Polymerase Chain Reaction and Enzyme-Linked Immunosorbent Assay as Diagnostic Tools for Detection of Hepatitis B Virus Infection, KHMC Experience

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ABSTRACT

Objective: To compare serological and molecular assays for detection hepatitis B virus infection.

Methods: A total of two hundreds patient were included with age range between 20 years and 60 years. Samples were obtained to detect serological markers of HBV infection using Enzyme linked immunosorbent assay (ELISA) and for HBV DNA quantitation through performing real time polymerase chain reaction (PCR).

Results: Of two hundreds samples tested by ELISA for HBsAg, 186 (93%) were positive and 14 (7%) samples were negative, the results had been confirmed by PCR where 1 sample showed false positive results and 2 false negative. The specificity, sensitivity, positive predictive value, and negative predictive value for ELISA were calculated: 92%, 99%, 99%, and 86% respectively.

Conclusion: ELISA is a reliable and relatively not expensive method for detection HBV, though PCR quantitation represents the gold standard assay for this purpose. Combining both methods in clinical practice ensure applying best laboratory practice if enough resources are available.

Key words: Elisa, Hepatitis B Infection, Polymerase chain reaction.

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Introduction

Viral hepatitis is an inflammation of the liver caused by several viruses including HBV. About two billion individuals have been infected with HBV; of which 350 million were chronically infected and became carriers.The World Health Organization (WHO) had divided the world into three areas depending on the prevalence of chronic hepatitis B virus: first area with high prevalence (> 8%), the second with intermediate prevalence (2-8%), and the third with low prevalence (< 2%).[1]

Hepatitis B virus is a double stranded DNA virus belongs to hepadnaviridiae family. The routes of transmission are perinatal, parenteral, and sexual. The disease usually asymptomatic, but may be acute or chronic with fatal or self-limited outcomes.[2] 25 % of chronic hepatitis B virus infection can progress to liver cirrhosis and hepatocellular carcinoma.[3]

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The correct diagnosis of the hepatitis B virus (HBV) infection is important because it represents a serious disease and major global health problem. There are two modalities for laboratory diagnosis of acute hepatitis B virus infection, first through serological markers detection, which include the presence of IgM antibody isotype to core antigen (HBc-IgM) or the presence of surface antigen (HBsAg), and the presence of hepatitis e antigen (HBeAg). Second is the molecular modality, which relies on the presence of HBV DNA in patient blood. Regarding chronic HBV infection, the diagnosis is based on the presence of HBsAg, HbeAg, HBV DNA, and at the same time absence of HBc IgM.[4]

For diagnosis of hepatitis B virus infection enzyme linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) are used. ELISA is a widely used and cost-effective laboratory procedure for detection of HBV antigens and antibodies. In addition to qualitative of HBV DNA in serum, PCR provides the privilege of quantitation of HBV DNA.[5] Detection of HBV DNA concentration in the serum is a reliable indicator of active viral replication, high infectivity rate, and response to antiviral treatment.[6] HBV DNA concentration plays an important role in the diagnosis and treatment of the disease. Several international liver societies (the American Association for the study of liver diseases, the Asia Pacific Association for the study of the liver, and the European Association for the study of the liver) published guidelines for management of chronic hepatitis B virus infection depending on HBV DNA level.[7-10]

Serological markers measurement is the first and main step in diagnosis of HBV infection, where the presence of HBsAg is very crucial except in the window period. Moreover indication of high concentration of virus in serum can be obtained by presence of HBeAg, however different HBV genotypes may have high replication rate without secretion of HBeAg. These two drawbacks of the serological tool could be overcomed by using the more reliable molecular method.[11,12]

Methods

Our study had been approved by the ethical committee of the Royal Medical Services, Amman- Jordan. The study was conducted at Princess Iman Center for research and laboratory sciences in a time period between 1/1/2013 and 28/2/2014 in a prospective manner. A total of two hundreds patient were included with age range between 20 years and 60 years, 25 % of which were female and 75 % male. Suspected cases of hepatitis B virus infection that were referred to gastrointestinal clinic were included in this study.

From each individuals two tubes (each 10 ml) of blood was collected. One yellow-top tube to detect serological markers of HBV infection using Enzyme linked immunosorbent assay (ELISA) and an another yellow-top tube for HBV DNA quantitation through performing real time polymerase chain reaction (PCR) techniques as follows:

1. ELISA

Hepatitis B virus serological markers including HBsAg, HBsAb, HBeAg, HBeAb, HBc-IgM, and HBc-IgG were tested for each samples using commercial kit from Bio-Rad, USA. The procedure type is enzyme linked immunosorbent assay, which is based on antigen- antibody interaction. ELISA is a five-step procedure: 1) coat the microtiter plate wells with antigen; 2) block all unbound sites to prevent false positive results; 3) add primary antibody (rabbit monoclonal antibody) to the wells; 4) add secondary antibody conjugated to an enzyme ; 5) reaction of a substrate with the enzyme to produce a colored product, thus indicating a positive reaction. The optical densities of samples were measured using spectrophotometer and the cut-off were calculated using specific formula for each marker. The negative and positive controls were included in each run.

2. HBV DNA real time PCR:

From 200 µl blood the minimal concentration (50µl) of HBV DNA was extracted using QIA DNA blood Mini kit (Qiagen, Hilden, Germany). 50µl of DNA was mixed with lyophilized reagents (Sacace™ HBV Real-TM Quant kit

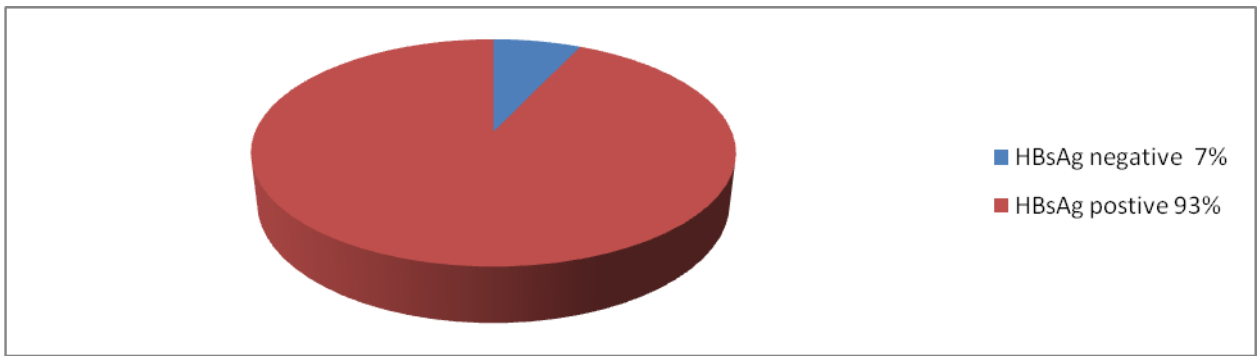

Fig. 1: Frequency of HBsAg positively by ELISA

Table I: Frequency of true and false results for ELISA in comparison with PCR results. Total number = 200

	True negative	False negative	True positive	False positive
No.	12	2	185	1

Table II: The specificity, sensitivity, positive predictive value, and negative predictive value for ELISA

	True negative	False negative	True positive	False positive
No.	12	2	185	1
	specificity	sensitivity	positive predictive value	negative predictive value
No. (%)	92%,	99%	99%	86%

Table III: The concentration of HBV DNA in patient samples

	Age ≤ 14 years HBV DNA concentration (IU/ML)				Age ≤ 14 years HBV DNA concentration (IU/ML)			
	50- 10000	11000-100000	101000-1000000	> 1000000	50- 10000	11000-100000	101000-1000000	> 1000000
No.	6	2	2	6	120	23	15	10

from Sacace Biotechnologies, Italy) and the amplification mixture carried out in Rotor-Gene™ 6000/Q instrument (thermocycler). According to manufacturer's protocol the PCR conditions were run.

For each run the negative and positive controls as well as HBV calibrators 1 and 2 were included.

The amplified product was detected by using fluorescent dyes specific for HBV. The fluorescence measured at 60° C. Samples with 50 IU concentration and more considered as positive for HBV DNA.

Statistics

The sensitivity, specificity, positive predicitive value and negative predictive value were calculated using Bayesian model.

Results

Of two hundreds samples tested by ELISA for HBsAg, 186 (93%) were positive and 14 (7%) samples were negative Fig. 1, the results had been confirmed by PCR where one sample showed false positive results (positive by ELISA though negative by PCR) and 2 false negative (negative by ELISA though positive by PCR), Table I.

According to the results shown in Table I, the specificity, sensitivity, positive predictive value, and negative predictive value for ELISA were calculated: 92%, 99%, 99%, and 86% respectively, Table II. The P-value was 0.0000 which is significant.

Depending on age of patients the samples were divided into 2 groups, the first was patients with age less than or equal 14 years (17 patients-8.5%) and the second more than 14 years (183-91.5%). The mean concentration of HBV DNA was higher ($56.0*10^7$ iu/ml) in first group when compared with second group ($16.0*10^7$ iu/ml). Table III

Table IV: The mean of viral load in HbeAg positive and negative samples

State	Mean of HBV DNA concentration (IU/ML)
HbeAg positive	19,543,455
HbeAg negative	565,660

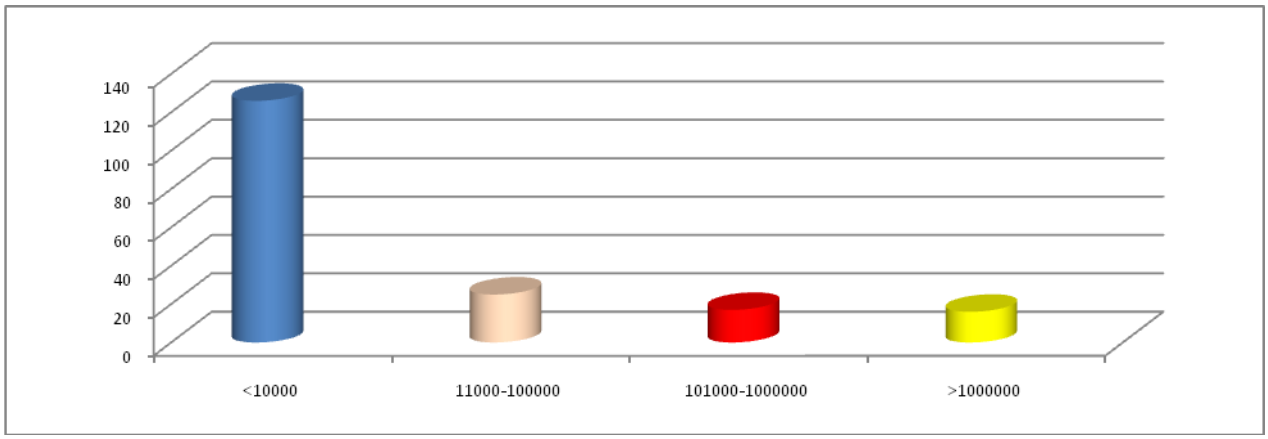

Fig 2: Frequency of HBV DNA concentration (IU/ml) by PCR Total number 184

Sixteen patients were negative by PCR.68.5% (126/184) of HBV DNA positive patients had viral load less than 10000 iu/ml. Fig. 2.

The HBV DNA level was correlated with HbeAg. Hepatitis B virus load was higher in samples having positive results for HbeAg. Table IV

20.4 % of HBsAg positive patients had positive result for HbeAg and 97.8% had HBV DNA in their blood.

Discussion

The study focuses on the correlation between enzymes linked immunosorbent assay and real time-polymerase chain reaction that used for diagnosis of hepatitis B virus infection. ELISA is a fundamental technique for detection of HBV serological markers. HbsAg, despite being a diagnostic marker of HBV infection, does not assort information about active replication and infectivity rates of the virus. Present study showed that ELISA test achieved 99% sensitivity and 92% specificity. The specificity is the probability of being test negative when disease absent, while sensitivity is the probability of being test positive when disease present (as confirmed by PCR). Positive predictive value and negative predictive value were executed 99% and 86% respectively. The positive predictive value indicates the percentage of patients with positive test that have the disease, whereas the negative predictive value is the percentage of patients with negative test who do not have the disease. The sensitivity and positive predictive value were excellent in our study and ELISA failed to diagnose only two samples (1.08%) of PCR positive samples.

In present study the accuracy of ELISA to detect HBV infection was 98.5% and this high accuracy rate due to low rate of false negative (two samples) and false positive (one sample). Comparing our results with results obtained from study conducted in Egypt (92%) we have higher accuracy rate.[5] Also Another study conducted in Brazil by Pereira1 *et al* showed lower figures; the accuracy, sensitivity and specificity of ELISA for HBsAg were 90.6%, 50% and 94.8 respectively.[13] Durgadevi S *et al* screened 30 HBsAg positive cases tested by ELISA, out of which 22 samples were found positive of HBV DNA by PCR, which includes 2 samples with only surface (HBsAg) and antibody to core antigen (IgM anti HBc) positive.[14]

Regarding viral replication we reported 20.6% (38/184) and 79.4% (146/184) positive rate by polymerase chain reaction in replicative (HbeAg positive) and non-replicative (HbeAg negative) hepatitis B virus disease respectively, whereas Rodrgues *et al* reported the 44% positive rate in non-replicative disease,[15] also Baker *et al* reported 78% in non-replicative HbeAg negative disease.[16] The presence of HBV DNA in non-replicative HBV disease may be due to low

viremia or mutations that lead to HbeAg negative cases.

The concentration of HBV DNA was calculated and the viral load below 10000 iu/ml constitutes the majority of cases with frequency rate 68.5% (126/184). Ghanim *et al* reported about 50% of cases having viral load below 10000 in/ml.[17]

The highest concentration of HBV DNA in this study was found in HbeAg positive individuals and lowest in HbeAg negative. Ferruccio *et al* study conducted in Italy showed that the HbeAg positive patients had higher HBV DNA level than HbeAg negative which consistent with our results.[18]

HbsAg is an important serological marker used for diagnosis of HBV infection, but it does not give information about replication and infectivity of the virus. For these purposes HbeAg and HBV DNA are mandatory. Present study showed only 20.4% of HbsAg positive individuals had positive results for HbeAg and 97.8% had HBV DNA in their blood.

Prospective study in HBV infection including relevant clinical and laboratory data could explore on pathogenesis and prognosis of HBV infection. More clinical samples comparing both ELISA and PCR are warranted to have more solid results. Moreover, another study for HBV DNA genotyping could touch base on contradicting serological and immunological results.

Conclusion

ELISA is a reliable and relatively inexpensive method for detection HBV, though PCR quantitation represents the gold standard assay for this purpose. Combining both techniques in clinical practice ensures applying the best laboratory practice if enough resources are available.

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