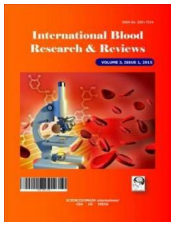

International Blood Research & Reviews
7(1): 1-6, 2017; Article no.IBRR.31171
ISSN: 2321–7219

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Molecular Detection of Hepatitis B Viruses (HBV)

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Authors' contributions

This work was carried out in collaboration between all authors. Author Samander Kaushik designed the study and contributed in writing protocol and manuscript. Authors DK and DD contributed in writing manuscript. Author VS did the laboratory work and contributed in writing manuscript. Author Sulochana Kaushik managed the analyses of the study and literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/IBRR/2017/31171
Editor(s):
(1) Dharmesh Chandra Sharma, Incharge Blood Component & Aphaeresis Unit, G. R. Medical College, Gwalior, India.
Reviewers:
(1) Darko Nozic, Millitary Medical Academy, Belgrade Serbia & General Hospital BelMedic, Serbia.
(2) Dhastagir Sheriff, MAPIMS, Medical Sciences and Research, Melmaruvathur, India.
(3) Li Wang, Infectious Disease Hospital of Yantai, Shandong, China.
Complete Peer review History: http://www.sciencedomain.org/review-history/17828

Short Research Article

Received 24th December 2016
Accepted 7th February 2017
Published 14th February 2017

ABSTRACT

Aims: HBV causes both acute and chronic infections and is transmitted through blood or other body fluids. The present study deals with the molecular detection of HBV using PCR.
Study Design: Confirmed positive samples of HBV were used to standardize the molecular diagnostic assay. DNA was extracted and used to standardize diagnostic PCR.
Place and Duration of Study: Centre for Biotechnology, Maharshi Dayanand University, Rohtak, Haryana, India, between January 2015 and July 2015.
Methodology: Positive Samples were obtained from Department of Medicine, Maulana Azad Medical College (MAMC), New Delhi. DNA was extracted from these positive samples and used for standardization of conventional PCR reaction. The results were checked by gel electrophoresis.
Results: Positive samples of HBV were detected by standardize PCR. Both the samples showed strong band of 259 bp and there is no amplification in the negative control.
Conclusion: Rapid tests have low sensitivity and specificity while molecular assays are rapid, sensitive and specific. Conventional PCR is rapid, specific, sensitive and it is also less costly than Real-Time PCR. Cost of an assay is an important factor in controlling a disease in developing countries.

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Keywords: Hepatocellular carcinoma; conventional PCR; viral hepatitis; hepatitis B virus.

ABBREVIATIONS

HBV: Hepatitis B virus; PCR: Polymerase chain reaction; HBsAg: hepatitis B surface antigen; HBcAg: hepatitis B core antigen.

1. INTRODUCTION

Viral hepatitis is a common infectious disease found worldwide caused by the many viruses including hepatitis B virus (HBV). HBV causes both acute and chronic infections and is transmitted through blood or other body fluids. About 240 million people are infected with chronic HBV and about 0.7 million people die every year due to complications of HBV [1]. About 25% to 40% of chronic infections develop into serious complications including cirrhosis and hepatocellular carcinoma (HCC) [2]. Clinical symptoms of HBV include vomiting, fever, anorexia, jaundice and right upper quadrant pain [3]. HBV is a member of *Hepadnavirus* family and is one of the smallest viruses (about 42 nm) known to infect humans. HBV is also an oncogenic virus. HBV is DNA which encodes hepatitis B surface antigen (HBsAg), hepatitis B core antigen (HBcAg), viral polymerase and HBx protein [4]. HBV DNA can be detected in serum and is used to monitor viral replication. HBV is transmitted by infected blood and various body fluids, including saliva, menstrual, vaginal, and seminal fluids [5]. Sexual transmission of HBV may occur. There is a risk of transmission of HBV from infecting mother to child. Risk of developing chronic infection is higher during perinatal infection but decreases with increasing age and vaccination [6,7]. Nine genotypes of HBV (A-I) have been identified on the basis of more than 8% difference in their genome sequences [4,8,9]. Higher rates of HCC have been found in persons infected with genotypes C and F. Several viral factors, especially co-infections with HIV, HCV and HDV and personal factor such as alcohol consuming, may increase the severity of diseases [10-13]. An effective and safe vaccine is also there which reduces the incidence and prevalence of HBV but in most part of world, HBV is still a problem [14-16]. Diagnosis is one important aspect for HBV infection control and early patient management. Primarily, Hepatitis B viruses are detected by presence of antigens like HBsAg, HBeAg and presence of antibodies against these antigens. Molecular detection methods of HBV include PCR, qPCR/realtime PCR or isothermal amplification based methods such as nucleic acid sequence based amplification (NASBA, loop mediated isothermal amplification (LAMP). The present study has been conceptualized for HBV detection by PCR from standard strains of HBV. There is no effective system available for culture of HBV which hamper the antigenic and molecular characterization of viruses in details. There are effective vaccine and few antiviral available in the market which seems to be very effective against HBV, if given in early stage of the HBV infection. Hence a rapid accurate and sensitive diagnostic assay is crucial for HBV to help in early diagnostic and better patient management.

2. METHODOLOGY

2.1 Positive Samples of Hepatitis B Virus

The two HBV positive serum samples were obtained from the Department of Medicine, Maulana Azad Medical College (MAMC), New Delhi. At MAMC, these two samples were positive for highly prevalence HBV genotype D and their results were also confirmed by sequencing at MAMC. HBV/D genotype is responsible for majority of infection in India. DNA was isolated from these two positive samples and was used to standardize conventional PCR assay.

2.2 DNA Extraction and Conventional PCR

Viral DNA from these two positive serum samples was extracted using Thermo scientific verso kit following the manufacturer's instructions. DNA was eluted in the elution buffer (final volume 50µl) and was either used immediately in standardization of PCR or stored at -80℃.

Conventional PCR was standardized in two separate tubes for each HBV strains using the forward and reverse primer and DNA from the reference genotypes of HBV. Different concentration of Primers, dNTPs, $MgCl_2$ and Taq DNA polymerase were used.

2.2.1 Primers

Published primers were selected from the surface antigen synthesizing gene region of Hepatitis B Virus [17]. The sequences of forward and reverse primer are CAAGGTATGTTGCCCGTTTG and AAAGCCCTGCGAACCACTGA respectively and they amplified segment a 259bp region from surface antigen synthesizing gene region of HBV.

2.2.2 Conventional PCR for HBV

Conventional PCR assay was carried out with 5 µl sample DNA in a total volume of 25 µl containing 0.2 µM each of forward and reverse primers of HBV, 200 µM of dNTPs, Taq Polymerase (0.5U), 1.5 mM $MgCl_2$, 12.5 µl 10X reaction buffer and sterile milliQ water. DNA amplification was performed using initial denaturation at 95℃ for 3 minutes, forty cycles of denaturation at 95℃ for 45 sec; annealing temperature of 50℃ for 45 seconds; extension temperature of 72° for 45 sec and final extension at 72℃ for 7 minutes in thermocycler.

2.3 Agarose Gel Electrophoresis

Amplicon were visualized on 2.5% agarose gel. The agarose gel was prepared in TAE (Tris acetate EDTA) buffer and the amplicons were loaded with the help of loading dye buffer in the wells. Electrophoresis was performed for about sixty minute at a constant voltage of 80 V in running buffer containing TAE and ethidium bromide (1 µg/ml). After electrophoresis, gel was visualized in a gel documented system.

3. RESULTS

3.1 Specificity of Primers

Specificity of forward and reverse primer pair of Hepatitis B Virus was checked by applying standardized assay on nucleic acids (RNA/DNA) of the other related viruses. This assay did not show any amplification of other related viral RNA/DNA hence this assay is specific only for the detection of HBV.

3.2 Standardized Conventional PCR for HBV

PCR products were visualized on 2.5% agarose gel. Gel was run along with DNA marker (100 bp). Both amplicons for the positive samples of HBV showed strong band of 259 bp while there was no band in the negative controls. Results of PCR amplification are shown in Fig. 1.

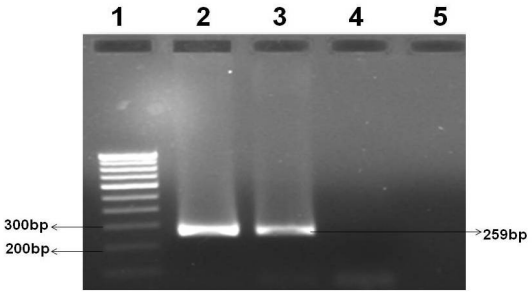

Fig. 1. Conventional PCR for HBV. Lane 1,100bp DNA marker; lane 2 and 3, HBV positive samples; lane 4 and 5, negative controls

4. DISCUSSION

Common viruses responsible for viral hepatitis are HAV, HBV, HCV, HDV and HEV. Among these, HBV, HCV, HDV are transmitted by blood or body fluid and HAV and HEV are transmitted by fecal-oral route. Viral hepatitis infections are of two types; acute and chronic viral infections. About 2 billion people have been infected with the HBV globally and about 350 million people are chronic carriers. Comparatively chronic hepatitis stage is more dangerous than the acute viral hepatitis stage. Annually more than 0.6 million people die due to acute or chronic viral hepatitis at world level [1]. There are no virus surveillance systems in developing countries like, India, so it is difficult to predict the disease burden. Higher prevalence is found in the developing countries than developed part of world where more awareness and facility are available. Disease spectrum of HBV is variable from mild to life threatening. HBV are responsible for more than 45% of cases of hepatocellular carcinoma (HCC) and 30% of liver cirrhosis with much higher proportions in low- and middle-income countries [14,18]. HCC is major causes of death, significant economical loses and accounts for 5-10% of liver transplants [12,19,20]. Due to significant role of hepatitis B virus in viral hepatitis, there is a need of rapid, sensitive, specific and cost effective assay for timely and correct diagnosis of HBV. All hepatitis viruses produce similar clinical symptoms therefore can be differentiate by laboratory diagnosis only. The laboratory diagnosis for

viruses is done by rapid commercial kits, virus culture/isolation, serological assays and molecular diagnostic techniques. There is no suitable animal model or specific cell-line for hepatitis B virus which also hamper further research of hepatitis B virus. Presently laboratory diagnosis of HBV is done by conventional biochemical markers like alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), gamma glutamyle transferase (GGT), alkaline phosphatase (AP), and bilirubin [21]. These biochemical assays give the similar indication for every hepatitis viral infections while the virulence of each hepatitis viruses (HAV, HBV, HCV, HDV and HEV) is quite different. Recently few companies introduce some rapid commercial kits for the detection of HBV but these commercial kits have poor sensitive and specificity. Molecular assays like conventional PCR and Real Time PCR are rapid, sensitive and specific but Real Time PCR is costly than the conventional PCR. Loop mediated Isothermal amplification (LAMP) is also used to detect viruses DNA/RNA [22]. LAMP is a novel method for detection of viral pathogens and also it has similar sensitivity and specificity as real time PCR [22-26]. Serological assays based on HBsAg are very rapid and cost effective in the diagnosis of HBV infection but sensitivity and specificity of serological assays are poor when compared with molecular methods. There are evidences that some patients with persistent HBV infection do not have detectable HBsAg in their serum. And also a significant fraction of blood donors whose sera are HBsAg negative by most of diagnostic tests, transmit HBV infection to recipients of their blood [27]. PCR techniques are providing good correlation (100%) between circulating or active HBV DNA and the disease [28]. PCR and other molecular techniques can be applied on any type of samples like, blood, serum or any other fluids while serological techniques can be applied on the serum samples only. Sample requirement of molecular assays is very low as compared to other techniques. Molecular techniques are highly sensitive and are able to detect occult HBV infections and window period infections. Multiple pathogens detection in a single assay is possible with PCR while other assays like serological assays are uni-target. Multiplexicity of an assay is not only more informative but also cost effective. Significant advances have been made in the diagnosis and treatment of chronic HBV infection and the HBV DNA amplification assays serve as valuable tools.

5. CONCLUSION

Presently, Hepatitis B viruses are detected by classical assay like biochemical assays, rapid tests and modern molecular assays. Rapid tests have low sensitivity and specificity while molecular assays are rapid, sensitive and specific but costly. Cost of assay is an important factor in developing countries like India. Effective vaccine and few antiviral are also available for hepatitis B viruses. Still Hepatitis B virus is a big problem and affect large segment of the population world-wide. A rapid sensitive, specific, and cost effective assay for HBV will surely be useful for better patient management and to curb the infection in early stages. There is still need to development of some specific therapeutic agent for Hepatitis B Virus.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

ACKNOWLEDGEMENTS

Authors are thankful to Council of Scientific & Industrial Research, New Delhi, India for providing financial support in form of SRF to Vikrant Sharma and to Haryana State Council for Science & Technology (HSCST), Haryana, India for financial assistance.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
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