Original Article

Comparison of Reverse Transcription Loop-Mediated Isothermal Amplification Method with SYBR Green Real-Time RT-PCR and Direct Fluorescent Antibody Test for Diagnosis of Rabies

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SUMMARY: Rabies as an endemic disease in most Asian and African countries, especially in remote areas, and requires a reliable diagnostic method. This study aimed to develop a reverse transcription loop-mediated isothermal amplification (RT-LAMP) method for rapid detection of rabies virus RNA in the brain samples, compared to SYBR Green real time RT-PCR test as a molecular technique and direct fluorescent antibody test as a serological method. In this study, RT-LAMP was developed to diagnose rabies. Six primers were designed based on the nucleoprotein (N) of rabies virus. The sensitivity and specificity of SYBR Green real-time RT-PCR and RT-LAMP methods were also determined. RT-LAMP was optimized at 58 °C for 60 min. The sensitivity and specificity of RT-LAMP and SYBR Green real-time RT-PCR were 91.2% and 84.2%, and 94.12% and 88.9%, respectively. The slight difference between the sensitivity and specificity of RT-LAMP and that of SYBR Green Real-Time RT-PCR demonstrated that RT-LAMP could be used as a reliable and cost-effective method for the diagnosis of rabies.

INTRODUCTION

Rabies is one of the fatal infectious diseases causing viral encephalitis in humans. This infection results from the rabies virus that belongs to *Lyssavirus* genus of the *Rhabdoviridae* family (1). Most underdeveloped countries have limitations in terms of laboratory facilities for rabies diagnosis. Therefore, without an accurate and useful risk assessment system, rabies will remain under-reported in many regions (2). The early detection of rabies in animals can enhance the prevention and control of the infection, and promote appropriate treatment that can be useful to reach prognosis and preventive care in humans. Laboratory diagnosis of rabies can also help to identify the properties of the infectious agent and its potential sources, particularly in cases with no documented evidence of contact with a rabid animal. Thus, others who have been exposed to the same source of infection can be identified (3, 4). The rapid diagnosis of the infection is essential to determine the adequate time for vaccination in rabies reservoirs. Therefore, the development of faster and more sensitive methods such as nucleic acid-based techniques requires new research (5).

The three main methods recommended by the World

Health Organization (6) for the diagnosis of rabies are direct antibody fluorescent (DFA), tissue culture infection test (RTCIT), and mouse inoculation test (MIT) (7). Although the DFA test is considered as the gold standard, there are serious challenges with this method including fresh sample collection without a cold chain and requirement of specialized equipment such as fluorescent microscopes and trained staff (8). In addition, the RTCIT and MIT methods are time-consuming.

SYBR Green real-time RT-PCR has high sensitivity for pre and post-mortem diagnosis of rabies in clinical specimens; however, it relies on expensive equipment. Therefore, rabies diagnosis requires the development of a low-cost method in developing countries. The loop-mediated isothermal amplification (LAMP) method is used to detect various pathogens based on strand displacement by *Bst* polymerase and the formation of stem-loop structure by four specific primers that can recognize six distinct regions on the target gene sequence. This reaction is performed under isothermal conditions using a water bath or heat blocks for an hour. Since RT-LAMP method emphasizes on cheapness and simplicity (9), it is suitable for detecting the rabies virus in humans and animals in both developing and underdeveloped countries, where it is difficult to perform DFA, RT-PCR, and Real-Time PCR tests (10).

The aim of our study was to detect the RNA of rabies virus in the brain samples using the RT-LAMP method compared to SYBR Green Real-Time RT-PCR test and DFA as a serological method recommended by the WHO.

Received January 20, 2019. Accepted August 5, 2019.

J-STAGE Advance Publication August 30, 2019.

DOI: 10.7883/yoken.JJID.2019.009

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MATERIALS AND METHODS

Ethical statement: All the brain specimens used in this study were obtained from animals that had died of rabies. These samples had been forwarded to the National Center for Reference and Research on Rabies, Pasteur Institute of Iran for routine diagnostic activities. The center repository has been registered for research purposes in accordance with the license from the Iran Veterinary Organization.

Virus isolates and sample collection: The fixed RABV strain, CVS batch1 (35009_RABV_CVS, accession number LT839616.1), donated by ANSES Nancy, France, ($LD_{50} > 10^{-8}$) was utilized in this study. In addition, 50 specimens of the brain tissue stored at -80°C in the archive of National Center for Reference and Research on Rabies at Pasteur Institute of Iran over a period of two years (2015–2017) were randomly selected. The result of the rabies test was 34 positive cases (68%) in terms of presence of rabies virus antigen using DFA. A healthy brain sample was considered as a negative control and a brain sample that was positive in DFA as a positive control.

RNA extraction and cDNA synthesis: Total RNAs were extracted using the Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. Total RNA was quantified using a Nanodrop at OD 260/280 nm. Viral RNA of 50 to 100 ng was first converted to cDNA using reverse transcriptase polymerase chain reaction and 2.5 μM of random hexamer. This was subjected to 70°C temperature condition for 5 min and was later snap cooled on ice and spun down briefly. The RevertAid First Strand cDNA synthesis kit (Thermo scientific, Lithuania) was used for the cDNA synthesis. Reverse transcriptase (Thermo scientific, Lithuania) mix was prepared and subjected to temperature conditions of 37°C for 60 min, 70°C for 5 minutes and chilling on ice for 5 minutes in a thermal cycler (Bio-Rad). Nanodrop Spectrophotometer (Nanodrop Technologies, CA) was used to measure cDNA concentration and quality in the ratio of OD 260/280.

RT-PCR amplification for controlling RNA extraction process: The quality of the RNA template was assessed by the amplification of the housekeeping gene *GAPDH*, in each sample using RT-PCR technique. Primer GAPDH_LONG_F1 [forward (5'-ACCACAGTCCATGCCATCAC-3')] and GAPDH_LONG_R1 [reverse (5'-TCCACCACCCTGTTGCTGTA-3')] targeting the *GAPDH* gene were previously described

(11) (2008). RT-PCR was performed using 2x EasyTaq PCR SuperMix (+dye) (Yekta Tajhiz Azma, Iran), according to the manufacturer's protocol. The results were visualized under ultraviolet light using a transilluminator device following 2% agarose gel electrophoresis.

Real-Time RT-PCR: We developed SYBR Green real-time RT-PCR to detect rabies nucleoprotein (N) gene using 5 μl cDNA (template), 10 μl SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 0.25 pmol of each primers Taq5long [(forward (5'-TATGAGAAATGGAACAAYCAYCA-3'))] and Taq16revlong [reverse (5'-GATTTTGAAAGAACTCATGKGTTC-3')] (12), and distilled water to a total volume of 20 μl . Amplification was performed using Rotor-Gene Q (Qiagen, Hilden, Germany) instrument under the following conditions: 10 min hot start at 95°C , 40 cycles of 95°C for 15 s, 55°C for 1 min, and 65°C for 30 s. This was followed by one cycle each of 95°C for 15 s, 55°C for 1 min, and 65°C for 10 min. In addition, analysis of the melting curve was performed to ensure false positive results, such as those due to primer and dimers were not included in any results.

Primer design, RT-LAMP set-up and optimization: We selected three different sets of primers based on the criteria described by Notomi et al (13). The primers were designed using the LAMP primer designing software Primer Explorer V5 (<https://primerexplorer.jp/e/>). Six primers including two external primers (F3 and B3), two loop primers (LB and LF), and two internal primers (FIP and BIP) (Table1) were designed specifically for the nucleoprotein (N) of rabies virus (35009_RABV_CVS, accession number LT839616.1) (Fig. 1). For optimizing RT-LAMP reaction conditions, the temperature range (56 – 61°C), time (15 min, 30 min, 45 min, and 60 min), and template volume (2 μl , 3 μl , and 5 μl) were tested. According to the optimization results, RT-LAMP was carried out in a tube containing 0.2 μM F3 and B3, 1.6 μM FIP and BIP, 0.4 μM FLP and BLP primers, 6 μM MgSO_4 , 1.4 mM dNTPs, 2.5 μl 10X Bst buffer, 1 μl enzyme mixture containing avian myeloblastosis virus (AMV) reverse transcriptase and Bst DNA polymerase, 2 μl template, and distilled water up to 25 μl final volume.

Sensitivity and specificity of RT-LAMP compared to SYBR Green Real Time RT-PCR: For establishing and validating RT-LAMP method, the results were compared to those of SYBR Green Real-Time RT-PCR as a molecular method for detecting rabies virus. Serial 10-fold dilutions of virus stock solution (10^6 FFU/

Table 1. Sequences of designed primers for RT-LAMP assay

Primer name	Sequence
F3	5' GAATGTTTGAGCCAGGC 3'
B3	5' GAAGAATTCCTCTCCAGAT 3'
FIP	ACCAACAGCATTTGATGAATAAGGATCACTCTTATTTTCATCCACTTCC
BIP	TCACTTTGTAGGATGCTATATGGGTCAATTCATGAGGAGCACATG
FLP	5' TCCCACTCAAGCCTAGTGAAC 3'
BLP	5' CAAGTCAGATCCCTAAATGCAACG 3'

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881 agaatgttg agccagggca ggagacagct gtctctcact ctatttcat ccactccgt tcactaggct tgagtgggaa
 <===== F3 =====> <===== F2 =====> <===== LF =====>
 961 atctccttat tcatcaaatg ctgttgggtca cgtgttcaat ctattcact ttgtaggatg ctatatgggt caagtcagat
 <===== F1 =====> <===== B1 =====> <===== LB =====>
 1041 ccctaaatgc aacgggtatt gctgcatgtg ctcctcatga aatgtctgt ctagggggct atctggggaga ggaattcttc
 =====> <===== B2 =====> <===== B 3 =====>

Fig. 1. Locations of the primers used in RT-LAMP based on rabies virus nucleoprotein gene (Accession number of NC_001542.1). The nucleotide sequences of primers are underlined.

Table 2. The results of SYBR Green Real-Time RT-PCR and RT-LAMP tests in comparison with DFA test

Species (Number)	DFA test		Real Time PCR		RT-LAMP	
	+	–	+	–	+	–
Dog (24)	18	6	17	7	17	7
Cow (12)	8	4	8	4	7	5
Sheep (4)	2	2	2	2	2	2
Wolf (3)	2	1	2	1	2	1
Jackal (2)	1	1	0	2	0	2
Cat (2)	1	1	1	1	1	1
Goat (1)	0	1	0	1	0	1
Donkey (1)	1	0	1	0	1	0
Horse (1)	1	0	1	0	1	0
Total (50)	34	16	32	18	31	19

Fig. 2. The analytical sensitivity of SYBR Green Real-Time RT-PCR and RT-LAMP methods. Amplification plot (A) and melting curve of Real-Time RT-PCR (B). The results of RT-LAMP was loaded on 2% agarose gel electrophoresis (C). Lane M, 1000 bp DNA ladder; 1, 10⁵ particles; 2, 10⁴ particles; 3, 10³ particles; 4, 10² particles; and 5, 10 particles. The analytical sensitivity of both assays was detected 10³ FFU/ml.

ml) were used for RNA extraction and were tested by both RT-LAMP and SYBR Green real-time RT-PCR to determine the detection limits of each assay. The specificity of RT-LAMP assay was evaluated using the DNA extracted from HBV and HSV-1, and the RNA extracted from HCV and HIV isolated from clinical samples.

RESULTS

Optimization of RT-LAMP reaction: Thirty-four rabies DFA positive and 16 rabies negative brain specimens from dog (24, 48%), cow (12, 24%), sheep (4, 8%), wolf (3, 6%), jackal (2, 4%), cat (2, 4%), and goat, donkey, and horse (each of them 1, 2%) were used for RNA extraction (Table 2). In this study, 32 out of 34 DFA positive samples were SYBR Green real-time RT-PCR positive and all of the control samples were negative. In RT-LAMP optimization procedures, the best results were achieved at 58 °C for 60 min with 2 µL of the cDNA template.

The sensitivity and specificity of SYBR Green Real Time RT-PCR and RT-LAMP: According the number of rabies positive and negative specimens (Table 2), the sensitivity and specificity tests were respectively obtained as 94.12% and 88.9%, for SYBR Green real-time RT-PCR, and 91.2% and 84.2% for RT-LAMP.

The analytical sensitivity and specificity of SYBR Green Real Time RT-PCR and RT-LAMP: The viral serial dilution of up to 10^3 FFU/ml could be detected by SYBR Green real-time RT-PCR as well as RT-LAMP (Fig. 2). The RT-LAMP primers amplified only rabies virus RNA; no RT-LAMP products were detected with other organisms (HBV, HSV-1, HCV, and HIV) (Fig. 3). Using the NCBI Primer-BLAST (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>), we confirmed that the sequences of the rabies virus RT-LAMP and SYBR Green real-time RT-PCR primers were matched with the rabies virus sequences circulating in Iran and some neighboring countries, as deposited in the GenBank database. The sequence similarity of the RT-LAMP and SYBR Green Real-Time RT-PCR primers with one of the sequences deposited in GenBank is shown in Table 3.

DISCUSSION

In this study, RNA samples (n = 50) were successfully extracted from the brain samples of dogs, cows, sheep, wolves, jackals, cats, a goat, a donkey, and a horse over a period of two years (2015–2017) from the archive of National Center for Reference and Research on Rabies at Pasteur Institute of Iran. The results of 34 (68%) samples were positive by the DFA method (Table 2). In our study, RT-LAMP assay showed 91.2% sensitivity

Fig. 3. The analytical specificity SYBR Green Real-Time RT-PCR and RT-LAMP methods. Amplification Curve (A) and Melting Curve of Real-Time RT-PCR (B). The results of RT-LAMP was loaded on 2% agarose gel electrophoresis (C). Lane 1, 1000 bp DNA ladder; 2, rabies virus; 3, HBV; 4, HCV; 5, HIV; 6, HSV-1. The results show that only the positive control (rabies virus isolate 35009) has been detected in both methods.

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Table 3. The location and differences of primers based on the conserved region of the N gene sequences of rabies virus genotypes circulating in Iran and some neighboring countries

RT-LAMP Primers				SYBR Green Real Time RT-PCR Primers					
Forward		Reverse		Forward		Reverse			
	812	829	1031	1050		7194	7216	7288	7311
Iran Sheep	GAATGTTTGAGCCAGGGC		GAAGAATTCCTCTCCCAGAT		Iran Jackal	TATGAGAAATGGAACAAYCAYCA		GATTTTGAAAGAACTCATGKGTYC	
	C.....		C.....			G.....		A C...C.....	T.....
Iran Wolf	GAATGTTTGAGCCAGGGC		GAAGAATTCCTCTCCCAGAT		Iran Sheep	TATGAGAAATGGAACAAYCAYCA		GATTTTGAAAGAACTCATGKGTYC	
	C.....		C.....			G.....		A C...C.....	T.....
Iran Jackal	GAATGTTTGAGCCAGGGC		GAAGAATTCCTCTCCCAGAT		Iran Jackal	TATGAGAAATGGAACAAYCAYCA		GATTTTGAAAGAACTCATGKGTYC	
	C.....		C.....			G.....		A C...C.....	T.....
Iran Wolf	GAATGTTTGAGCCAGGGC		GAAGAATTCCTCTCCCAGAT		Iran Jackal	TATGAGAAATGGAACAAYCAYCA		GATTTTGAAAGAACTCATGKGTYC	
	C.....		C.....			G.....		A C...C.....	T.....
Iran dog	GAATGTTTGAGCCAGGGC		GAAGAATTCCTCTCCCAGAT		Forward		Reverse		
	C.....		C.....		7193	7215	7287	7319	
Iran Jackal	GAATGTTTGAGCCAGGGC		GAAGAATTCCTCTCCCAGAT		Iran Wolf	TATGAGAAATGGAACAAYCAYCA		GATTTTGAAAGAACTCATGKGTYC	
	C.....		C.....			G.....		A C...C.....	T.....
Iran Sheep	GAATGTTTGAGCCAGGGC		GAAGAATTCCTCTCCCAGAT		Forward		Reverse		
	C.....		C.....		7263	7285	7357	7380	
Iran Jackal	GAATGTTTGAGCCAGGGC		GAAGAATTCCTCTCCCAGAT		Turkey Dog	TATGAGAAATGGAACAAYCAYCA		GATTTTGAAAGAACTCATGKGTYC AC	
	C.....		C.....			G.....		C.....A.....	T.....
Iran Jackal	GAATGTTTGAGCCAGGGC		GAAGAATTCCTCTCCCAGAT		Turkey Fox	TATGAGAAATGGAACAAYCAYCA		GATTTTGAAAGAACTCATGKGTYC AC	
	C.....		C.....			G.....		C.....A.....	T.....
Challenge Virus Standard rabies strain (LT839616)	GAATGTTTGAGCCAGGGC		GAAGAATTCCTCTCCCAGAT		Turkey Fox	TATGAGAAATGGAACAAYCAYCA		GATTTTGAAAGAACTCATGKGTYC AC	
	C.....		C.....			G.....		C.....A.....	T.....
Turkey Cow	GAATGTTTGAGCCAGGGC		GAAGAATTCCTCTCCCAGAT		Turkey Fox	TATGAGAAATGGAACAAYCAYCA		GATTTTGAAAGAACTCATGKGTYC AC	
	C.....		C.....			G.....		C.....A.....	T.....
Forward				Reverse					
	882	899	1101	1120					
Iraq Cow	GAATGTTTGAGCCAGGGC		GAAGAATTCCTCTCCCAGAT		Turkey Fox	TATGAGAAATGGAACAAYCAYCA		GATTTTGAAAGAACTCATGKGTYC AC	
	C.....		C.....			G.....		C.....A.....	T.....
					Turkey Dog	TATGAGAAATGGAACAAYCAYCA		GATTTTGAAAGAACTCATGKGTYC AC	
						G.....		C.....A.....	T.....
					Turkey Dog	TATGAGAAATGGAACAAYCAYCA		GATTTTGAAAGAACTCATGKGTYC AC	
						G.....		C.....A.....	T.....
Forward				Reverse					
	7264	7286	7358	7381					
Turkey Fox	TATGAGAAATGGAACAAYCAYCA		GATTTTGAAAGAACTCATGKGTYC AC		Turkey Fox	TATGAGAAATGGAACAAYCAYCA		GATTTTGAAAGAACTCATGKGTYC AC	
	G.....		C.....A.....			G.....		C.....A.....	T.....
Turkey Fox	TATGAGAAATGGAACAAYCAYCA		GATTTTGAAAGAACTCATGKGTYC AC		Turkey Fox	TATGAGAAATGGAACAAYCAYCA		GATTTTGAAAGAACTCATGKGTYC AC	
	G.....		C.....A.....			G.....		C.....A.....	T.....
Turkey Fox	TATGAGAAATGGAACAAYCAYCA		GATTTTGAAAGAACTCATGKGTYC AC		Turkey Fox	TATGAGAAATGGAACAAYCAYCA		GATTTTGAAAGAACTCATGKGTYC AC	
	G.....		C.....A.....			G.....		C.....A.....	T.....
Turkey Fox	TATGAGAAATGGAACAAYCAYCA		GATTTTGAAAGAACTCATGKGTYC AC		Turkey Fox	TATGAGAAATGGAACAAYCAYCA		GATTTTGAAAGAACTCATGKGTYC AC	
	G.....		C.....A.....			G.....		C.....A.....	T.....
Turkey Fox	TATGAGAAATGGAACAAYCAYCA		GATTTTGAAAGAACTCATGKGTYC AC		Turkey Fox	TATGAGAAATGGAACAAYCAYCA		GATTTTGAAAGAACTCATGKGTYC AC	
	G.....		C.....A.....			G.....		C.....A.....	T.....
Turkey Dog	TATGAGAAATGGAACAAYCAYCA		GATTTTGAAAGAACTCATGKGTYC AC		Turkey Dog	TATGAGAAATGGAACAAYCAYCA		GATTTTGAAAGAACTCATGKGTYC AC	
	G.....		C.....A.....			G.....		C.....A.....	T.....
Turkey Fox	TATGAGAAATGGAACAAYCAYCA		GATTTTGAAAGAACTCATGKGTYC AC		Turkey Fox	TATGAGAAATGGAACAAYCAYCA		GATTTTGAAAGAACTCATGKGTYC AC	
	G.....		C.....A.....			G.....		C.....A.....	T.....
Turkey Fox	TATGAGAAATGGAACAAYCAYCA		GATTTTGAAAGAACTCATGKGTYC AC		Turkey Fox	TATGAGAAATGGAACAAYCAYCA		GATTTTGAAAGAACTCATGKGTYC AC	
	G.....		C.....A.....			G.....		C.....A.....	T.....
Turkey Dog	TATGAGAAATGGAACAAYCAYCA		GATTTTGAAAGAACTCATGKGTYC AC		Turkey Dog	TATGAGAAATGGAACAAYCAYCA		GATTTTGAAAGAACTCATGKGTYC AC	
	G.....		C.....A.....			G.....		C.....A.....	T.....

and 84.2% specificity compared to the SYBR Green real-time RT-PCR with 94.12% sensitivity and 88.9% specificity. The obtained results showed a slight difference between the clinical sensitivity and specificity to SYBR Green real-time RT-PCR, which targets the N gene, in post-mortem specimens. However, RT-LAMP assay has no need for a thermocycler and expensive reagents for the diagnosis of rabies; therefore, it is more efficient in field studies and deprived areas. This reaction has fewer steps than SYBR Green real-time RT-PCR and DFA, and is therefore, a timesaving method. Moreover, the results of RT-LAMP assay are obtained within 1 h, whereas the other methods last for 2-4 h.

Rabies is prevalent in the populations with limited access to healthcare seen in developing countries. The disease is also grossly underreported in these areas because most victims die at home (14) and most of the suspected cases are not referred to healthcare centers. Animal rabies occurs in a wide variety of species and it has dispersed geographically in Iran. Thus, an accurate and rapid diagnostic method is needed to be used in remote areas. Many suspected rabies cases may not be delivered to diagnostic centers due to a wrong clinical diagnosis, lack of cooperation by livestock owners, and inaccessible delivery facilities (15).

Different methods are available for the diagnosis of rabies among animals including DFA, MIT, RTCIT (rabies tissue culture infection test), direct rapid immunohistochemical test (dRIT), and PCR (16). Some of these methods are time-consuming or require special equipment (17). Recent studies have shown that the sensitivity of PCR is much higher than the diagnostic method based on finding a specific antigen or cell culture (18). However, a qualitative study with real-time PCR can be the most reliable method to identify infectious agents. Some studies showed that molecular-based techniques for rabies identification are easy, fast, and efficient. In other reports, real-time PCR was introduced as a highly sensitive and specific method for rabies diagnosis. Although DFA is the gold standard for detecting rabies virus, some studies reported that real-time PCR has the same sensitivity as DFA (19).

The RT-LAMP assay was initially developed by Notomi et al. (13) and was recently recommended for the diagnosis of diseases at the fields (20, 21). In the present study, we applied and evaluated RT-LAMP assay as a fast, sensitive, specific, and cost-effective method for rapid detection of rabies virus in animal brain samples. RT-LAMP is a simple diagnostic technique with the reaction occurring in a single tube containing target DNA, buffer, *Bst* DNA polymerase, and primers. This technique has no dependency on the thermocycler, which is expensive equipment. Therefore, a simple thermal procedure is used in performing RT-LAMP assay in a conventional laboratory water bath or heat block that maintains a constant temperature (22).

RT-LAMP assay was optimized with high sensitivity for primers. In our study, the selected range of 56–61 °C temperature in our study was related to the heat-resistant property of *Bst* polymerase with its maximum activity achieved at the mentioned thermal range. The optimal temperature for the tube containing RT-LAMP reaction mixture was 58 °C, which differed from the result of Boldbaatar et al. (63 °C) (10). This was probably due

to the difference in the primer sequences. Suzuki et al. suggested 63–65 °C as the best temperature range for identification of cytomegalovirus (CMV) (23). In addition, Ihira et al. (24) established RT-LAMP assay at 63 °C for the identification of camel smallpox and HHV-6 viruses, respectively. According to some studies on RT-LAMP technique, a range of 55 °C–65 °C was recommended for incubating the mixture tube (25), which is in line with the current study. These results also showed that at least 45 min is required for adequate amplification of the target sequence so that its product can be detected. However, Nagamine et al. suggested that if circular primers are used, RT-LAMP reaction could be performed in 30 min (26). The study by Wang et al. also identified CMV virus at 64 °C in 30 min with RT-LAMP reaction (27).

The optimized RT-LAMP assay had high sensitivity (91.2%) and specificity (84.2%) for rabies virus RNA detection. Our results showed that the percentage of true positive cases is lower than that in the SYBR Green real-time RT-PCR assay. There are several factors, which cause low sensitivity of RT-LAMP compared to the Green real-time RT-PCR assay. The conditions that cause optimum activity of *Bst* polymerase enzyme may not always be maintained, leading to lower sensitivity and specificity.

In this study, we developed the RT-LAMP diagnostic method using new primers that were designed based on sequences of rabies virus circulating in the Middle East countries. According to the obtained data, like DFA, this method can be used as a rapid and cost-effective alternative method instead of SYBR Green real-time RT-PCR in rabies diagnostic laboratories and epidemiological fields worldwide, especially in developing countries.

Acknowledgments This study was supported by Pasteur Institute of Iran (grant number 1394/850). The authors would like to acknowledge the staffs of the National Center for Reference and Research on Rabies, Pasteur Institute of Iran especially Dr. Rouzbeh Bashari (director of the center) and Dr. Behzad Pourhossein for their cooperation and kind supports in this project.

Conflict of interest None to declare.

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