

*Full Length Research Paper*

# Loop mediated isothermal amplification (LAMP) for rapid detection of HBV in Iran

Elham Moslemi<sup>1\*</sup>, Mohammad Hassan Shahhosseiny<sup>2</sup>, Gholamreza Javadi<sup>1</sup>, Kazem Praivar<sup>1</sup>,  
Taher Nejad Sattari<sup>1</sup> and Hossein Keyvani Amini<sup>3</sup>

<sup>1</sup>Islamic Azad University, Science and research branch, Cellular and molecular Biology department, Tehran, Iran.

<sup>2</sup>Islamic Azad University, Quds unit, Microbiology department, Tehran, Iran.

<sup>3</sup>Keivan Virology Laboratory, Tehran, Iran.

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Hepatitis B virus (HBV) is one of the most important factors for hepatocellular carcinoma and a liver disease that seems 350 - 400 million persons are infected with all over the world. Loop mediated isothermal amplification (LAMP) is a novel nucleic acid amplification technique with high specificity and sensitivity which has been done under isothermal condition. In this study we tried to apply LAMP technique for rapid, accurate, and cost-effective diagnosis of HBV in patient's serum samples. 104 HBV quantities sera were supplied. Six LAMP specific primers were designed for HBs region of HBV. LAMP and PCR reactions were optimized. At the end of the LAMP reaction, SYBR Green was used for identifying negative and positive results. The PCR sensitivity up to 40 particles was observed and the LAMP sensitivity test was verified up to 4 particles. Among the 104 quantities sera, only 95 cases were PCR positive but 101 cases were LAMP positive. 9 cases were PCR negative among these, 6 cases were reported as LAMP positive. In 3 cases LAMP and PCR were both negative. In comparison, between LAMP and PCR, the LAMP technique in spite of its simplicity, high sensitivity and specificity, could be an appropriate replacement for PCR.

**Key word:** Hepatitis B, PCR, LAMP.

## INTRODUCTION

Hepatitis B virus (HBV) is one of hepadnaviridea family which has a great role in foundation of hepatocellular carcinoma and liver disease (Dienstag, 2008). Today's infection by HBV has become as a world wide problem which seems 350 - 400 million people are infected in all over the world (Alavian et al., 2008). At the first stages of infection, a great amount of HBV particles are observed in blood that the power of transmission at this level is very high (Pawlotsky et al., 2000). HBV Diagnostic methods are divided in two groups;

1. Common methods.
2. Molecular methods.

Common methods include serodiagnostic, liver biopsy, clinical and pathological finding and abnormal liver activity

test such as ALT (Dienstag, 2008). The usual serological diagnostic methods detect virus surface antigen (HBsAg) which is based on Ag-Ab reaction and because of the mutation, can not be very reliable (Weber and Berge, 2005; Behbahani et al., 2006). As some percentage of HBsAg negative bloods in blood's donators has caused the transition of disease (Pourazar et al., 2005).

Therefore although serological methods are well documented but they are not appropriate indicators for detection, as a result using specific molecular methods are evadible (Notomi et al., 2000).

At the first stages of revealing disease, detection methods based on antibody and biopsy are not recognizable (Dienstag, 2008). Molecular detection methods based on isolation and amplification of virus nucleic acid such as PCR (Polymerase Chain Reaction), Isothermal amplification, and Hybridization are capable to recognize infection at early stages (Vernet, 2004; Shahhosseiny and Tehrani, 2005).

Hybridization based on b-DNA (branched DNA) is

\*Corresponding author. E-mail: [elham\\_moslemi60@yahoo.com](mailto:elham_moslemi60@yahoo.com).

another molecular technique which occurs by the usage of b-DNA florescent probes which provide the quantitative DNA virus recognition in patient's serums. But using this method has been limited to some research institute meanwhile the sensitivity of this method had been 1 up to 2 log less than PCR (Shahhosseiny, 2005; Shahhosseiny and Rahimi, 2007; Nagamine et al., 2002b).

Despite its advantages, each amplification method has its own problems. For example using PCR against expanded development and high accuracy because of needing to improved instrument like thermal cycler have not been distributed in all diagnostic center as a routine test up to now (Zhang et al., 2001). Although thermal cycler in Isothermal amplification methods such as RCA (Rolling circle amplification), NASBA (Nucleic acid sequence based amplification), TMA (Transcription based amplification system, 3SR (Self sustaining sequences replication) are not required, but because of low reaction temperature or non specific sequences amplification these test dose not have sufficient sensitivity and specificity for diagnostic tests (Nagamine et al., 2001). Loop mediated isothermal amplification (LAMP) is an isothermal amplification method which is performed in isothermal condition, thereby obviating the need of thermal cycler whether has a grate specificity and sensitivity (Mori et al., 2001).

The reaction will take place without using template denaturation by the assistance of DNA polymerase with strand displacement activity. Also the reaction required a set of six specially designed primers called inner- outer and loop primers (Mori et al., 2006; Nagamine et al., 2002b). In addition gate amount of DNA (10 - 30 microgram per 25 micro liters) in a short time (15 - 60 min) with high specificity can be synthesized; it means that all process from amplification to detection will be done at the same temperature. Since stem-loop structures are created by designing special probes for these structures it can be used for hybridization without heat denaturation after amplification. Also when reaction combined with reverse transcription, RNA can be amplified too (Parida et al., 2004, 2005, 2007).

So there is no need to next stages for proving the neither reaction nor observations of the product in agarose gel and by adding 0.1% SYBR Green to each reaction tube and appearance of green color under U.V light positive result will be obtained.

## MATERIAL AND METHODS

### Sampling

104 HBV positive sera which were quantities by COBAS Amplicor HBV Monitor kit were supplied.

### DNA extraction

DNA was extracted from patient's serum by using CinnaGen DNP kit (cat: DN811530) in following process. 5 µl protein's-K (10 mg/ml) were added to 100 µl of each serum sample and incubated in 72°C for 10 min. Then 400 µl lysis buffer added to each tube and

subsequently 300 µl precipitate buffer (Isopropanol) also added then cen-trifuged at 12000 rpm for 10 min. After decanting, 1 ml of 70% ethanol was used for washing which then was centrifuged at 12000 rpm for 5 min. Finally ethanol was completely poured off and dried, at last DNA pellet was dissolved in 30 µl distilled water.

### PCR and LAMP Primers

PCR primers were designed for HBsAg; the amplified region has 262 bp lengths. LAMP primers were designed on the basis of the published sequence of common strain in Iran (Gen bank accession no. AY741794) by employing the LAMP primer designing support software program (primer explorer V4; <http://primerexplorer.jp/e/>). The location and sequences of primers are shown in Figure 1.

### PCR reaction

PCR reaction was carried out in a total 25 µl reaction mixture containing 2 mM dNTP, 1.5 mM MgCl<sub>2</sub>, 1 micromole of each forward and reverse primers, 1XPCR buffer, 1.25 unit of Taq DNA polymerase enzyme, and 5 µl templates DNA. PCR cycling parameters incubation cycles consisting of denaturation at 93°C for 40 s, annealing at 60°C for 40 s and extension at 72°C for 40 s, repeated for 40 cycles. The PCR product was electrophoresed in 1.5% agarose gel (0.5X TBE) stained with Ethidium bromide (CinnaGen).

### PCR sensitivity and specificity

For determination PCR sensitivity, different dilutions of DNA virus from 4 million to 40 particles were provided. The DNA's of Mice, human, *Toxoplasma gondii*, Hepatitis C virus, *Mycobacterium tuberculosis*, *Saccharomyces cerevisiae*, and *Escherichia coli* were used to verify PCR specificity.

### Cloning of PCR product as a positive control

After purification, the PCR product was cloned in pTZ57R vector by using T/A cloning kit (Fermentas, cat: K1214).

### The LAMP reaction

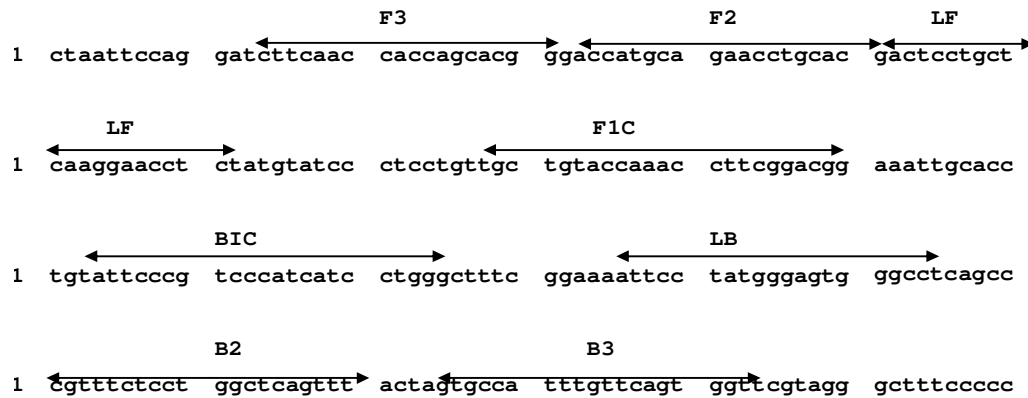
The LAMP reaction was made in 25 µl by mixing 0.2 µM F3/B3, 1.6 µM FIP/BIP, 0.8 µM LF/LB, 20 Mm Tris-Hcl, 10 Mm Kcl, 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 9 mM MgSO<sub>4</sub>, 1.4 mM dNTP, 0.8 M Betain (Sigma-Aldrich), 8 u Bst DNA polymerase (New England Biolabs). The mixture was incubated at 66 for 1 h.

### The LAMP reaction evaluation and Electrophoresis

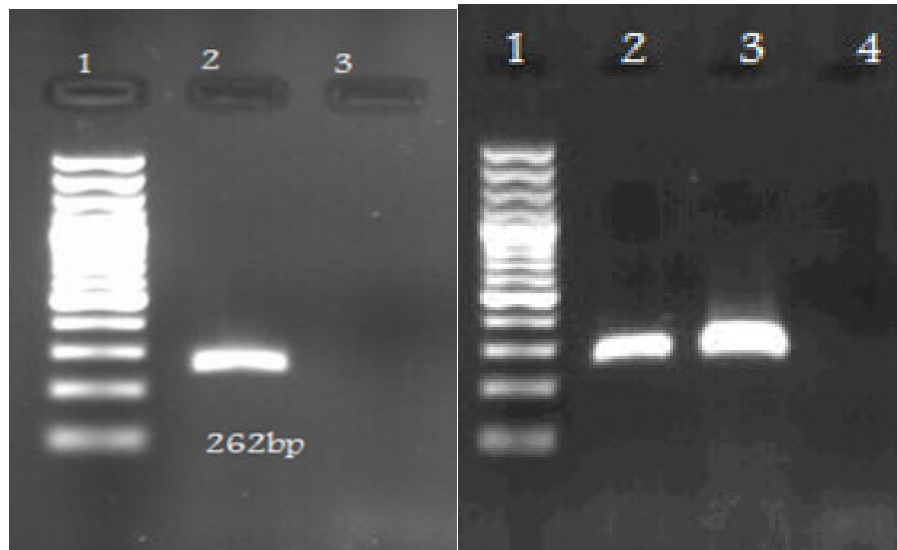
0.1% SYBR Green (Invitrogen lot: 49743A) is added to each reaction tubes and were observed under U.V light (302 nm). The positive tubes were in green color and the negative one was in orange colors. LAMP product was electrophoresis in 2% agaros gel (0.5X TBE) stained with Ethidium Bromide.

### LAMP sensitivity and specificity

To obtain LAMP sensitivity test same as PCR, the different dilution of virus's DNA from 4 million to 4 particles were provided. The DNA's of Mice, human, *Toxoplasma gondii*, *Hepatitis C virus* (cDNA), *Mycobacterium tuberculosis*, *Saccharomyces cerevisiae*, and *Escherichia coli* were used to identify LAMP specificity.

**LAMP Primer location on target sequences:**

**Figure 1.** F3: CTT-CAA-CCA-CCA-GCA-CGG, B3: ACC-ACT-GAA-CAA-ATG-GCA-, F1C: CGT-CCG-AAG-GTT-TGG-TAC-AGC-<A>-, F2: CCA-TGC-AGA-ACC-TGC-ACG, B1C: ATT-CCC-GTC-CCA-TCA-TCC-TGG-<G>, B2: AAA-CTG-AGC-CAG-GAG-AAA-CG, LF: GAG-GTT-CCT-TGA-GCA-GGA-GT, LB: ATT-CCT-ATG-GGA-GTG-GGC-C

**PCR product electrophoreses**

**Figure 2. (a)** Lane 1; 100bp DNA Ladder PLUs (Fermentas), 2; PCR product of target DNA (262 bp), 3; negative control. **(b)** Lane 1; 100 bp DNA Ladder PLUs (Fermentas), 2; Positive control, 3; PCR product of pTZR57 plasmid, 4; negative control.

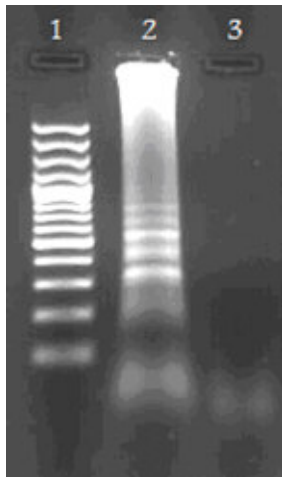
**RESULTS**

The PCR products were loaded on 1.5% agaros gel, the fragment's size obtained by using specific primers was 262 bp (Figure 2a) The PCR product was cloned in pTZR57 plasmid. White colonies were isolated and verified by PCR (Figure 2b) The LAMP reaction was incubated at 66 C for 1 h. The Electrophoresis of the LAMP products showed different size fragments in smear shape

(Figure 3). PCR and LAMP had high specificity showed no reaction to the other infectious agents except DNA of hepatitis B virus (Figure 4a, 4b). The PCR sensitivity up to 40 particles was observed (Figure 5a) and the LAMP sensitivity test was verified up to 4 particles (Figure 5b, 5c).

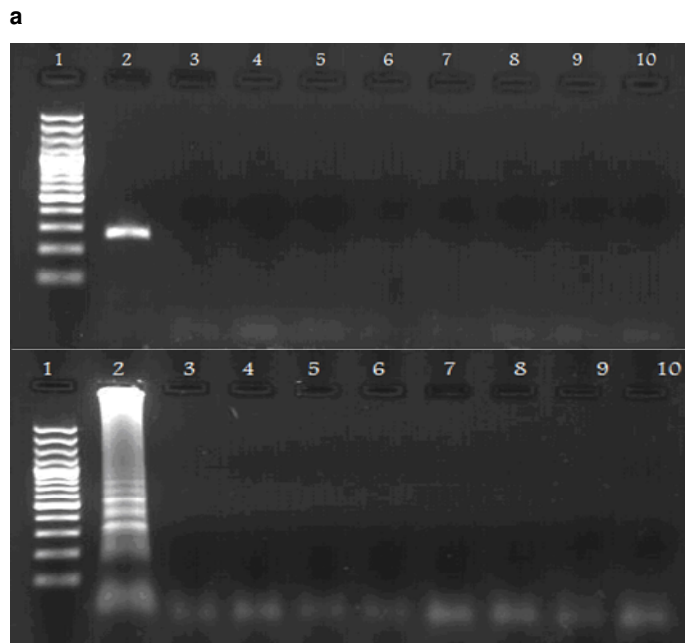
Among the 104 COBAS PCR positive quantities serum only 95 cases were PCR positive in our study, but 101 cases were LAMP positive.

## LAMP product electrophoreses



**Figure 3.** Lane 1; 100 bp DNA Ladder PLUs (Fermentas), 2; LAMP product of target DNA, 3; negative control.

## PCR and LAMP specificity



**Figure 4. (a)** Lane 1; 100 bp DNA Ladder PLUs (Fermentas), 2; positive control (HBV PCR product), 3; PCR product of Mice DNA, 4; PCR Human DNA, 5; PCR product of *E. coli* DNA, 6; *Saccharomyces cerevisiae* DNA, 7; Hepatitis C virus DNA, 8; *Mycobacterium tuberculosis* DNA, 9; *Toxoplasma gondii* DNA, 10; negative control. **(b)** Lane 1; 100 bp DNA Ladder PLUs (Fermentas), 2; positive control of LAMP (HBV PCR product), 3; Mice DNA, 4; Human DNA, 5; *E. coli* DNA, 6; *Saccharomyces cerevisiae* DNA, 7; Hepatitis C virus DNA, 8; *Mycobacterium tuberculosis* DNA, 9; *Toxoplasma gondii* DNA, 10; negative control.

9 cases were PCR negative, among this 9 cases 6 cases were reported as LAMP positive. In 3 cases LAMP PCR were both negatives.

## DISCUSSION

Hepatitis B virus infection has become a worldwide problem as seems that about 2.5 million people are infected by hepatitis B virus in Iran (Alavian et al., 2008).

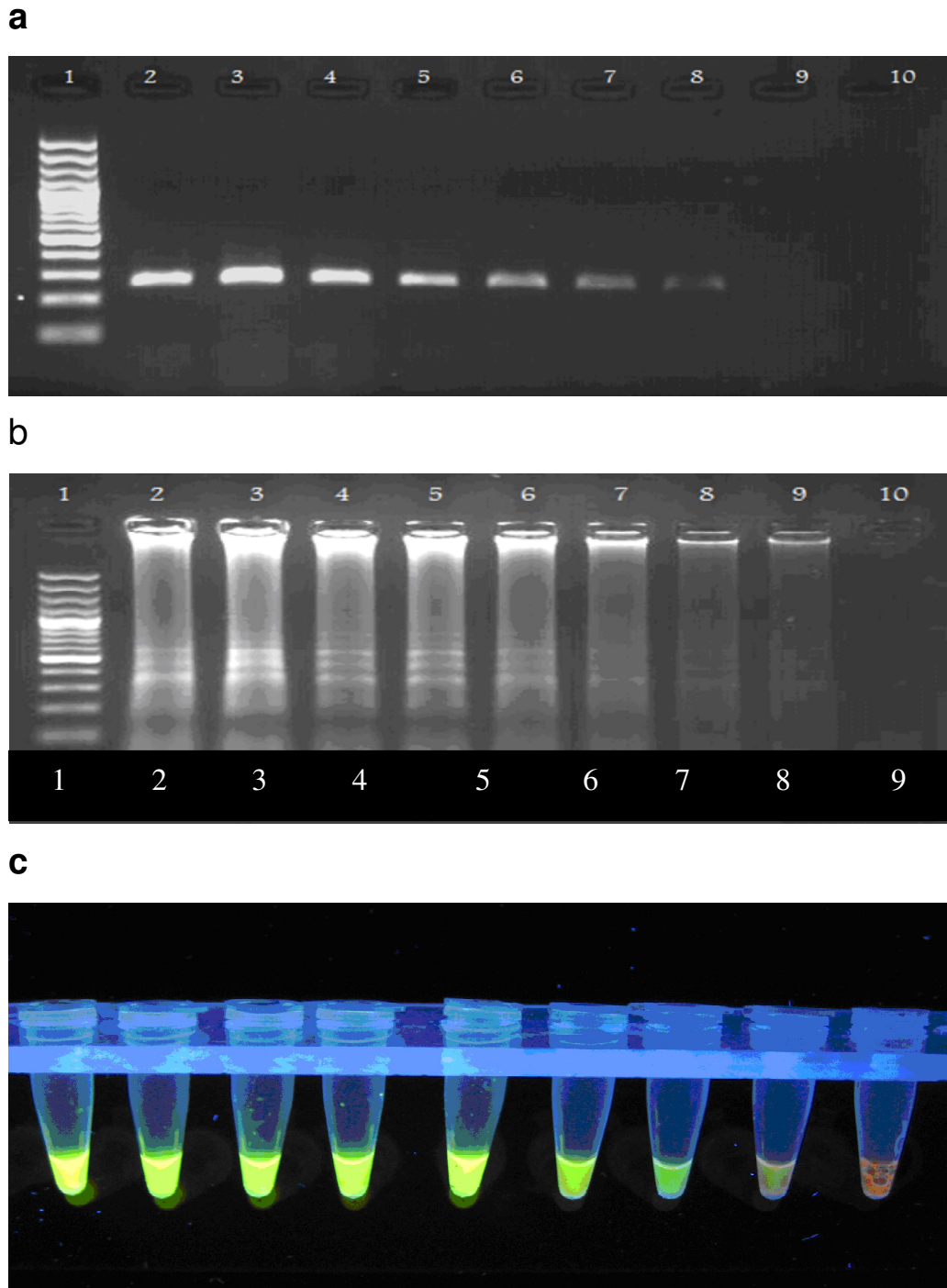
Even though the serological HBV detection methods are known very well but they aren't considered as a good indicator for virus recognition so molecular methods must be substituted (Shahhosseiny, 2005). One of the most common molecular techniques is PCR; although it has high sensitivity and specificity, it has not been used in all diagnostic centers until now. The Need for improved instruments like thermal cyclers is the main reason for this limitation.

LAMP New novel technique is one of the very simple gene amplification methods which were performed under isothermal condition. In contrary with its simplicity it has high specificity and sensitivity with obviating the need for using thermal cycler (Notomi et al., 2000; Mori et al., 2009).

The reaction is administered external primers (F3, B3) and internal primers (FIP, BIP) which each one recognizes 2 separate distinct site and also two special loop primers which recognize a total of 8 distinct sequences in the DNA target. The amplification products are stem-loop DNA structure with several inverted repeats and cauliflower-like structure with multiple loops (Nagamine et al., 2001; Nagamine et al., 2002a; Mori et al., 2001).

At present time in all over the world grate studies have been developed to recognize infectious agent by LAMP technique. Although most of these research are about RNA viruses detection such as African Trypanosomes (Kuboki et al., 2003), Tomato yellow leaf curl virus (Fukuta et al., 2003), Varicella-zoster virus (Okamoto et al., 2004), West Nile Virus (Parida et al., 2004), Newcastle disease virus (Pham et al., 2005), Respiratory syncytial virus (Ushio, 2005), MUMPS virus (Okafuji, 2005), Herpes Simplex Virus (Enomoto et al., 2005), Encephalitis virus (Parida et al., 2006), Influenza A and B (Ito et al., 2006), Epstein-Bar Viruse (Iwata et al., 2006, Chikun-gunya Virus (Parida et al., 2007), Ebola Virus (Kurosaki et al., 2007), Human Papillomavirus type 6, 11, 16, 18 (Hagiwara et al., 2007), HIV-1 virus (Curtis et al., 2009), Monkeypox virus (Iizuka et al., 2009) but also there are many investigation which approved the detection of bacteria and parasites like *Mycobacterium tuberculosis* (Iwamoto et al., 2003), *M. pneumoniae* (Saito et al., 2005), *Cryptosporidium Oocysts* (Karanis, 2007), *Enterococcus faecalis* (Kato et al., 2007), *Actinobacillus actinomycetemcomitans* (Osawa et al., 2007), *Trypanosoma brucei rhodesiense* (Njiru et al., 2008a,b) *Filarial* parasites (Aonuma et al., 2009), *Lepospira* (Lin et al., 2009), *Toxoplasma gondii* (Zhang et al., 2009) by LAMP.

## PCR and LAMP sensitivity



**Figure 5.** (a) Lane 1; 100 bp DNA Ladder PLUs (Fermentas), 2; positive control of PCR (HBV PCR product), 3; serum with 4,000,000 particle, 4; serum with 400,000 particle, 5; serum with 40,000 particle, 6; serum with 4,000 particle, 7; serum with 400 particle, 8; serum with 40 particle, 9; serum with 4 particle, 10; negative control. (b) Lane 1; 100 bp DNA Ladder PLUs (Fermentas), 2; positive control of LAMP (HBV PCR product), 3; serum with 4,000,000 particle, 4; serum with 400,000 particle, 5; serum with 40,000 particle, 6; serum with 4,000 particle, 7; serum with 400 particle, 8; serum with 40 particle, 9; serum with 4 particle, 10; negative control. (c) Lane 1; positive control of LAMP (HBV PCR product), 2; serum with 4,000,000 particle, 3; serum with 400,000 particle, 4; serum with 40,000 particle, 5; serum with 4,000 particle, 6; serum with 400 particle, 7; serum with 40 particle, 8; serum with 4 particle, 9; negative control.

LAMP has verity characteristics and application besides diagnosis, including SNP typing and DNA quantification as real-time detection and particularly in development of g-POCT devices (Parida et al., 2008). In this study we designed specific primers for HBs region of HBV by awy2 serotype, D genotype which is the most common sero-type in Iran. This technique has a very high sensitivity and specificity in comparison with PCR and also against high sensitivity and specificity improved instruments was not required and the reaction was verified in a short time merely by using a simple heater.

Among 104 cases with define viral load, 95 cases were PCR positive whether 101 cases were LAMP positive. 9 cases were PCR negative which these cases had low viral load and were collected many times ago.

Only 3 cases were LAMP negative that these cases had slightly viral load (129, 1200, 1600) and also stored for long time.

Probably decreasing of viral loads with respect to initial low virus particles, and also long storage, are the main reasons for negative results in these 3 cases in both LAMP and PCR tests. Therefore because of significant LAMP sensitivity in 6 cases with similar condition, LAMP test was able to detect truly result which seems PCR test had not this property.

So it can be mentioned that in this study HBV particles can be detected even thought in old samples with at least viral load by using LAMP technique.

Although improved instrument are not required in this technique but it has grate sensitivity and accuracy that reaction can be done simply by applying just a dry-plate. By adding 0.1% SYBR\_Green and observing under U.V light the result could be obtained, so needing to post amplification processes like as electrophoresis were obviate.

Optimistically It is no far that in near future by using LAMP technique, HBV detection possibility without administering improved instrument with significant sensitivity and specificity and also cost saving will be available in all diagnostic centers.

## Conclusion

With respect to this result and comparing PCR with LAMP can be stated that LAMP technique in spite of simplicity and not needing to improved instruments has higher sensitivity over PCR and could be a proper replacement for it in future.

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