Original Article

Evaluation and Optimization of Loop-Mediated Isothermal Amplification (LAMP) Technique for Capripoxvirus Diagnosis and Its Comparison with PCR Method

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ABSTRACT

Sheep pox (SP), goat pox (GP), and lumpy skin disease (LSD) are caused by subspecies of the capripox virus (CaPVs). They are significant pathogens of sheep, goats, and cattle. The causative agent is the capripox virus (CaPV), which was first isolated in South Africa. The viruses responsible for sheep pox (SP), goat pox (GP), and lumpy skin disease (LSD) are morphologically indistinguishable and have been adapted to different host species (4). Serologically, distinguishing between these viruses is challenging, and cross-immunity exists among them (2). The present study reports the evaluation and optimization of a novel loop-mediated isothermal amplification (LAMP) technique for the rapid detection of capripox viruses (CaPVs) and compares it with the polymerase chain reaction (PCR) method. LAMP primers were selected from the P32-protected gene of CaPV. The Safe-Red fluorescent dye was used to monitor the color change from red to bright yellow at a wavelength of 320 nm in positive cases, and the final results were documented through electrophoresis. The proposed LAMP test for the capripox virus demonstrated high specificity and did not cross react with other viruses in the Poxviridae family that present similar clinical symptoms. The optimized LAMP test was then compared with the PCR. The diagnostic sensitivity of LAMP and PCR was found to be identical (100%). The specificity of the LAMP test was evaluated using 30 samples of cow skin that were suspected of lumpy skin disease, along with16 additional samples, including nine positive references, fivenegative references, and two negative controls. A negative reference sample was used to assess the diagnostic sensitivity of LSDV. The proposed LAMP test is simple to implement, cost-effective, and highly sensitive, making it particularly well-suited for the detection of the capripox virus in less developed regions, laboratories, and facilities with limited resources.

Keywords: LSDV, LAMP, PCR, Capripoxvirus.

1. Introduction

Lumpy skin disease (LSD) is a highly infectious disease of cattle that results in significant economic losses. It is caused by a virus belonging to the Capripoxvirus genus of the family Poxviridae (1). The disease is typified by the presence of fever, large skin nodules, and necrotic plaques in the mucous membranes, with the most common sites of involvement being the upper respiratory tract, the mammary gland, and the lymph nodes (2). The causative agent is the LSD virus (LSDV), which was initially isolated in South Africa in 1957 (3). The viruses responsible for sheep pox (SPPV), goat pox (GPPV), and lumpy skin disease (LSD) are similar in appearance and have been adapted to different host species (4). It is difficult to differentiate these viruses serologically, and cross-immunity exists between them. It has been demonstrated that immunity to one virus can provide some protection against the others (2). The original virus strain designated the Neethling virus, serves as the reference strain for researchers. The World Organization for Animal Health (WOAH) has identified lumpy skin disease (LSD) as a significant threat to cattle due to its contagious nature and high economic impact (5, 26). Consequently, countries that are free of the disease are obliged to report any confirmed cases to the World Organization for Animal Health (WOAH) within 24 hours (5). Infected herds typically exhibit an infection rate of 5 to 45 percent, with mortality occurring in more than 10 percent of infected animals (6). The initial clinical manifestation is fever, which persists for 1–3 days and is accompanied by increased nasal and pharyngeal secretions, lacrimation, enlargement of lymph nodes, anorexia, general depression, and a disinclination to move. The most conspicuous clinical manifestation of the disease is the appearance of skin nodules, which manifest during the second stage, when the body temperature rises and fever develops. The nodules are randomly distributed and measure between one and two centimeters in size. They affect the epidermis, dermis, and, in some cases, even the hypodermis. An initial diagnosis can be made on the basis of the clinical signs (7, 8). The confirmation of a diagnosis can be achieved through electron microscopy with negative staining, a process that can be completed in less than an hour. This method involves the staining of the affected skin or affected mucus membrane (9). Immunohistochemical methods, such as immuno-peroxide staining of tissue samples, can be employed in cases of both acute and chronic disease (10). The LSD virus can be cultivated in a variety of conventional culture media, including lama testicular and kidney cells, and will exhibit a high titer. Additionally, the virus can be cultivated and reproduced in embryonic cells and the chorioallantoic membrane (CAM) of the embryonated chicken egg. The LSD virus is capable of growth in a multitude of common culture media, including lama testicular cells and lama kidney cells, and will demonstrate a high titer. Additionally, the virus is capable of growth and reproduction in embryonic cells and the chorioallantoic membrane (CAM) of the embryonated chicken egg (11). Bacterial and fungal infections are frequently observed in the affected skin samples. The interpretation of serological results in cases where the antibody titer is low, in the context of vaccination, or the context of a mild form of the disease presents a significant challenge (7). A virus neutralization test has been a widely utilized diagnostic tool; however, its efficacy is limited in scenarios where the number of samples is considerable (12). A number of papers have been published utilising ELISA methodology for the detection of antigens or antibodies (12). To date, four countries—Morocco, Tunisia, Algeria, and Libya—have recently reported cases of LSD. In 2012, Israel reported LSD outbreaks, and in 2013, additional outbreaks were documented in the West Bank of the Jordan River, Lebanon, Jordan, Turkey, and Iraq (7). In 2014, Iran and Syria also reported the disease (13). Laboratory tests, including cell culture, serum neutralization, ELISA, and PCR, are indispensable for the precise diagnosis of LSD. However, the aforementioned tests necessitate the presence of specialized laboratories and trained personnel. Consequently, these tests may not be accessible in impoverished or isolated regions, where the identification of such pathogens is vital to avert substantial economic losses. This limitation underscores the necessity for the development of alternative diagnostic methods that can be utilized in these regions (14). It is therefore necessary to develop a simpler and more efficient method that can be performed in both well-equipped laboratories and smaller, less specialized facilities. LAMP (Loop-mediated isothermal amplification) is a variant of PCR that completes all requisite steps in less than an hour (between 15 and 60 minutes) under isothermal conditions. This is achieved through the use of a set of six primers that have been specifically designed to identify eight sequences within the target gene. All requisite steps and reactions are conducted within a single tube. Presently, this technology is available in the form of commercial kits for a multitude of pathogens, including bacteria, viruses, parasites, and so forth. LAMP is a rapid, sensitive, highly specific, and cost-effective test that can be performed with simple equipment requirements, allowing for the visual detection of the white opacity of magnesium phosphate. The aforementioned features result in a significant reduction in cost and the elimination of the need for well-equipped laboratories. Consequently, LAMP can be readily employed in epidemiological and diagnostic studies (15). The method has been employed for the diagnosis of other diseases, including salmonellosis. In this context, LAMP has been demonstrated to be 100 times more sensitive, three times faster, and 10 times cheaper than PCR for the diagnosis of salmonella (16). LAMP represents a distinctive approach to nucleic acid amplification, whereby the DNA polymerase enzyme with strand displacement activity recognizes the target sequence and generates a substantial quantity of amplified product. LAMP does not necessitate thermocycling, as the amplification process occurs at a constant temperature

between 60°C and 65°C (17). LAMP employs a set of four to six primers, including a forward inner primer (FIP) and a backward inner primer (BIP), outer primers (F3 and B3), and loop primers (LF and LB). The aforementioned primers are capable of identifying six distinct areas within the sample gene. The inclusion of forward loop primer (FLP) and backward loop primer (BLP) is optional and will facilitate the acceleration of the reaction and enhance the sensitivity of the test. The LAMP test exhibits high specificity, progressing only when at least four target genes have been identified by the primers (15).

In comparison to the polymerase chain reaction (PCR) and real-time PCR, the detection time of the loop-mediated isothermal amplification (LAMP) method is significantly reduced, with results obtained in less than an hour. This is due to the fact that the target gene is amplified under isothermal conditions, eliminating the need for complex temperature cycles or devices. LAMP is a more specific method than PCR due to the use of four or six primers, which enhance the specificity of the process. LAMP approaches have demonstrated tolerance to inhibitory substances present in biological samples. Furthermore, their simple and rapid extraction methods allow users to circumvent the necessity for complicated DNA refinement protocols. Due to its high amplification capacity, the number of copies obtained can exceed 10^{39} in less than an hour of incubation (17). LAMP can be documented in many ways. The following methods may be employed for LAMP documentation:

1) Gel electrophoresis
2) Measurement of turbidity resulting from pyrophosphate manganese deposition
3) Measurement of fluorescence using DNA fluorescent dyes such as Safe-Red (18) or a color that binds to metals with fluorescent properties such as Calcein, and
4) Colors that bind to metals such as hydroxyl naphthol blue (HNB) or
5) the naked eye (19).

The present study evaluates and optimizes the LAMP technique for diagnosing Capripoxvirus in Iranian cattle, with results compared to those obtained through PCR.

2. Materials and Methods

2.1. Virus Isolation and Culture

In the Animal Pox Disease Reference Laboratory at the Razi Vaccine and Serum Research Institute in Iran, a number of strains of capripoxvirus were isolated and cultured, including Rm65 SP, Gorgan strain GP, and Neethling LSDV. These samples were utilized as positive controls (n=3), in addition to unrelated poxviruses, such as orf virus and camelpox virus, which were employed as negative controls (n=2). To ensure accuracy, samples of healthy skin and virus-free cell culture were utilized as negative controls (n=2). In the study, positive tissue samples were obtained from seven cows suspected to have LSD (n=7), while two negative tissue samples free from capripox were approved by the RAZI Institute (n=2). A total of nine positive reference samples and seven negative reference samples were utilized in the study. In response to a national call from the Iranian Veterinary Organization, the Razi Institute received clinical samples from cows suspected to have LSD. The samples included dermatological specimens from lesions resulting from the disease. The appropriate protocols were employed for the collection and preparation of the samples. The capripox virus was cultivated in lamb kidney cells (LK) following a protocol provided by the WOAH (25). LK cells were cultured in Dulbecco's minimal essential medium (DMEM) (Gibco, Grand Island, NY) with 10% calf serum (Atlas Biological, Fort Collins, CO, USA) at 37°C and 5% CO_2 at 100% humidity. Monolayer LK cells reach confluence within a two-to-three-day incubation period. To isolate the virus, the infected LK cells were maintained in a medium comprising 10 ml DMEM and 4% FBS in cell culture flasks of 25 cm^2 at 37°C and 5% CO_2.

2.2. Loop-Mediated Isothermal Amplification (LAMP)

2.2.1. Primer Design

The development of cytopathic effect (CPE) was observed microscopically on a daily basis to examine the infected cells. CPE included cell lysis and loss of the monolayer of the cultured cells. The cytopathic effect (CPE) typically manifested between three and five days after the initiation of the cell culture. The DNA of all samples, including homogenized tissue and cell culture, was extracted using tissue and blood DNA extraction kits (Roche, Germany). LAMP primers were designed using the Primer Explorer V4 software, which is available from EIKEN (Japan). The software settings were adjusted for AT-rich samples, which included lower melting points, longer primer lengths, and a reduced distance between primers. The P32 gene (Neethling 2490 Orf074 (Genbank accession no. AF325528.1)), which has previously been identified in the capripox virus genome as a highly conserved segment, was used as the basis for the design of the LAMP primers (14). Initially, the software was unable to design primers for FIP, BIP, and FLP due to the low Tm. Accordingly, the primers proposed by Murray et al. (2013) were employed. The nucleotide sequences are presented in Table 1 for reference.

2.2.2. LAMP optimization

LAMP was conducted at temperatures ranging from 50 to 65 degrees Celsius and with varying concentrations of Bst DNA Polymerase (New England Biolab, USA), MgSO4 (NEB, USA) (4, 6, 8 and 10 mM), and betaine (Geneon, Germany) (1, 1.5, 2 and 4 mM). The concentrations of the primers employed were 0.1–0.2 µM for F3 and B3, 0.4 µM for FIP and BIP, and 1–4 µM for FLP and BLP. The conditions for optimization of isothermal amplification were as follows: The reaction was conducted at 65°C for one hour with a concentration of 0.2 µM for F3 and B3 primers, 1.6 µM for FIP and BIP primers, and 2 µM for FLP and BLP primers. The reaction was conducted with 2 µl of Bst DNA Polymerase (equivalent to 16 units) and 2.5 µl of 10X Termopol buffer (containing 20 mM tris hydrochloride with a pH of 8.8, 10 mM (NH4)2SO4, 10

mM potassium chloride, and 0. The aforementioned solutions were combined with 10 mM $MgSO_4$, 1 M betaine, 1.4 mM dNTP (with a concentration of 10 mM for each nucleotide), 2.5 µl of the extracted DNA, and nuclease-free water to achieve a final volume of 25 µl. To improve the primer's ability to adhere to the target sequence, the sample was subjected to a 5-minute heating process at 95°C prior to the addition of Bst DNA Polymerase. Subsequently, the sample was placed on ice for five minutes to reduce the temperature and facilitate optimal connection of the primers to their complementary sequences on the target DNA. To assess the analytical sensitivity of the test, one of the positive reference strains was subjected to serial dilution (0.05, 0.1, 0.2, 0.4, and 4 ng dilutions) with nuclease-free water. The isothermal amplification reactions were conducted using a PCR thermal cycler (Biorad S1000, USA), and the LAMP products were separated using 2% gel electrophoresis. Subsequently, the amplified products were subjected to staining with ethidium bromide and Safe-Red fluorescent dyes, followed by detection via ultraviolet illumination at a wavelength of 320 nm using a UV illuminator from USA. Subsequently, Bst DNA Polymerase was introduced to commence the LAMP reaction. To assess the efficacy of the LAMP test, Safe-Red (Invitrogen, USA) was incorporated into the final product at a concentration of 0.01 following amplification. The resulting solution was then subjected to UV light at 320 nm for detection.

2.2.3. Polymerase Chain Reaction

A comparison was conducted between the PCR and LAMP tests. Primers were designed for the conserved P32 gene in order to facilitate the detection of Capripoxviruses. The master mix utilized for PCR consisted of 0.2 µm forward and reverse primers, 5 µl of 5X buffer, 0.2 mmol of each dNTP, 0.1 U of DNA polymerase, 2.5 µl of the sample DNA, and an adequate quantity of nuclease-free water to reach the final volume of 25 µl (5). The polymerase chain reaction (PCR) was conducted using the Biorad S1000 thermal cycler. All tests were conducted in triplicate. The P32 gene was employed for the execution of PCR and the estimation of the analytical sensitivity of the PCR. The master mix comprised 0.9 µM of forward and reverse primers, respectively. The PCR cycling conditions involved a two-minute cycle at 95°C (denaturation), 35 cycles at 94°C for one minute to separate the two strands of DNA, an annealing phase at 48°C for one minute, and an extension phase at 68°C for two minutes (5).

3. Results

The efficacy of LAMP with and without loop primers was assessed using the Rm/65 positive reference sample. The results were subsequently evaluated following gel electrophoresis and a color change assessment using UV light. The utilization of the loop primer resulted in a reduction of the test time to one hour, whereas the absence of the primer would have resulted in an increase of the test time to one and a half hours. Figure 1 illustrates the outcomes of the LAMP reaction at varying dilution levels.

Table 1: Primers were utilized for the detection of Capripox virus from the P32 gene of LSD virus, Neethling 2490 Orf 074.

Target region	Primer	Length (BP)	Position	Sequences 5`-3`
P32 Gene (lumpy skin – Neethling 2490- Orf074) GenBank accession no. AF325528	FIP	58	F1c610-639,F2 559-582	TTCAAAACTCAAACTGGTAGAAATACCTTTGTAATTAGATTA TCGTCTGCCATA
	BIP	56	B1c667-696, B2 724-745	CTCAATAGACAAGTTTTAAATGACTCATCTCGTTAGCTCTTT TTTTTGACAA
	F3	24	532-555	GGATATGATTTTACCTTATCTGCA
	B3	23	754-776	CCAACTCTATTCCATATACCGTT
	F Loop	13	590-602	ATAATTTCGTTTA
	B Loop	15	706-720	CTTCACAATACTAAG
	PCR Forward primer(B68)	25	B68	5' - CTA AAA TTA GAG AGC TAT ACT TCT T- 3'
	PCR Backward primer(B69)	21	B69	5' - CGA TTT CCA TAA ACT AAA GTA-3'

All LAMP primers used in this study were purified via high-pressure liquid chromatography (HPLC) (Metabion, Germany).

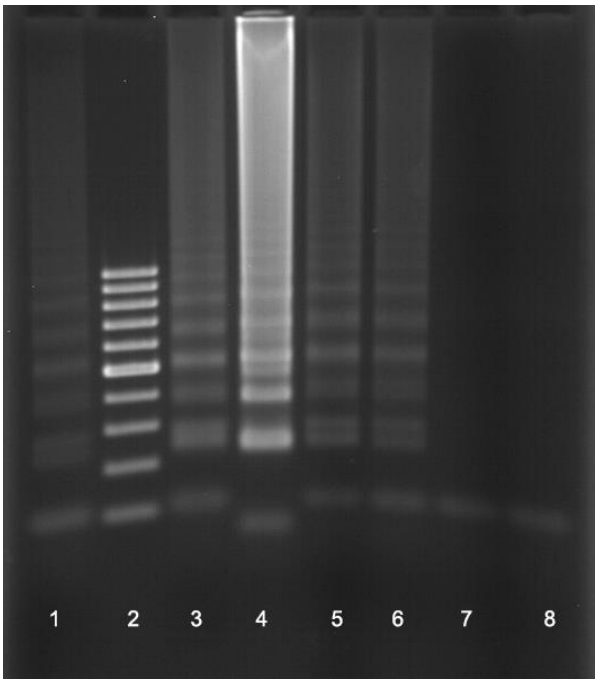

Figure 1. LAMP at different dilutions 1- 0.1ng dilution, 2- marker 100 kb, 3- 4ng dilution, 4- undiluted, 5- 0.4ng dilution, 6-0.2ng dilution 7- negative control, and 8- 0.05ng dilution.

The specificity of LAMP was evaluated using DNA extracted from culture media containing various capripox viruses, including RM/65GPV, Gorgan strain, and Neethling strain, which were used as positive reference samples. All of the aforementioned strains were subjected to gel electrophoresis and stained with Safe-Red, yielding positive results. However, no turbidity was observed following the completion of the reaction. The LAMP tests conducted in the present study were evaluated using two distinct methods: color change detection with Safe-Red and gel electrophoresis. The negative reference strains, including the ORF virus and camelpox, exhibited no reaction in either electrophoresis or Safe-Red staining. A healthy skin sample and a virus-free cell culture sample were employed as negative controls, which also exhibited no reaction in the LAMP assay (Figure 4). The results demonstrated that the designed LAMP assay exhibited 100% specificity in detecting the Capripox virus. To assess diagnostic sensitivity, LAMP and PCR tests were conducted using a positive reference strain sample of the LSD virus. The diagnostic sensitivity of both tests was reported to be equal to 0.2 ng (Figures 1 and 2). A positive reference sample of LSD disease was subjected to an LAMP test utilizing safe-red dye and UV with a wavelength of 320 nm within a microtube environment. The diagnostic sensitivity of this method was reported to be 0.4 ng (Figure 3).

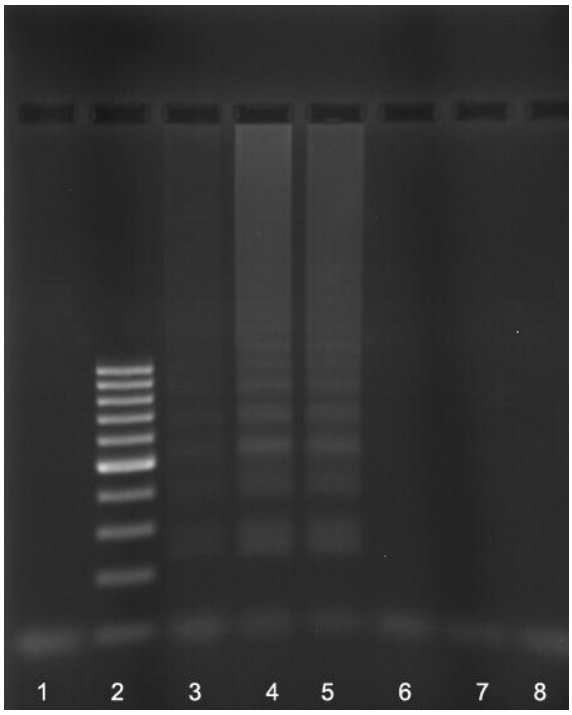

Figure 4. The LAMP test was conducted on a series of samples, arranged from left to right in the following order: 1) negative control, 2) marker 100 kb, 3) sheep pox RM/65 strain, 4) goat pox Gorgan strain, 5) LSD Neethling strain, 6) camelpox, 7) ecthyma, and 8) healthy cattle skin sample.

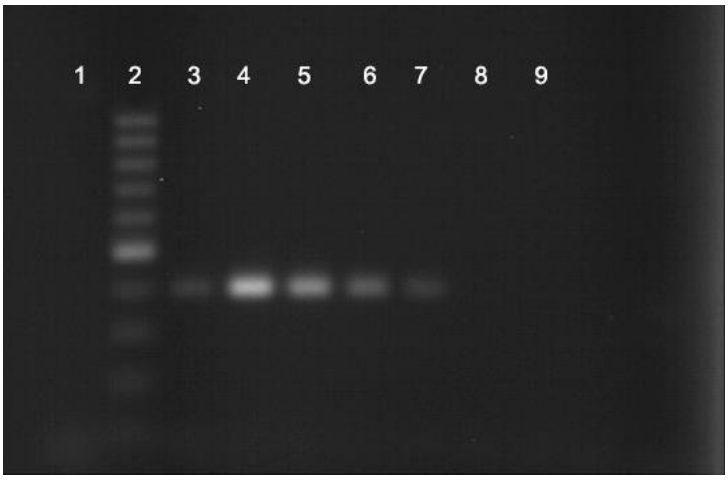

Figure 2. Perform PCR with different dilutions: 1) negative control, 2) markers kb100, 3) dilutions 0.1 ng, 4) Not diluted, 5) 4 ng dilution, 6) 0.4 ng dilution, 7) 0.2 ng dilution, 8) 0.05 ng dilution, and 9) 0.001 ng dilution

Figure 3: The LAMP test was conducted with varying dilutions: 1) negative control, 2) 0.05 ng dilution, 3) 0.1 ng dilution, 4) 0.2 ng dilution, 5) 0.4 ng dilution, 6) 4 ng dilution, and 7) undiluted.

4. Discussion

Molecular diagnosis by amplifying DNA and RNA using PCR represents the gold standard for detecting a number of viral diseases, particularly those caused by capripox viruses (2, 3). A number of PCR-based methods are available for the detection of capripox viruses, including PCR and real-time PCR (2). The principal advantages of PCR are the high speed and sensitivity of the test. However, it requires expensive, high-precision instruments and specialized training for operation and data analysis (27). Although virus isolation represents the gold standard for viral disease diagnosis, its use in capripoxvirus diagnosis necessitates a lengthy period of time, typically between 10 and 14 days, for the generation of results (13). In light of these limitations, it is imperative to develop an alternative assay that is cost-effective, rapid, user-friendly, and requires minimal maintenance. The LAMP test employs isothermal DNA amplification at temperatures between 60 and 65 degrees Celsius, necessitating only the most basic laboratory equipment, such as a water bath. Furthermore, the LAMP test exhibits high specificity, employing six primers that can identify eight regions within the studied sample's gene. In certain instances, the amplification process proposed by LAMP can be more rapid than that of PCR (15), as LAMP does not necessitate thermocycling at disparate temperatures. The polymerase chain reaction (PCR) requires a minimum of an hour and a half, which is a longer processing time than that required for the loop-mediated isothermal amplification (LAMP) method, which takes approximately an hour. However, the advantage of the PCR method is that the DNA polymerase is added to the master mix prior to the two-stranded DNA separation step, which is known as denaturation. In contrast, the addition of DNA polymerase in the LAMP test is conducted subsequent to the denaturation step. Such an approach may increase the risk of contamination. Nevertheless, several reports have indicated that LAMP is more sensitive than PCR in identifying a range of pathogens, including viruses, bacteria, parasites, protozoa, and fungi (20, 23). Furthermore, our findings demonstrate that LAMP is resilient to biological inhibitors (21). As detailed in the Materials and Methods section, LAMP primers for capripox detection were designed using EIKEN's free web-based software, version 4 (24). Initially, the software was unable to design a primer from the desired gene sequence. Consequently, primers were extracted from Murray's paper (14) to facilitate the process. One of the software's inherent constraints is its inability to design primers from samples with a GC content below 40%. The GC content of the LSDV, Neethling 2490 strain, is 25.91%, and that of the P32 gene is 25.55%. Accordingly, the EIKEN software was unable to design the primer with such a low GC content. In such circumstances, it is advised that the length of the primers be increased manually. This will result in an increase in the melting temperature of the primers, bringing it into the desired range. The LAMP test was conducted using the Safe-Red, which was potentiated by connecting to DNA grooves and creating a fluorescent color at 320 nm. The colorant was incorporated into the final product subsequent to the conclusion of the testing procedure. In instances where the test yielded a positive result, the final product exhibited a notable change in color, appearing bright yellow under ultraviolet light. In the event of a negative result, the orange color will lack the requisite fluorescent properties. The color change is a more sensitive indicator than turbidity measurement in the LAMP assay, given that LAMP does not exhibit any visible turbidity following the amplification reaction. The turbidity observed in LAMP reactions can be attributed to the formation of insoluble magnesium pyrophosphate, a byproduct that arises during the synthesis of DNA by Bst DNA polymerase. A high level of turbidity is indicative of a high quantity of DNA synthesis (18). The absence of turbidity in the LAMP assay for the capripox virus may be attributed to inadequate DNA amplification. The high sensitivity of the LAMP assay when employing a color change indicator in comparison to turbidity measurement has been previously documented by other researchers (22). Furthermore, Safe-Red was incorporated into the product following isothermal amplification, which is less prone to environmental contamination in comparison to gel electrophoresis. In the present study, the diagnostic efficacy of LAMP and PCR assays for the capripox virus was evaluated. The analytical sensitivity of LAMP was found to be comparable to that of PCR. LAMP demonstrated a higher degree of specificity than PCR in the detection of the capripox virus, exhibiting no cross-reactivity with other viruses within the same family or those exhibiting analogous clinical symptoms.

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Authors' Contribution

Study concept and design: H. V.
Acquisition of data: M. E.
Analysis and interpretation of data: M. E
Drafting of the manuscript: M. E
Critical revision of the manuscript for important intellectual content: S. S.
Statistical analysis: M.E
Administrative, technical, and material support: H. V

Ethics

The study design was approved by the ethics committee of the Islamic Azad University, Tehran, Iran.

Conflict of Interest

The authors certify that they have no conflicts of interest.

Data Availability

The data that support the findings of this study are available on request from the corresponding author.

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