Cihan University-Erbil Scientific Journal (CUESJ)

Research Article

Significance of Hepatitis B Virus Diagnosis by Real-time Polymerase Chain Reaction over Serological Markers in Hepatitis B Virus Patients

Amer A. Khaleel[1]*, Salah T. Jalal[1], Saeed G. Hussain[2]

[1]Department of Medical Microbiology, College of Health Sciences-Hawler Medical University, Erbil, Iraq, [2]Department of Medical Microbiology, College of Medicine-Hawler Medical University, Erbil, Iraq

ABSTRACT

Hepatitis B virus (HBV) is the leading cause of viral hepatitis, as currently over 2 billion people have HBV infection worldwide. Nucleic acid assay and quantitative hepatitis B surface antigen (HBsAg) have been developed for diagnostic and therapeutic monitoring of patients with HBV infection. These tests might also show correlation between HBV DNA and HBs serostatus. The study aimed to find and analyze the frequency and impact of HBsAg seropositivity among patients revealed HBV DNA negative level through quantitative estimation of both seromarkers. Real-time polymerase chain reaction (RT-PCR) and Elecsys assays were used for quantitative estimation of HBV DNA and HBs antigen, respectively. A total of 256 blood samples were used from patients referred for either diagnostic purpose and/or HBV viral load monitoring after antiviral therapy. Blood profile analysis showed 12.26% HBs antigen seropositivity among patients revealed negative for nucleic acid assay for HBV DNA. Positive HBs antigen titers ranged from 1000–50,000 COI, with seronegative anti-HBs antibody test for all samples tested positive for HBs antigen. This study delineated that negative or undetectable quantitation of HBV DNA level does not exclude HBV infection; as the level might fluctuate in different phases of HBV replication. This gives an impression and raising a question about significance of replacing test for HBsAg with quantitation of HBV DNA PCR assay. Thus, the study refers to a special HBV profile outside the classical pattern.

Keywords: Component, hepatitis B surface antigen, hepatitis B virus, hepatitis, quantitative hepatitis B virus DNA

INTRODUCTION

Hepatitis B virus (HBV) is the leading cause of viral hepatitis, as currently over 2 billion people have HBV infection worldwide. Approximately 600,000 deaths occur every year as a result of the acute and chronic consequences of HBV infection.[1] Chronic HBV affects nearly 350 million patients worldwide and may further progress to cirrhosis and/or hepatocellular carcinoma (HCC) in 15–40% of cases.[2] HBV infection is a dynamic process characterized by replicative and non-replicative phases based on virus-host interaction, which are present in some form of all infected patients.[3] To measure HBV DNA (viral load), a laboratory measures how many HBV DNA units are found in 1 ml of blood. The result is written in international units per ml (IU/ml). High levels of HBV DNA indicate a high rate of HBV replication; however, low or undetectable levels–<2000 IU/ml indicate an "inactive" infection.[4] During HBV infection, markers can be detected in sera samples using immunoassays. For example, patient sera that contain anti-HBs indicates immunity from past infections or vaccinations, and a patient with anti-HBe antibodies in their sera is considered as spontaneous resolution of infection or therapy-induced improvement.[5,6] One of the serological methods using for the diagnosis of HBV is enzyme immune assays. The hepatitis B surface antigen (HBsAg) and HBV DNA levels were considered to be a risk factor of hepatocarcinogenesis in untreated HB patients.[7] The levels of quantified HBsAg are considered as a marker with which to evaluate the host immunological control of infection and HBV replication.[8] Low HBsAg levels in some HBV patients are considered to indicate a high likelihood of HBV clearance and lower hepatitis activity.[7,9] The aim of this study was to estimate HBV-DNA positivity states among serologically screened patients and to compare the sensitivity of molecular and serological methods for the diagnosis of HBV infections.

Corresponding Author:
Amer A. Khaleel, College of Health Sciences-Hawler Medical University, Erbil, Iraq. E-mail: ameralikhaleel@yahoo.com

Received: Mar 31, 2019
Accepted: Apr 17, 2019
Published: Jan 20, 2020

DOI: 10.24086/cuesj.v4n1y2020.pp52-56

MATERIALS AND METHODS

For this cross-sectional study, a total of 256 blood samples were collected from HBV patients referred to Erbil Central Laboratory, from June 2017 to September 2017. Both sexes were included (154 males and 102 females), with age range between 20 and 60 years. Real-time polymerase chain reaction (RT-PCR) as a molecular and Elecsys assay as a serological method were used for the diagnosis and comparing their sensitivity purposes. For RT-PCR technique, 5 ml of venous blood were collected using EDTA tubes and centrifuged at 1000 rpm for 5 min and patients' plasma was separated. Viral DNA was extracted from a 200 μl aliquot of serum using a Qiagen mini blood kit (Qiagen, Hilden, Germany) according to the manufacture instructions. Viral loads of HBV DNA were estimated by RT-PCR (HBV RG PCR artus, Germany). The amplification was performed in a 50 μl reaction mixture containing 30 μl of mixture of HBV RG Master mix (buffer, dNTP, primer, probe, and enzymes) and HBV RG inhibition control mixed with 20 μl of DNA template to each reaction. The primers HBV-Taq I (forward primer): CAA CCT CCA ATC ACT CAC CAAC and HBV-Taq 2 (reverse primer): ATA TGA TAA AAC GCC GCA GAC AC were used from the sequences of the conserved regions of the HBV surface gene used previously by Weinberger *et al*.[10] For performing RT-PCR, Rotorgene 3000 (a machine used routinely in Erbil Central Laboratory for detection of variety of DNAs and RNAs) was used following manufacture's instructions. The RT-PCR cycling parameters consisted of denaturation at 95°C for 15 s, 55°C for 30 s, and 72°C for 15 s. The results were considered positive if a signal was detected in Cycling A FAM, whereas no signal detection indicated as negative results. The quantitation of HBV DNA was performed for all blood samples and the results were considered positive and significant if viral load was more than 1×10^5 viral copies/ml. Titers <50 IU were considered as negative for HBV DNA. The screened results were categorized according to positive HBV DNA titers into inactive, gray zone, and active carriers.

Quantitation of HBsAg was measured using the Elecsys HBsAg quantitative assay. Detection of HBsAg by Elecsys utilizes a sandwich principle and this assay has two stages: First, a complex is formed with two monoclonal HBsAg-specific antibodies, one of which is biotinylated and the other labeled with a ruthenium complex. This complex joins to the solid phase through the interaction of biotin and streptavidin after the attachment of streptavidin-coated microparticles.[11] The mixture is subsequently aspirated into a measuring cell, where application of a voltage induces chemiluminescent emission, which is measured by a photomultiplier. All serum samples were tested at a dilution of 1:400. The Elecsys HBsAg quantitative assay is calibrated to give results in IU/ml.[11]

Statistical Analysis

Statistical analysis was done using SPSS 23.0. HBV titers expressed as means ± standard error. Comparisons between active and inactive carriers use *t*-tests. Comparisons between multiple groups were performed by ANOVA. $P \leq 0.05$ was considered as statistically significant.

RESULTS AND DISCUSSION

Of the 256 cases, 154 (60%) were male and 102 (40%) were female, with age range between 20 and 60 years. RT-PCR results showed that 163 of 256 samples were HBV DNA negative, from them, 20 samples showed positive results for HBsAg quantitation [Table 1].

The Asian Pacific Association for the Study of the Liver, European Association for the Study of the Liver, and the guidelines of the American Association for the Study of Liver Diseases refer that the disappearance of HBsAg is the ideal and cornerstone during natural course of HBV infection.[12-14] From the clinical standpoint of view, the progression from chronic hepatitis B to cirrhosis is increasingly signified through the HBV viral load.[8] Meanwhile, quantitative HBsAg level was regarded as marker for clearance, hepatitis activity, and immunological host response to control HBV replication and the likelihood of inactive virus carrier.[9,7,15] However, little is known about correlation between HBV replication and low levels of HBsAg, as reported in some patients with chronic HBV infection.[16-18]

The results of the present study showed 12.26% HBs seropositivity (range 1000–5000 COI) among patients seronegative for HBV DNA [Table 2], with highly significant difference ($P \leq 0.001$) between mean of HBV DNA seropositive

Table 1: Baseline patient's characteristics

Variables	All patients (*n*=256)
Gender	
Male	154 (60%)
Female	102 (40%)
HBV DNA IU/ml	256
<50 (negative)	163 (63.67%)
101–500	22 (24.44%)
501–1000	10 (11.11%)
1001–2000	10 (11.11%)
2001–20,000	20 (22.22%)
>20,000	31 (34.44%)
qHBsAg tested	163/256 (63.67%)
<10 COI	143 (55.85%)
1000–2000 COI	3 (15%)
2001–3000 COI	4 (20%)
3001–4000 COI	8 (40%)
4001–5000 COI	5 (25%)
Anti-HBsAb tested	20
Positive	0.00
Negative	20 (100%)
HBs positive, HBV DNA negative	20/163

qHBsAg: Quantitative hepatitis B surface antigen, HBV: Hepatitis B virus

Table 2: Frequencies of HBV DNA seropositivity among patients negative for HBV DNA assay

Number screened	HBV DNA PCR +ve	HBV DNA PCR−ve (*n*=163)	
		HBs +ve	HBs −ve
n=256	93 (36.32%)	20 (12.26%)	143 (87.73%)

HBV: Hepatitis B virus, PCR: Polymerase chain reaction

and negative titers [Table 3]. Regarding to the gender positivity for PCR test, in total of 154 male patients 65 (42%) were positive and of 102 Female patients only 28 (27%) were positive [Table 4]. The seromarkers profile among patients negative for HBV DNA revealed anti-HBs antibody seronegative among the total 20 patients positive for HBsAg [Table 5].

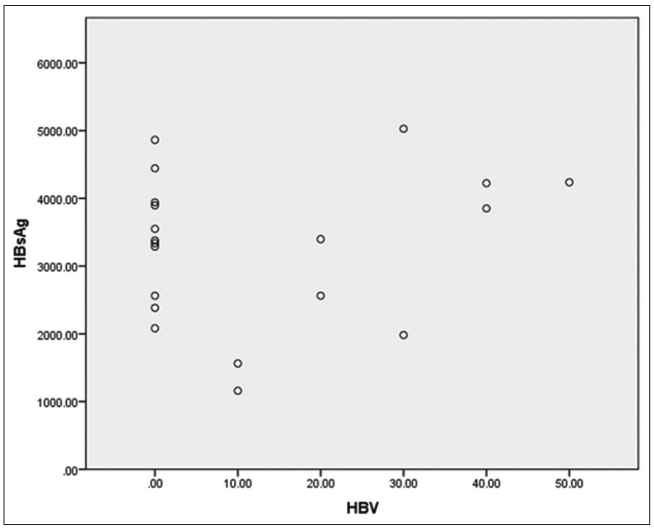

Figure 1: Pearson correlation between quantitative hepatitis B virus DNA level and hepatitis B surface antigen. $R = 0.229$, $P = 0.331$

Table 3: Mean titers of HBV DNA among patients screened

Number screened	HBV DNA PCR +ve (*n*=93)	HBV DNA PCR −ve (*n*=163)	*P*-value
n=256	18,582,661.24±5,934,488.97	31.12±4.45	0.000

HBV: Hepatitis B virus, PCR: Polymerase chain reaction

Table 4: Frequencies of HBV DNA positivity according to the gender

Gender	Positive PCR	Negative PCR	Total	*P*-value
Male	65 (42.20%)	89 (57.79%)	154	≤0.001
Female	28 (27.45%)	74 (72.54%)	102	
Total	93 (36.3232%)	163 (63.67%)	256	

HBV: Hepatitis B virus, PCR: Polymerase chain reaction

However, results of anti-HBs titers among patients negative for HBV DNA showed 20 samples were positive showing different titers [Table 6]. During the immune-tolerant phase, HBsAg level is the highest one, and the level decreases progressively from immune tolerance to inactive phase that reflects a low replicative phase of immune control.[19] The detection of HBV infection is vital for diagnosis our group published a paper that delineated the impact of PCR DNA assay for the diagnosis of HBV infection.[20]

The data of this study are agree with other studies[21] for the serologic profile of HBsAg seropositivity among patients screened negative for HBV DNA. They reported 6% of their samples gave HBsAg positive among donations negative for HBV DNA level, others[8] referred that in nine patients with HCC; HBV DNA was undetectable but the level of HBsAg was ≥2000 COI. Others reported 31.4% HBsAg positive among patients negative for HBV DNA.[22] However, data from another study showed that 100 of 140 patients were seronegative for HBV DNA.[23] Another study[24] reported that 69% of confirmed HBsAg-positive donations had undetectable HBV DNA levels. However, others[25,26] found that none of the HBsAg-positive donors with very low or negative for HBV DNA were anti-HBs antibody positive.[24,27]

Among explanation for such unusual HBV serologic profile, the patients may be chronically infected with HBV particularly; they are in the non-replicative phase as part of the natural course of infection.[22] The question might be raised here is about such serologic profile of HBsAg positive among patients negative for HBV DNA, are these patients infectious and transmissible for community,[21] referred for the need of larger sample volume (5–10 ml) necessary for extraction and concentration to determine the real HBV DNA status among samples negative for DNA.

In this study, Pearson's correlation analysis revealed no significant correlation between serum HBV DNA and HBsAg levels ($r = 0.331$; $P > 0.3$) [Figure 1]. Different studies reported discrepant results regarding correlation between HBV DNA and HBsAg levels, our data were disagree with other studies who reported positive correlation.[28-34] Meanwhile, our results agree with others for no correlation between HBV DNA and HBsAg levels.[35,36] Such correlation is only weak in patients negative for HBsAg.[19,30,37,38] This is because the level of HBV DNA fluctuates from undetectable to >2,000,000 IU/ml.[12] This is why the level of HBsAg does not fall in the same proportion or level when correlated with HBV DNA level as a marker for

Table 5: HBsAg and anti-HBs seromarkers profile among patients negative for HBV DNA

Serological profile	HBs		Anti-HBs (*n*=20)	
	>10 COI +ve	<0.9 COI −ve	<10 IU/ml −ve	>10 IU/ml +ve
HBV DNA PCR negative (*n*=163)	20 (12.27%)	143 (87.73%)	20 (100%)	0.00

HBV: Hepatitis B virus, PCR: Polymerase chain reaction, HBsAg: Hepatitis B surface antigen

Table 6: Distribution of HBsAg and anti-HBs titers among patients negative for HBV DNA

HBs titers COI					Anti-HBsAb IU/ml	
Non-reactive <0.9 COI	1000–2000	2001–3000	3001–4000	4001–>5000	Non-reactive <10	Reactive >10
0.00	3 (15%)	4 (20%)	8 (40%)	5 (25%)	20 (100%)	0.00

HBV: Hepatitis B virus, PCR: Polymerase chain reaction, HBsAg: Hepatitis B surface antigen

disease progression.[4] Martinot-Peignoux *et al*.[39] referred that HBV DNA diminished with time from seroclearance; however, covalently closed circular DNA as integrated HBV DNA remained detectable in liver biopsies. It is thus critical for clinicians to be aware of the significance of HBV DNA serial measurement and lifelong follow-up to establish and confirm that inactive carrier condition is maintained.[4] Among major guidelines, if HBV DNA is undetectable, either prophylaxis with lamivudine or strict follow-up every 1–3 months for ALT and HBV DNA level is indicated.[13]

CONCLUSION

Serology will undoubtedly continue to be widely used in the detection of HBV infection; however, HBV DNA assays can serve as an important tool, especially in detecting low levels of HBV DNA and in patients with the past HBV infection. Data from the present study also concluded that negative or undetectable quantitation of HBV DNA level does not exclude HBV infection; as the level might fluctuate in different phases of HBV replication. This gives an impression and raising a question about significance of replacing test for HBsAg with quantitation of HBV DNA PCR assay. Thus, the study refers to a special HBV profile outside the classical pattern.

REFERENCES

1. World Health Organization. "World Health Organization Hepatitis B Factsheet". Geneva: World Health Organization, 2017.
2. S. Baig, A. A. Siddiqui, W. Ahmed, H. Qureshi and A. Arif. "The association of complex liver disorders with HBV genotypes prevalent in Pakistan". *Virology Journal*, vol. 4, no. 1, p. 128, 2007.
3. G. Fattovich. "Natural history of hepatitis B". *Journal of Hepatology*, vol. 39, pp. 50-58, 2003.
4. I. Pita, A. M. Horta-Vale, H. Cardoso and G. Macedo. "Hepatitis B inactive carriers: An overlooked population?" *GE Portuguese Journal of Gastroenterology*, vol. 21, no. 6, pp. 241-249, 2014.
5. L. M. Villar, H. M. Cruz, J. R. Barbosa, C. S. Bezerra, M. M. Portilho and L. de Paula Scalioni. "Update on hepatitis B and C virus diagnosis". *World Journal of Virology*, vol. 4, no. 4, p. 323, 2015.
6. I. J. Radii and A. M. Saud. "Comparative assessment between serological and molecular diagnosis for patients groups with hepatitis b virus". *International Journal of Current Microbiology and Applied Science*, vol. 6, no. 3, pp. 742-748, 2017.
7. T. C. Tseng, C. J. Liu, H. C. Yang, T. H. Su, C. C. Wang, C. L. Chen and J. H. Kao. "High levels of hepatitis B surface antigen increase risk of hepatocellular carcinoma in patients with low HBV load". *Gastroenterology*, vol. 142, no. 5, pp. 1140-1149, 2012.
8. M. Kawanaka, K. Nishino, J. Nakamura, T. Oka, N. Urata, D. Goto and G. Yamada. "Quantitative levels of hepatitis B virus DNA and surface antigen and the risk of hepatocellular carcinoma in patients with hepatitis B receiving long-term nucleos (t) ide analogue therapy". *Liver Cancer*, vol. 3, no. 1, pp. 41-52, 2014.
9. T. C. Tseng, C. J. Liu, T. H. Su, C. C. Wang, C. L. Chen, P. J. Chen and J. H. Kao. "Serum hepatitis B surface antigen levels predict surface antigen loss in hepatitis B e antigen seroconverts". *Gastroenterology*, vol. 141, no. 2, pp. 517-525, 2011.
10. K. M. Weinberger, E. Wiedenmann, S. Böhm and W. Jilg. "Sensitive and accurate quantitation of hepatitis B virus DNA using a kinetic fluorescence detection system (TaqMan PCR)". *Journal of Virological Methods*, vol. 85, no. 1-2, pp. 75-82, 2000.
11. H. J. Lee, S. Y. Kim, S. M. Lee, J. Heo, H. H. Kim, C. L. Chang and H. C. Son. "Elecsys hepatitis B surface antigen quantitative assay: Performance evaluation and correlation with hepatitis B virus DNA during 96 weeks of follow-up in chronic hepatitis B patients". *Annals of Laboratory Medicine*, vol. 32, no. 6, pp. 420-425, 2012.
12. A. S. Lok and B. J. McMahon. "Chronic hepatitis B: Update 2009". *Hepatology*, vol. 50, no. 3, pp. 661-662, 2009.
13. European Association for the Study of the Liver. "EASL clinical practice guidelines: Management of chronic hepatitis B virus infection". *Journal of Hepatology*, vol. 57, no. 1, pp. 167-185, 2012.
14. Y. F. Liaw, J. H. Kao, T. Piratvisuth, H. L. Y. Chan, R. N. Chien, C. J. Liu and D. Amarapurkar. "Asian-Pacific consensus statement on the management of chronic hepatitis B: A 2012 update". *Hepatology International*, vol. 6, no. 3, pp. 531-561, 2012.
15. H. Lik-Yuen Chan, G. Lai-Hung Wong, C. H. Tse, H. Y. Chan and V. Wai-Sun Wong. "Viral determinants of hepatitis b surface antigen seroclearance in hepatitis b e antigen-negative chronic hepatitis b patients". *Journal of Infectious Diseases*, vol. 204, no. 3, pp. 408-414, 2011.
16. J. Cheng, C. Sun, Y. Chen, Y. Dai, X. U. Zhiliang, G. Sun and L. I. Xiaojun. "Molecular analysis on chronic hepatitis B patients with low-level HBsAg". *Chinese Journal of Laboratory Medicine*, vol. 32, no. 10, pp. 1128-1132, 2009.
17. J. Cheng, C. Sun, Y. Chen, Z. Xu, G. Wang, G. Sun and X. Li. "Analysis on clinical features and immunity in chronic hepatitis B virus infected patients with low-level HBsAg". *African Journal of Microbiology Research*, vol. 4, no. 7, pp. 547-550, 2010.
18. A. Jeffery-Smith, J. Hubb, A. Oliver and C. Y. Tong. "An apparent low level of hepatitis B surface antigen (HBsAg) in the presence of significant viral replication". *Journal of Clinical Virology: The Official Publication of the Pan American Society for Clinical Virology*, vol. 77, p. 111, 2016.
19. T. Nguyen, A. J. Thompson, S. Bowden, C. Croagh, S. Bell, P. V Desmond and S. A. Locarnini. "Hepatitis B surface antigen levels during the natural history of chronic hepatitis B: A perspective on Asia". *Journal of Hepatology*, vol. 52, no. 4, pp. 508-513, 2010.
20. S. T. Balaky, S. G. Hussain, A. A. Khaleel, F. T. Sabeer and A. H. Mawlood. "Molecular diagnosis in differentiating active and inactive forms of hepatitis b virus carriers". *Journal of University of Babylon for Pure and Applied Sciences*, vol. 26, no. 10, pp. 41-45, 2018.
21. M. C. Kuhns, S. H. Kleinman, A. L. McNamara, B. Rawal, S. Glynn and M. P. Busch. "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*, vol. 44, no. 9, pp. 1332-1339, 2004.
22. A. S. K. Al-Suraifi, A. D. J. Al-Rubaie, S. M. G. Al-Mayahie and N. M. M. Al-Abedy. "Unusual HBV mixed genotype infections among hepatitis type B Iraqi patients in Wasit Province/Iraq". *International Journal of Biomedical Engineering and Clinical Science*, vol. 2, no. 1, pp. 1-7, 2016.
23. I. A. A. Khaled, O. M. Mahmoud, A. F. Saleh and E. A. Baioumi. "Prevalence of HBV genotypes in Egypt among hepatitis patients". *Journal of American Science*, vol. 6, no. 11, pp. 185-190, 2010.
24. W. K. Roth, M. Weber, D. Petersen, C. Drosten, S. Buhr, W. Sireis and E. Seifried. "NAT for HBV and anti-HBc testing increase blood safety". *Transfusion*, vol. 42, no. 7, pp. 869-875, 2002.
25. S. Sato, W. Ohhashi, H. Ihara, S. Sakaya, T. Kato and H. Ikeda. "Comparison of the sensitivity of NAT using pooled donor samples for HBV and that of a serologic HBsAg assay". *Transfusion*, vol. 41, no. 9, pp. 1107-1113, 2001.
26. H. Hennig, I. Puchta, J. Luhm, P. Schlenke, S. Goerg and H. Kirchner. "Frequency and load of hepatitis B virus DNA in first-time blood donors with antibodies to hepatitis B core antigen". *Blood*, vol. 100, no. 7, pp. 2637-2641, 2002.
27. M. C. Kuhns, A. L. McNamara and R. Perrillo. "Intermittent PCR positivity for hepatitis B viral DNA in surface antigen negative anti-core positive individuals". *Transfusion*, vol. 39, no. 10, pp. 95S-95S, 1999.
28. Y. J. Kim, H. C. Cho, M. S. Choi, J. H. Lee, K. C. Koh, B. C. Yoo and S. W. Paik. "The change of the quantitative HBsAg level during

the natural course of chronic hepatitis B". *Liver International*, vol. 31, no. 6, pp. 817-823, 2011.
29. J. Jaroszewicz, B. C. Serrano, K. Wursthorn, K. Deterding, J. Schlue, R. Raupach and M. Cornberg. "Hepatitis B surface antigen (HBsAg) levels in the natural history of hepatitis B virus (HBV)-infection: A European perspective". *Journal of Hepatology*, vol. 52, no. 4, pp. 514-522, 2010.
30. A. Alghamdi, N. Aref, M. El-Hazmi, W. Al-Hamoudi, K. Alswat, A. Helmy and A. A. Abdo. "Correlation between hepatitis B surface antigen titers and HBV DNA levels". *Saudi Journal of Gastroenterology: Official Journal of the Saudi Gastroenterology Association*, vol. 19, no. 6, p. 252, 2013.
31. P. P. Primadharsini and I. Wibawa. "Correlation between quantitative HBsAg and HBV-DNA in chronic hepatitis B infection". *Indonesian Journal of Gastroenterology, Hepatology, and Digestive Endoscopy*, vol. 14, no. 1, pp. 9-12, 2013.
32. M. Z. Hong, W. Q. Huang, F. Min, J. C. Xu, Z. Lin, K. N. Fang and J. S. Pan. "Enhanced HBsAg synthesis correlates with increased severity of fibrosis in chronic hepatitis B patients". *PLoS One*, vol. 9, no. 1, p. e87344, 2014.
33. O. Günal, S. Barut, I. Etikan, F. Duygu, U. Tuncel and M. Sünbül. "Relation between serum quantitative HBsAg, ALT and HBV DNA levels in HBeAg negative chronic HBV infection". *Turkish Journal of Gastroenterology*, vol. 25, no. 1, pp. 142-146, 2014.
34. G. Li, J. Wang, Y. Bao, L. Zheng, K. Ge, X. Zhou and G. Chen. "Clinical significance of serum HBsAg levels, HBsAg/HBV DNA ratio, and association with liver inflammation activity in HBeAg-positive chronic hepatitis B". *Zhonghua Gan Zang Bing Za Zhi=Zhonghua Ganzangbing Zazhi=Chinese Journal of Hepatology*, vol. 23, no. 1, pp. 40-45, 2015.
35. A. Ganji, A. Esmaeilzadeh, K. Ghafarzadegan, H. Helalat, H. Rafatpanah and A. Mokhtarifar. "Correlation between HBsAg quantitative assay results and HBV DNA levels in chronic HBV". *Hepatitis Monthly*, vol. 11, no. 5, p. 342, 2011.
36. R. M. Mukherjee, J. Arava, P. B. Reddy, P. N. Rao, R. Gupta and D. N. Reddy. "Role of hepatitis b virus surface antigen quantification in E antigen negative chronic hepatitis B infection". *Global Journal of Science Frontier Research*, vol. 13, no. 1, pp. 1-5, 2013.
37. H. Park, J. M. Lee, J. H. Seo, H. S. Kim, S. H. Ahn, D. Y. Kim and J. Y. Park. "Predictive value of HBsAg quantification for determining the clinical course of genotype C HBeAg-negative carriers". *Liver International*, vol. 32, no. 5, pp. 796-802, 2012.
38. A. J. Thompson, T. Nguyen, D. Iser, A. Ayres, K. Jackson, M. Littlejohn and G. K. Lau. "Serum hepatitis B surface antigen and hepatitis B e antigen titers: Disease phase influences correlation with viral load and intrahepatic hepatitis B virus markers". *Hepatology*, vol. 51, no. 6, pp. 1933-1944, 2010.
39. M. Martinot-Peignoux, M. Lapalus, C. Laouénan, O. Lada, A. C. F. Netto-Cardoso, N. Boyer and P. Marcellin. "Prediction of disease reactivation in asymptomatic hepatitis B e antigen-negative chronic hepatitis B patients using baseline serum measurements of HBsAg and HBV-DNA". *Journal of Clinical Virology*, vol. 58, no. 2, pp. 401-407, 2013.