, surgery and psychiatry and members of the medical staff (pre-vaccination). A total of 5520 (19.3 %) individuals were tested negative for HBsAg and positive for anti-HBc. Among the 5520 anti-HBc positive samples, 643 (11.6 %) were isolated anti-HBc reactive, the prevalence in the population tested (n=28,596) was 2.2 % [10].

In selected individuals, depending on the prevalence of HBV infection and patient group investigated, 1 to 32 % of positive anti-HBc results are isolated positive findings [10, 13]. Isolated anti-HBc is frequently observed in intravenous drug abusers, HIV infected individuals [40, 41], prisoners [42], HBV and hepatitis C virus (HCV) co-infected patients [43]. The prevalence of "anti-HBc alone" is highest in intravenous drug abusers (IDU) (> 40 %) [44]. Isolated detection of anti-HBc is more common in HIV-positive than in HIV-negative IDUs [45]. Despite their progressive immunosuppression, both anti-HBs loss and HBV reactivation are rare in HIV-infected IDUs. In a follow-up study, the incidence of anti-HBs loss was 1.8 cases/100 person-year in HIV-positive and in HIV-negative IDU. Incidence of anti-HBs development was 17.6 cases/100 person-year in HIV-positive and 25.6 cases/100 person-year in HIV-negative IDU. In a survey from Mezzelani *et al.* [46], 141 isolated anti-HBc positive IDUs were been followed for a period of time: 77 % remained without any marker change and 23 % showed the appearance of anti-HBs. Subsequently, other 75 IDUs became carrier only of the anti HBc, for the following reasons: loss of anti-HBs (57 %) or HBsAg (15 %) appearance only of anti-HBc (28 %). No carrier of only anti-HBc seroconverted for HBsAg.

Isolated anti-HBc reactivity and HBV transmission

Several reports have shown that blood of individuals positive solely for anti-HBc can transmit HBV via blood transfusion or from mother to child (for a review see [28]). Anti-HBc positive organ donors are a potential source of transmission of HBV to their recipients [47]. The infectivity of these individuals can be confirmed by the detection of HBV DNA by hybridisation [48] or PCR [36, 40, 48–50]. The presence of HBV DNA can be demonstrated in 3 to more than 40 % of isolated anti-HBc positive individuals, depending on the sensitivity of HBV DNA detection protocol, sample volume and the population tested [51, 52]. Jilg *et al.* [43] observed that 32.9 % of individuals with isolated anti-HBc reactivity were HBV DNA PCR positive, the majority of them showing very low hepatitis B virus concentrations. Similar high HBV DNA prevalence rates were found in groups consisting of more than 50 % of high-risk individuals [36, 40, 51]. Since the prevalence of isolated anti-HBc reactivity is estimated to be about 1.4 % in the German population and at least 3 % of the cases are low-level viraemic, this means that a minimum of one out of 3000 individuals is a potential infectious carrier of HBV, without being detectable by standard screening measures. The risk of transmission by blood transfusion, according to a recent study from Alain *et al.* [53] seems to be lower since the frequency of HBV transmission by chronic carriers negative for hepatitis B surface antigen is estimated to be 1 in 52,000 donations (CI 0.3–7.8/100,000) from HBsAg-negative donors, and in this survey, all isolated anti-HBc positive donors were HBV DNA negative. Such potentially positive HBV infectious donations may not be detected by DNA amplification [53, 54] since HBV DNA has been found to be only positive temporarily, when patients are followed for years [36]. HBV DNA positive individuals are mostly chronic HBV carriers and show very low virus concentrations in their sera, but nevertheless can transmit the infection [47, 55, 56].

Since the viral load in chronic isolated anti-HBc positive carriers is low, there is a potential risk for failure of HBV DNA detection with pool-PCR in blood donors. Anti-HBc screening would reduce the residual risk [57–59]. Moreover, donors who have experienced hepatitis B infection should be lifetime excluded from further blood donations. Actually, in Germany, blood donors can donate again after five years if infectiosity can be excluded.

Chronic hepatitis B and HCV co-infection

High HCV seroprevalence rates (>30 %) are observed in isolated anti-HBc positive individuals since most of them are from high risk groups, i. e. haemodialysis patients, organ transplant recipients, intravenous drug addicts, and HIV-infected individuals HCV suppresses the replication of HBV and also substantially suppresses the expression of HBV surface proteins in vitro and in vivo [60]. Concurrent HCV infection can enhance the termination of the HBsAg carrier state in chronic HBsAg carriers [61].

Isolated anti-HBc reactivity and liver disease

Although, limited clinical data are available, the majority of isolated anti-HBc positive individuals seem to be healthy. If clinical symptoms or elevated liver enzymes are present, the role of chronic HBV infection is difficult to ascertain, since co-infection with HCV or HIV is frequent or other underlying diseases are present.

In a collective of 104 isolated anti-HBc reactive individuals, median values for ALT, AST and γGT were 14 U/l (range 3–365 U/l), 5 U/l (range 1–385 U/l) and 18.5 U/l (range 4–347 U/l), respectively. Elevated ALT, AST and γGT values were observed in 15 (14.4 %), 5 (4.8 %) and 37 (35.6 %) of the patients, respectively. Seven individuals were HIV-1 antibody positive. HCV antibodies were present in 68 patients. HCV and HIV co-infection was observed in 4 cases [14]. Little is known about the late outcome, most seem to remain healthy carriers, however the risk of progression to cirrhosis and hepatocellular carcinoma (HCC) does exist [11].

In a Japanese study, the prevalence of patients with HCC who were positive for only anti-hepatitis B core (anti-HBc) antibody among 284 patients was 5.6 % for anti-HBc alone positive, 6.7 % for HBsAg positive and 83.1 % for anti-HCV positive patients. Significant differences between the HCC-anti-HBc alone positive and HCC-HBsAg positive groups were that age at diagnosis was higher in the anti-HBc alone positive group (72.1 yr) than in the HBsAg positive group (56.2 yr), the serum alpha-fetoprotein concentrations were lower in the anti-HBc positive alone group (8.2 ng/ml) than in the HBsAg positive group (43 ng/ml) ($p = 0.0488$). However, there was no significant difference in the cumulative survival rate [62].

Patients with HCV chronic hepatitis and isolated anti-HBc show a poor response to IFN-alpha, irrespective of the HCV genotype [63].

Conclusions

The most probable explanations for isolated anti-HBc reactivity are resolved or chronic HBV infection and to a lesser extent false positive test results. Isolated anti-HBc reactivity therefore needs to be confirmed and staging of HBV infection needs to be performed in order to exclude chronic infection. In case of chronic hepatitis B (presence of HBV DNA, elevated ALT and histological signs of active hepatitis), therapy should be considered (11). There are no clear recommendations for the reintroduction of anti-HBc testing for blood donor screening, although it may be the only positive marker in chronic infectious carriers. The probably relatively poor cost/benefit relation and the crucial issue of increased loss of donors by poor specificity or incorrect result interpretation are major arguments against the routine testing of blood donors, especially in countries with low incidence of HBV infection and where donor screening by pool HBV DNA testing is already performed. In order to permit a more cost-effective screening of blood donations, combined assays for anti-HBc/HBsAg need to be developed. In the field of HIV screening, combined antigen/antibody assays have achieved a high sensitivity and specificity now after five years of experience and technical improvements (Weber *et al.*, 2002). Combined anti-HBc/HBsAg should be more easily achievable than p24Ag/HIV antibody detection from a technical point of view.

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