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COMPARISON OF ANALYTICAL SENSITIVITY AND SPECIFICITY OF REVERSE TRANSCRIPTION LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (RT-LAMP) AND REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR) FOR DETECTION OF LOCAL PESTIVIRUS A

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ABSTRACT

The main aim of this study was to compare reverse transcription loop-mediated isothermal amplification (RT-LAMP) and reverse transcription polymerase chain reaction (RT-PCR) in order to determine their analytical sensitivity and specificity using local characterized Pestivirus A. In order to compare the sensitivity of RT-LAMP and RT-PCR, serial 10 fold dilutions containing 9.91×10^{10} to 9.91×10^{-2} copies of cDNA of Pestivirus A, were prepared and tested. RT-LAMP proved more sensitive and was able to detect 9.91×10^{0} copies of cDNA compared to RT-PCR (9.91×10^{1}). Both RT-LAMP and RT-PCR were found equally specific as no cross reaction with bovine herpes virus was observed. Present study showed that RT-LAMP assay is highly sensitive, specific, rapid and can be used as an alternative to conventional RT-PCR, for the detection of Pestivirus A.

Key words: Pestivirus A, Sensitivity, Specificity, RT-LAMP, RT-PCR.

INTRODUCTION

Bovine viral diarrhea virus (BVDV) is a single stranded positive sense RNA virus that causes lower milk production in cattle and has worldwide distribution (Ridpath 2010). The virus belongs to the family flavividiae and among the members of flaviviridae, bovine viral diarrhea virus is largest comprising about 12.5 kb. For translation of viral protein it contains internal ribosomal entry site present in 59 un-translated region of genome of BVDV. Thirty nine amino acids are coded by single open reading frame, these proteins are processed laterally into twelve functional proteins by both viral and host protease. These functional proteins include NS5B, NS5A, NS4A, NS4B, NS2, NS3, P7, E1, E2, Erns and Npro-C. The last encoded protein Npro-C is unique protease which is responsible for cleavage core and Npro protein. Actually Npro is cysteine protease and for proteolytic activity it requires cysteine. For polyprotein processing NS3 protease require NS4A as a cofactor (Lai *et al*. 2000). On the basis of genotyping, this virus has two genotypes that are BVDV 1 and BVDV 2 (Vilcek *et al*. 2001). Recently, international committee of taxonomy of viruses (ICTV) has developed a new classification of viruses on the basis of host independent manner, BVDV 1 has been named as Pestivirus A while BVDV 2 named as Pestivirus B (Smith *et al*. 2017). There are serious economic losses that occur due to this virus and various eradication programs are being conducting internationally (Duncan *et al*. 2016). In order to reduce the economic losses, cost effective diagnostic technique needed for control of bovine viral diarrhea virus (Moennig and Bencher, 2018).

The diagnosis of Pestivirus A is usually made by enzyme linked immunosorbent assay (ELISA) and reverse transcription polymerase chain reaction (RT-PCR) using specific primers. Due to advancement in diagnostic technology, RT-LAMP can also be used for the rapid detection of Pestivirus A. RT-LAMP is more specific and sensitive molecular technique as compared to RT-PCR (Fan *et al*. 2012). Loop mediated isothermal amplification (LAMP) is a well-known novel molecular technique developed by Notomi to amplify the DNA sequences (Notomi *et al*. 2000). This technique has been established efficiently for the detection of various infectious agents including bacteria and viruses affecting humans and animals, emerging pathogens having zoonotic concerns (Dhama *et al*. 2014; Dhama *et al*. 2017; Ramees *et al*. 2017). The main principle of this technique is the strand displacement using novel DNA polymerase and two to three pairs of designed primers, named as outer primers, inner primer and loop primers which are further divided into of forward outer and inner primers, reverse outer and inner primers, forward and reverse loop primers (Notomi *et al*. 2000). By using the loop primers, amplification reaction speed up and reduces upto half of the original LAMP. By the strand displacement technique, synthesis of DNA occurs due to

attachment of DNA strand loops with exception of loops that formed after inner primer hybridization (Nagamine *et al*. 2002). In this technique, four to six pairs of primers are used which target six to eight sites of the genome, so specificity of this technique increases as compared to conventional PCR (Enomoto *et al*. 2005). Amplification of the DNA occurs isothermally from 60°C to 65°C which gives benefit to this assay. Cauliflower-like structure is formed by the amplification of DNA, due to attachment of stem loops DNA strands with the various repetitive target gene sequence (Nagamine *et al*. 2001). Ladder like pattern appears on the agarose gel indicates the final end amplified product (Lee *et al*. 2012).

Moreover, visualization of the final end product can also be monitored by the indication of white precipitate of pyrophosphate as by products, which makes the reaction turbid. So, indication of turbidity in real time visualization shows the successful amplification of the DNA (Mori *et al*. 2001). When DNA is being synthesized, then a large quantity of pyrophosphate ions are released as by product. The divalent metallic ions react with the released ions and an insoluble salt is formed. The results can be visualized within one hour with the naked eye due to variation of fluorescence's intensity by the addition of a combination of calcein and manganese ions as a fluorescent dye to the reaction mixture (Tomita *et al*. 2008). As in this technique, amplification of DNA occurs at single temperature so a simple water bath or heat block can be used, due to which cost of this technique is also reduces as compared to other molecular techniques such as conventional PCR etc. for the diagnostic purpose (Chen *et al*. 2008). Another advantage of this technique is that amplification of the target DNA from the swab samples can be done directly without DNA extraction by just immersing the swab samples in the water bath (Higashimoto *et al*. 2008). In the samples, there are many inhibitory components which does not effect to the LAMP as compared to the conventional PCR. These were the perfect features of any test to be implemented in the developing world as well as in clinical laboratories (Francois *et al*. 2011).

Keeping in view the economic importance of BVD virus, this study was designed to develop a cost effective assay for the rapid detection of local Pestivirus A.

MATERIALS AND METHODS

Source of Virus: Characterized local Pestivirus A (MK084980.1) isolated from Holestein cattle during 2018 was used in this study. Bovine Herpesvirus 1 (BoHV-1) strain named IBRV HB06 were used as a negative control to observe any cross reaction.

Extraction of RNA: RNA was extracted by using commercially available QIAamp Viral RNA Extraction kit (QIAGEN GmbH, Germany) as per recommendations of manufacturer. RNA Concentration was checked through NANODROP 1000 (Thermo Scientific, USA) and stored at -80°C.

Synthesis of cDNA: Commercial cDNA Kit (NEB Inc., USA) was used for the synthesis of cDNA and reaction mixture of 20ul was synthesized which containing 6ul of total RNA , 2ul of D(T)23 VN (50uM), 10ul of M-MuLV Reaction Mix and 2ul of M-MuLV Enzyme Mix. The reaction mixture was placed at 42°C for 60min and 80°C for 5min. After synthesis of cDNA, 30ul of nuclease free water was dispensed to reaction mixture, which dilutes reaction mixture to 50ul. NANODROP 1000 (Thermo Scientific, USA) was used for determining purity and quantification of the extracted cDNA.

Calculating the number of copies of template: The number of copies present in 1ul of cDNA was calculated through calculator given in the link http://cels.uri.edu/gsc/cndna.html. The calculator based on the equation (Whelan *et al*. 2003) given as:

$$\text{number of copies} = \frac{(\text{amount of cDNA (ng)} \times 6.022 \times 10^{23})}{(\text{length of template (bp)} \times 1 \times 10^{9} \times 650)}$$

Reverse Transcription Polymerase Chain Reaction (RT-PCR): The RT-PCR of Pestivirus A was optimized by using previously described primers (Vilcek *et al*. 1994) (Table 01) and commercially available Green Taq Master mix (Thermo Fisher Scientific, USA). The reaction mixture of 25ul was prepared, containing 12.5ul of Green Taq Master Mix 2X , 9.5ul of nuclease free water, 1ul of Primer F ,V324 (20pmole/ul), 1ul of Primer R ,V326 (20pmole/ul) and 1ul of cDNA while no cDNA was added in the negative control. The reaction mixture was followed by one cycle of initial denaturation at 95°C for 5 minutes then followed by 40 cycles of denaturation at 95°C for 45 seconds, annealing at 56°C to 59°C for 60 seconds, extension at 72°C for 60 seconds and then followed by one cycle of final extension at 72°C for 7 minutes.

Sensitivity of RT-PCR: The sensitivity of RT-PCR was determined by serial 10 fold dilutions containing 9.91 x 10^{10} to 9.91 x 10^{-2} copies of cDNA; the 1ul of cDNA from each dilution were taken and put into the reaction mixture. The reactions mixtures were prepared as per described in previous section. The reaction mixture was followed by one cycle of initial denaturation at 95°C for 5 minutes then followed by 40 cycles of denaturation at 95°C for 45 seconds, annealing at 57°C for 60 seconds, extension at 72°C for 60 seconds and then followed by one cycle of final extension at 72°C for 7 minutes. The reaction mixture 'N' acts as negative control by adding the nuclease free water.

Specificity of RT-PCR: Specificity of RT-PCR was also determined by making three reaction mixtures 'D1', 'D2'

and 'N'. In 'D2' reaction mixtures, 1ul of bovine herpes virus DNA was added and in 'D1' 1ul of Pestivirus A cDNA was added, while 'N' reaction mixture acts as negative control by adding the nuclease free water. The reactions mixtures were prepared and incubated as per described in previous section.

Reverse Transcriptase Loop-mediated Isothermal Amplification (RT-LAMP): RT-LAMP was optimized by using previously published sets of primers (Aebischer *et al*. 2014) (Table 02), with conditions which targeting the 5'UTR region (Figure 01). Reaction mixture was prepared by using Warm Start LAMP kit (NEB Inc., USA) as per recommendations of manufacturer. Briefly, RT-LAMP was done in a reaction mixture of 25ul in PCR grade 0.2ml microfuge tube. Reaction mixture was containing 12.5ul of LAMP Master Mix 2X, 1ul of Primer F3 (2.5pmole), 1ul of Primer B3 (2.5pmole), 1ul of Primer FIP (25pmole), 1ul of Primer BIP (25pmole), 1ul of Primer LF (12.5pmole), 1ul of cDNA, 0.5ul of LAMP Fluorescent Dye 50X and 6ul of Nuclease Free Water while no cDNA was added in the negative control. Then optimization of RT-LAMP was done in eight reaction mixtures by changing the temperature. These reaction mixtures were placed at temperature from 61°C to 68°C. The reaction mixture 'N' acts as negative control by adding the nuclease free water.

Sensitivity of RT-LAMP: To determine the sensitivity of RT-LAMP, serial 10 fold dilutions of calculated quantity of cDNA from 9.91×10^{10} to 9.91×10^{-2} were performed; the 1ul of cDNA from each dilution were taken and put into the reaction mixtures. The reactions mixtures were prepared as per described in previous section. The reaction mixtures were incubated at 63°C for 60 min. Reaction mixture 'N' act as negative control by adding the nuclease free water.

Specificity of RT-LAMP: Specificity of RT-LAMP was determined by making three reaction mixtures 'D1', 'D2', and 'N'. In 'D2' reaction mixture, 1ul of bovine herpes virus DNA was added and in 'D1' 1ul of Pestivirus A cDNA was added, while 'N' reaction mixture acts as negative control by adding the nuclease free water. The reactions mixtures were prepared as per described in previous section. These reaction mixtures were incubated at 63°C for 60 min.

Analysis of Amplified Products

Real time visualization: RT-LAMP amplified products were visualized by naked eye under UV-Trans illuminator and see the fluorescent green color in positive cases.

Gel Electrophoresis: RT-LAMP and RT-PCR amplified products were also be analyzed by gel electrophoresis by preparing 1.6% agarose gel in 1X TAE (Tris-acetate-EDTA) buffer. PCR products band was measured by using 100bp DNA Ladder (Thermo Fisher Scientific, USA).

RESULTS AND DISCUSSION

RT-LAMP and RT-PCR were optimized for Pestivirus A using various annealing temperatures. The RT-LAMP product was observed like a ladder on the agarose gel after incubation at 63 °C for 1 hr (Figure 05 & 06). The RT-PCR amplified 288 bp band by targeting sequence of 5'UTR at 57°C (Figure 02). In order to compare the sensitivity of RT-LAMP and RT-PCR, serial 10 fold dilutions of calculated quantity of cDNA from 9.91×10^{10} to 9.91×10^{-2} were prepared and tested. RT-LAMP assay detected 9.91×10^{0} copies of cDNA (Figure 09& 10) as compared to RT-PCR that detected 9.91×10^{1} (Figure 04). Both RT-LAMP and RT-PCR were found equally specific as no cross reaction with bovine herpes virus was observed (Figure 03, 07& 08).

There are various techniques that have been developed for the identification of various infectious pathogens including viruses in clinical laboratories in the last two decades. Various types of serological tests including ELISA, are usually used for identification of infectious pathogens.

In the current study, the focus was to use newly emerging molecular technique such as reverse transcription loop-mediated isothermal amplification (RT-LAMP) as an alternative to RT-PCR. In this technique, isothermal amplification of the nucleic acid occurs. In our hands, RT-LAMP detected 9.91×10^{0} copies of cDNA as compared to RT-PCR that detected 9.91×10^{1}. This study showed that RT-LAMP has more sensitivity as compared to RT-PCR. This finding is in concordance with the results of research conducted earlier (Fan *et al*. 2012) in which RT-LAMP was found higly sensitive and capable to detect 4.67×10^{0} copies of BVDV RNA present in the sample as compared to RT-PCR which detected 4.67×10^{3} copies. Our study results are also in agreement with the results of another study where RT-LAMP was able to detect 10^{1} copies of RNA as compared to conventional RT-PCR that detected 10^{3} copies of BVDV RNA present in contaiminated commercial bovine sera samples (Zhang *et al*. 2014).

In this study, both assays used were found equally specific as no cross reaction was evidenced with bovine herpes virus. This finding is in accordance with results of research conducted earlier (Zhang *et al*. 2014) in which amplification of samples containing other closely related bovine pathogenic viruses and classical swine fever produced negative results. Our study results are also in agreement with the results of another study where no cross reaction with other bovine viruses were observed (Fan *et al*. 2012).

Figure 01. Location of LAMP primers on 5'UTR sequence of Bovine Pestivirus.

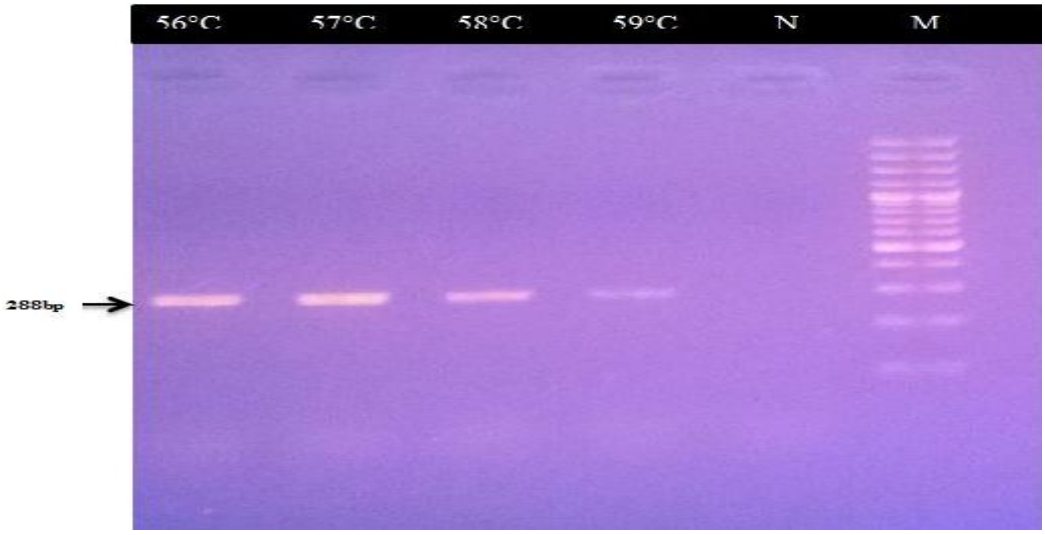

Figure 02. Optimization of RT-PCR for Bovine Pestivirus

Table 01. PCR Primers for Bovine Pestivirus.

Sr.	Name of Primer	Sequence (5' – 3')	Length (bp)
1.	V324	ATGCCCWTAGTAGGACTAGCA	21
2.	V326	TCAACTCCATGTGCCATGTAC	21

Table 02. LAMP Primers for Identification of Bovine Pestivirus.

Sr.	Name of Primer	Sequence (5' – 3')	Length (bp)
1.	BVDV1–F3	CATGCCCTTAGTAGGACTAGC	21
2.	BVDV1–B3	TTTTGTTTGTAWGTTTTGTATAAAAGTTCATT	32
3.	BVDV1-FIP (F1c-F2)	GGCRTGCCCTCGTCCACGTG-TGGATGGCTKAAGCCCTGAG	40
4.	BVDV1-BIP (B1c-B2)	TGATAGGRTGCTGCAGAGGCCCAC-ATGTGCCATGTACAGCAGAG	44
5.	BVDV1-LF	CGTCGAACCACTGACGACTAC	21

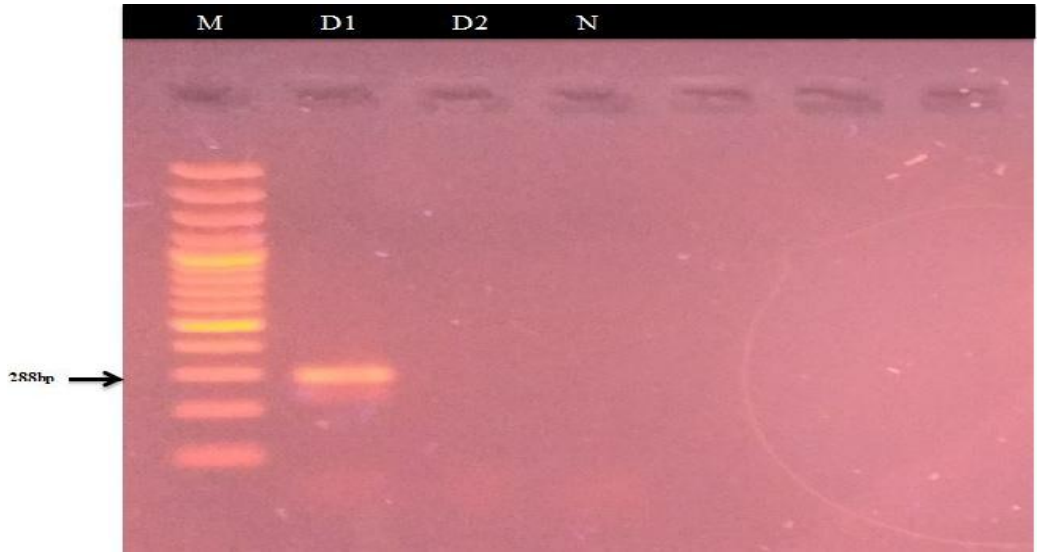

Figure 03. Specificity of RT-PCR for Bovine Pestivirus

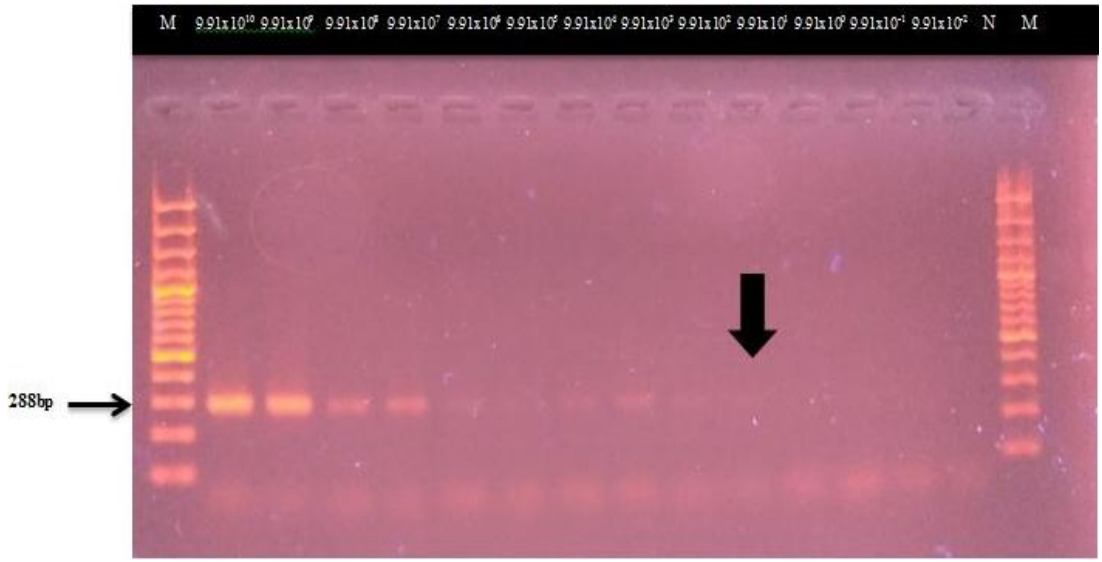

Figure 04. Sensitivity of RT-PCR for Bovine Pestivirus

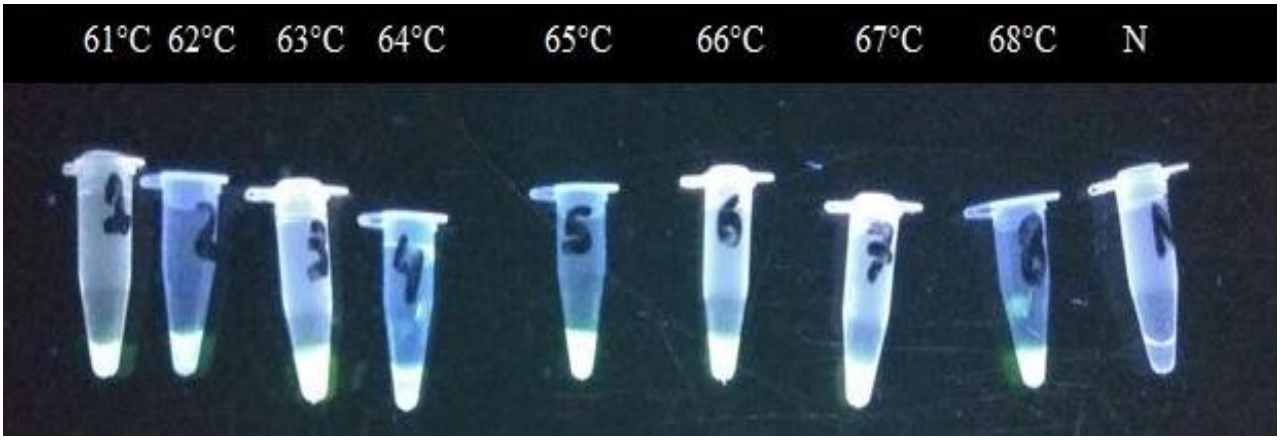

Figure 05. Optimization of RT-LAMP (Real time visualization of amplified RT- LAMP products under UV Trans-illuminator).

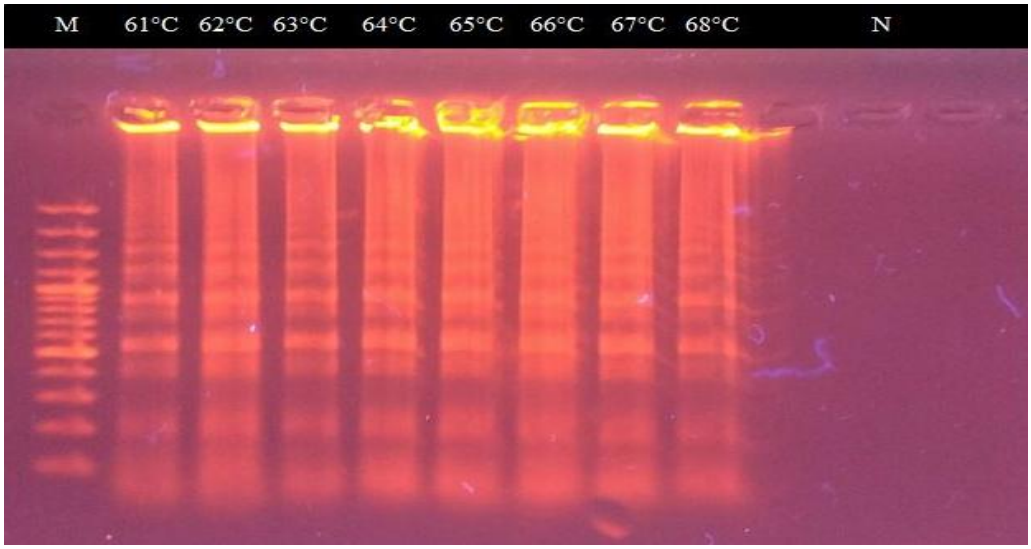

Figure 06. Optimization of RT-LAMP (Gel Electrophoresis)

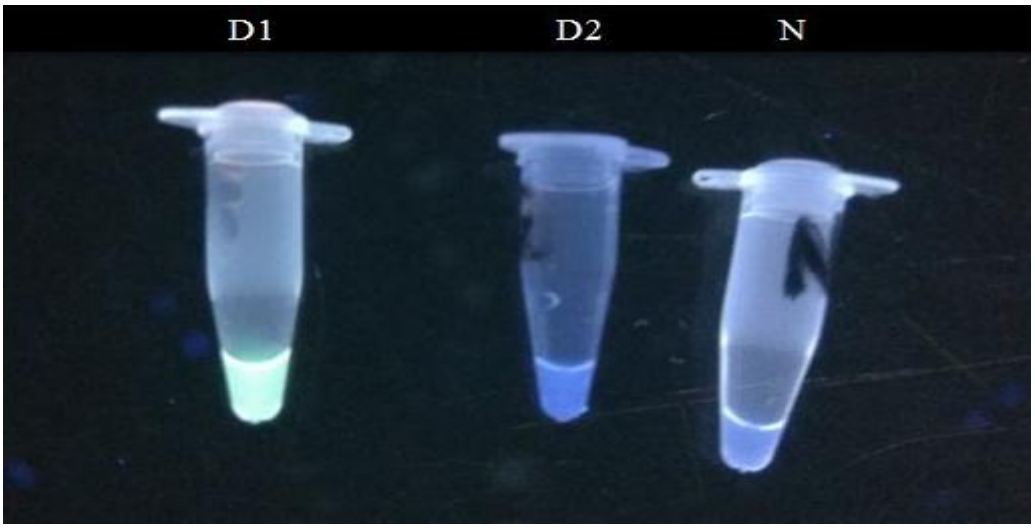

Figure 07. Specificity of RT-LAMP (Real time visualization of amplified RT- LAMP products under UV Trans-illuminator)

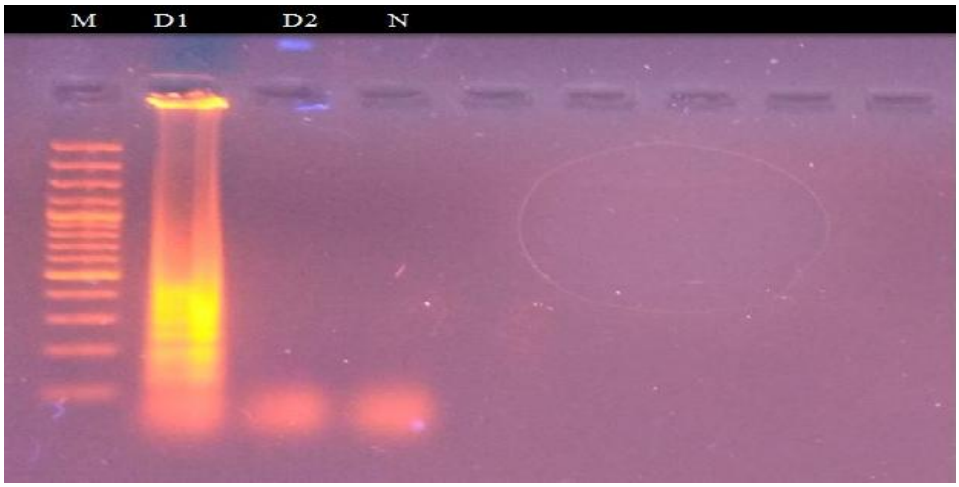

Figure 08: Specificity of RT-LAMP (Gel Electrophoresis)

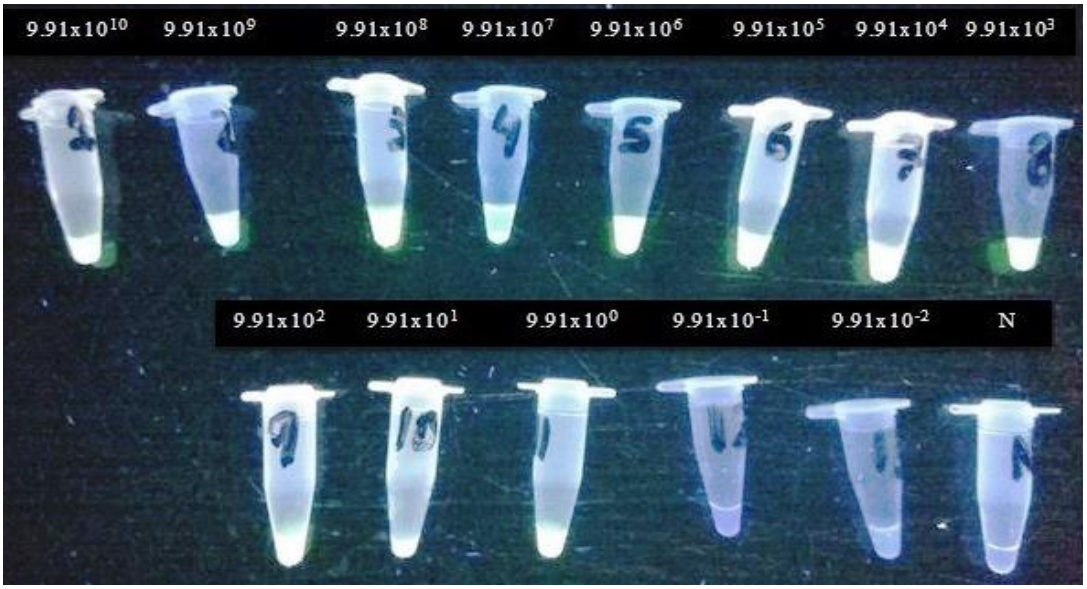

Figure 09. Sensitivity of RT-LAMP (Real time visualization of amplified RT- LAMP products under UV Trans-illuminator)

Figure 10: Sensitivity of RT-LAMP (Gel Electrophoresis)

Based on this study finding, it can be concluded that RT-LAMP can be performed in a simple water bath/heat block or thermocycler at single temperature and can be used in resource limited setting or in the field for the detection of Pestivirus A. RT-LAMP is the better choice as compared to RT-PCR, because it does not require any sophisticated equipment like thermocycler and can be performed rapidly (Dhama *et al*. 2014). Moreover, RT-LAMP has advantages over RT-PCR because amplification of RT-LAMP can be done in 30 min to one hour while RT-PCR takes 2-3 hours for amplification. In addition, there is less chances of cross-contamination because there is only one step for amplification. Furthermore, RT-LAMP is more specific as compared to RT-PCR because RT-LAMP reaction takes 4 to 6 distinct primers which reduce chance of non-specific amplification and incorrect target being mistakenly amplified. In last, only the nucleic acid of virus, whose amplification is to be done, amplified within the presence of other cellular and nucleic acids present in the sample. Therefore, based on this study results, it is concluded that the RT-LAMP assay, can be used as an alternative to conventional RT-PCR and, may be helpful for the molecular detection of Pestivirus A in an economical way and in limited time in the field.

REFERENCES

Aebischer, A., K. Wernike, B. Hoffmann and M. Beer (2014). Rapid genome detection of Schmallenberg virus and bovine viral diarrhea virus by use of isothermal amplification methods

and high-speed real-time reverse transcriptase PCR. J. Clin. Microbiol. 52(6): 1883-1892.

Chen, H., J. Zhang, D. Sun, L. Ma, X. Liu, X. Cai and Y. Liu (2008). Development of reverse transcription loop-mediated isothermal amplification for the rapid detection of H9 avain influenza virus. J Virol Methods. 151: 200-203.

Dhama, K., K. Karthik, S. Chakraborty, R. Tiwari, S. Kapoor, A. Kumar and P. Thomas (2014). Loop-mediated isothermal amplification of DNA (LAMP) – a new diagnostic tool lights the world of diagnosis of animal and human pathogens: A review. Pakistan J. Biol. Sci. 17(2): 151-166.

Dhama, K., N. Kumar, M. Saminathan, R. Tiwari, K. Karthik, M.A. Kumar, M. Palanivelu, M.Z. Shabbir, Y.S. Malik and R.K. Singh (2017). Duck virus enteritis (duck plague) - a comprehensive update. Vet. Quarterly. 37(1):57-80.

Duncan, A., G. Gunn and R. Humphry (2016). Difficulties arising from the variety of testing schemes used for bovine viral diarrhoea virus (BVDV). Vet. Rec. 178, 292.

Enomoto, Y., T. Yoshikawa, M. Ihira, S. Akimoto, F. Miyake, S. Suga, K. Suzuki, T. Kawana and Y. Nishiyama (2005). Rapid diagnosis of herpes simplex virus infection by a loop-mediated isothermal amplification method. J Clin Microbiol. 43: 951-955.

Fan, Q., Z. Xie, L. Xie, J. Liu, Y. Pang, X. Deng, Z. Xie, Y. Peng and X. Wang (2012). A reverse transcription loop-mediated isothermal amplification method for rapid detection of bovine viral diarrhea virus. J. Virol. Methods. 186(1): 43-48.

Francois, P., M. Tangomo, J. Hibbs, E.J. Bonetti, C.C. Boehme, T. Notomi, M.D. Perkins and J. Schrenzel (2011). Robustness of a loop-mediated isothermal amplification reaction for diagnosis applications. Ferns Immunol. Med. Mic. 62: 41-48.

Higashimoto, Y., M. Ihira, A. Ohta, S. Inoue, C. Usui, Y. Asano, and T. Yoshikawa (2008). Discriminating between varicella-zoster virus vaccine and wild-type strains by loop-mediated isothermal amplification. J. Clin. Microbiol. 46(8), 2665-2670.

Lai, V.C., W. Zhong, A. Skelton, P. Ingravallo, V. Vassilev, R.O. Donis, Z. Hong and J.Y. Lau (2000). Generation and characterization of a hepatitis C virus NS3 protease-dependent bovine viral diarrhea virus. J. Virol. 74(14): 6339-6347.

Lee, C., Y. Lin, C. Tsang, Y. Chung (2012). A loop mediated isothermal amplification (LAMP) assay for rapid detection of Anaplasma phagocytophilum infection in dogs. Turk. J. Vet. Anim. Sci. 36.

Moennig, V., and P. Becher (2018). Control of bovine viral diarrhea. Pathog. 7(1), 29.

Mori, Y., K. Nagamine, N. Tomita, T. Notomi (2001). Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. Biochem. Bioph. Res. Comp. 289: 150-154.

Nagamine, K., T. Hase and T. Notomi (2002). Accelerated reaction by loop-mediated isothermal amplification using loop primers. Mol. Cel. Prob. 16: 223-229.

Nagamine, K., K. Watanabe, K. Ohtsuka, T. Hase and T. Notomi (2001). Loop-mediated isothermal amplification reaction using non denatured template. Clin. Chem. 47: 1742-1743.

Notomi, T., H. Okayama, H. Masubuchi, T. Yonekawa, K. Watanabe, N. Amino and T.Hase (2000). Loop-mediated isothermal amplification of DNA. Nucleic Acids Res. 28(12): e63-e63.

Ramees, T.P., K. Dhama, K. Karthik, R.S. Rathore, A. Kumar, M. Saminathan, R. Tiwari, Y.S. Malik and R.K. Singh (2017). Arcobacter: an emerging food-borne zoonotic pathogen, its public health concerns and advances in diagnosis and control - a comprehensive review. Vet Quarterly. 37(1):136-161.

Ridpath, J. F. (2010). Bovine viral diarrhea virus: global status. Vet. Clin. North Am. Food Anim. Pract. 26(1): 105-121.

Smith, D.B., G. Meyers, J. Bukh, E.A. Gould, T. Monath, A.S. Muerhoff, A. Pletnev, R. Rico-Hesse, J.T. Stapleton, P. Simmonds and P. Becher (2017). Proposed revision to the taxonomy of the genus Pestivirus, family Flaviviridae. J. Gen. Virol. 98(8): 2106.

Tomita, N., Y. Mori, H. Kanda and T. Notomi (2008). Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products. Nat. Protoc. 3: 877-882.

Vilcek, S., D. Paton, B. Durkovic, L. Strojny, G. Ibata, A. Moussa, A. Loitsch, W. Rossmanith, S. Vega and M. Scicluna (2001). Bovine viral diarrhoea virus genotype 1 can be separated into at least eleven genetic groups. Arch. Virol. 146(1): 99-115.

Vilcek, S., A. Herring, J. Herring, P. Nettleton, J. Lowings and D. Paton (1994). Pestiviruses isolated from pigs, cattle and sheep can be allocated into at least three genogroups using polymerase chain reaction and restriction endonuclease analysis. Arch. Virol. 136(3): 309-323.

Whelan, J. A., N.B. Russell, and M.A. Whelan (2003). A method for the absolute quantification of cDNA using real-time PCR. J Immunol Methods, 278(1-2), 261-269.

Zhang, S.Q., B. Tan, P. Li, F.X. Wang, L. Guo, Y. Yang, N. Sun, H.W. Zhu, Y.J. Wen and S.P. Cheng (2014). Comparison of conventional RT-PCR, reverse-transcription loop-mediated isothermal amplification, and SYBR green I-based real-time RT-PCR in the rapid detection of bovine viral diarrhea virus nucleotide in contaminated commercial bovine sera batches. J. Virol. Methods. 207: 204-209.