

# Visual Detection of *Curly Top Virus* by the Colorimetric Loop-Mediated Isothermal Amplification

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## Abstract

*Curly Top Virus* (CTV) in sugar beet, belonging to the family *Geminiviridae*, genus *Curtovirus* is a considerable problem in semiarid areas of the Iran and other semiarid parts of the world. There are several diagnostic methods to detect CTV, but these techniques take a long time for 3 h to tow day, requiring sophisticated tools. The aim of this study, for the first time, was to reduce the time required to detect CTV in sugar beet, using colorimetric loop-mediated isothermal amplification (LAMP) technique requiring only an ordinary water bath or thermoblock. DNA was extracted from infected naturally leaf tissues. Samples were tested for the presence of *Curtovirus* species by PCR and LAMP reactions to amplify replication-associated protein (rep) gene using species primers. LAMP was optimized to amplify CTV DNA under isothermal conditions by incubation at 63°C for 30 min. LAMP products were detected visually using the different dyes. The LAMP amplification products had a ladder-like appearance when electrophoresed on an agarose gel and also positive results using the different dyes were a color change. Results confirmed LAMP with different dyes provides a rapid and safe assay for detection of CTV in sugar beet. Since with other molecular methods, equipping laboratories with a thermocycler or expensive detector systems is unavoidable, this assay was found to be a simple, cost-effective molecular method that has the potential to replace other diagnostics in primary laboratories without the need for expensive equipment or specialized techniques. It can also be considered as a reliable alternative viral detection system in further investigations.

**Keywords:** *Curly top virus*; Colorimetric assay; LAMP reaction; Sugar beet

## Introduction

Curly top is one of the most devastating DNA viruses, causing curly top diseases in a wide range of plants. It can be caused by a number of closely related species in the genus *Curtovirus* of the family *Geminiviridae*. These viruses are transmitted by the beet leafhopper (*Circulifer tenellus* Baker) and can infect dicotyledonous hosts from 44 families and at least 300 species [1,2]. The viruses responsible for curly top diseases have been separated into three distinct species, based largely on sequence variation in select regions of the curtovirus genome, as well as some differences in host range and disease severity [3]. Curly top disease (CTD) of sugar beet and several crops such as tomato, pepper, bean, cucurbits, tobacco and potato is a known disease affecting these crops for over a century [4,5]. Different symptoms caused by curly top viruses include stunting, leaf curling, vein swelling and yellowing, crumpling, and hyperplasia of the phloem. Early infection causes early death in plants [6,7]. CTD is caused by several curtoviruses, including *beet curly top virus* (BCTV), *Beet Severe Curly Top Virus* (BSCTV), *Beet Mild Curly Top Virus* (BMCTV), *Horseradish Curly Top Virus* (HrCTV), *Spinach Curly Top Virus* (SpCTV) and *Pepper Curly Top Virus* (PepCTV). Furthermore, two divergent curly top viruses, *Beet Curly Top Iran Virus* (BCTIV) and *Turnip Curly Top Virus* (TCTV), have been reported from Iran in the past five years [8-12]. Recombination has also been suggested to have played a part in the evolution of species in this group [13]. BCTV may have arisen by recombination between a whitefly-transmitted geminivirus, which may have donated the complementary genes involved in virus replication, and a leafhopper-transmitted geminivirus, which donated the coat protein gene [14].

Curtovirus species possess a monopartite genome with circular single-stranded DNA of approximately 3 kb with three overlapping virion sense genes (*V1*, *V2*, and *V3*), four overlapping complementary

sense genes (*C1*, *C2*, *C3*, and *C4*), and an intergenic region containing the origin of replication [15-18]. During the period from the 1950s to the 1970s a broad range of curly top isolates were described and some were noted to have increased in severity [19]. Some alternative approaches were gradually developed including polymerase chain reaction (PCR), Rolling-circle amplification (RCA), Restriction fragment Length Polymorphism (RFLP) and Chromatin immunoprecipitation (ChIP) assays, all of which were unfortunately time-consuming and require expensive or carcinogenic materials to visualize DNA amplification [20-23].

Among various isothermal amplification systems developed over the recent years, the most frequently applied approach seems to be loop-mediated isothermal amplification (LAMP), implemented first by Notomi et al. [24]. Due to its enormous rate of amplification paired with a very high specificity, sensitivity, rapidity and low artifact susceptibility, the method together with its modifications have been strongly recommended for detection of a great number of strains of bacteria as well as viruses worldwide [25]. Briefly, each reaction is carried out by the use of a DNA polymerase with strand displacement activity and set of four different primers: F3 (forward outer primer), B3 (backward outer primer), FIP (forward inner primer), BIP (backward

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inner primer), designed specifically to recognize six distinct regions on the target sequence in conjunction with two loop primers: LF (loop forward) and LB (loop backward) to accelerate the reaction [24,26]. LAMP assay, alternately, can also amplify nucleic acid under isothermal condition in the range of 60°C-65°C, all turbidity and fluorescent based detections, as well as agarose gel electrophoresis system are applied to visualize suspicious samples [24-26]. LAMP has been shown to be as sensitive and specific as real-time PCR, and several LAMP assays have been developed for the detection of different plant viruses and viroids [27-29]. Here, for the first time, LAMP technique based on visualization system was consequently employed to detection of CTV in sugar beet.

## Materials and Methods

### Plant samples and extraction of total DNA

Samples were collected from the sugar beet production areas in Iran such as East-Azarbaigan where curly top has been observed. Sugar beet (*Beta vulgaris* L.) leaf samples symptomatic for curly top were collected between 2012 and 2013.

Leaf tissue was sampled by punching out a disk with the cap of a sterile 2 ml microcentrifuge tube and stored at -80°C. Individual tubes containing the frozen tissue were dipped in liquid nitrogen and then pulverized using a Retsch MM 301 mixer mill (Retsch, Inc., Newton, PA) with 5 mm stainless steel beads. DNA was extracted using a DNeasy Plant Mini Isolation kit (Qiagen, Inc., Valencia, CA) following standard protocols suggested by the manufacturer, except DNase and RNase free water (W4502, Sigma-Aldrich, St. Louis, MO) was used in place of Buffer AE in the final steps. The DNA was stored at -20°C.

### PCR and LAMP primers

PCR and LAMP assays were developed using specific primers designed based on replication-associated protein (rep) gene (GenBank accession AF379637.1). PCR (Forward and Backward) and LAMP specific primers were designed using the Oligo7 and Primer ExplorerV4 software (specific for LAMP) respectively (Table 1). In addition, Figure 1 shows the position of the PCR and LAMP primers on rep gene. At LAMP assay, six primers recognizing eight distinct regions in the target sequence were used, including Outer primers (F3 and B3), Inner primers (FIB and BIP) and Loop primers (LF and LB). Moreover, each primer was tested for similarities with other sequences available in the GenBank databases (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) using the BlastN algorithm.

### PCR reaction

PCR were performed in volumes of 25 µl with the components in the master mix at the following concentrations: 1×PCR buffer (GeneAmp 10×PCR Gold buffer with 150 mM Tris-HCl, pH 8.0, 500 mM KCl; Applied Biosystems, Foster City, CA), 2.5 mM MgCl<sub>2</sub> (Applied

Biosystems), 1.4 mM dNTPs (Applied Biosystems), 0.2 M each of F and B primer, 0.625 U AmpliTaq Gold (Applied Biosystems), and 2 µl DNA. Amplification consisted of 5 min at 95°C followed by 34 cycles 95°C for 1 min, 54°C for 1 min, and 72°C for 1 min. After the last cycle, the reaction was held at 72°C for 5 min and then 4°C. PCR amplification products were visualized with agarose gel electrophoresis, 1.5% in TAE buffer and visualized by staining with ethidium bromide under UV light. To determine specificity of the primers, LAMP and PCR reactions were carried out to BCTV (*Beet Curly Top Virus*) DNA as positive control.

### Colorimetric LAMP reaction

In order to select the most appropriate condition, LAMP reaction was conducted according to procedures published previously [24-27]. The amplification reaction was performed at 63°C for 30 min in water bath by mixing 1.6 M each of FIP and BIP primer, 0.2 M each of F3 and B3 primer, 0.8 M each of LF and LB primer, 1.4 mM dNTPs, 0.8 M betaine (Sigma-Aldrich, Saint Luis, MO, USA), 8U of BstDNA polymerase (New England Biolabs, Hertfordshire, England, UK) using the supplied buffer (Thermopol buffer containing 2 mM of MgSO<sub>4</sub>) and 2 µl DNA. As an additional proof, products from the reaction were also analyzed by agarose gel electrophoresis. Various colorimetric assays including magnesium sulfate (MgSO<sub>4</sub>), Calcium chloride (CaCl<sub>2</sub>), SYBR® Premix Ex Taq™ II, Hydroxyl naphthol blue (HNB), GeneFinder™, SYBR Green I and Ethidium bromide have been proposed and used in several investigations [30-41]. Here, thus, the validations of positive LAMP reactions were justified by means of these seven staining approaches.

### Magnesium sulfate

Like other metal indicators, magnesium sulfate must be added before reaction. At the end of the amplification process, positive reactions were accompanied by a visible darker phase in the tubes in consequence of the formation of magnesium pyrophosphate which can be easily visualized with the naked eye [24,35]. It is noticeable that the turbidity of the positive samples is stable but just for a short time, which should be consequently judged soon after taking out of the samples either from the water bath or the thermal cyclor.

### Calcium chloride

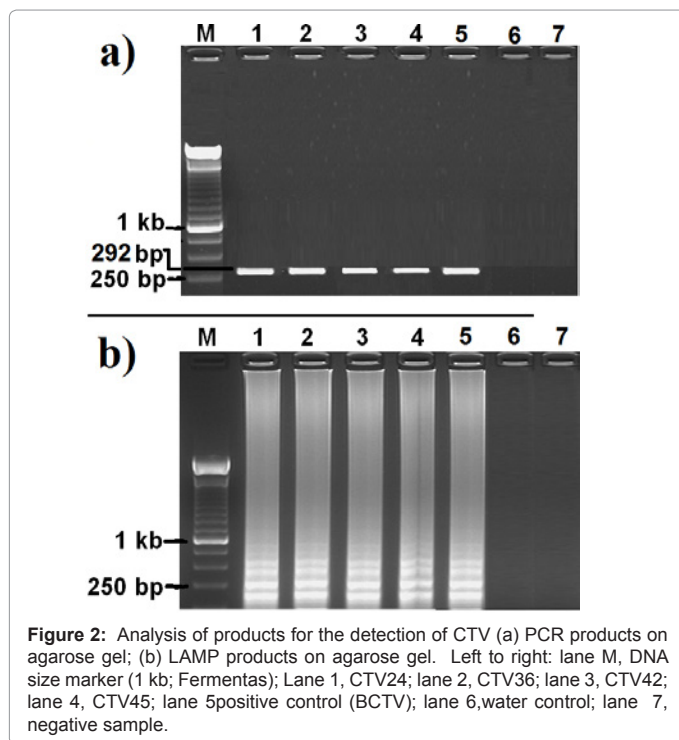
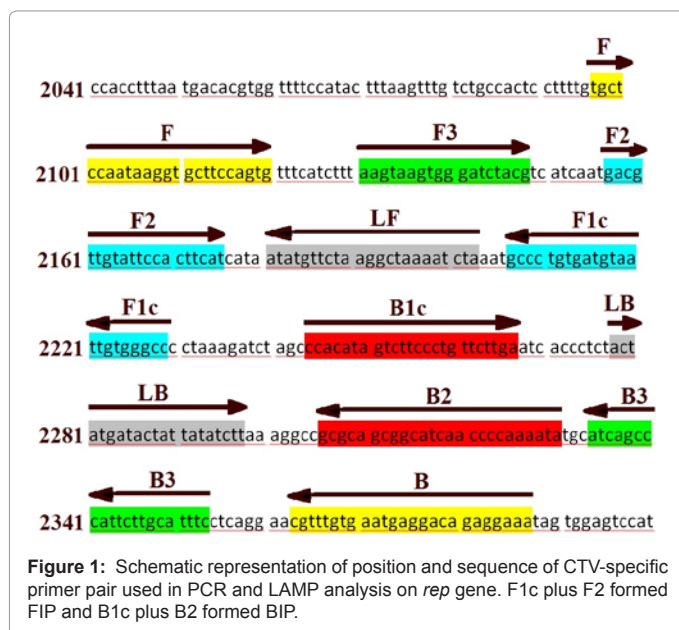
Use of MgSO<sub>4</sub> in LAMP reaction in order to create turbidity has some drawbacks such as, low stability and concentration. In this research, we used CaCl<sub>2</sub> to address these disadvantages as described previously by Almasi et al. [38]. Thus, 2 mM CaCl<sub>2</sub> was added to LAMP mixture to improve both stability and concentration of turbidity in the reaction.

### SYBR® Premix ExTaq™ II

To conquer time-dependent instability of magnesium or calcium

Reaction	Primer	Type	Length of Primer	Sequence(5'-3')	Length of Product
PCR	F	Forward	24 nt	TGCTCCAATAAGGTGCTTCCAGTG	292 bp
PCR	B	Backward	25 nt	TTTCTCTGTCCTCATTACAAACG	
LAMP	F3	Forward outer	18 nt	AAGTAAGTGGGATCTACC	Fragment with different sizes
LAMP	B3	Backward outer	21 nt	GAAATGCAAGAATGGGCTGAT	
LAMP	FIB	Forward inner	47 nt	GGCCACAATTACATCACAGGGCTTTGACGTTGTATTCCACTTCAT	
LAMP	BIP	Backward inner	53 nt	CCACATAGTCTTCCCTGTTCTTGATTTTATTTTGGGGTTGATGCCGCTGCCG	
LAMP	LF	Loop forward	23 nt	TAGATTTTAGCCTTAGAACATAT	
LAMP	LB	Loop backward	21 nt	ACTATGATACTATTATCTT	

Table 1: Oligonucleotide primers used for PCR and LAMP to detection of CTV.



pyrophosphate-based detection method, an alternative visual system using SYBR® Premix Ex Taq™ II was employed [34,42]. Hence, 2 µl SYBR® Premix Ex Taq™ II (Perfect Real Time, Takara Bio Co., Ltd., RR081A) was added into each completely finished LAMP reaction containing 25 µl LAMP products.

### Hydroxynaphthol blue (HNB)

In this protocol, 1 µl of the hydroxynaphthol blue dye (3 mM, Lemongreen, Shanghai, China) is mixed prior to amplification; all positive reactions can be easily identified using the naked eye, interestingly with no probable cross contaminations which usually arise from opened tubes after amplification [31-33,38,41].

### GeneFinder™

An obvious Green fluorescence pattern was observed to confirm positive LAMP products through visual observation with the naked eye when 1 µl of GeneFinder™, diluted to 1:10 with 6×loading buffer (Biov. Bio. Xiamen, China), was added to each reaction as described previously [30,36,39,41].

### SYBR Green I

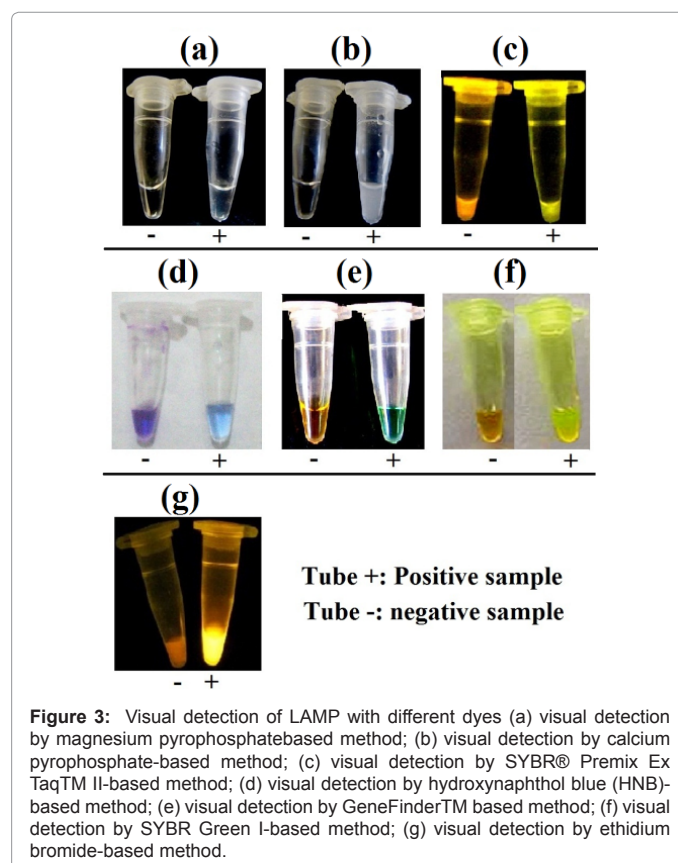
About 1 µl of SYBR Green I (Invitrogen, Sydney, Australia) diluted to 1:10 with 6×loading buffer was separately added to each reaction as described previously [33,38]. After addition of SYBR Green I, the products can be visualized directly by the naked eye.

### Ethidium bromide

Under a UV transilluminator, positive products will be consequently marked if a detectable yellow colour pattern is observed [24]. Ethidium bromide is a polycyclic fluorescent dye that binds to double-stranded DNA molecules by intercalating a planar group between the stacked base pairs of the nucleic acid [34-37]. About 0.5 µg ethidium bromide/ml (Sigma) was added to each tube as designed previously by Tsai et al. [35].

### Results

On the whole, 4 out of 83 leaf samples suspicious of having infection with CTV (CTV24, CTV36, CTV42 and CTV45) (4.8%) showed positive responses. When PCR was used for the detection of CTV, band of approximately 292 bp was amplified and visualized under UV light (Figure 2a). The LAMP technique can from amplified products of various sizes, consisting of alternately inverted repeats of the target





sequence on the same strand. Therefore, the LAMP primers generated many fragments with different sizes and formed a ladder pattern on an agarose gel (Figure 2b). LAMP amplicons were able to be detected with the naked eye by adding different visual dyes followed by color changing in the solutions. In this regard, all used visual components could successfully make a clear distinction between positive infected samples and negative ones. In the LAMP reaction when DNA polymerizes, producing pyrophosphate ions and interaction between pyrophosphate and magnesium or calcium lead to produce magnesium pyrophosphate or calcium pyrophosphate (white precipitate) which can be observed and detectable as turbidity in positive samples. In contrast, negative control remains transparent without any turbidity (Figure 3a and 3b). The CaCl<sub>2</sub> was improved both stability and concentration of turbidity in contrast of MgSO<sub>4</sub>. Under UV illumination (302 nm), a green color pattern is an identical characteristic of all positive reactions was monitored in this study after was used SYBR® Premix Ex Taq™ II (Figure 3c). When HNB was used for detect, a sky blue color pattern implies the existence of the reference virus, whereas a violet color change is observed when the control(s) are taken into consideration (Figure 3d). An obvious green fluorescence pattern was observed to confirm positive LAMP products through visual observation with the naked eye when GeneFinder™ or SYBR Green I were added to each reaction. Remarkably, concerning negative reaction, the original orange color could be observed (Figures 3e and 3f). Under a UV transilluminator, also a detectable yellow color pattern is observed when ethidium bromide was applied to detection of positive reaction (Figure 3g). As a result, the intensity of the fluorescent emissions moves up in positive tubes, while the reverse is true regarding negative tubes with no amplified fragments. More interestingly, in some cases, just a little fluorescent emission could be observed in negative tubes, presumably arising from the presence of primers and/or DNA templates, leading to an increase to false positive outputs.

## Discussion

*Curly top virus* (CTV) and related viruses (collectively known as curtoviruses) have caused significant problems to irrigated agriculture in Iran. CTV is known to infect a broad range of crop and weed hosts in many plant families. Crop hosts for which natural CTV infection has been reported include sugar beet, tomato, pepper, bean, spinach, and cucurbits [7,22]. Molecular methods including polymerase

chain reaction (PCR), Rolling-circle amplification (RCA), Restriction fragment length polymorphism (RFLP) and Chromatin immunoprecipitation (ChIP) assays, are specific and accurate approaches, but most are technically demanding and time-consuming techniques, needing post-process stage or costly detection equipment to visualize amplified DNA [20-22]. Thus, an inexpensive and rapid diagnostic method is required, especially in developing countries with insufficient facilities. The LAMP assay was first applied for detection of yam mosaic virus [27]. In this study, for the first time, a colorimetric LAMP method with the use of different dyes was developed and optimized. In reality, LAMP overall requires just 30 min to accomplish while PCR method 3 h should be served (Table 2). This, in turn, would simplify the detection procedure and result in saving of significant time needing for separating of the amplified products on the gel and the analyzing of the data. Safety regarding a number of detection methods, application of gel electrophoresis systems has emerged as a routine approach with enough potential to observe related amplicons. Just the same, such visual methods not only involve some expensive instruments but also during a period of time, exposure to the UV ray (because it is harmful to the eyes, even watching for a short period would irritate eyes and cause symptoms similar to conjunctivitis) as well as ethidium bromide could accompany a number of serious negative effects on researchers who use these methods [34-43]. More surprisingly, in LAMP, amplified products can be easily visualized by means of different in tube color indicators with no essential requirement of additional staining systems; thus, toxic staining materials would be significantly avoided. Simplicity, cost and user-friendly equipped labs with some molecular instruments as well as trained personnel are prerequisites to perform other assays, all of which are undoubtedly costly. On the contrary, LAMP can be easily accomplished just in a water bath or temperature block with no need of thermocycler and gel electrophoresis [27,44].

According to our results, despite the precise detection of positive LAMP products using all dyes, some were significantly superior when the time of stability, cost and the safety were taken into consideration (Table 3). For instance, regardless of its reasonable color change stability (≥ 2 weeks), when employing ethidium bromide, a UV transilluminator must be available, both of which are toxic, resulting in deleterious consequences on human health and the environment [34,43]. As the

Assay	Total time	Detection method	Safety	Need to UV ray	Need to detect instruments	Cost	User Friendly	Accuracy
PCR	3 hours	Gel Electrophoresis	No	Yes	Yes	High	Low	High
LAMP	30 minutes	Visual+ Gel Electrophoresis	Yes	No and Yes	No and Yes	Low	High	High
Colorimetric LAMP	30 minutes	Visual	Yes	No	No	Low	Very high	High

Table 2: Comparison of PCR, LAMP and colorimetric LAMP assays for detection of CTV.

Visual system	Detection method	Safety	Positive reaction	Negative sample	Need to UV ray	Stability	Cross contamination	Use time
magnesium Sulfate	Visual	Yes	Turbidity	No turbidity	No	5-10 seconds	No	Before the amplification
Calcium chloride	Visual	Yes	Turbidity	No turbidity	No	40-60 seconds	No	Before the amplification
SYBR® Premix Ex Taq™ II	Visual	Yes	Green	Red	Yes	1-3 days	Yes	After the amplification
Hydroxynaphthol blue	Visua	Yes	Sky blue	Violet	No	2-3 weeks	No	Before the amplification
GeneFinder™	Visual	Yes	Green	Red	No	2-3 weeks	No	Before the amplification
SYBR Green I	Visual	Yes	Green	Red	Yes	2-3 weeks	No	Before the amplification
Ethidium bromide	Visual	No	Yellow	No color	Yes	2 weeks	Yes	After the amplification

Table 3: Comparison of colorimetric assays together.

last drawback, the corresponding lab(s) should be equipped with such instruments which are commonly costly. Alternatively, to remedy such problems, the first metal dye called  $MgSO_4$  and  $CaCl_2$  was utilized, but just an ephemeral color change (i.e. turbidity; no more than a few seconds or minutes) was observed in tubes [24,35]. Even though short stability of the colour change probably cannot be a significant problem as long as just a few numbers of suspicious samples are used, during assessment of a great quantity of infected samples, most of the time, inaccurate results will be accordingly achieved [36-41]. The method, nevertheless, since in one hand is exploited prior to the reaction, leading to a significant reduction on probable contaminations, and on the other there is no need of toxic devices, could be thereby attributed as a reliable visual observation approach. Anyway, to provide a situation to increase time stability, the second fluorescent dye coined SYBR<sup>®</sup> Premix Ex Taq<sup>™</sup> II was employed, leading to a clear green colour pattern. Notably, regardless of observing a significant growth on time stability (about 1–3 days), the method not only requires UV transilluminator but also its stability is more negatively sensitive when amplified reactions are exposed to the daylight. Indeed, since the dye must be added after the amplification so requires opening of the tubes, the occurrence of cross contamination risk will be accordingly enhanced [42]. To avoid such contaminations, using separate rooms can be a solution for LAMP setup and analysis [31]. Interestingly, HNB, SYBR Green I and GeneFinder<sup>™</sup> dye-based assays were accompanied by several remarkable advantages compared with other colorimetric-based methods in that of which are mixed prior to amplification, a need to open the assayed samples to add the dye is thereby omitted, and the risk of cross-contamination will be excluded drastically [31-33,42]. Meanwhile, the visual inspection of LAMP products by means of the dyes was seen as advantageous as there was no need for electrophoresis and subsequent staining with carcinogenic ethidium bromide [30]. Lastly, the color brightness and stability of the both HNB, SYBR Green I and GeneFinder<sup>™</sup> in the solutions with positive/negative reactions were remained constant after 2 weeks of exposure to ambient light, whereas detection by SYBR<sup>®</sup> Premix Ex Taq<sup>™</sup> II was stable only 1–3 days. For example, at the study of Goto et al. [31], HNB was reported as the best visual system, while the brightness of SYBR green fluorescence and calcein fluorescence was significantly weaker than that of HNB. It is noticeable that since the color presented by HNB was light blue for positive results and dark blue for negative results, which cannot be discriminated precisely [42], so such based detection methods involve a little more attention to provide accurate decision. From the present study it is concluded that colorimetric LAMP could be a reliable and rapid technique that can help at the maximum to this direction.

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