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Original Paper

A reverse transcriptase-loop mediated isothermal amplification assay (RT-LAMP) for rapid detection of bovine viral diarrhea virus 1 and 2

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ABSTRACT

Bovine viral diarrhea virus (BVDV) is a pathogen that infects cattle, and is globally important. It causes substantial financial losses to the livestock industry. In the current study, a one-step reverse transcriptase-loop-mediated isothermal amplification (RT-LAMP) assay was set up for rapid and efficient detection of BVDV. For this purpose, four primers were designed to recognize six distinct regions on the target RNA based on a highly conserved sequence in the 5′ UTR of the BVDV genome. Eighty blood specimens were collected from bovines suspected to suffer from BVDV infection, and were tested in parallel by RT-LAMP and RT-PCR. Twenty four of these samples were positive by RT-LAMP, while twenty were positive by RT-PCR. The RT-LAMP detection limit was estimated to be approximately 70PFU /mL of virus. Comparison of RT-PCR with RT-LAMP in this study revealed the recent developed RT-LAMP a highly sensitive and specific for BVD virus detection in the clinical samples.

Keywords: Bovine viral diarrhea; Reverse Transcriptase-Loop mediated isothermal amplification assay; Reverse Transcriptase-Polymerase Chain Reaction

Conception et évaluation de la méthode de transcriptase inverse combinée à une réaction d'amplification isothermique par loupe pour détecter le virus de la diarrhée virale bovine

Résumé: Le virus de la diarrhée virale bovine (BVDV) est un agent pathogène chez la vache et a un impact économique important dans le monde. Cette maladie engendre des coûts énormes dans l'industrie du bétail. La méthode de transcriptase inverse en une étape suivie d'une amplification isothermique par loupe (RT-LAMP) a été conçue pour une identification rapide et efficace du virus BVD. Quatre amorces ont été conçues selon la zone non protégée et non traduite de l'extrémité (5'UTR)5' du génome du virus BVD afin de détecter six emplacements différents de l'ARN ciblée. Quatre-vingt échantillons de sang provenant d'animaux soupçonnés d'être porteur du BVD ont été simultanément recueillis et testés en utilisant les deux méthodes de RT-PCR et RT-LAMP. Parmi ces derniers, 24 et 20 échantillons positifs ont été respectivement détectés par les méthodes RT-LAMP et RT-PRC. La limite de détection a été estimée à environ 70PFU/mL pour la méthode RT-LAMP. La comparaison des deux méthodes RT-PRC et RT-LAMP a montré que le RT-LAMP représente une méthode sensible et spécifique pour détecter le virus BVD dans des échantillons cliniques.

Mots clés: la diarrhée virale bovine, transcriptase inverse et l'amplification isothermique par loupe, transcriptase inverse-réaction en chaîne de polymerase

INTRODUCTION

Bovine viral diarrhea virus [BVDV) is a pestivirus of the family *Flaviviridae*. It causes globally a serious disease in the cattle that have significant financial impact on the livestock industry (Houe, 1999; OIE, 2008; Lanyon et al., 2014). Based on the genetic and antigenic variations, BVDV was classified into two distinct genotypes (types 1 and 2) (Vilcek et al., 2001; Lanyon et al., 2014). On the other hand, according to the recent classification of International Committee of Taxonomy of Viruses (ICTV), it is divided into two species BVDV-1 and BVDV-2. Both species of BVDV are seen in cytopathogenic (cp) and noncytopathogenic (ncp) forms (biotypes), based on the production or non-production of visible modification in cell cultures, respectively (Gamlen et al., 2010). Susceptibility to viral infection is common in cattle of all ages. The clinical signs of the disease vary from subclinical infection to the fatal disease known as mucosal disease (MD) (Baker, 1995). Acute form of the disease in non-immune cattle might be asymptomatic or be presented with pneumonia, diarrhea, transient leucopenia, thrombocytopenia and fever (Carman et al., 1998). However, most infections in the young calves are not severe and remain asymptomatic (OIE, 2008). Besides, the vertical transmission of the virus has an important role in epidemiology and pathogenesis of the disease (OIE, 2008). If the virus infects the bovine fetus, it may result in abortion, stillbirth, teratogenic effects, or persistent infection (PI) in the neonatal calves (Baker, 1995; Moennig and Liess, 1995; Brownlie et al., 1998). The PI animals may be clinically unrecognized and later develop MD, which only appears in persistently infected animals (Brownlie, 1985). The PI calves continuously shed the virus, so they are the hidden source of virus transmission to susceptible animals and spread of the disease in the herd (OIE, 2008; Lanyon et al., 2014). The serological surveys in some provinces of Iran have showed different prevalence rate of BVDV infection in the cattle from 20% to 100% (Talebkhan Garoussi et al., 2009; Shirvani et al., 2012). Detection and removal of PI calves from herds is an essential component of the programs for successful control and eradication of BVD (OIE, 2008). Several diagnostic tests are applied for detection of BVDV in the PI calves including cell culture (Saliki et al., 1997); serological assays such as ELISA (Katz and Hanson, 1987; Paton et al., 1991; Horner et al., 1995) and serum neutralization test (SN) (Edwards, 1990); and some molecular methods such as RT-PCR (Schmitt et al., 1994; Vilcek et al., 1994; Hamel et al., 1995; Fulton et al., 1999), real- time PCR (Zhang et al., 2011) and hybridization assays. Each of these methods has the pros and cons. A suitable diagnostic test for virus detection should be economic, accurate, robust, and time saving to be usable in the fields or small laboratories with limited equipments. A new molecular assay was described by Notomi *et al*. called "loop mediated isothermal amplification test" (LAMP) that can amplify an specific nucleic acid up to 10^9 fold with high specificity and sensitivity in less than an hour (Notomi et al., 2000). This reaction is done by the *Bst* DNA polymerase with strong strand displacement activity, under isothermal conditions. The LAMP employs 4-6 specific primers that can recognize 6-8 distinct regions on the template DNA. For detection of RNA targets, reverse transcription is combined with LAMP. The *Bst* polymerase combined with reverse transcriptase (RTase) amplifies RNA templates (Notomi et al., 2000). In the current study, a uni-step RT-LAMP assay was developed for fast, sensitive and specific detection of BVDV.

MATERIALS AND METHODS

Reference isolates, samples and other pathogens. As a positive control, Iran-Razi-Khedmati Bovine Kidney (IRKHBK) cells (Razi Vaccine and Serum Research Institute, Karaj, Iran) were infected with cpBVDV-NADL and two clinical isolates (BVDV-1 and 2). The viruses were inoculated to IRKHBK cell line. Then the cells grown in RPMI 1640 media (Sigma, USA) mixed with 2% fetal bovine serum free of BVDV antibodies (FBS; Sigma) at 37°C under 5% CO_2. In the next step, the blood samples were collected

in EDTA tubes from 80 cattle presented clinical signs of BVD (fever, respiratory manifestations, diarrhea, conjunctivitis, lethargy and/or abortion). These cases were randomly selected from the different bovine farms in Khuzestan province, south west of Iran. Two rectal swabs, one nasal mucus swab, two human blood samples and two blood samples taken from healthy bovines used as negative controls. Five other bovine pathogens were also used to analyze the specificity of the assay. Buffy coat of the blood samples was separated by centrifuging at 1000g for 10min.

RNA and DNA extraction. RNA was extracted from the supernatant of IRKHBK cells infected with the BVDV-NADL, foot and mouth disease virus (FMDV) infected cells, none infected IRKHBK cells of 80 suspected blood samples and two clinical isolates as reference samples. RNA was extracted using a commercial kit (CinnaGen, Tehran, Iran) according to the manufacturer's instructions. DNA isolation of *Mycobacterium bovis*, *Escherichia coli* O157:H7, *Pasteurella multocida* and Infectious bovine Rinotheracheitis (IBR) was carried out using DNA extraction kit (CinnaGen, Tehran, Iran) according to the manufacturer's instruction. All the extracted RNA and DNA were stored at minus20°C until the next phase.

Primers design. The 5′-untranslated region (5′UTR) and the NS3 (p80 gene) are conserved sequences of the BVDV genome and recommended for primer design (OIE, 2008). In this study, RT-LAMP primers were designed for 5′UTR of the genome. Twenty five 5′UTR genomic sequences of BVDV from different regions (fifteen BVDV-1 and ten BVDV-2 isolates) were retrieved from the NCBI GenBank. These sequences were aligned using the ClustalX® (version 1.83) and the conserved sequences for BVDV-1 and 2 were selected. A set of four primers comprising of two outer and two inner primers, which recognizing six distinct regions on the target sequence, was designed. The outer primers help in strand displacement and are called the forward and backward outer primers (F3 and B3, Respectively). The two inner primers are called the forward and backward inner primers (FIP and BIP, respectively). The FIP primer contains F1 complementary (F1c) and F2 sequences. The BIP contains B1 complementary (B1c) and B2 sequences. The RT-LAMP primers were designed by Primer Explorer version 4 (http :// primerexplorer .jp /e /) according to the5′UTR of BVDV-NADL sequence (accession No. M31182). To ensure the specificity of primers, they were analyzed by BLASTN program (http://blast. ncbi. nlm. nih. gov / Blast. cgi). All the primers were synthesized by CinnaGen Company (CinnaGen, Tehran, Iran). The primers are shown in Table 1 and Figure 1.

Table 1. RT-LAMP primers and target regions used for amplification of BVDV

Primer name		Sequence (5′→3′)[a]	Length (Nucleotide)	Genome Position
F3		AGCGAAGGCCGAAAAGAG	18	83-100
B3		GGTTAAGATGTGCYKTGG	18	270-253
FIP (F1c+F2)	F1c	TTMRGCCATCCAACGAACTCA-	40	173-153
	F2	CTAGCCATGCCCTTAGTAG		102-120
BIP (B1c+B2)	B1c	CCCTGAGTACAGGGTAGTCG-	40	175-194
	B2	CCCTCGTCCACRGTGGCATCT		247-228

[a] BVDV strain NADL (GenBank accession no M31182),(Y: C or T); (K: T or G); (M: A or C); (R: A or G)

RT-LAMP conditions. The RT-LAMP assay was performed at a 25µl volume with various concentrations of $MgSO_4$ (2, 4, 6, 8 and 10mM), primers (each of F3, B3/FIP, BIP primers: 0.1/0.8, 0.2/0.8, 0.2/1.6 and 0.4/1.6µM) and betaine (0, 0.5, 1 and 1.5 M) (Sigma, USA). This reaction contained 2.5 µL of 10x Thermo buffer, 8U of *Bst* DNA polymerase (New England BioLabs), 50U of avian myeloblastosis virus reverse transcriptase (CinnaGen, Tehran, Iran), 1.4 mM of each deoxy ribonucleotide triphosphate and 2µL of template RNA. The Nuclease-free water was added to adjust the final reaction volume to 25µL. To determine the optimal incubation temperature, the

reaction mixture was incubated at nine different temperatures from 59-67 °C for 40, 50, 60 and 70 min in a thermal cycler (Bio-Rad iCycler™, USA). Two methods were used to detect the positive and negative control samples. In the first method, RT-LAMP products were electrophoresed on 2% gel agarose, stained with safe stain (CinnaGene, Tehran, Iran) and visualized under UV light. The samples with a pattern of multiple bands of different molecular weights or smears were considered positive (Notomi et al., 2000). In the second method, 1µL of fluorescent dye GelRed (Biotium, USA), was added to the samples and then

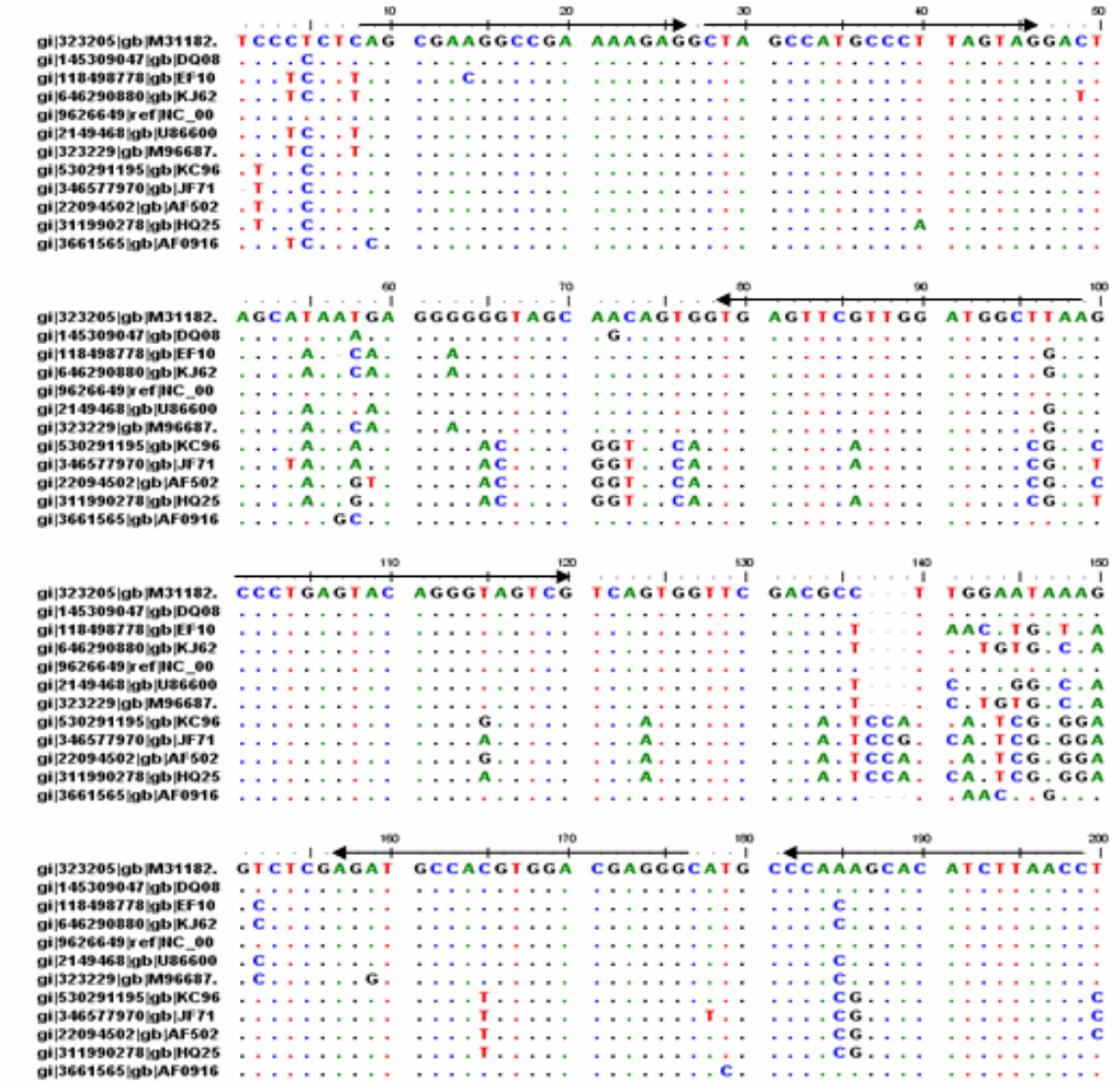

Figure 1. Multiple sequenses alignment of BVDV 5′ UTR and position of primers; Sequenses of gi|323205|, gil145309047l, gil118498778l,gil646290880l, gil9626649l, gil2149468 gil323229l, andgil3661565lare related to BVDV-1 and Sequenses of gil530291195l,gil346577970l, gil22094502l and gil311990278l are related to BVDV-2.

visualized under UV light (320nm). The samples that emitted yellow fluorescence were considered positive, while the samples with red color were considered negative. The clinical samples were analyzed by two methods of agarose gel electrophoresis and GelRed™ staining.

RT-PCR. The extracted RNA was reverse transcribed to cDNA as follows: 10µL of extracted RNA, 1µLof Reverse primer (10pmol/µL) and 1µL of distilled H_2O were mixed and incubated at 65 °C for ten minutes and then cooled on ice pack. Afterwards, 2µL dNTPs (10mM), 2µL 10x RT buffer, 0.5µL RNase inhibitor, 100UM-MuLV reverse transcriptase and 3µl distilled H_2O were added and incubated at 42 °C for an hour and then 70 °C for ten minutes. The cDNA was stored at minus 20 °C until further use. The PCR was done according Vilcek et al. (1994) method.

Analytical specificity and Sensitivity. In addition to BVDV, two other viruses that might cause diseases in cattle (FMDV and IBR), three pathogenic bacteria in cattle (*M. bovis*, *E. coli* O157:H7, and *P. multocida*), IRHKBK cell line and two human blood sample were used to investigate the specificity of the assay. RNA or DNA was extracted from these samples and subjected to RT-LAMP. To determine the sensitivity of the RT-LAMP method, ten fold serially diluted RNA from 10^{-4} to one was prepared. The RNA was extracted of BVDV grown in IRHKBK cell line with the titer of the 0.7 $\times 10^5$PFU/mL. The Triplicate templates at identical RNA concentrations were compared with RT-PCR and RT-LAMP.

Reproducibility. Three positive templates and BVDV-NADL were chosen to test the reproducibility of the RT-LAMP assay. Each sample was tested for three times using the designed RT-LAMP assay and then analyzed by agarose gel electrophoresis.

Relative diagnostic sensitivity. To determine the relative diagnostic sensitivity of the test, the eighty clinical samples with suspected BVDV infection were tested in parallel by RT-PCR and RT-LAMP. The blood samples were collected from calves and bovines between one and 48 months. The assays were performed as described above and then the results of two assays were compared with each other.

RESULTS

Optimum RT-LAMP reaction. For the RT-LAMP reaction, the optimal concentrations of $MgSO_4$, inner and outer primers were 2mM, 0.2µM and 0.8µM, respectively. Betaine did not have any considerable effect on the quality of the reaction. The optimal incubation temperature for *Bst* DNA polymerase was found to be 63°C for 40 min. The amplified products on agarose gel electrophoresis were seen as a series of bands with different molecular weights; while the negative samples did not demonstrated any bands (Figure 2a). For the GelRed staining method, the positive samples emitted yellow fluorescence under UV light, while the negative samples presented red color (Figure 3).

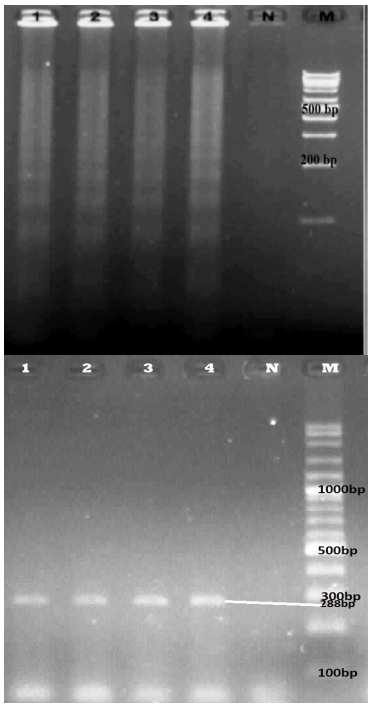

Figure 2. Agarose gel electrophoresis of RT-LAMP (a) and RT-PCR (b) amplified products. (a) Results of RT-LAMP analysis; positive RT-LAMP products (lanes 1-4), negative control (N), ladder maker 100 bp(M) (CinnaGen, Iran) (b) Results of the RT-PCR analysis, positive RT-PCR products (288 bp) (lanes 1-4), negative control (N), ladder marker 100 bp (M)

Analytical sensitivity and specificity of RT-LAMP. By the BLASTN program it was shown that all primers were specific for BVDV-1 and 2 but not for the other species of pestiviruses) Border disease and Swine

classic fever .(This RT-LAMP assay was performed on two viruses and three pathogenic bacteria of bovine cell culture and also on five samples from healthy bovines . However ,no amplified product was observed in these specimens ,in this assay ,BVDV-NADL and two clinical isolate samples were positive. These results indicated the specificity of this assay for BVDV. To evaluate the sensitivity of the RT-LAMP assay, serial ten fold dilutions of the extracted RNA were tested in parallel by RT-LAMP and RT-PCR, and the results were compared with each other. The RT-LAMP assay could detect 0.7×10^{2}PFU/mL of virus (Figure 4), while RT-PCR detected 0.7×10^{4}PFU/mL. Therefore RT-LAMP showed 100-fold higher sensitivity compared with RT-PCR.

Reproducibility and relative diagnostic sensitivity of RT-LAMP. The tested samples for evaluation of reproducibility, all yielded positive results in all iterations. This confirmed the reproducibility of the designed RT-LAMP assay. To investigate the diagnostic accuracy of the RT-LAMP, the eighty samples were subjected to RT-LAMP and RT-PCR assays in parallel. Out of 80 specimens, 20 were positive for BVDV by RT-PCR and RT-LAMP assay (Figure 2). However, four samples were positive by RT-LAMP while they were not detected by RT-PCR. Blood sample was collected again from one of these suspected bovines and tested by RT-PCR. This case was again positive by RT-PCR a week afterward. Based on these data, the relative sensitivity and specificity of RT-LAMP was 100% and 93.75%, respectively in comparison with RT-PCR. The two selected detection methods (agarose gel electrophoresis and GelRed staining) reporterd the same findings for all positive and negative samples in the RT-LAMP method.

DISCUSSION

The gold standard method for isolation and detection of BVDV is cell culture (OIE, 2008); however, this method is time-consuming and also has poor sensitivity. On the other hand, Real-time PCR is a sensitive and accurate method for detection of the virus, but the requirement for expensive instruments and highly trained personnel prevents this method to become the routine diagnostic tool in private clinics. Conventional RT-PCR is more common for detection of the BVDV in many laboratories; therefore in this study we compared the RT-LAMP and RT-PCR for detection of BVDV. LAMP has a higher specificity for amplification of the target sequence, since uses four specific primers. Four primers recognize six independent sequences in the initial stages of the LAMP reaction; then in the later steps, two primers recognize four distinct sequences (Notomi et al., 2000), this is while in PCR only two sequences are recognized. Therefore, specificity of LAMP is expected to be higher than conventional PCR (Notomi et al., 2000). Primers for RT-LAMP and Vilcek primer's for RT-PCR could detect 5´UTR of BVDV (Vilcek et al., 1994). A highly conserved region in the genome of all types of BVDV is 5´UTR (OIE, 2008; Lanyon et al., 2014). For this reason, the primers designed for this region can theoretically target all types of BVDV. In addition to DNA, RNA could also be amplified in the LAMP reaction, if reverse transcriptase is used in combination with DNA polymerase (Notomi et al., 2000). In this study, RNA of BVDV was amplified with high efficiency in one step. Similarly, Fan et al. (2012) used RT-LAMP for detection of BVDV by other primers (Fan et al., 2012). In their study, this method also had high sensitivity and specificity. Their primers were designed based on 5´UTR and polyprotein gene, while in our study primers only identified 5´UTR BVDV. Zhang et al. (2014) designed RT-LAMP for detection of BVDV and suggested that this method is sensitive and specific for detection of BVDV in biological samples and might be used for quality control of biomaterials. The sensitivity of the RT-LAMP assay for BVDV detection was 100-fold higher than RT-PCR; therefore, this reaction could be used as an alternative method for detection of the virus. The RT-LAMP could detect the virus in primary stages of infection, in

which viral load is very low. This reaction has fewer steps than RT-PCR and is therefore, a time-saving method. Moreover, results of RT-LAMP assay are obtained within an hour, whereas it takes 2-4 h in the conventional RT-PCR. Four positive blood samples by RT-LAMP were not detected when were tested by RT-PCR method. One of these samples was positive by RT-PCR after a week. Unfortunately, three other cases were not available for re-test. Perhaps, these positive RT-LAMP samples had low number of viruses, which were undetectable by RT-PCR assay. The RT-LAMP only requires four primers, reverse transcriptase, a DNA polymerase and a heat block or water bath for performing the reaction (Notomi et al., 2000; Rovira et al., 2009; Jiang et al., 2011). Therefore, this method is easy to perform. These benefits demonstrate that LAMP can be used in small or regional laboratories where access to equipment is limited and/or on-site testing in the filed (Yang et al., 2012). For example, to control the prevalence of BVDV, the animals newly entering in the herd should be screened for the virus. This method may lead to faster detection of the virus in such cases.

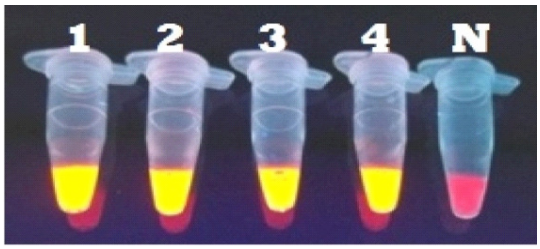

Figure 3. Visual observation of RT-LAMP amplified products under UV light with a fluorescent detection reagent. Positive samples (micro tubes 1-4 were fluorescent yellow under UV light, while negative samples (micro tube N was red color.

The amplified products in LAMP can be detected by some alternative methods in addition to agarose gel electrophoresis. Adding SYBR Green I to the LAMP products could help in detecting the positive products by unaided eyes (Chen et al., 2011; Yang et al., 2012). Hence, the formation of magnesium pyrophosphate as a white precipitate in the reaction tube shows the presence of the reaction (Chen et al., 2011; Shi et al., 2011). In this study, a fluorescent dye GelRed was used for detection of the amplified products. The color change in the positive samples is observed under UV light. In these samples the red color of the dye changes to yellow, while the color of reaction stays red in the negative samples. In summary, the aforementioned RT-LAMP method for detection of BVDV is a simple, timesaving and economic method. The designed RT-LAMP assay has high sensitivity and specificity, and can be used for detection of BVDV in the field.

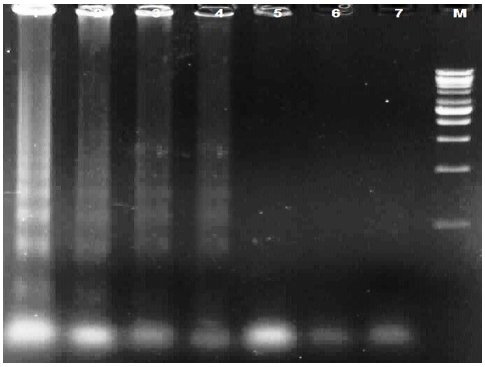

Figure 4. The electrophoresis diagram of RT-LAMP with virus serial dilution. Lanes 1-7 are target RNA at 0.7×10^5, 0.7×10^4, 0.7×10^3, 70, 7, 0.7, and 0 PFU/ml respectively and lane 8 ladder maker 100 bp (M) (CinnaGen, Iran).

Ethics

I hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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