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DEVELOPMENT OF A REVERSE TRANSCRIPTION-LOOP MEDIATED ISOTHERMAL AMPLIFICATION ASSAY FOR RAPID DETECTION OF INFECTIOUS BURSAL DISEASE VIRUS IN CHICKEN

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Abstract–Infectious bursal disease (IBD) is an immunosuppressive disease caused by infectious bursal disease virus (IBDV) that affects chickens, results in significant economic losses to the poultry industry worldwide. With the objective of developing a simple and rapid method to detect IBDV, a reverse transcription-loop mediated isothermal amplification assay (RT-LAMP) was developed and evaluated in this study. This method targets the well-conserved RNA dependent RNA polymerase (VP1) gene of IBDV and has the potential to detect diverse IBDV isolates. RT-LAMP amplified products were successfully detected by naked eye using SYBR Green I and when subjected to agarose gel electrophoresis revealed ladder-like pattern. The sensitivity of the RT-LAMP was compared with other genome amplification methods such as RT-PCR and real time RT-PCR methods. It was observed that RT-LAMP was more sensitive than RT-PCR but as sensitive as real time PCR. Furthermore, this method exhibited excellent specificity without any cross reaction to other common avian pathogens. This method also exhibited superior practical applicability when tested on tissue samples suspected for IBDV. Overall, the IBDV VP1-gene based RT-LAMP assay developed in this study is a rapid, simple and sensitive assay suitable for less-equipped laboratories and can be utilized in field conditions as a screening test for rapid detection of IBDV.

INTRODUCTION

Infectious bursal disease (IBD) is a highly contagious disease of young chickens caused by infectious bursal disease virus (IBDV) belonging to the family Birnaviridae (Muller, 1986). Chicks of 2 to 8 weeks age are susceptible to the infection. Two serotypes (serotype I and serotype II) have been recognized to naturally infect chickens, of which IBDV strains belonging to serotype I are considered pathogenic for chickens (Jackwood *et al.,* 1985). In serotype I, the Larger genomic segment A encodes for 4 viral proteins, two capsid proteins VP2 (48 kDa) and VP3 (32–35 kDa), viral protease VP4 (24 kDa), and a nonstructural protein VP5 (17–21 kDa), while the smaller segment B encodes VP1 (94 kDa), an RNA-dependent RNA polymerase. The level of conserved nature of different pathotypes of IBDV VP1 ranging from 96.8 to 100 per cent.

Currently, there are several conventional methods that can be used to diagnose the IBDV pathogen. Some of these include agar gel precipitation test (AGPT) (Hirai *et al.,* 1972), virus neutralization test (VNT) (Weisman and Hitchner, 1978), enzyme-linked immunosorbent assay (ELISA) (Marquardt *et al.,* 1980), dot blot hybridization assay (Henderson

and Jackwood, 1990) and electron microscopy (Harkness *et al.,* 1975). In addition, RT-PCR (reverse transcriptase-polymerase chain reaction) (Lee *et al.,* 1994), real-time RT-PCR (Tham *et al.,* 1995), RT-PCR-RE (analysis of PCR product by restriction endonuclease) (Moody *et al.,* 2000) and RT-PCR-RFLP (analysis of PCR product by restriction fragment length polymorphism) (Jackwood and Nielson, 1997) have also been commonly used as molecular diagnostic methods for detection and discrimination of IBDV. Of all the available methods, RT-PCR is the most convenient method for detection of IBDV, because it allows testing of a large number of samples. However, traditional RT-PCR method requires a relatively sophisticated laboratory with well-trained staff and involves multiple reaction steps to amplify the nucleic acid. Further, the sensitivity of this method can be affected by concentration of RNA in the template. Hence, there is a need for an alternative assay that is more efficient, rapid with higher sensitivity and specificity, as well as economical for use under field conditions for screening and early diagnosis of IBDV infection (Lee *et al.,* 1994).

Loop Mediated Isothermal Amplification (LAMP) is a novel amplification method developed by Notomi *et al.* (2000) and can be carried out under isothermal conditions (60–65°C) without sophisticated equipments and produces a large amount of DNA. The LAMP assay was based on the principle of autocycling strand displacement DNA synthesis performed by the *Bst* DNA polymerase and a set of two inner and two outer primers that recognized 6–8 regions of target DNA. LAMP had high specificity as the primers recognized six specific regions of the target amplicons (Tsai *et al.,* 2012). LAMP technique has been successfully applied for detection of viruses such as west Nile virus (Parida *et al.,* 2004), Respiratory syndrome virus (RSV) (Ushio *et al.,* 2005), haemorrhagic septicemia virus (Soliman and El-matbouli, 2006), Newcastle disease virus (Chen *et al.,* 2008) and H3 Swine influenza virus (Gu *et al.,* 2009). RT-LAMP detection methods have been previously utilized for detection of IBDV by few research groups using VP2, VP3 or VP5 regions of IBDV (Xu *et al.,* 2009; Lee *et al.,* 2011 and Wang *et al.,* 2011). However, there are no reports on use of RT-LAMP targeting VP1 gene for detection of IBDV. The present study was undertaken to develop RT-LAMP for detection of IBDV and to compare its sensitivity and specificity with real time PCR, RT-PCR and assess its applicability under field conditions.

MATERIALS AND METHODS

Processing of tissue samples

A total of 180 suspected tissue samples collected from fifteen commercial broiler flocks from southern states of India with IBD symptoms during the year 2013-2016 were subjected for analysis. The bursal tissue samples collected were homogenized in Phosphate Buffered Saline (PBS). The suspension was centrifuged at 10,621 g for 5 min and the supernatant was collected in 1.5 ml eppendorf tubes and stored at-70ºC until used.

RNA extraction and reverse transcription-polymerase chain reaction. For RNA isolation, 250 µl of clarified bursal homogenate was mixed with 750 µl of Trizol reagent (RNA isoplus, Takara). To this, 200 µl of chloroform was added and mixed well. The aqueous phase was separated by centrifugation at 10, 621 g for 15 min at 4 °C. RNA was precipitated by adding equal volume of ice chilled isopropanol followed by overnight incubation at -70ºC. RNA was pelleted by centrifugation at 10, 621 g for 20 min and washing was carried out with 70% ethanol and air dried. The pellet was resuspended in 10 µl of nuclease free water and stored at-70 °C until used.

The reverse transcription reaction to synthesize cDNA were carried out using RevertAid RT Reverse Transcription Kit (Thermoscitific, USA) as per manufacturer instructions with slight modifications. Briefly, 9 µl of RNA extract, with 1µl of random hexamer primer were incubated at 65 °C for 5 min and chilled on ice. To this, a mixture containing 4 µl reaction buffer, 2 µl of 10 mM dNTP, 2 µl of DMSO, 1 µl of ribolock RNase inhhibitor, and 1 µl of revert aid H minus M-MuLV reverse transcriptase enzyme was added and incubated for 5 min at 25 °C followed by 60 min at 42 °C. The reaction was terminated by denaturation at 70 °C for 5min and chilled on ice. The cDNA was used as template for all RT-PCR and real time PCR purposes. For RT-PCR, a 749 bp region of VP1 gene was amplified using 749-FP-5′-CTACGGGAGTGGGACCTACA-3′ and 749-RP- 5′-ACCACGTGTTGGAGTGAACA-3′ primer set (Ashraf *et al.,* 2007) and, a 743 bp region of the VP2 hypervariable region was amplified using 743-FP- 5′-GCCCAGAGTCTACACCAT-3′ and 743-RP- 5′-CCCGGATTATGTCTTTGA-3′ primer set (Jackwood and Sommer-Wagner, 2005).

Development of Reverse Transcription-Loop Mediated Isothermal Amplification (RT-LAMP) assay

LAMP primers were designed from conserved region in the VP1 gene after aligning the following sequences: Winterfield 2512 (AF083092), D-78(AF499930), Edger (AY459320), IM (AY029165), GLS (AY368654), OKYM (D49707),UK661 (X92761), IL3 (AF083093).

RT-LAMP primers (Table 1) were designed using primer explorer version 4 (http://primerexplorer.jpg/lamp4.0.0/index.html). The RT-LAMP reaction was carried out in a total volume of 25 µl. The reaction mixture consisted of 10 pmol each of F3 and B3 primers and 40 pmol each of FIB and BIP primers,1.4 mM of dNTPs, 0.8 M of betaine, 10 mM of $MgSO_4$, 3 µl of cDNA (1-5ìg concentration) as template, 8 U of *Bst* DNA polymerase with 1X thermopol buffer (NEW England Biolabs, Germany) and NFW was added to make the volume up to 25 µl. The reaction mixtures were incubated at 55 to 60 °C for 60 to 120 minutes followed by enzyme inactivation at 80 °C for 10 min in a heating block/water bath. The RT-LAMP products were detected by naked eye and visualized under ultraviolet light by addition of 1 µl of 1:1000 SYBR Green I reagent (Invitrogen, USA) to the LAMP reaction mixture. In addition, 10 µl of LAMP product was subjected to gel electrophoresis on a 2 % agarose gel and visualized under ultraviolet light after ethidium bromide staining.

To evaluate the sensitivity of RT-LAMP assay, the detection limit was determined using 10-fold serial dilutions of IBDV RNA template (Georgia intermediate vaccine) with an initial RNA concentration of 8.90 ng/µl. The same RNA concentrations with 10 fold serial dilution were also subjected to RT-PCR and real time RT-PCR assays to compare the sensitivities of all three assays.

To assess the specificity of RT-LAMP for IBDV, and potential cross reactivities with other common poultry pathogens, like NDV, IBV, MDV, S. *typhi*, *B. abortus* and *E. coli* were used. The genomic RNA of IBDV- Georgia intermediate vaccine strain was used as the positive control.

Real time RT-PCR assay. The primers for real time RT-PCR was designed targeting the VP1 gene in segment B of IBDV available from NCBI by using Beacon Designer™ (PREMIER Biosoft, USA). To amplify a 194 bp region, the forward primer RT-VP1 FP-5′-TAGTCCAGACAGCGAAGAG-3′ and reverse primer RT-VP1 RP-5′-GCAGACCATCCGAGTAGG-3′ were used. Real time RT-PCR was carried out using Mastercycler® ep realflex* (Eppendorf). The reaction mixture contained 5 µl of 2X RT-PCR Takara SYBR Green Mix, 1 µl of forward and reverse primer (each 10 pmol/µl), 1 µl of cDNA and 2 µl of nuclease free water was added to obtain a final reaction volume of 10 µl. In the negative control 1 µl of NFW was added instead of template. The reaction conditions consisted of initial denaturation step at 95 °C for 2 min and 40 cycles of denaturation at 95 °C for 15 sec, annealing at 55 °C for 15 sec and elongation at 68 °C for 45 sec. Following amplification, a melting curve was determined to the RT-PCR products.

RESULTS

Standardization and optimization of RT-LAMP assay.

In order to determine the optimal conditions of RT-LAMP, genomic RNA of Georgia intermediate vaccine strain was used as the target template along with the newly-designed VP1 primer set as the LAMP primer. Several reaction parameters such as incubation temperature (55-60° C), incubation time (30-90 min), $MgSO_4$ concentration (4-12 mM), betaine concentration (0.4 to 1M), and dNTP concentration (1-3 mM) were tested for optimization of the assay. Following agarose gel electrophoresis, maximum amplification was observed at 60 °C and consisted of the typical ladder pattern. The intensity

Table 1. The RT-LAMP primers designed for IBDV detection

Name	Type	Length	Position	Sequence (5′-3′)
F3	Forward outer	19	1087-1105	GACAAAGCACATGGCTCACCTAA
B3	Reverse outer	20	1287-1306	GGCCTTGGGTTCATCC
FIP	Forward inner	47	1161-1182-	GGACATCACGGGCCAGGTTATCT
	(F1c-TTTTT-F2)		TTTTT-1107-1126	TTTTCAAGACCCGCAACATATGGT
BIP	Reverse inner	47	1203-1224-TTTTT-	GAACATTGAGGGGTGTCCGTCAT
	(B1c-TTTTT-B2)		1264-1283	TTTTGCCATTATCCACTCCACGAT

of the amplicons was significantly lower in other temperatures than that observed at 60 °C. The subsequent assays were carried out in 60 °C which is considered as the optimal temperature for IBDV RT-LAMP (Fig. 1).

Results from $MgSO_4$ optimization revealed that maximum amplification was observed at 10 mM concentration (Fig. 2). Our results also indicated that the optimal concentrations of other parameters were 8 U for *Bst* DNA polymerase and 1.4 mM for dNTPs (data not shown).

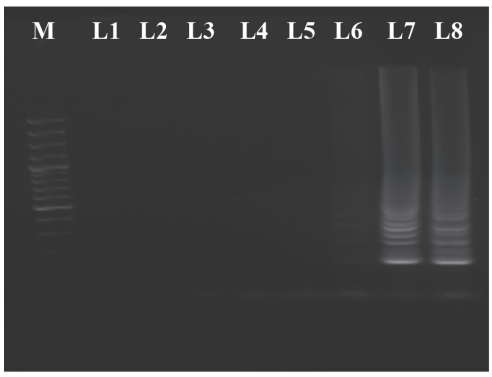
In order to determine the optimal reaction time, the RT-LAMP reaction was carried out at various incubation periods such as 30, 45, 60 and 90 min at 60 °C. No amplification of product was found in the reaction time of 30 min but amplification was found at reaction times of 45, 60 and 90 min. The amplified product was detected as early as 45 min of reaction time and it increased with time upto 90 min. However, maximal amplification was observed at 60 min. Therefore, optimized RT-LAMP conditions were maintained at 60 °C for 60 min in further studies.

Sensitivity of LAMP and PCR assays

The sensitivity of the RT-LAMP assay for the detection of IBDV VP1 gene was determined and compared with PCR based assays. The concentration of RNA in the undiluted sample was 8.90 ng. The lowest concentration of IBDV RNA detected by RT-PCR was 890 pg (Fig.2a) while RT-LAMP and real time RT-PCR had a detection limit of 8.90fg (Fig. 2b, 2c and 2d). Therefore, the developed RT-LAMP has 100 times more sensitive than RT-PCR and equally sensitive to real time RT-PCR for detecting IBDV.

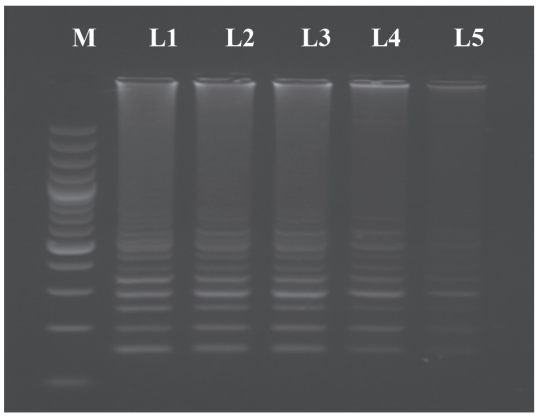
Specificity of RT-LAMP assay

The specificity of RT-LAMP reaction was studied with common viral and bacterial pathogens and Georgia intermediate vaccine strain of IBDV. A characteristic ladder like pattern was observed only with IBDV when the RT-LAMP amplification products were run on an agarose gel, while the other viruses and bacteria produced negative results (Fig. 3). The specificity results were also confirmed using SYBR Green I fluorescence dye and the positive results of LAMP assay were directly visualized and



M – 100bp Ladder
L1 – Negative control
L2 – 55°C
L3 – 56°C
L4 – 57°C
L5 – 58°C
L6 – 59°C
L7 – 60°C
L8 – 61°C

Fig. 1. Optimization of Temperature for RT-LAMP



M – 100 bp Ladder
L1 – 3.0 µl of 100mM $MgSO_4$
L2 – 2.5 µl of 100mM $MgSO_4$
L3 – 2.0 µl of 100mM $MgSO_4$
L4 – 1.5 µl of 100mM $MgSO_4$
L5 – 1.0 µl of 100mM $MgSO_4$

Fig. 2. Optimization of $MgSO_4$ concentration for RT-LAMP

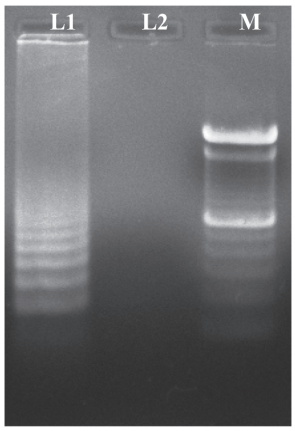


Fig. 3. Standardization of RT-LAMP

discriminated from negative results by SYBR Green I fluorescence dye. Positive samples were green while negative samples remained orange.

Validation of RT-LAMP assay

The RT-LAMP assay developed in the present study was validated for its ability to detect IBDV from 180 field tissue samples collected from chicken suspected of IBDV. Our results revealed that the newly-developed RT-LAMP assay had a detection efficiency of 37.22% (67/180 samples) while real time RT-PCR assay showed an efficiency of 45% and RT-PCR targeting VP1 showed a detection efficiency of 36.11% while that targeting VP2 was 35.56% (Table 2). Chi-square test of significance revealed a highly significant difference (16.22**) among various detection methods developed for detection of IBDV.

DISCUSSION

In this study, a simple, rapid and sensitive RT-LAMP assay based on the VP1 gene was successfully developed for the detection of IBDV infection in broiler chickens. Two set of primers that bind to the VP1 gene of IBDV segment B was designed and used in this assay. Several reaction parameters were evaluated and optimal conditions for this newly-developed RT-LAMP were determined. Reaction conditions determined from our study agree well with findings from other researchers (Parida *et al.,* 2005; Francois *et al.,* 2011; Zanoli and Spoto, 2013). Since VP1 that encodes for the viral RNA dependent RNA polymerase is highly conserved across IBDV isolates, we selected VP1 as the target region for this method and it could detect various IBDV isolates prevalent in India.

Interestingly, this method was able to detect a spectrum of viruses that include classical laboratory strains, vaccine strains, very virulent strains of IBDV (vvIBDV) and reassortant viruses (Raja *et al.,* 2016) indicating its broad applicability under field

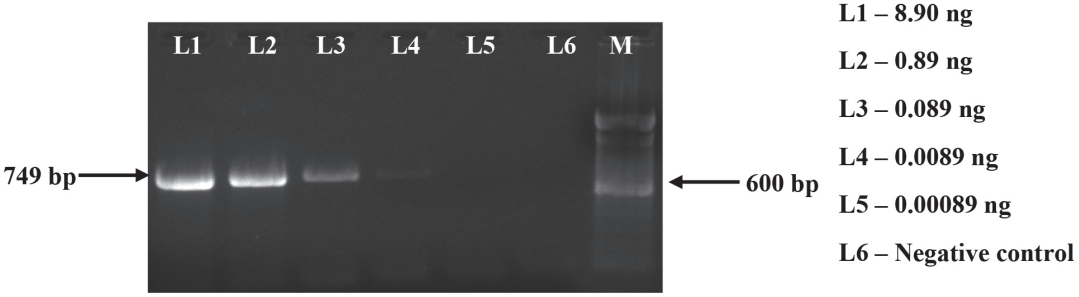


Fig. 4. Sensitivity of RT-PCR

Table 2. Comparison of embryonated egg inoculation, RT-PCR, RT-LAMP and Real time RT-PCR assays for detection of IBDV from field samples

Sl. No.	Age groups	Result of the test		Total
		Positive	Negative	
1.	Embryonated egg inoculation	57(31.67)	123(68.33)	180(100.00)
2.	VP1-RT-PCR	65(36.11)	115(63.89)	180(100.00)
3.	VP2 –RT-PCR	64(35.56)	116(64.44)	180(100.00)
4.	VP1 RT-LAMP	67(37.22)	113(62.78)	180(100.00)
5.	VP1 Real time PCR	81(45.00)	99(55.00)	180(100.00)

χ^2 (between different type of test and result of the test) = 16.22**

Figures in parentheses indicate per cent to row total** – Significant (P<0.01)

conditions.

Several researchers have reported RT-LAMP assay for detection of IBDV. Targeting VP3 (Xu *et al.,* 2009), VP2 (Lee *et al.,* 2011; Tsai *et al.,* 2012) or VP5 (Wang *et al.,* 2011) regions of the IBDV genome. To the best of our knowledge this is first report of RT-LAMP assay targeting VP1 gene of IBDV segment B. In general, RT-LAMP assays use 6-8 primers in the reaction. Future studies exploring increased number of primers (Loop Primers) to the VP1 could be interesting in terms of the performance of this assay.

LAMP technique is characterized by high specificity, sensitivity, speed (45-60 min), single reaction temperature, and high robustness (Francois *et al.,* 2011). A major advantage of this method is that it is easy to apply to clinical samples and thereby very helpful during epidemiological investigations. Isothermally-amplified DNA can be visualized directly by naked eye (due to increase in turbidity) or visualization can be enhanced by addition of DNA-binding dyes such as SYBR green. Furthermore, if required, the amplicons from the RT-LAMP can also be subjected to gel electrophoresis. Xu *et al.* (2009) reported that real-time PCR is more sensitive than conventional PCR because of the increased sensitivity due to the added probe. Real time PCR assay could be accomplished with equal or higher sensitivity and specificity. However, this requires flurogenic primers and probes as well as expensive detection equipment. The high cost of the instrument required to perform the real-time assays restricted their use to referral laboratories (Parida *et al.,* 2005). From our study, we found that the newly-developed RT-LAMP has superior sensitivity than conventional RT-PCR or virus isolation methods. Our assay could detect as low as 800 fg of IBDV RNA compared to RT-PCR which had a lower limit of detection (80pg). Several research groups have also reported that RT-LAMP was more sensitive than RT-PCR (Xu *et al.,* 2009; Lee *et al.,* 2011; Wang *et al.,* 2011). Furthermore, when validated with clinical samples, the RT-LAMP assay was able to detect IBDV with better efficiency than RT-PCR. Detection of vvIBDV in the tested specimens using the newly-developed assay suggests the prevalent severity of vvIBDV in India and underscores the usefulness of this assay in detecting vvIBDV.

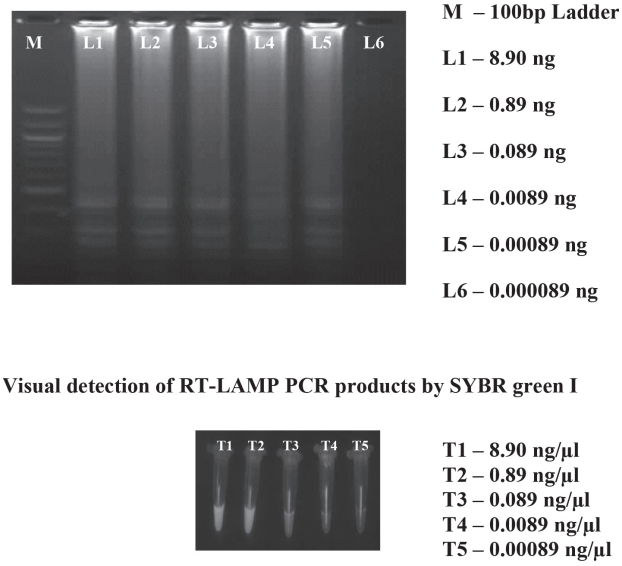


Fig. 5. Sensitivity of the RT-LAMP assay

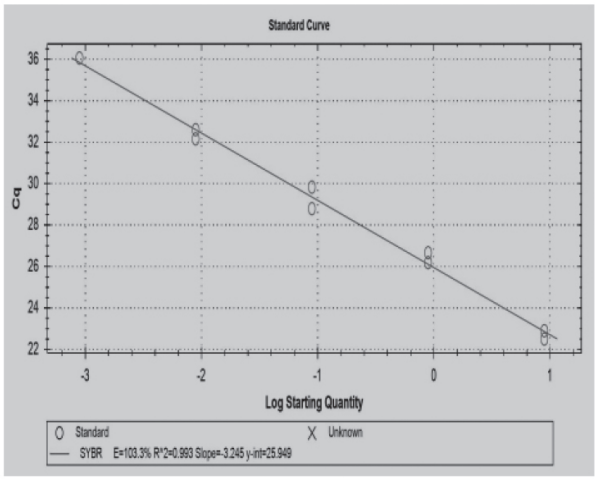


Fig. 6. Standard curve for IBDV in Real time PCR showing sensitivity is same as RT-LAMP

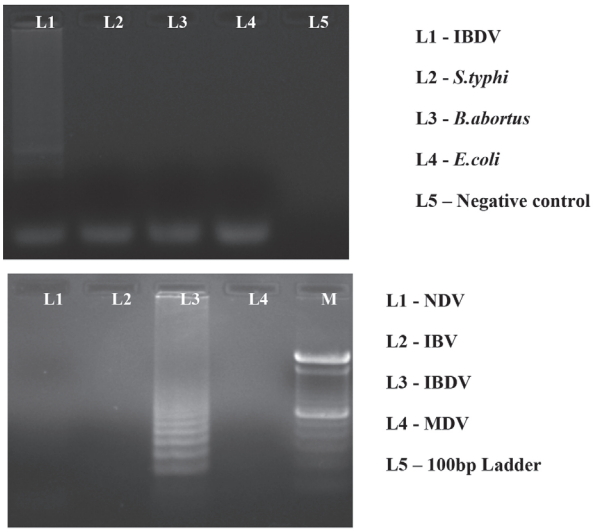


Fig. 7. Specificity of the RT-LAMP assay

CONCLUSION

Poultry production plays an important role in India and accounting about 70,000 crores to the national GDP of India (Chatterjee and Rajkumar, 2015). IBDV is an important viral pathogen that often causes severe economic losses to the poultry industry. Routine vaccination and disease surveillance are some of the critical measures needed to control this important pathogen. In spite of routine vaccinations, there are several instances of IBDV outbreaks leading to severe losses to poultry farmers. There are several instances of vvIBDV breaking through even high levels of maternal antibodies in commercial flocks. Although there are several methods to detect IBDV, availability of a simple and rapid assay that does not require highly sophisticated laboratory equipments could detect IBDV isolates belonging to different pathotypes could be very helpful especially under field conditions. Overall, the RT-LAMP technique developed targeting VP1 gene is a valuable alternative for the screening and sensitive detection of IBDV. This method can be performed with cost-effective equipment, requiring only a conventional water bath or heat block for incubation under isothermal conditions and hence can be used for filed level detection of IBDV.

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Conflict of Interest : The authors declare that they have no conflict of interest

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