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Molecular detection of hepatitis B virus: recent developments

Molekularer Nachweis des Hepatitis B-Virus: Aktueller Entwicklungsstand

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Abstract

Highly sensitive qualitative and quantitative automated nucleic acid amplification tests (NATs) that are commercially available for the detection of hepatitis B virus (HBV) infection have been developed only in the last few years. The potential indications for HBV NATs are: follow-up of chronic hepatitis B, therapy and antiviral resistance monitoring, determination of infectivity and transmission risk, detection of occult (HBsAg-negative and HBV DNA-positive) infection and mutant virus which may escape serologic diagnosis, blood donor screening, and resolution of unusual or discordant serologic constellations. Although NATs are now widely implemented in the routine diagnosis of clinical laboratories, there are several important issues which need to be further investigated. Standardisation of NATs used for the monitoring of antiviral therapy and follow-up of chronic infection is still lacking, and the clinical significance of HBV DNA levels needs to be clarified. The influence of genetic variability in terms of genotype variation has been poorly investigated so far. Although there are highly sensitive automated NATs for blood donor screening available, their implementation is still subject to discussion and certain countries rejected HBV DNA testing for blood donation for reasons of poor cost-effectiveness.

Keywords: anti-HBc; blood donor screening; diagnostic window; HBsAg; HBV; nucleic acid amplification test (NAT); surface antigen mutant; viral load.

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Zusammenfassung

Hoch sensitive, automatisierte, qualitative und quantitative Nukleinsäureamplifikationstests (NATs), die kommerziell verfügbar sind für den Nachweis einer Hepatitis B-Virus (HBV)- Infektion, wurden erst in den letzten Jahren entwickelt. Die potentiellen Anwendungsgebiete für HBV NATs beinhalten die Verlaufskontrolle der chronischen Hepatitis B und der antiviralen Therapie, die Überprüfung der Resistenzentwicklung gegenüber antiviralen Chemotherapeutika, Beurteilung der Infektiosität und des Übertragungsrisikos, den Nachweis einer okkulten (HBsAg negativ und HBV DNA positiv) Infektion und diagnostischer Ausweichsmutanten, das Blutspenderscreening und die Abklärung von ungewöhnlichen oder diskrepanten Serokonstellationen. Obwohl NATs heutzutage weitestgehend in diagnostischen Labors etabliert sind, besteht noch ein erheblicher Klärungsbedarf für folgende Punkte: NATs zum Monitoring der antiviralen Therapie und Verlaufskontrolle der chronischen Infektion sind nicht standardisiert. Die klinische Bedeutung der verschiedenen HBV DNA-Plasmaspiegelbereiche ist nicht eindeutig geklärt. Der Einfluss der genetischen Variabilität unter besonderer Berücksichtigung der genotypischen Variation ist bis zum heutigen Zeitpunkt nur spärlich untersucht worden. Obwohl hochsensitive NATs für das Blutspenderscreening verfügbar sind, wird ihr Einsatz im Blutspendewesen weiterhin kontrovers diskutiert. Verschiedene Länder haben die HBV DNA-Testung für Blutspender aus Gründen der schwachen Kosteneffizienz abgelehnt.

Schlüsselwörter: anti-HBc; HBsAg; Blutspenderscreening; diagnostisches Fenster; HBV; Nukleinsäureamplifikationstest (NAT); Oberflächenantigen-Mutante; Viruslast.

Introduction

In recent years, methods for the detection of viral genome (nucleic acid testing, NAT) have considerably extended the diagnostic repertoire of virological laboratories and proven to be superior to conventional techniques in many circumstances. In addition to qualitative analysis, quantitative NATs – in the beginning mainly

used for research purposes – have been established in routine diagnostic virology. Sensitive NATs for qualitative and quantitative detection of human immunodeficiency virus (HIV) and hepatitis C virus (HCV) have been established for about a decade. For hepatitis B virus (HBV), the quantification of viral DNA was performed mostly with relatively insensitive hybridisation assays and NATs with a low detection limit have only been introduced in the last years. The main reasons for the delayed commercial development of molecular assays for HBV infection were the availability of serological markers and hybridisation assays for the follow-up of infection and monitoring of antiviral therapy with interferon and the expectation of a low cost-effectiveness in screening for HBV DNA in blood donors. However, highly sensitive and reproducible quantitative NAT and genotyping assays are necessary for the accurate monitoring of antiviral therapy, especially in the presence of a low viral load, and for the detection of viral rebound associated with resistance. In order to reduce the residual risk of transfusion-transmitted hepatitis B, highly sensitive NATs for the screening of single donations or mini-pools have been developed and are under evaluation in many countries. Their implementation as routine assays, however, is still controversially discussed.

Recent developments in molecular detection of HBV

HBV DNA may be detected in serum or plasma by hybridisation with or without signal amplification or with nucleic acid amplification tests (NATs). To meet the needs of the routine-diagnostic laboratory and for blood donor screening, amplification and the detection of amplification products have been automated. Sample preparation constitutes the major weakness in NAT. Conventional nucleic acid extraction protocols have been largely replaced by rapid methods, which are available as ready-to-use sample pre-treatment kits, either separately or as a part of entire kits [1]. Manual sample preparation, however, still constitutes a major limiting factor for high throughput sample analysis and represents the contamination-prone part of a molecular assay. Recently, new automated preparation instruments developed for sample preparation have been found suitable for inclusion in NAT for the detection of RNA and DNA viruses [2]. With automated nucleic acid extraction, the reproducibility of the results is increased and the risk of contamination is significantly reduced [3].

Detection can be qualitative, semi-quantitative or quantitative. NATs include polymerase chain reaction (PCR) and transcription-mediated amplification assays, which are commercially available or have been established as home brew tests. NAT is performed as a single or multiplex assay in individual or pooled samples (see section on blood donor screening). Detection of the amplification products is performed by probes which bind to the target DNA at one or more different genomic regions. Recently, commercially available assays based on real-time NAT have been introduced on the international market [4]. Real-time NATs are designed to allow the detection of amplification products during all phases of the reaction, since the hybridisation step is carried out during amplification and does not require time-consuming post-amplification handling and thus reduces the overall handling and the risk of carry-over. Quantitative analysis is performed during the exponential phase of the amplification reaction. The use of fluorescent dye-labelled probes increases the sensitivity and gives rise to a linear relationship between copy number and signal. As a consequence, the dynamic range and the precision of real-time NAT are higher than the ones of conventional amplification assays.

Qualitative, highly sensitive assays with detection limits ranging between 3 and 6 IU/mL have been developed for blood donor screening in order to enable the detection of low level HBV DNA at the end of the incubation period, or in chronic inactive carriers in order to reduce the residual risk of transfusion-transmitted HBV infection (see also section on HBV blood donor screening). So far, only one qualitative PCR assay with automated amplification and detection is available on the market, the Cobas Amplisscreen HBV v2.0 (Roche Diagnostics). The lower limit of detection is 3 IU/mL, the assay is designed for screening plasma mini-pools of 24 donations and is the first HBV NAT to be accepted for review by the Federal Drug and Food Administration (FDA). A fully automated (extraction, amplification, and detection) multiplex (HBV, HCV, and HIV) transcription-mediated amplification (TMA) assay (Procleix Ultrio, developed by Gen-Probe Incorporated in collaboration with Chiron, all Emmeryville, MA, USA) for blood donor screening in individual plasma samples will be launched in Europe in 2005.

Table 1 gives an overview on quantitative hybridisation assays and NAT that are commercially available for the follow-up of chronic hepatitis B and therapy monitoring. The quantitative tests have different lower detection limits ranging from approximately 1–2 copies/mL (NAT) to more than 10^5 copies/mL for hybridisation assays. The assays also have different linearity ranges. The influence of genetic variability on the sensitivity of NAT is a very critical issue that needs to be further investigated (see chapter on genetic variability). The coefficient of variation ranges between 6–15% for hybridisation assays and 12–44% for NAT. Differences or variations of less than 0.5 log (less than three-fold) should not be taken into account. Since the assays are not standardised, the follow-up of patients should always be performed by the same method. Proficiency panel testing with international standards should contribute to a better inter-laboratory comparability of the results. There is also no international consensus for the DNA units. The World Health Organisation (WHO) has established an international standard for the universal standardisation of HBV DNA quantification units, and an HBV DNA international unit (IU) has

Table 1 Commercially available quantitative HBV DNA assays.

Assay	Manufacturer	Principle	Procedure	Lower detection limit copies/mL	Linearity	Genotypes detected
HBV Digene Hybrid Capture I	Digene	Hybridisation	Manual	7×10^5 copies/mL	7×10^5–6×10^8	A-D
HBV Digene Hybrid Capture II	Digene	Hybridisation with signal amplification	Manual	1×10^5 copies/mL	1×10^5–2×10^9	A-D
Ultra-sensitive HBV Digene Hybrid Capture II	Digene	Hybridisation with signal amplification after centrifugation	Manual	5×10^3 copies/mL	5×10^3–6×10^7	A-D
VERSANT HBV DNA 2.0 assay	Bayer Diagnostics	Hybridisation with signal amplification, bDNA	Manual	7×10^5 copies/mL	7×10^5–5×10^9	?
VERSANT HBV DNA 3.0 assay	Bayer Diagnostics	Hybridisation with signal amplification, bDNA	Semi-automated	2×10^3 copies/mL	2×10^3–1×10^8	A-F
Amplicor HBV Monitor	Roche Diagnostics	PCR	Semi-automated	1×10^3 copies/mL	1×10^3–4×10^7	?
Cobas Amplicor HBV Monitor	Roche Diagnostics	PCR	Automated	3×10^2 copies/mL	3×10^2–2×10^5	?
Cobas TaqMan HBV	Roche Diagnostics	PCR (real-time)	Automated	3.5×10^1 copies/mL 6 IU/mL	3.5×10^2–1×10^8	A-F
RealArt HBV TM PCR	Artus	PCR (real-time)	Automated	2 copies/mL 0.4 IU/mL	0.4–4×10^8 IU/mL	?

been defined [5]. This IU should be preferred to any other quantitative unit and implemented in all commercial HBV DNA quantitative assays to establish clinically relevant thresholds and recommendations for clinical decisions based on HBV DNA load [6].

In practice, depending on the manufacturer, results are expressed in pg/mL, copies/mL, genome equivalents/mL, Eurohep units/mL, and IU/mL. The conversion rate is: 1 pg/mL = 283.000 copies ≈ 300.000 genome equivalents ≈ 10^5 IU/mL. The multiplication factor, however, may vary depending on the manufacturer and assay, e.g., for the Taqman HBV PCR, 1 IU/mL is equivalent to 5 copies/mL.

With sensitive NAT assays, HBV DNA may be detected in HBsAg-positive individuals considered to be inactive HBsAg carriers. More than 80% of HBsAg-containing samples are HBV DNA-positive with NAT with a detection limit of $\leq$20 IU/mL [7]. The decreasing proportion of HBsAg-harbouring samples that are negative for HBV DNA as NAT sensitivity improves suggests that most HBsAg-positive samples in inactive carriers contain HBV DNA [7]. Of 200 HBsAg-positive anti-HBc-reactive blood donations, 97% were tested positive with a highly sensitive PCR with a detection threshold (95% positivity rate) of 6 copies/mL [8].

Low copy numbers of HBV DNA may also be detected in subjects with isolated anti-HBc reactivity after acute, resolved hepatitis B in anti-HBs-positive individuals. The frequency of HBV DNA detected in HBsAg-negative samples varies considerably according to the prevalence of the infection [9] and lower detection limit of NAT [7].

The clinical significance of different levels of HBV DNA still needs to be clarified. It appears that the level below which hepatitis B is inactive is 10^4–10^6 copies/mL, which corresponds to the typical limit of detection in the non-NAT-based assays used in many past clinical studies [10]. However, patients can have advanced liver disease even if they have HBV DNA levels persistently $<10^4$ copies/mL [11]. Patients with $<10^4$ copies/mL may still be at risk for biochemical, histological, and clinical progression of the disease, although to a lesser extent than those with $\geq10^4$ copies/mL. Fluctuations in HBV DNA levels occur frequently during the course of infection, and the assessment of clinical disease or infectivity has to be based on serial determinations. Table 2 shows the possible relatedness between HBV DNA levels and disease activity and infectivity for blood donation, household contacts, and intimate partners.

Genetic variability of HBV and NAT

Genotyping methods (in-house and commercial) have also been developed for HBV genotype determination since the association between HBV genotypes, severity of disease, cirrhosis, hepatocellular carcinoma (HCC), and response to treatment has become the focus of several investigations, especially in Asia. Protocols for sequence analysis, restriction fragment length polymorphism (RFLP), muliplex PCR amplification and hybridisation have been published, an expert overview on the results, advantages, and disadvantages of different HBV genotyping methods has recently been published by Bartholomeusz and Schaefer [12].

The influence of genetic variability on the sensitivity of NAT has been poorly investigated so far. Genotypes A to

Table 2 Relatedness between HBV DNA levels, disease activity and infectivity for household contacts and intimate partners (modified from (57).

HBV DNA* copies/mL	Viremia	Hybridisation (without signal amplification)	Possible stage of infection/immune response	Infectivity				
				Blood donation	Vertical	Intimate contact	Transmission by health care workers	Household contact
10^8–10^{11}	Very high	+++	End of incubation period/ immunosuppression/ immunotolerance	++++	+++	++	+++	++
10^6–10^7	High	++	Acute or chronic active infection	+++	+ or −	+ or −	++	− or +
$>10^3$–10^5	Low	−	Inactive and non-progressive infection	++	−	−	+ or −	−
10–10^3	Very low	−	Inactive and non-progressive infection	+	−	−	−	−

*Because of the fluctuating course of chronic HBV infection, serial determinations are necessary to ascertain HBV replication status of individual patients.

D seem to be detected with an equal sensitivity by hybridisation assays [11]. HBV DNA concentrations were all detected within 0.5 $\log_{10}$ of expected values throughout a 4-$\log_{10}$ quantitative range for HBV genotypes A to F with a newly developed hybridisation test with signal amplification [13]. Several recently published HBV-PCR procedures use primers which contain genotype-specific mismatches at the 3′ end that may lead to a failure in detection of HBV DNA [14]. Amplicor HBV Monitor and Cobas Amplicor HBV Monitor (both Roche Diagnostics, Basel, Switzerland) seem to have a lower sensitivity for genotype A. The real time TaqMan PCR should detect and quantify DNA from genotypes A to G equivalently [15]. These findings remain to be confirmed by evaluation of a higher number of samples harbouring HBV strains of different genotypes.

Monitoring of antiviral resistance

The emergence of HBV variants resistant to lamivudine and adefovir therapy has strengthened the need for rapid and sensitive assays able to detect mutations before or early in treatment, which could assist in optimising the antiviral therapy regimen. The major sites of mutation for lamivudine resistance are situated in the highly conserved motif, tyrosine (Y), methionine (M), aspartate (D), aspartate (D) (YMDD) of the catalytic (C) domain of the reverse transcriptase. Various protocols for genotyping resistance mutations have been described. Sequencing is the most reliable method, as it is based on the greater amount of information and is the only method suitable for the detection of new mutations or recombination. The major limitation of this method is its inability to detect mixed populations, due to its lack of sensitivity. A commercial direct sequencing assay (TRUGENE HBV; Visible Genetics, Cambridge, UK) based on the CLIP single tube chemistry has a detection threshold of 50.000 IU/mL.

INNO-LiPA HBV DR is a commercial assay based on the reverse hybridisation principle. Biotinylated amplification products hybridise to specific oligonucleotide probes that are immobilized as parallel lines on membrane-based strips. This assay detects the presence of mutations located at amino acid positions 180, 204, and 207 in the HBV polymerase protein [16]. The test is rapid and relatively easy to perform, shows a good agreement with sequencing and can detect the mutant population earlier than sequencing, since it allows simultaneous detection of key mutations and wild-type sequences. The ability of INNO-LiPA to detect mutant virus populations long after cessation of lamivudine treatment permits appropriate selection of antiviral agents before retreatment [16]. A single nucleotide difference may affect the binding and the hybridisation result. The assay needs to be updated at regular intervals with the characterisation of new mutations and the introduction of new antivirals, and the reagents are costly.

Follow-up of chronic infection and antiviral therapy

Serum HBV DNA is evolving as the most useful measurement for the follow-up of patients with chronic hepatitis B. A review of 26 prospective studies found significant correlations between viral load levels or viral load changes and histological and serological markers for disease activity [17]. Management of chronic hepatitis B requires the use of NAT to establish an accurate baseline HBV DNA level. The continued use of HBV DNA assays during antiviral therapy is necessary to most accurately measure response and viral rebound associ-

ated with resistance. For significant or high-level HBV replication, hybridisation assays suffice, for detecting low levels of viral DNA, however, sensitive NATs are needed.

There is no international consensus for treatment recommendations in chronic hepatitis B. In HBeAg-positive patients, for example, the European Association for the Study of Liver Diseases (EASL) recommends a follow-up of 6 months before starting therapy [10], the United States panel advises a viral load $\geq 10^5$ copies/mL as a reasonable threshold for determining candidates for treatment [11]. The US panel suggests that HBeAg-positive patients with a serum HBV DNA level $\geq 10^5$ copies/mL be considered for treatment, depending on their alanine aminotransferase (ALT) levels. Under therapy, ALT should be monitored every one to 3 months during the first six months and every 6 months thereafter [10]. Among patients with HBeAg-positive chronic hepatitis, those treated with interferon (IFN) should be monitored for HBV DNA, HBeAg and anti-HBe at the end of treatment and 6 months thereafter to assess the virological response. In HBeAg-negative patients, the determination of viral load is the only way of assessing the virological status.

Resistance is defined as a $\geq$1-$\log_{10}$ increase in serum HBV DNA level from the patient's lowest on-treatment level occurring on two sequential occasions [11]. An increase in serum HBV DNA load may also be caused by a lack of adherence.

Patients under lamivudine therapy should be monitored for resistance every 3 to 6 months. Since the rate of adefovir resistance is significantly lower, monitoring for resistance should be performed only every 6 months after the first year of therapy.

The durability of virological response should be established by testing every 1 to 3 months for 1 year after stopping therapy and every 6 months thereafter for ALT, HBV DNA, HBeAg, and anti-HBe for initially HbeAg positive patients. HBsAg should be determined annually in patients with a sustained virological response [11].

HBV blood donor screening

The transmission of HBV by blood transfusion is minimised by HBsAg and anti-HBc testing (in some countries) and the exclusion of high-risk donors prior to transfusion. In the USA, the use of HBsAg coupled with anti-HBc testing has resulted in very low estimates of residual risk [18].

Still, there is, however, a small residual risk of transmission of HBV from undetected donors with early acute infection, resolving infection, silent (occult) infection, or infection with atypical variants or mutants. In immunocompetent adults, inoculation with a low dose of HBV is mostly associated with asymptomatic self-limiting acute HBV infection with consecutive immunity [19]. HBsAg is present at low concentrations and during a very short time period in unapparent acute hepatitis B and may therefore not be detected by HBsAg screening in blood donors. Infected individuals may thus unwittingly present as blood donors. In order to improve the safety of blood donor screening, several approaches to reduce the diagnostic window period before detection of the first HBV specific marker have been conceived.

Screening of blood donations with more sensitive CE-certified HBsAg assays reduces the diagnostic window period by 2 to 9 days in comparison to so far FDA-licensed tests [20, 21]. The time delay in comparison to the first positive HBV PCR result varies from 0 to 15 days for new HBsAg assays with improved sensitivity. Newer HBsAg tests would be expected to detect an additional 15 to 21 infected units per 10^7 donations in the USA [21].

NAT has been introduced in industrialised countries for the screening of HIV and HCV in pooled plasma samples and/or individual donations. Technical improvements and automation of all steps of NAT, including sample preparation, make it possible to perform nucleic acid amplification for the three most relevant blood-borne infections, HIV, and hepatitis B and C in mini-pools or single donations [22]. While in most countries there is a consensus to perform NAT for HIV and HCV, the utility in terms of cost-effectiveness for HBV NAT screening is still controversially discussed and some European countries are opposed to blood donor screening for HBV DNA. About 95% of HBV infections are self-limiting and the mean survival rate of blood recipients is relatively low. Due to the severe underlying disease or condition, which necessitates blood transfusion, the incremental costs for reducing the transfusion risk are high but the benefit for the patient in terms of quality of life is relatively poor [23].

HBV DNA is detected 3 to 5 weeks after infection. HBV DNA, in contrast to HIV and HCV RNA, rises slowly and circulates at relatively low levels (30 to 300 IU/mL) in the pre-ramp-up phase during the early HBsAg seronegative window period [24]. HBV NAT should have a detection threshold below 30 IU/mL in single donations in order to detect a low copy number of virus nucleic acid in the HBsAg-negative window phase, to further reduce the NAT diagnostic window period and to detect low-level chronic HBV carriers. Ten- to twenty-fold more sensitive NAT protocols could be used as an alternative for the testing of 8- or 16-member mini-pools in order to achieve a maximal reduction of the residual risk. NAT screening on individual donations or on 8–16 member mini-pools with highly sensitive single or multiplex NAT may reduce the diagnostic window by 24 to 36 days [21].

In some circumstances, the cost-effectiveness of enhanced sensitivity HBsAg assays, combined with anti-HBc screening and pooled NAT, would be within acceptable ranges for new public health interventions. This strategy would permit to detect not only acute HBV infection in asymptomatic blood donors with a very short HBsAg positive period, but also occult HBV infection in chronic carriers who are HBsAg-negative and may be negative for all the other HBV-specific markers [25].

Since the viral load in chronic inactive isolated anti-HBc-positive carriers is low, there is a potential risk for failure of HBV DNA detection with pool- or mini-pool- or even single samples NAT in blood donors [26–28]. Anti-HBc screening would reduce the residual risk [7, 29], since it has the potential to exclude the vast majority of occult HBV infection, leaving only the probably rare cases with HBV DNA alone. The frequency of HBV DNA positive in anti-HBc donations is highly variable ranging from 0.24% for US [27] to 15% for German first-time blood donors [30] (Table 3). In probably more than two-thirds of the cases, viral loads are extremely low with less than 30 IU/mL. However, this approach has two main drawbacks:

1. Anti-HBc testing does not detect the pre-seroconversion window-period infections.
2. Anti-HBc testing would not be practical in most parts of the world where the prevalence of anti-HBc is $>10\%$, as too many donors will be ineligible [7]. In those situations, high titre anti-HBs coupled with anti-HBc may indicate immunity of the donor, especially if HBV DNA is negative. It is therefore common for many blood centres performing anti-HBc screening to perform anti-HBs testing if the donor has previously been exposed to HBV and has resolved infection. However, low levels of HBV DNA may circulate in subjects with the serological profile of resolved hepatitis B (Table 4).

Anti-HBc screening might carry a higher cost-effectiveness than NAT in areas of low HBV prevalence, while NAT is probably the only choice in countries where the prevalence is moderate to high [7]. However, most of these countries do not have the financial resources to implement NAT screening of blood donors.

There are no clear recommendations for the reintroduction of anti-HBc for blood donor screening in those countries where it has been discontinued, 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.

In order to permit a more cost-effective screening of blood donations, combined assays for anti-HBc/HBsAg need to be developed for low-endemic areas. In the field of HIV screening, combined antigen/antibody assays have now, after 5 years of experience and technical improvements, achieved a high sensitivity and specificity [31].

Determination of infectivity

Another important application field for HBV viral load determination is the assessment of the infectivity of hepatitis B carriers. Without intervention, more than 90% of HBeAg-positive female chronic HBV carriers transmit the virus to their infants [32]; of these, 85–90% develop chronic HBV infection – in most cases asymptomatically-thus perpetuating the infection in high-endemicity settings. Immediate post-partum immunisation of the infant efficiently prevents transmission. It has to be considered that the level of viraemia present in maternal serum can only be approximated with the HBeAg assay. Recent quantitative evaluation of sera for HBV DNA by using molecular hybridisation technology has shown that wide fluctuations in the concentration of the virus exist in HBeAg-positive carriers. Vertical transmission is rarely documented with maternal HBV DNA levels below 10^7 geq/mL (5 pg/mL) [33, 34]. In a recent study, no cases of transplacental transmission of HBV were observed with maternal HBV DNA levels of 6.0×10^5 geq/mL or lower. In mothers with HBV DNA levels of 10^6/10^7 geq/mL and 10^8 geq/mL, 2% and 22% of the children, respectively, had HBsAg detected in the blood within 24 hours after birth (=transplacental transmission) [35].

HBeAg-positive physicians are not allowed to perform exposure-prone procedures. This prevention measure is inadequate, since HBeAg-negative pre-core mutants of HBV may achieve high titers of circulating virus in the peripheral blood. HBV transmission from four HBeAg negative surgeons to patients has been documented [36]. A more reliable estimate of the infectivity can be obtained by testing serum concentrations of HBV DNA [37].

The European consensus panel [38] now proposes a maximum HBV DNA level of 10^4 geq/mL, but does not supply scientific data supporting this suggestion [39].

Detection of HBV DNA in occult infection and in unusual serologic constellations

HBV infection can be detected with HBV NAT in virus carriers with immunosilent infection (absence of any serological marker) or presenting an “unusual serologic constellation” as listed below:

- Individuals with isolated anti-HBc reactivity
- Patients with discordant results between HBsAg assays
- Patients seronegative for HBsAg but HBeAg-positive
- Presence of both HBsAg (HBeAg) and anti-HBs (mostly at low titer <100 mUI/mL)

An HBV infection is considered to be an occult infection if HBsAg is not detected, but HBV DNA is present independently of the other serological markers. Occult infection may also be present in patients with “recovery” from infection, defined by the presence of anti-HBs. The reasons for the presence of HBV DNA, mostly at low viral load, are the following (in order of importance):

1. After many years of chronic HBV carriage, the level of HBsAg in the peripheral blood declines below the detection limit of immunoassays, giving rise to the

Table 3 Prevalence of occult HBV infection in anti-HBc-positive individuals, HBV DNA load and frequency of diagnostic escape mutants.

Population	Geographic location	Isolated anti-HBc positive (%)	HBV DNA-positive (%)	HBV DNA load (copies/mL)	Frequency of escape mutants (%)	Lower detection limit of NAT	Author(s)
First-time blood donors	Germany	20 (0.14)	3 (15)	ND	ND	28.7 IU/mL	Hennig et al. [30]
Blood donors	Ghana	107	14 (13)	ND	ND	20 IU/mL	Alain et al. [51]
Blood donors	USA	387	4 (3.7)	10–100	ND	<50 copies/mL	Kleinman et al. [27]
Blood donors	Indonesia	67	20 (29.9)	ND	6 (9.0)		Thedja et al. [52]
General	Germany	81 (1.5)	5 (7.7)	100–1000	ND	100 copies/mL	Jilg et al. [53]
Hospitalised	Germany	184 (1.4)	6 (3.2)		ND	5× copies/mL	Berger et al. [54]
Hospitalised	Germany				ND	400 copies/mL	Weber et al. [29]
	UK	151	6 (4.0)		1 (0.7)	100–400 copies/mL	Alhababi et al. [45]

ND, not determined.

isolated anti-HBc positive reactivity. The second most frequent reason for the solely anti-HBc reactivity is the disappearance of anti-HBs in individuals who have recovered from HBV. Table 3 gives an overview of the prevalence of occult HBV infection in anti-HBc positive individuals, HBV DNA load, and frequency of diagnostic escape mutants.

2. Amino acid substitution within the "a" determinant of the HBsAg can lead to conformational changes, which can affect the binding of neutralising antibodies with the possible consequence that mutant viruses may escape detection by certain commercial HBsAg kits and/or give discordant results when tested with HBsAg assays that show different sensitivities for surface mutants. The great danger represented by HBV S gene variants/mutants is that they can be transmitted horizontally by the peripheral blood of asymptomatic carriers, with no known risk factors of HBV infection. The presence of G145R mutant in the PBL may be associated with immunosilent carriage (HBsAg and anti-HBc-negative [40]). Immunosilent HBV infection may also be observed in HBV carriers of double mutants with amino acid substitutions in the "a" determinant of the S antigen as well as in the C gene leading to truncated HBeAg and core proteins [41]. There is great concern that surface mutants may be spread by blood transfusion, since HBV DNA detection by nucleic acid amplification technology is not mandatory for blood donor screening in many countries. This is true especially in areas where the prevalence of escape mutants is expected to be high. HBV mutants in blood donors negative for HBsAg by one or several screening assays have been described [42, 43]. A mutation in position 144 gave a negative result in the HBsAg assay based on monoclonal capture and tracer antibodies in an HBV DNA positive blood donor. When retested with an alternative assay, which used polyclonal tracer antibody, HBsAg could be detected.

The sensitivity of HBsAg assays for mutant detection is continuously improved. However, the performance of immunoassays for mutant virus detection is highly variable, ranging from 57.5% for a screening assay based on monoclonal capture and tracer antibodies and to 80.6–97.4% of mutants detected for immunoassays that use polyclonal tracer antibodies [44].

If the level of antigenaemia is low, as it is generally the case in healthy blood donors with inactive hepatitis B, low levels of circulating HBsAg mutants may not be diagnosed, even if the immunoassay is capable of detecting the recombinant form of the corresponding mutant [45, 46]. The analytical sensitivity of HBsAg assays should therefore be further improved. On the basis of available data, monoclonal antibody-based assays show a poor sensitivity for mutants.

Table 4 Prevalence of occult HBV infection in individuals with a serological profile of resolved infection.

Population	Geographic location	Resolved infection	HBV DNA positive (%)	HBV DNA load (copies/mL)	Follow-up after onset of acute self-limiting HBV infection	Lower detection limit of NAT	Author(s)
First-time blood donors	Germany	169	3 (1.8)	ND	ND	28.7 IU/mL	Hennig et al. [30]
General	Luxembourg	137	10 (7.3)	40–50.000	ND	6 IU/mL	Weber et al. [47]
	Japan	12	3 (25)		4.2 years (median)	?	Yuki et al. [55]
Blood donors	Japan	11	10 (90.9)	<1000	19 months	?	Yotsuyanagi et al. [48]
General	Sweden	63	20 (31.7)	100–700		200 copies/mL	Noborg et al. [56]

ND, not determined; ?, no data available.

Table 5 Occult HBV infection in individuals with a serological profile of resolved infection: Viral load and anti-HBs titer classes.

Anti-HBs titre	HBV DNA tested	HBV DNA-positive (%)	Viral loads (IU/mL)
10–100	38	3 (7.9)	7.5; 155; 679
100–1000	15	1 (6.6)	10.8
>1000	84	6 (7.1)	11.6; 26; 46; 49; 805; 9230
Total	137	10 (7.3)	

Table 6 Possible causes for the presence of HBV DNA in patients presenting the following serological profiles.

Seroconstellation	Possible causes for the presence of HBV DNA	Relative probability	Comments
Isolated anti-HBc positive	Resolved infection with loss of anti-HBs	+++	Consider algorithm for the resolution of isolated anti-HBc reactivity [57].
	Low-level HBsAg under detection limit of EIAs	+++	
	Inhibition of HBsAg synthesis by HCV co-infection	++	Several causes, e.g. presence of low-level **and** mutant HBsAg may be responsible for this seroconstellation [45].
	Variability of anti-HBs assays	++	
	False-positive result	+	
	S-gene mutant	+	
Resolved infection	Insufficient cytotoxic T-lymphocyte response that is not able to completely clear the produced virus and presence of immune complexes	+++	Infectivity is probably low due to the presence of immune complexes.
	S-gene mutant	+	
Discordant results between HBsAg assays	Low-level HBsAg carrier tested with EIAs of different sensitivities	++	Several causes, e. g. presence of low-level **and** mutant HBsAg may be responsible for this seroconstellation.
	Presence of a genotype not recognised by one of the EIAs	++	
	S-gene mutant not recognised by one of the EIAs	++	
Presence of both HBsAg and anti-HBs	Heterotypic immune response: sequential infection with antibodies directed against subtypic and not "a"determinant	+++	This pattern is observed in about 10% of chronic HBsAg carriers.
	S-gene mutant	+	Mutations close to the "a" determinant may induce changes in the structural configuration so that it is not efficiently neutralized by anti-HBs antibody but still recognised by the antibody of the anti-HBs antibody of the diagnostic enzyme immunoassay [58].
Immunosilent infection	Double S and C gene mutants	?	Amino acid substitutions in the "a" determinant of the S antigen and in the C gene leading to truncated proteins [41].
	Others: HCV-co-infection?	?	

+++ high probability; ++ moderate probability; + low probability; ? unknown.

HBsAg assays capable of detecting mutants may potentially reduce the frequency of occult infection, since in isolated anti-HBc reactive HBV DNA-positive individuals, mutants of the "a" determinant may be present. As the viral load in this seroconstellation is generally low, the HBsAg concentration may be below the detection limit of the immunoassay, even if it is capable of recognising the corresponding recombinant mutant form [45]. Consequently, these assays will not replace NAT and/or anti-HBc screening of blood donors.

3. Individuals who have recovered from hepatitis B and produced neutralising anti-HBs even at high levels (Tables 4 and 5, data from [47]) may continue to replicate HBV DNA at low levels that are detectable in the liver, peripheral blood, mononuclear cells or serum for years [7]. The persistence of HBV might be explained by an insufficient cytotoxic T-lymphocyte response which is unable to completely clear the produced virus. The percentage of HBV DNA carriers is highly variable depending on the geographical location, the population tested, and the study design. Most of the data published so far are only preliminary since small numbers of samples have been investigated. The frequency of low-level HBV DNA carriers in follow-up surveys of individuals with self-limiting acute hepatitis seems to be very high. There is probably a statistical bias, since in cases of acute symp-

tomatic hepatitis, the inoculated viral load may be high in contrast to subclinical or asymptomatic acute HBV infection, which may explain why in individuals who recovered from infection, HBV DNA may persist for a longer time, and/or the immune response is not efficient enough to completely eliminate circulating virus. HBV DNA is not only present in individuals with low anti-HBs (<100 IU/L), but is observed with a similar frequency in the presence of high anti-HBs titres (Table 5).

It is admitted that the circulating virions are probably mostly wild-type. However, due to the selective pressure of a "subliminal" immune response, there might be an ideal breading ground for surface mutant emergence in patients with the serological profile of resolved hepatitis B. The infectivity of carriers with recovered infection is probably weak, since the copy number of HBV DNA is low and a variable part of the virions is trapped in immune complexes with anti-HBs [48].

4. Low levels of HBV DNA may be detected in individuals with no marker of HBV (immunosilent) infection. The frequency of this kind of occult infection and the mechanism leading to the loss of serological markers are not known. Immunosilent HBV infection may be observed in HBV carriers of double mutants with amino acid substitutions in the "a" determinant of the S antigen as well as in the C gene leading to truncated HBeAg and core proteins [41]. In cases of HCV infection, several reports have found low levels of HBV DNA in the absence of serological HBV markers [7, 49]. HBV DNA has also been recovered from vaccinated children in which anti-HBs were no longer detectable [50].

   Table 6 gives a summary of the possible causes for the presence of HBV DNA in "unusual" seroconstellations and immunosilent infection.

Conclusions

HBV NAT blood donor screening in individual samples or mini-pools will probably be performed with fully automated highly sensitive multiplex assays in most industrialised countries, despite the relatively high incremental costs for reducing the transfusion risk and the modest benefit for the patient in terms of quality of life.

Automation of quantitative NAT for monitoring of chronic infection and therapy will be further optimised in order to offer easy-to-use assays for the medium-sized clinical laboratory. PCR and TMA assays as alternatives to those actually available are on the verge of being commercialised. With the number of NAT increasing, the goal of standardisation of HBV DNA quantification will be difficult to achieve. Studies are also needed on the clinical significance of low serum HBV DNA levels in relation to the natural history of hepatitis B and the relation between serum HBV DNA levels and clinical outcome. The clinical value of quantitative anti-HBc-IgM and HBsAg and HBeAg as easy-to-use and low-cost surrogate markers in the era of highly sensitive NAT needs to be re-assessed.

With the introduction of new antiviral agents and combination therapies, the number of resistance mutations will increase. Gene arrays will represent an alternative to sequencing or reverse hybridisation assays for mutation genotyping. Treatment algorithms need to be developed in order to optimise treatment strategies and to prevent the recurrence or reactivation of HBV infection.

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