African Journal of Microbiology Research Vol. 6(42), pp. 7003-7007, 6 November, 2012
Available online at http://www.academicjournals.org/AJMR
DOI: 10.5897/AJMR12.1181
ISSN 1996-0808 ©2012 Academic Journals

Full Length Research Paper

Comparison of SYBR Green and turbidimetry methods for loop mediated isothermal amplification (LAMP) product detection in diagnosis of hepatitis B virus (HBV)

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Accepted 6 September, 2012

Hepatitis B virus (HBV) is one of the leading causes of hepatocellular carcinoma and liver diseases. The HBV serological and molecular detection methods have some limitations; therefore, use of these methods is not feasible in many diagnosis centers. In this study, SYBR Green was compared with spectrophotometry for turbidity assay for final detection of LAMP reaction product in diagnosis of HBV. Two hundred HBsAg positive serum samples were obtained from 3 different groups. DNA was extracted by using DNP kit and six specific primers for LAMP technique were designed. The sensitivity and specificity tests were performed and test was optimized on samples. Loop mediated isothermal amplification (LAMP) product were evaluated by absorption and SYBR Green fluorescence studies and using electrophoresis measurements. Among 200 samples with known viral load and varied titers, 172 samples (about 86%) were LAMP positive. The analysis of the reaction products by SYBR Green fluorescence, electrophoresis and spectrophotometry showed similar results. As reaction goes to completion, along with the DNA synthesis, MgP_2O_3 value is increased. This fact resulted in an increase of turbidity in the reaction. in comparison to conventional polymerase chain reaction (PCR) technique, LAMP technique had more sensitivity and specificity. In addition, comparison of the three final detection methods showed that the final detection of the LAMP product using SYBR Green is easier and more cost-effective, also no need for electrophoresis and other post amplification methods.

Key words: Hepatitis B, Loop-mediated isothermal amplification (LAMP), spectrophotometer.

INTRODUCTION

Human hepatitis B virus (HBV) is a member of the Hepadnaviridae family. HBV infection is a major public health problem worldwide causing 500,000 to 1.2 million deaths per year, due to chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC) (WHO, 2002). HBV infection is still a global public health problem. So far, over 2 billion people across the globe have been exposed to HBV and 350 up to 400 million are chronic carriers of HBV. Indirect estimation of HBV rate in Iranian population shows that about 1.5 to 2.5 million people are suffering from HBV infection (Alavian et al., 2008), and unintentionally up to 70 to 80% develop chronic hepatitis, indicating that HBV alone is the leading cause of chronic liver disease in Iran (Zali et al., 1996; Alavian et al., 2006). Due to the mutation incidence on the virus surface antigen (HBsAg), the usual serological diagnostic methods which are working based on Ag-Ab reaction are not absolutely reliable. In other words, although serological methods are well documented, they are not appropriate indicators of HBsAg detection. This necessitates the use of specific molecular methods (Gilbert et al., 1999). Molecular detection methods based on isolation and amplification of virus nucleic acid such as polymerase chain reaction (PCR), isothermal

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amplification, and hybridization are capable to recognize infection at early stages (Compton, 1991). Loop-mediated isothermal amplification (LAMP) provides a convenient method to simply amplify specific nucleic acid (Notomi et al., 2000). The main characteristic of the LAMP method is its ability to amplify nucleic acid under isothermal conditions in the range of 65°C (Nagamine et al., 2001). As the LAMP method uses 6 primers recognizing 8 distinct regions on the template DNA, its specificity is extremely high. The LAMP method synthesizes 10 to 20 mg of specific DNA for 25 ml of reaction mixture in 30 to 60 min. In the reaction of DNA polymerization by DNA polymerase, pyrophosphate ion is released from dNTP as a byproduct (Nagamine et al., 2002a). A large amount of pyrophosphate ion reacts with magnesium ion in the LAMP reaction buffer producing a precipitate (Mori et al., 2001). As a first step, a stem-loop DNA structure, in which the sequences of both DNA ends are derived from the inner primer, is constructed as the starting material.

Subsequently, one inner primer hybridizes to the loop on the product in the LAMP cycle and initiates strand displacement DNA synthesis, yielding the original stem-loop DNA and a new stem-loop DNA with a stem twice as long (Walker et al., 1992). The final products are stem-loop DNAs with several inverted repeats of the target DNA and cauliflower-like structures with multiple loops amplified to the amount of 109 copies of the target. These reactions occur in less than an hour (Ushikubo, 2004; Parida et al., 2004). There is no need to run the product on agarose gel to separate and visualize DNA fragments. After adding 0.1% SYBR Green to each reaction tube, green color is formed under ultraviolet light which is an indicator of positive results (Mori and Notomi, 2009). There is a relation between DNA concentration and turbidity. The aim of this study is to compare the relation between use of SYBR green and turbidometery assay for obtaining best final result of LAMP reaction.

MATERIALS AND METHODS

Sampling

Two hundred serum samples were collected and based on laboratory source were classified into 3 different groups. First group included 66 serum samples generously given by Keyvan virology laboratory (equipped with COBAS amplicor system, FDA approved) with known viral load. Second group contained 36 serum samples provided by both Azadi and Resalat laboratories. HBsAg ELISA test were positive on these samples. Third group consisted of 98 serum samples with defined number of virus particles which was donated by Gholhak laboratory.

LAMP reaction

Primers which play the role of starters for initiation of *in vitro*reactions are one of the most important factors in LAMP reaction. LAMP primers were designed for HBV surface antigen using primer explorer V4 software. Target sequences were common sero and genotype (accession No. Ay771794) in Iran (awy2, D).

Total reaction volume were 25 µl containing 0.2 µM external primer (F3/B3), 1.6 µM internal primer (FIP/BIP), 0.8 µM loop primer (LF/LB), 20 mM Tris-Hcl, 10 mM Kcl, 10 mM $(NH_4)_2SO_4$, 9 mM $MgSO_4$, 1.4 mM dNTP, 0.8 M Betain (Sigma-Aldrich), 1 µl (8 Unit) Bst DNA polymerase (New England Biolabs) and finally 5 µl of template DNA . The mixture was incubated at 66°C for 1 h.

LAMP production detection

LAMP product were detected by adding 0.1% SYBR green per each reaction and visualized under ultraviolet light (302 nm). Green tubes represent positive results, while orange ones indicate negative results. To determine the amount of MgP_2O_3 as a byproduct of the reaction, optical absorption was measured at 400 nm within 5 min time intervals from first to the end of reaction using spectrophotometer (Bio-Rad). DNA concentration was determined by measuring optical absorption during reaction by Eppendorf spectrophotometer at 260 nm.

RESULTS

DNA was extracted from HBV positive serum samples obtained from Keyvan laboratory with 4,000,000 viral particle loads by DNP kit (Cinnagen). After adding SYBR Green to each reaction and visualization under ultraviolet light (302 nm), positive reactions developed green color, while negative reactions were seen as orange (Figure 1). MgP_2O_3 byproduct caused turbidity in reaction which was measured by spectrophotometer. As reaction goes to completion, MgP_2O_3 value was increased to maximum level. Optical absorption of MgP_2O_3 during reaction at 400 nm is as shown in Table 1. Increased turbidity due to formation of MgP_2O_3 over the reaction period is as shown in Figure 2.

As the reaction proceeds, the turbidity was found to increase due to accumulative production of MgP_2O_3 complexes. Measuring optical density of reaction mixture at 260 nm in different time intervals showed that DNA synthesis has also elevated (Table 1). Simultaneous increase of turbidity and DNA concentration in reaction is as shown in Figure 3. As observed, turbidity raised followed by increase in DNA concentration level.

Among 66 positive serum samples belonging to the first group, 61 were LAMP positive and 5 were negatives. LAMP technique sensitivity on the basis of this result was 92/42%. Twenty four out of 33 samples of the second group were LAMP positive, while two were negative. It is obvious that known false positive results are common errors in serological methods. Some of these 12 negative results, which were ELISA positive, were probably false positive. Among 98 samples of third group, 11 were LAMP negative, while the others were positive. LAMP technique sensitivity was 88.9% for this group.

DISCUSSION

LAMP novel technique is one of the simple gene amplification methods which are performed at the same

Table 1. Absorption value of samples at 400 nm with DNA concentration at 260 nm.

Sample	Time	Absorption in 400 nm	DNA concentration in µg/25 µl
S1	5	0.032	0.5
S2	10	0.044	0.9
S3	15	0.076	2.45
S4	20	0.152	5.9
S5	25	0.264	7
S6	30	0.28	7.85
S7	35	0.336	9.35
S8	40	0.388	9.68
S9	45	0.476	14.46
S10	50	0.552	14.7
S11	55	0.792	15.14
S12	60	1.024	16.02

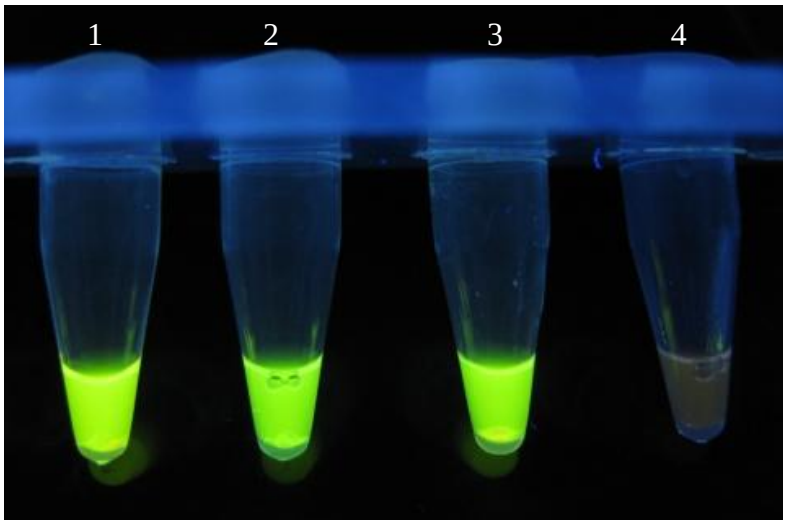

Figure 1. Lamp products: tubes 1 to 3, LAMP positive product; tube 4, LAMP negative control.

temperature throughout the entire reaction period (Francois et al., 2011). Despite the simplicity, LAMP is a very sensitive and specific technique with high accuracy. In addition, it does not need any improved instrument like thermo cycler and is more economical (Notomi et al., 2000; Kirunda et al., 2012). Overall, it could be noted that in near future, LAMP will be one of the most beneficial molecular technique for detection of infectious agents in developing countries such as Iran. Problem of nonspecific amplification and false positive results are solved by designing 6 specific primers for 8 distinct regions of target sequences. Non-specific product caused by low reaction temperature is the most limiting factor in isothermal amplification technique (Parida, 2008). However, the drawback of having nonspecific results has been overcome in LAMP technique by using 6 specific primers with high specificity (Mori et al., 2004; Mori et al., 2006).

There is a direct correlation between DNA synthesis and MgP_2O_3 production during reaction. Increment in DNA synthesis caused increases in producer price index (PPi) value and after that MgP_2O_3 complex concentration will increase which results in turbidity increases (Nagamine et al., 2002b; Mori et al., 2006; Parida et al., 2005).

A tremendous amount of studies demonstrated that LAMP technique in spite of simplicity has high specificity and could be a suitable alternative for PCR (Parida et al., 2007; Kurosaki et al., 2009; Zhang et al., 2009). The result of this study showed that LAMP technique is able to detect very low viral load (as mentioned, the obtained viral particle limitation was 5 particle) even in old stored samples. This method is convenient to use and cost-effective; there is no need for sophisticated instrument

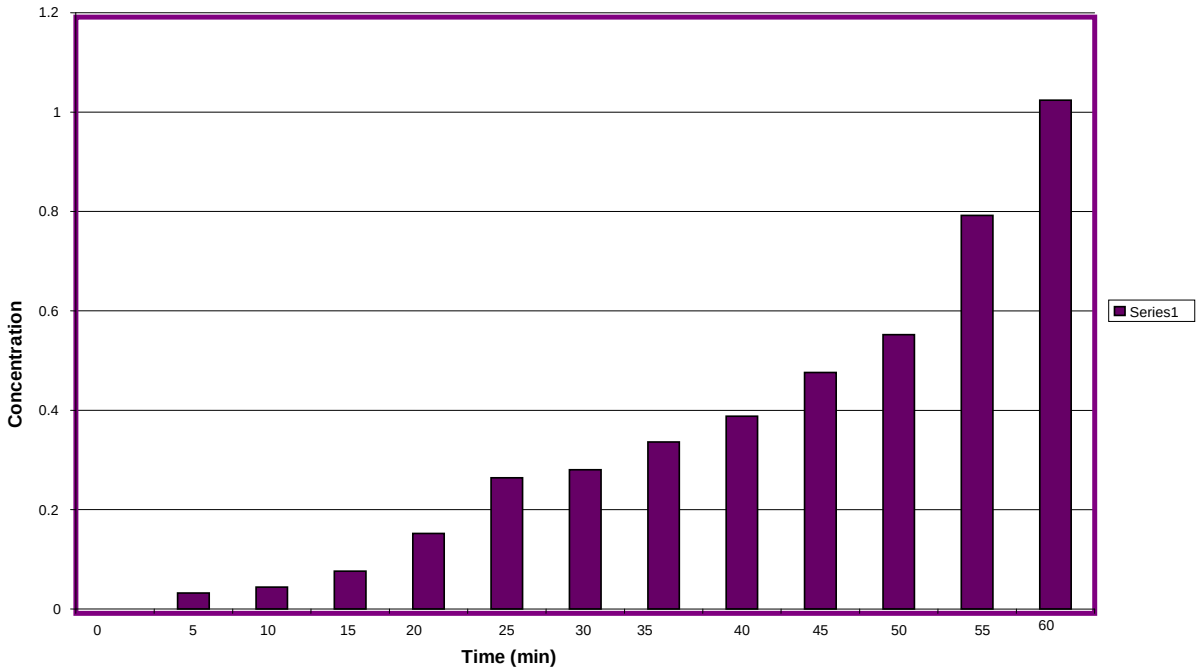

Figure 2. Turbidity concentration during reaction in 400 nm (X) against time of reaction (Y).

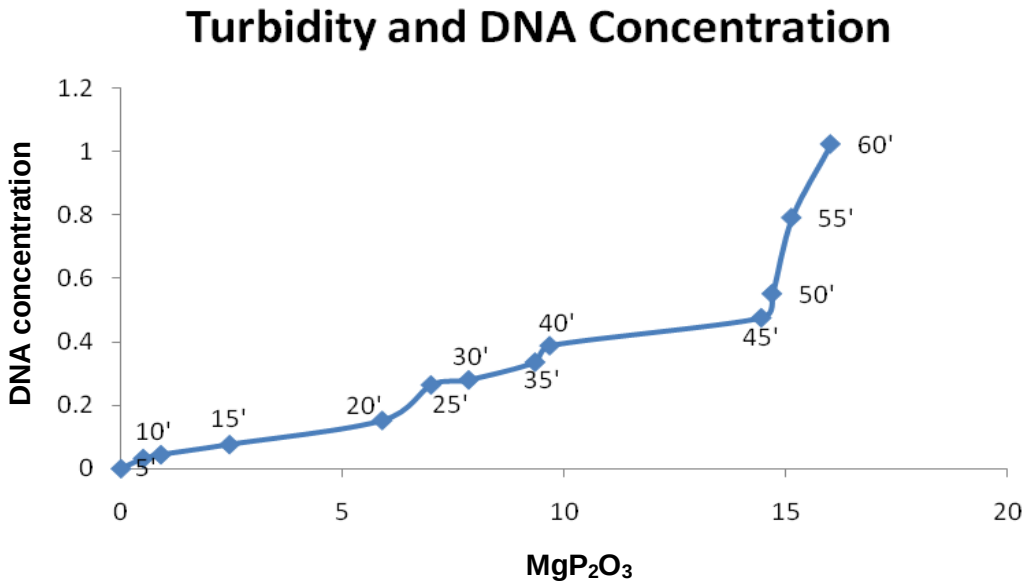

Figure 3. Optical absorption of DNA concentration (260 nm) (X) against optical absorption of MgP_2O_3 (400 nm) (Y).

and it is simply carried out in a heater block. Also, after adding SYBR Green, the DNA products were visualized under ultraviolet light. In addition, as optical absorption result were the same as florescent results, it can be concluded that this cost effective molecular detection method with high specificity, sensitivity, and accuracy can be used for rapid diagnosis of HBV in rural areas.

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