Detection Technology and Clinical Applications of Serum Virological Products of Hepatitis B Virus (HBV) Infection

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Abstract: Serum virological products of hepatitis B virus (HBV) infection, such as HBV DNA, HBV RNA, hepatitis B surface antigen (HBsAg), hepatitis B e antigen (HBeAg), and hepatitis B core-related antigen (HBcrAg), are important non-invasive indicators that are critical for the rapid diagnosis of the diseases. Improving the accuracy of these products is essential, and the development of corresponding detection technologies is pivotal in achieving this goal. The aim of this article is to provide a comprehensive and systematic overview of the life cycle of HBV, including the latest research progress. In addition, classical and novel methods for detecting HBV serum virological products with high accuracy are analyzed and compared, and their clinical applications are discussed. Ultimately, the goal of this article is to provide valuable insights and references for effectively monitoring chronic hepatitis B (CHB) infection.

Keywords: HBV; Serum virological products; Detection technology; Diagnosis and treatment

1.Introduction

Hepatitis B virus (HBV) is a small (3.2 kb), partially double-stranded DNA virus that belongs to the Hepadnaviridae family [1]. The discovery of the "Australian antigen" as the hepatitis B virus surface antigen (HBsAg) by Dr. Baruch Blumberg in 1967 paved the way for the diagnosis, prevention, and treatment of HBV infection [2]. The history of serum virological products of hepatitis B virus is shown in Figure 1. As shown in Table1, the European Association for the Study of the Liver (EASL) classified chronic HBV infection into the following categories: HBeAg-positive chronic infection (formerly known as the immune tolerance period), HBeAg-positive hepatitis B infection (formerly known as the HBeAg-positive immune activity period or immune clearance period), HBeAg-negative chronic infection (formerly known as the inactive carrier phase or low replication phase), and HBeAg-negative chronic hepatitis B infection (formerly known as the HBeAg-negative immune active phase or reactivation phase) based on the evaluation of HBV related liver disease indicators [3, 4]. However, around 40% of patients cannot be categorized under the above-mentioned stages [4], resulting in a new category of infection which is referred to as the "uncertain period" of chronic HBV infection. Furthermore, the risk of progression of chronic hepatitis B infection during the "uncertain period" is still high [4].

Over the past 30 years, there has been extensive research on the HBV life cycle[5]. The virus binds to liver-specific receptors such as sodium taurocholate cotransporting polypeptide (NTCPs) and heparan sulfate proteoglycans (HSPGs), resulted in endocytosis and the release of HBV core particles[6, 7]. These particles are transported to the nucleus, where the HBV genome is released and gets converted from relaxed circular double-stranded DNA (rcDNA) to covalently closed circular DNA (cccDNA)[8], which is highly stable and contributes to the persistence of chronic HBV infection and low cure rates[9]. HBV cccDNA interacts with host transcription factors to produce pgRNA, HBx mRNA, precore mRNA, and preS/S

mRNA [10]. The HBV mRNA is primarily transported to the cytoplasm and translated without splicing. The pgRNA is responsible for the expression of core proteins and is translated into core proteins and viral polymerase in the cytosol, which are then assembled into immature core particles and released into the blood[11]. It has been studied that the serological HBV RNA level might reflect cccDNA translational activity in liver tissue[12-15]. The mutation of the HBV genome occurs through reverse transcription of the HBV pgRNA by HBV DNA polymerase to rcDNA in core granules [16, 17]. Mature core particles can be enveloped by virus surface proteins and secreted through the multivesicularbody (MVB) pathway, or these can migrate to the nuclear pore complex [18]. Early assessment of histological damage and residual levels of new infection caused by viral activity during the HBV life cycle is crucial for preventing hepatitis recurrence, liver disease progression and ultimately achieving a functional cure against chronic hepatitis B (CHB) infection.

2. Advancements in the detection methods for HBV serum virological products

In clinical practice, based on the nature of the specific products, valuable information for evaluating clinical phase of HBV infection can be extracted by quantifying serum virological products. Currently, there are ongoing debates within the scientific community about the optimal test method for different serological products as the detection methods are constantly getting innovated. Classical methods of detection include the following: Quantitative Real-time polymerase chain reaction (qPCR), enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), and enzyme immunoassay (EIA). In recent years, stable and highly precise new detection technologies including Chemiluminescence immunoassay (CLIA) and chemiluminescent particle immunoassay (CMIA), emerged [19].Among them, five methods for classical HBV serum biomarker detection are shown in Table 2.

Quantitative Real-time PCR (qPCR) is a powerful detection method that involves the addition of a fluorescent gene to the PCR reaction system. This allows for real-time monitoring of the entire PCR process using fluorescence signal accumulation, enabling accurate of an unknown template by using a standard curve [20]. In compared to clinical testing, biological chemistry, and other assays, quantitative PCR

testing does have a relatively low degree of automation and requires higher levels of expertise from operators and careful management of experimental conditions[21, 22]. The precision and accuracy of the La Roche Light Cycler 480 qPCR system for HBV-DNA detection have been evaluated, and its performance has been verified. Using TaqMan probes, HBV RNA in serum samples can also be quantified utilizing quantitative Real-time RT-PCR[23]. However, during qPCR operation, several parameters must be taken into consideration, such as the background of homologous and heterologous DNA and the specificity of oligonucleotide hybridization, as these factors may contribute to the quantitative deviation of real-time fluorescence quantitative PCR reactions. Therefore, to ensure the accuracy and reliability of the results, it is crucial to optimize the experimental conditions during the operation [24, 25].

In medical laboratories, in vitro diagnostic product manufacturers industries, regulatory agencies, and external quality assessment and capability verification organizations, enzyme-linked immunosorbent assay (ELISA) has become a ubiquitous technology [26]. ELISA is an immunological detection commonly used to detect the presence of the hepatitis B virus. However, while ELISA is a useful tool for detecting the virus, it cannot provide precise information on virus replication, infection, and associated risks. This method can also yield false positive and false negative results [27].

Chemiluminescence immunoassay (CLIA) is a widely used technology in clinical medicine for detecting trace amounts of antigens and antibodies. It is a labeled immunoassay that combines luminescence analysis with the reaction of immune system[28]. CLIA is a detection technique developed behind enzyme-linked immunoassay, radioimmunoassay and fluorescence immunoassay. Because it has the advantages of simple operation, convenient marking, high stability and detection sensitivity, fast speed, no pollution to the environment and so on, it has been praised by medical examiners and doctors in clinical practice.

Electrochemiluminescence immunoassay (ECLIA), a highly sensitive detection technology, which combines electrochemical luminescence with immunoassay, is

tested by chemiluminescence reaction [29]. Its significant stability and sensitivity make it ideal for detecting trace substances. However, the results obtained from ECLIA tests that seem suspicious should be further confirmed using additional testing methods. For instance, owing to the increased sensitivity of HBeAg, a considerable proportion (21.24%) of HBeAg and HBeAb can be detected simultaneously during the recognition of HBeAg and HBeAb [30, 31]. However, the classification of some HBeAg positive test results obtained using current domestic EIA test standards as "false positive" necessitates further discussion[30, 32]. Microparticle enzyme immunoassay (MIEA) is an immunoassay technology in which intraparticle factors are used to form complexes with substances to be tested. The complexes then react with an alkaline phosphatase conjugate compound to produce a fluorescent product. It offers various advantageous features, such as high sensitivity, strong specificity, good repeatability, and simple operation. Chemiluminescent particle immunoassay (CMIA) is a technique that involves two methods, namely, competitive and double antibody sandwich. The small solid-phase magnetic particles used in this instrument, have a diameter of only 1.0 μm. This small size increases the coating surface area, amount of adsorption of the antigen or antibody, and reaction speed and decreases the amount of pollution and the probability of cross-contamination [33]. In CMIA, the antigen or antibody is labeled with alkaline phosphatase (ALP), which undergoes luminescence reactions by reacting with its substrate, dioxane phosphate [34]. CMIA provides offers a versatile selection of serum immunoassays with high sensitivity to detect HBsAg, capable of reaching 0.1ng/ml, good repeatability and specificity[35, 36]. However, the cost of the instrument cost is high [35]. In summary, these detection technologies are valuable tools in clinical medicine because of their high sensitivity, specificity, and good repeatability [37]. Nevertheless, if the results seem to be suspicious, further confirmatory tests should be.

3.Routine serum virological products of HBV and their detection methods

A critical aspect of managing CHB infection involve monitoring the activity of HBV replication. Serological and histological products are typically detected during the diagnosis of HBV infection. Over the duration of the infection, cccDNA accumulates

in the nucleus, persists as a stable inclusion, and serves as a template for the transcription of viral genes[38]. HBV has a relatively short half-life [39, 40], but its persistence remains a significant challenge in eradicating HBV infection. One of the major challenges in HBV cccDNA research is the absence of an efficient method for detecting cccDNA in biopsy tissues with high sensitivity, significant specificity, and accurate quantification [41]. Liver biopsies, which are the most commonly used for detecting HBV cccDNA, are invasive and associated with risks, making them challenging to perform routine detection on a regular basis [42]. To address this issue, facilitate research on cccDNA, and detect and quantify cccDNA, numerous novel approaches have recently been applied, including polymerase chain reaction (PCR)-based methods, Invader assays, in situ hybridization, and surrogates, [43]. Serological detection is currently considered as an alternative, which is non-invasive, easy to operate, and cost-effective [44]. Finding an ideal serological index, therefore, that can reflect the presence of HBV cccDNA and its transcriptional activity is a crucial clinical requirement. In the process of gradually discovering HBV, HBV serum products play a crucial role in promoting the development of clinical medicine and have significant value in the diagnosis and treatment of HBV infection.

Currently, common serological products that are used to diagnose the infection of the HBV include the following: HBsAg, HBeAg, and HBV DNA [45]. Among these, HBsAg is widely used product for diagnosing hepatitis B and is considered superior to other products in indicating clinical cure after treatment with pegylated interferon and nucleoside (acid) analogs [46-48]. HBsAg is an antigen that is found on the surface of HBV. It’s composed of small, medium, and large HB proteins. It plays a crucial role in initiating the infection process of HBV by facilitating the attachment of the virus to the host cell membrane [49]. HBsAg is the earliest serological product to appear in acute hepatitis B infection. It’s also commonly detected in chronic carriers of disease [50], indicating ongoing viral transcriptional activity and rather than acute infection[51]. Automatic chemiluminescent enzyme immunoassay (CLEIA) [52] , CLIA or standard ELISA is generally performed to detect HBsAg [53]. Chronic HBV infection is defined as a persistent infection lasting for more than 6 months, during

which detectable HBsAg can be detected using detection technologies [54]. Patients with low levels of HBsAg may still experience active replication of the virus and associated liver injury. The patients may also be capable of spreading the infection to others [55, 56]. As diagnostic and treatment technologies for HBV continues to advance, researchers are increasingly focusing on individuals who have low levels of HBsAg [57]. It has been shown that a combination of HBsAg quantification and the expression of certain T-cell markers could be a potential predictor of HBsAg clearance in patients with chronic HBV infection within a period of 12 months [46].

Hepatitis B e antigen (HBeAg) is a soluble component of the hepatitis B core antigen. The presence of HBeAg indicates the risk of active replication of the HBV and transmitting the infection [58]. Clinical laboratories detect HBeAg using procedures like ELISA, ECLIA, or CLIA. Research studies focusing on these methods demonstrated that HBeAg quantification is a useful tool in predicting the antiviral efficacy of HBV reactivation. Additionally, HBeAg levels are higher in patients experiencing HBV reactivation compared to those with acute infection and HBeAg-positive chronic infection [59]. HBeAg seroconversion is a crucial goal of antiviral treatment for patients with CHB. When a patient undergoes HBeAg seroconversion, it indicates that HBV has entered the a replication stage, reducing the likelihood of progression to cirrhosis and liver cancer, as well as reducing the risk of infectivity [60]. However, several factors can influence the seroconversion of HBeAg. Numerous cytokines/chemokines, including IL-37, IP-10, IL-21, and CLEC18, have been correlated with HBeAg seroconversion in patients with CHB who are being treated with nucleos(t)ide analogues (NAs) [61-63]. Studies conducted on mice demonstrated that in liver-hosted macrophages KCs, HBeAg inhibits the transcription of NLRP3 and pro-interleukin (IL) 1β by reducing the phosphorylation level of NF-κB. Additionally, it inhibits caspase-1 activation and IL-1β maturation by blocking the production of reactive oxygen species [64].

The high level of HBV DNA in a patient’s serum indicates active replication of the virus in the liver, which is a critical factor in the progression of liver disease. Recent research suggests that even persistently low levels of HBV DNA can contribute to the

progression of chronic hepatitis B infection [65]. HBV DNA Low-Level Viremia (LLV) is a term used to describe the detection of HBV DNA in a patient's serum at levels ranging from 20 to 2000 IU/ML after 48 weeks of antiviral treatment [66, 67]. According to the guidelines of the American Association for Liver Research (AASLD) and the EASL, potent antiviral drugs such as propofol tenofovir (TAF), tenofovir (TDF), and entecavir (ETV) are recommended for the management of LLV, after excluding issues related to compliance and detection errors [68]. The detection of HBV DNA in a patient's serum is considered the most reliable method for determine hepatitis B viremia, and it is the most reliable product of active viral replication [69]. The level of HBV DNA in a patient's serum is closely associated with the risk of liver fibrosis and hepatocellular carcinoma in patients with CHB[70]. Occult HBV infection (OBI) is a term used to describe the presence of cccDNA in the liver and/or the presence of HBV DNA in the blood of individuals who have tested negative for HBsAg [71]. Detecting HBV DNA in the liver is the ideal method of diagnosis [72] and detection of anti-HBc in the blood is often used as a surrogate[69]. In occult infections, there may be an increased risk of progression to cirrhosis and HCC development despite complete loss of serum HBsAg and undetectable or very low[73]. Over the past decade, there have been significant advancements in the methods used to assess serum HBV DNA levels. These advancements have resulted in several sensitive tests. Currently, qPCR has become the most widely used method for detecting secreted HBV DNA, in addition to Nested PCR and Droplet Digital PCR (ddPCR) [74, 75].

4.Novel serum virological products of HBV and their detection methods

In recent years, there has been a growing trend to use novel serological viral nucleic acid products such as serum HBV RNA, for monitoring patients infected with HBV. Previous studies indicated that HBV RNA in serum is pgRNA, which is encapsulated in HBV-like virus particles, that can be secreted extracellularly. This suggests that HBV may have alternative form of virion, in which the nucleic acid is composed of RNA rather than DNA [76]. HBV RNA has been identified as an indicator of intrahepatic transcriptional activity of cccDNA and is found to be

associated with liver histological changes in patients with chronic hepatitis B who have been treated with nucleoside (acid) analogs [77]. Serum HBV RNA levels have emerged as a useful alternative product for assessing the transcriptional activity of cccDNA. A case-control study involving 104 patients receiving entecavir treatment revealed that after adjusting for various risk factors such as age, sex, presence or absence of cirrhosis, and duration of antiviral treatment, the level of HBV RNA detected during treatment was associated with an increased risk of developing HCC within the next two years [78]. To date, relatively few studies have been published focusing on the association between HBV RNA levels and the risk of developing HCC. It has been reported that HBV RNA serves as a predictive product not only for HBsAg response during early antiviral therapy but also for the risk of HBsAg reversal after discontinuing the treatment. At 24 weeks, levels of HBV RNA declined more rapidly in patients who received HBsAg serologic conversion compared to those who did not [79]. Several different techniques are available for quantifying RNA levels, including rapid amplification RT-qPCR based on cDNA terminal (RACE), standard RT-qPCR, and droplet digital PCR (ddPCR) [78]. Following that, standardization of HBV RNA detection methods is essential.

Hepatitis B core antigen (HBcrAg) includes several proteins, including HBV core antigen (HBcAg), HBeAg, and pre-core/core protein (p22cr), which has a molecular weight of 22 kDa. Its quantitative measurement is of great significance for guiding the clinical management of chronic HBV infection[49, 80, 81]. HBcrAg is emerging as a novel product for CHB that has been shown to correlate with current antiviral therapy responses to HBeAg-positive CHB and should be considered in addition to secreted HBV RNA when evaluating new antiviral therapies that target hepatic cccDNA activity directly or indirectly, with the goal of achieving functional cure [72]. The use of highly sensitive HBcrAg detection in patients with undetectable HBV DNA and HBsAg is anticipated to become a very useful prognostic factor for predicting the long-term prognosis of CHB[82]. It is reported that the predicted performance of HBcrAg may vary depending on the clinical endpoint being considered for CHB [83]. The clinically anticipated performance of HBcrAg is inconsistent, and there is a poor

correlation between HBsAg loss and antiviral treatment [84]. Therefore, the interpretation of the HBcrAg results should be made carefully in clinical practice [81, 85]. CLEIA is a method primarily used for the detection of HBcrAg in patient's serum [78].

5.Summary and prospect

Over the past decade, there has been significant progress in the assessment of non-invasive liver disease in patients with CHB infection [86]. Although existing antiviral drugs to treat hepatitis B can effectively control the progression of the disease, they cannot always completely eliminate the virus or achieve a functional cure [87], which is characterized as follows: negative HBsAg, undetectable HBV DNA, normal liver biochemical indicators, and improved liver tissue pathology [4, 88]. However, cccDNA may still exist in the nucleus of the liver of patients, increasing their likelihood of HBV reactivation and hepatocellular carcinoma [89, 90]. It is anticipated that novel HBV products, including HBV RNA and HBcrAg, that have emerged will help in predicting treatment response to new therapies [91]. However, the sensitivity and standardization of these products should be further optimized, and they may not necessarily be better than HBsAg at predicting treatment outcomes. To prevent affecting the impact of detection, it is crucial to select different detection methods and commercial kits for viral detection. Their effects may change with the introduction of new therapeutic drugs, and the progress in technology and ultra-sensitive detection may redefine the meaning of "functional cure" or even "partial functional cure"[72, 78]. Overall, the development of new HBV products has the potential to improve the evaluation of antiviral efficacy and prediction of relapse risk after drug withdrawal, thereby aiding clinicians to better treat patients with CHB.

There is a requirement for continuous improvement in detection sensitivity to prevent and treat hepatitis B effectively. Currently, the ELISA method is mostly in the detection of HBsAg; however, its operation is complex and susceptible to human error, which can lead to the possibility of missing the detection of low levels of HBsAg [92]. The MEIA method has high sensitivity and good repeatability, making it a safe and closed operation with a low risk of contamination. However, it is relatively expensive

compared to other methods of detection. As a result, CMIA methods are likely to replace MEIA methods, particularly in large environments, due to their sensitivity, speed, reliability, and capacity to combine immunology and biochemical tests. ECLIA method has ideal clinical application value. It has the advantages of high detection rate, high sensitivity, strong specificity and wide detection range. The reagent has the potential to remain stable for a long time without toxicity or contamination. CLIA is a non-radioactive immunoassay method developed rapidly in the past 30 years. It is an ultra-sensitive microassay technology developed after EIA, RIA, etc. With high sensitivity of chemiluminescence and good specificity of immunoassay, it has attracted wide attention in recent years. CLIA is widely used in clinical diagnosis and biochemistry, for the determination of tumor markers, cytokines, hormones, etc[93]. Currently imported chemiluminescence system is still the mainstream detection method, such as Abbott, Siemens, Roche, Beckman and other brands.

Research into new antiviral drugs with novel targets of action is ongoingly driven by an improved understanding of the replication cycle of the HBV. However, the existence of cccDNA and integrated DNA, as well as the complex regulation of HBV-specific immunity, poses major challenges for this study [67, 88]. To address these challenges, new detection methods have been developed, such as the quantification of HBsAg, HBcrAg, HBV RNA, and other products, as substitute indicators of liver cccDNA. These methods can aid in the confirmation of the targets of new drugs and the prediction of the serological conversion of HBsAg. With the development of new and more sensitive detection methods for quantifying HBV products, it is anticipated that these methods will become increasingly useful in guiding clinical treatment and improving outcomes for patients with HBV infection. The combination of new products with traditional products and clinical parameters can improve the specificity and sensitivity of the detection[72].

Reference

1. Li, X., et al., *The infection efficiency and replication ability of circularized HBV DNA optimized the linear HBV DNA in vitro and in vivo.* Int J Mol Sci, 2015. **16**(3): p. 5141-60.
2. Li, H., et al., *Hepatitis B Virus Infection: Overview.* Adv Exp Med Biol, 2020.

1179: p. 1-16.

3. Zhuang, H., *[Brief introduction of "Treatment algorithm for managing chronic hepatitis B virus infection in the United States: 2021 update"].* Zhonghua Gan Zang Bing Za Zhi, 2022. **30**(2): p. 190-195.
4. Chinese Society of Hepatology, C.M.A., *[Expert opinion on expanding anti-HBV treatment for chronic hepatitis B].* Zhonghua Gan Zang Bing Za Zhi, 2022. **30**(2): p. 131-136.
5. Safadi, R., et al., *Efficacy of Birth Dose Vaccination in Preventing Mother-to-Child Transmission of Hepatitis B: A Randomized Controlled Trial Comparing Engerix-B and Sci-B-Vac*. Vaccines (Basel), 2021. **9**(4).
6. Lim, S.G., et al., *The scientific basis of combination therapy for chronic hepatitis B functional cure.* Nat Rev Gastroenterol Hepatol, 2023.
7. Li, Q., et al., *Interferon and interferon-stimulated genes in HBV treatment.* Front Immunol, 2022. **13**: p. 1034968.
8. Wu, J.F. and M.H. Chang, *Natural history of chronic hepatitis B virus infection from infancy to adult life - the mechanism of inflammation triggering and long-term impacts.* J Biomed Sci, 2015. **22**: p. 92.
9. Tsukuda, S. and K. Watashi, *Hepatitis B virus biology and life cycle.* Antiviral Res, 2020. **182**: p. 104925.
10. Nassal, M., *HBV cccDNA: viral persistence reservoir and key obstacle for a cure of chronic hepatitis B.* Gut, 2015. **64**(12): p. 1972-84.
11. Olenginski, L.T., et al., *Virtual Screening of Hepatitis B Virus Pre-Genomic RNA as a Novel Therapeutic Target.* Molecules, 2023. **28**(4).
12. Tong, S. and P. Revill, *Overview of hepatitis B viral replication and genetic variability.* J Hepatol, 2016. **64**(1 Suppl): p. S4-S16.
13. Yu, T., et al., *Evidence of Residual Ongoing Viral Replication in Chronic Hepatitis B Patients Successfully Treated with Nucleos(t)ide Analogues.* J Infect Dis, 2022.
14. Zhao, Q., et al., *Pregenomic RNA Launch Hepatitis B Virus Replication System Facilitates the Mechanistic Study of Antiviral Agents and Drug-Resistant Variants on Covalently Closed Circular DNA Synthesis.* J Virol, 2022. **96**(24): p. e0115022.
15. Liu, Y.N., et al., *[Expert consensus on measurement and clinical application of serum HBV RNA in patients with chronic HBV infection].* Zhonghua Gan Zang Bing Za Zhi, 2022. **30**(5): p. 505-512.
16. Imam, H., et al., *N6-methyladenosine modification of hepatitis B virus RNA differentially regulates the viral life cycle.* Proc Natl Acad Sci U S A, 2018. **115**(35): p. 8829-8834.
17. Schadler, S. and E. Hildt, *HBV life cycle: entry and morphogenesis.* Viruses, 2009. **1**(2): p. 185-209.
18. Chigbu, D.I., et al., *Hepatitis C Virus Infection: Host(-)Virus Interaction and Mechanisms of Viral Persistence*. Cells, 2019. **8**(4).
19. Rong, X., et al., *Monitoring hepatitis B by using point-of-care testing: biomarkers, current technologies, and perspectives*. Expert Rev Mol Diagn,

2021. **21**(2): p. 195-211.
20. Siegel, S.R., M. Ulrich, and S.F. Logue, *Comparison qPCR study for selecting a valid single copy gene for measuring absolute telomere length.* Gene, 2023. **860**: p. 147192.
21. Liu, J., et al., *Countdown to 2030: eliminating hepatitis B disease, China.* Bull World Health Organ, 2019. **97**(3): p. 230-238.
22. Irshad, M., et al., *Multiplex qPCR for serodetection and serotyping of hepatitis viruses: A brief review.* World J Gastroenterol, 2016. **22**(20): p. 4824-34.
23. Ji, X., et al., *Serum Hepatitis B Virus RNA Levels Predict HBeAg Seroconversion and Virological Response in Chronic Hepatitis B Patients with High Viral Load Treated with Nucleos(t)ide Analog.* Infect Drug Resist, 2020. **13**: p. 1881-1888.
24. Wikstrom, P., L. Hagglund, and M. Forsman, *Structure of a Natural Microbial Community in a Nitroaromatic Contaminated Groundwater Is Altered during Biodegradation of Extrinsic, but Not Intrinsic Substrates*. Microb Ecol, 2000. **39**(3): p. 203-210.
25. Tay, S.T., et al., *Population Dynamics of Two Toluene Degrading Bacterial Species in a Contaminated Stream.* Microb Ecol, 2001. **41**(2): p. 124-131.
26. Lequin, R.M., *Enzyme immunoassay (EIA)/enzyme-linked immunosorbent assay (ELISA).* Clin Chem, 2005. **51**(12): p. 2415-8.
27. Hu, X., et al., *DropLab: an automated magnetic digital microfluidic platform for sample-to-answer point-of-care testing-development and application to quantitative immunodiagnostics.* Microsyst Nanoeng, 2023. **9**: p. 10.
28. Shen, Y., W. Prinyawiwatkul, and Z. Xu, *Insulin: a review of analytical methods*. Analyst, 2019. **144**(14): p. 4139-4148.
29. Kovac, M., et al., *EDTA-Anticoagulated Whole Blood for SARS-CoV-2 Antibody Testing by Electrochemiluminescence Immunoassay (ECLIA) and Enzyme-Linked Immunosorbent Assay (ELISA).* Diagnostics (Basel), 2020. **10**(8).
30. Yuan, Q., et al., *Molecular characteristics of occult hepatitis B virus from blood donors in southeast China*. J Clin Microbiol, 2010. **48**(2): p. 357-62.
31. Ocana, S., et al., *Diagnostic strategy for occult hepatitis B virus infection.* World J Gastroenterol, 2011. **17**(12): p. 1553-7.
32. Prieto, B., et al., *New quantitative electrochemiluminescence method (ECLIA) for interleukin-6 (IL-6) measurement.* Clin Chem Lab Med, 2010. **48**(6): p. 835-8.
33. Li, F., et al., *Human hepatocyte-enriched miRNA-192-3p promotes HBV replication through inhibiting Akt/mTOR signalling by targeting ZNF143 in hepatic cell lines.* Emerg Microbes Infect, 2022. **11**(1): p. 616-628.
34. Loglio, A., et al., *The combination of PIVKA-II and AFP improves the detection accuracy for HCC in HBV caucasian cirrhotics on long-term oral therapy.* Liver Int, 2020. **40**(8): p. 1987-1996.
35. Motyka, J., et al., *Plasma Levels of CXC Motif Chemokine 1 (CXCL1) and Chemokine 8 (CXCL8) as Diagnostic Biomarkers in Luminal A and B Breast*

Cancer. J Clin Med, 2022. **11**(22).

36. Hadziyannis, E., et al., *Performance characteristics of microparticle enzyme and chemiluminescence immunoassays for measurement of anti-HBc immunoglobulin M in sera of patients with HBeAg-negative chronic hepatitis B virus infection.* Clin Vaccine Immunol, 2008. **15**(2): p. 385-7.
37. Liu, W., et al., *Clinical application of Chemiluminescence Microparticle Immunoassay for SARS-CoV-2 infection diagnosis.* J Clin Virol, 2020. **130**: p. 104576.
38. Ding, J., et al., *Illuminating the Live-Cell Dynamics of Hepatitis B Virus Covalently Closed Circular DNA Using the CRISPR-Tag System.* mBio, 2023: p. e0355022.
39. Gao, L., et al., *[A short half-life of cccDNA offer or ignite hope for hepatitis B cure under nucleos(t)ide analogues treatment]*. Zhonghua Gan Zang Bing Za Zhi, 2022. **30**(1): p. 99-102.
40. Lu, F.M., et al., *[The potential use of serum HBV RNA to guide the functional cure of chronic hepatitis B]*. Zhonghua Gan Zang Bing Za Zhi, 2017. **25**(2): p. 105-110.
41. Tu, T., et al., *D e novo synthesis of hepatitis B virus nucleocapsids is dispensable for the maintenance and transcriptional regulation of cccDNA.* JHEP Rep, 2021. **3**(1): p. 100195.
42. Caviglia, G.P., et al., *Quantitation of HBV cccDNA in anti-HBc-positive liver donors by droplet digital PCR: A new tool to detect occult infection*. J Hepatol, 2018. **69**(2): p. 301-307.
43. Li, X., et al., *Detection of HBV Covalently Closed Circular DNA.* Viruses, 2017. **9**(6).
44. Zuo, D., et al., *Screening for Lipid-Metabolism-Related Genes and Identifying the Diagnostic Potential of ANGPTL6 for HBV-Related Early-Stage Hepatocellular Carcinoma.* Biomolecules, 2022. **12**(11).
45. Chen, L.Y., et al., *Hepatitis D: challenges in the estimation of true prevalence and laboratory diagnosis*. Gut Pathog, 2021. **13**(1): p. 66.
46. Wang, Z.L., et al., *An Ideal Hallmark Closest to Complete Cure of Chronic Hepatitis B Patients: High-sensitivity Quantitative HBsAg Loss.* J Clin Transl Hepatol, 2023. **11**(1): p. 197-206.
47. Lim, S.G., et al., *Comparative biomarkers for HBsAg loss with antiviral therapy shows dominant influence of quantitative HBsAg (qHBsAg).* Aliment Pharmacol Ther, 2021. **53**(1): p. 172-182.
48. Chuaypen, N., et al., *Predictive role of serum HBsAg and HBcrAg kinetics in patients with HBeAg-negative chronic hepatitis B receiving pegylated interferon-based therapy.* Clin Microbiol Infect, 2018. **24**(3): p. 306 e7-306 e13.
49. Lee, J.M. and S.H. Ahn, *Quantification of HBsAg: basic virology for clinical practice.* World J Gastroenterol, 2011. **17**(3): p. 283-9.
50. Kao, J.H., *Diagnosis of hepatitis B virus infection through serological and virological markers.* Expert Rev Gastroenterol Hepatol, 2008. **2**(4): p. 553-62.

51. de Almeida Ponde, R.A., *Detection of the serological markers hepatitis B virus surface antigen (HBsAg) and hepatitis B core IgM antibody (anti-HBcIgM) in the diagnosis of acute hepatitis B virus infection after recent exposure*. Microbiol Immunol, 2022. **66**(1): p. 1-9.
52. Takeda, K., et al., *Highly sensitive detection of hepatitis B virus surface antigen by use of a semiautomated immune complex transfer chemiluminescence enzyme immunoassay.* J Clin Microbiol, 2013. **51**(7): p. 2238-44.
53. Kim, S.H., *ELISA for Quantitative Determination of Hepatitis B Virus Surface Antigen.* Immune Netw, 2017. **17**(6): p. 451-459.
54. Salpini, R., et al., *Key mutations in the C-terminus of the HBV surface glycoprotein correlate with lower HBsAg levels in vivo, hinder HBsAg secretion in vitro and reduce HBsAg structural stability in the setting of HBeAg-negative chronic HBV genotype-D infection.* Emerg Microbes Infect, 2020. **9**(1): p. 928-939.
55. Moini, M. and S. Fung, *HBsAg Loss as a Treatment Endpoint for Chronic HBV Infection: HBV Cure.* Viruses, 2022. **14**(4).
56. Wu, Y., et al., *Evolutionary Analysis of Pre-S/S Mutations in HBeAg-Negative Chronic Hepatitis B With HBsAg < 100 IU/ml.* Front Public Health, 2021. **9**: p. 633792.
57. Wu, J., et al., *Research Progress on the Mechanism of Persistent Low-Level HBsAg Expression in the Serum of Patients with Chronic HBV Infection.* J Immunol Res, 2022. **2022**: p. 1372705.
58. Cornberg, M., et al., *The role of quantitative hepatitis B surface antigen revisited.* J Hepatol, 2017. **66**(2): p. 398-411.
59. Piermatteo, L., et al., *HBeAg Levels Vary across the Different Stages of HBV Infection According to the Extent of Immunological Pressure and Are Associated with Therapeutic Outcome in the Setting of Immunosuppression-Driven HBV Reactivation.* Biomedicines, 2021. **9**(10).
60. Hadziyannis, E. and A. Laras, *Viral Biomarkers in Chronic HBeAg Negative HBV Infection.* Genes (Basel), 2018. **9**(10).
61. Zhao, X.A., et al., *Baseline serum hepatitis B core antibody level predicts HBeAg seroconversion in patients with HBeAg-positive chronic hepatitis B after antiviral treatment.* Antiviral Res, 2021. **193**: p. 105146.
62. Guo, R., et al., *Slow reduction of IP-10 Levels predicts HBeAg seroconversion in chronic hepatitis B patients with 5 years of entecavir treatment.* Sci Rep, 2016. **6**: p. 37015.
63. Ma, S.W., et al., *High serum IL-21 levels after 12 weeks of antiviral therapy predict HBeAg seroconversion in chronic hepatitis B.* J Hepatol, 2012. **56**(4): p. 775-81.
64. Yu, X., et al., *HBV inhibits LPS-induced NLRP3 inflammasome activation and IL-1beta production via suppressing the NF-kappaB pathway and ROS production.* J Hepatol, 2017. **66**(4): p. 693-702.
65. Sun, Y., et al., *Persistent Low Level of Hepatitis B Virus Promotes Fibrosis*

Progression During Therapy. Clin Gastroenterol Hepatol, 2020. **18**(11): p. 2582-2591 e6.

66. European Association for the Study of the Liver. Electronic address, e.e.e. and L. European Association for the Study of the, *EASL 2017 Clinical Practice Guidelines on the management of hepatitis B virus infection*. J Hepatol, 2017. **67**(2): p. 370-398.
67. Terrault, N.A., et al., *Update on prevention, diagnosis, and treatment of chronic hepatitis B: AASLD 2018 hepatitis B guidance*. Hepatology, 2018. **67**(4): p. 1560-1599.
68. Lee, H., et al., *Cost-effectiveness of antiviral therapy in untreated compensated cirrhosis patient with serum HBV-DNA level < 2000 IU/mL*. Hepatol Int, 2022. **16**(2): p. 294-305.
69. Raimondo, G., et al., *Update of the statements on biology and clinical impact of occult hepatitis B virus infection*. J Hepatol, 2019. **71**(2): p. 397-408.
70. Kim, S.K., et al., *Hepatitis B Virus Treatment and Hepatocellular Carcinoma: Controversies and Approaches to Consensus*. Liver Cancer, 2022. **11**(6): p. 497-510.
71. Lasagna, A., et al., *Occult hepatitis B in patients with cancer during immunotherapy with or without chemotherapy: A real-life retrospective single-center cohort study.* Front Oncol, 2023. **13**: p. 1044098.
72. Kramvis, A., et al., *A roadmap for serum biomarkers for hepatitis B virus: current status and future outlook*. Nat Rev Gastroenterol Hepatol, 2022. **19**(11): p. 727-745.
73. Gish, R.G., et al., *Chronic hepatitis B: Virology, natural history, current management and a glimpse at future opportunities.* Antiviral Res, 2015. **121**: p. 47-58.
74. Zhang, H. and T. Tu, *Approaches to quantifying hepatitis B virus covalently closed circular DNA.* Clin Mol Hepatol, 2022. **28**(2): p. 135-149.
75. Saitta, C., T. Pollicino, and G. Raimondo, *Occult Hepatitis B Virus Infection: An Update.* Viruses, 2022. **14**(7).
76. Wang, J., et al., *Serum hepatitis B virus RNA is encapsidated pregenome RNA that may be associated with persistence of viral infection and rebound.* J Hepatol, 2016. **65**(4): p. 700-710.
77. Jiang, B., et al., *Evaluation of HBV serological markers in treatment-naive HBV mono-infected patients and HBV-HIV co-infected patients*. Virus Res, 2020. **290**: p. 198117.
78. Lok, J., et al., *Review article: novel biomarkers in hepatitis B infection.* Aliment Pharmacol Ther, 2022. **56**(5): p. 760-776.
79. Mak, L.Y., et al., *HBV pgRNA and HBcrAg reductions at week 4 predict favourable HBsAg response upon long-term nucleos(t)ide analogue in CHB.* Clin Mol Hepatol, 2022.
80. Ye, X., et al., *High Frequency Occult Hepatitis B Virus Infection Detected in Non-Resolved Donations Suggests the Requirement of Anti-HBc Test in Blood Donors in Southern China*. Front Immunol, 2021. **12**: p. 699217.

81. Adraneda, C., et al., *A critique and systematic review of the clinical utility of hepatitis B core-related antigen.* J Hepatol, 2022.
82. Watanabe, T., T. Inoue, and Y. Tanaka, *Hepatitis B Core-Related Antigen and New Therapies for Hepatitis B.* Microorganisms, 2021. **9**(10).
83. Tseng, T.C., et al., *Low Hepatitis B Core-Related Antigen Levels Correlate Higher Spontaneous Seroclearance of Hepatitis B Surface Antigen in Chronic Hepatitis B Patients With High Hepatitis B Surface Antigen Levels*. Gastroenterology, 2023.
84. Wong, D.K., et al., *A longitudinal study to detect hepatitis B surface and core-related antigens in chronic hepatitis B patients with hepatitis B surface antigen seroclearance using highly sensitive assays.* J Clin Virol, 2023. **160**: p. 105375.
85. Tseng, T.C., et al., *Hepatitis B Core-Related Antigen Stratifies the Risk of Liver Cancer in HBeAg-Negative Patients With Indeterminate Phase.* Am J Gastroenterol, 2022. **117**(5): p. 748-757.
86. Wu, J. and W. Mao, *Review of Serum Biomarkers and Models Derived from Them in HBV-Related Liver Diseases*. Dis Markers, 2020. **2020**: p. 2471252.
87. Watanabe, T., S. Hayashi, and Y. Tanaka, *Drug Discovery Study Aimed at a Functional Cure for HBV.* Viruses, 2022. **14**(7).
88. Fanning, G.C., et al., *Therapeutic strategies for hepatitis B virus infection: towards a cure.* Nat Rev Drug Discov, 2019. **18**(11): p. 827-844.
89. World Health, O., *Hepatitis B vaccines: WHO position paper, July 2017 - Recommendations.* Vaccine, 2019. **37**(2): p. 223-225.
90. Moro, P.L., et al., *Assessing the safety of hepatitis B vaccination during pregnancy in the Vaccine Adverse Event Reporting System (VAERS), 1990-2016.* Vaccine, 2018. **36**(1): p. 50-54.
91. Kuhns, M.C., et al., *Hepatitis B seroconversion revisited: new insights into the natural history of acute hepatitis B virus (HBV) infection from quantitative and highly sensitive assays and novel biomarkers.* Virol J, 2021. **18**(1): p. 235.
92. Kuhns, M.C., et al., *Lack of correlation between HBsAg and HBV DNA levels in blood donors who test positive for HBsAg and anti-HBc: implications for future HBV screening policy.* Transfusion, 2004. **44**(9): p. 1332-9.
93. Yang, N., et al., *In Situ Generation of Prussian Blue with Potassium Ferrocyanide to Improve the Sensitivity of Chemiluminescence Immunoassay Using Magnetic Nanoparticles as Label.* Anal Chem, 2019. **91**(7): p. 4906-4912.

Fig1 The discovery of HBV Serum Virological products