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Rapid detection of infectious bursal disease by loop-mediated isothermal amplification for field analysis

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Summary

Infectious bursal disease (IBD) is an immunosuppressive, acute and highly contagious illness of growing-poultry stock infected with infectious bursal disease virus (IBDV). It is common in Pakistan, causing potential economic losses throughout the year. The objective of the study is to propose a rapid, sensitive and specific diagnostic tool, and compare it with existing commonly used reverse transcriptase polymerase chain reaction (RT-PCR) method for IBDV. Different primers were used for RT-PCR and reverse transcriptase loop-mediated isothermal amplification (RT-LAMP) to target the IBD virus. RT-LAMP primers showed prodigious specificity without cross reaction to the other animal pathogens. Moreover, RT-LAMP was found to have 10 times higher selectivity for IBDV identification as compared to RT-PCR. RT-LAMP detected 9.2% more field samples than RT-PCR. Sequences of PCR products were determined and phylogenetic analysis of research isolates revealed its maximum similarity with indigenous and Indian IBDV isolates. RT-LAMP was found to be simple, specific, less laborious and a better technique as compared to RT-PCR for quick analysis. In general, RT-LAMP was declared positive on observing turbidity or adding fluorescence staining reagent such as SYBR Green I. The options of direct use of field sample homogenate and viewing directly the peaks in the graph shown on a monitor/laptop have made it much more convenient and time saving than gel based RT-PCR.

Key words: Detection, IBDV, Poultry farm diagnosis, RT-LAMP, RT-PCR

Introduction

Infectious bursal disease virus (IBDV) targeted B lymphocytes at immature stages, in the bursa of Fabricius resulted in significant immunosuppression (Müller *et al*., 1979; Umar *et al*., 2016) in young birds known as infectious bursal disease (IBD) (Khan *et al*., 2017). The fate of immunosuppression due to depletion of B lymphocytes led chickens to opportunistic pathogens and resulted in vaccination failure (Ingrao *et al*., 2013). The infected birds may be considered as a commendable spreader for other viral pathogens (Sarwar *et al*., 2015). Infectious bursal disease virus, with double-stranded segmented RNA genome, is highly prone to sudden mutations eliciting the different genotypic and phenotypic diversity (Xu *et al*., 2015). Therefore, the presence of IBDV throughout the world (Berg, 2000) and complex evolution of this highly mutating virus has been observed after its first discovery over 55 years ago (Aini, 1990). For prevention and proper control of this disease, a simple, reliable, rapid as well as sensitive method for the diagnosis of IBDV is required (Mori and Notomi, 2009).

Loop-mediated isothermal amplification (LAMP) is a novel technique that can rapidly amplify the target genome under isothermal conditions. Specifically, four to six primers are designed to recognize six to eight regions of DNA template, making the technique highly specific (Notomi *et al*., 2000). Two more primers, forward loop primer (F loop) and backward loop primer (B loop) are integrated if acceleration of reaction is required. This newly devised process has no denaturation step, which makes it efficient as compared to conventional polymerase chain reaction (PCR). It works under a constant isothermal temperature (60-65°C) as compared to different temperatures for denaturation, annealing and extension in PCR. Loop-mediated isothermal amplification is usually completed in 50-60 min while PCR takes 3-4 h based on different parameters for diagnosis of different samples (Notomi *et al*., 2000). There is a need for highly purified sample or template DNA for proceeding with PCR, otherwise impurities show false results (Dhama *et al*., 2014), while in case of LAMP, a robust and sensitive technique, samples can be integrated to the test (Francois *et al*., 2011).

Loop-mediated isothermal amplification has been effectively applied for quick and sensitive detection of IBDV and other important pathogens (Pham *et al*., 2005; Xu *et al*., 2009; Xue *et al*., 2009; Wang *et al*., 2011; Tsai *et al*., 2012; Dhama *et al*., 2014). In this study, field

isolates are confirmed with RT-PCR and the same results analyzed with RT-LAMP. Further, both techniques are compared in terms of sensitivity and specificity for efficient identification of IBDV.

Materials and Methods

Collection of clinical samples

Total numbers of 76 pathological samples (bursa) were collected from suspected flocks presenting typical signs of IBD after post mortem (PM) conduction. These samples were collected throughout Punjab province, Pakistan. After proper labeling, received samples were stored at -70°C for further processing.

Preparation of bursal homogenates

Bursal homogenates were prepared by placing bursae in pestle and mortar, cut into small pieces using a sterile scalpel blade, and ground using a pestle. Then ground tissue was added to a 1.5 ml microfuge tube, freeze-thawed for 2 or 3 times, centrifuged and the supernatant was removed and stored at -70°C with 50 µg/ml streptomycin (Brown and Mims, 1995) and 50 IU/ml penicillin.

One step RT-PCR

RNA extraction

RNA was extracted by FavorPrep® Viral Nucleic Acid Extraction kit (Favorgen, Biotech Corporation, Taiwan) according to manufacturer's protocol. Total RNA was eluted in 40 µL elution buffer.

cDNA synthesis

Complimentary DNA (cDNA) was synthesized by using Fermantas Revert Aid First Strand cDNA kit. The extracted RNA (5 µL) was heated for 5 min at 65°C with 1 µL random hexanucleotide (0.2 µg/µL), chilled on ice and incubated at 42°C for 1 h, in a 20 µL reaction containing 5 µL of 5X reaction buffer, 1 µL Moloney-murine leukemia virus (M-MuLV) reverse transcriptase (RT), 1 µL (20 U) and 2 µL of 10 mMdNTPs mix.

Polymerase chain reaction (PCR)

The synthesized cDNAs were used as templates for PCR for amplification. A pair of primers (Forward; CAG GGT CAG GGC TAA TTG TCT TTT, Reverse; CTC TGG GCC TGT CAC TGC TGT C) was used. The cycling conditions for designed oligonucleotides consisted of initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, annealing at 57.6°C for 30 s and extension at 72°C for 1 min. After completion of 30 cycles, a final extension at 72°C for 10 min was carried out. The primer pair elongated DNA amplicons of about 459 bp. PCR products were visualized under UV light following electrophoresis on a 1.5% agarose gel stained with Ethidium Bromide.

RT-LAMP reaction

Six primers specific for the *VP5* gene including two outer primers (F3 and B3), two inner primers (FIP and BIP) and two loop primers (F loop and B loop) which were reported by Wang *et al*. (2011) were used. The sequences of reported primers were enlisted in Table 1. The RT-LAMP reaction was carried out using RNA Amplification kit (Thermo Scientific Thermo, Cat # VO524). Each 25 µL reaction contained 10 µL of reconstituted reaction mixture, 5 µL of reaction buffer (5X), 1 µL of $MgSO_4$ (25 mM), 1 µL primer mix (40 pmol of each inner primer (FIP and BIP)), 20 pmol of each loop primer (F loop and B loop), 5 pmol of each outer primer (F3 and B3), and 1 µL template RNA and 7 µL nuclease free water. After initial optimization of reaction conditions under different temperatures (50-60°C) for various durations (15-60 min), 58°C incubation for 60 min yielded the best result, therefore, all the LAMP reactions in the study presented in "Results" were carried out at 58°C for 1 h, and recovered product was inactivated at 80°C for 5 min. The RT-LAMP product was inspected using ESE Quant Tube scanner (Fig. 1) and analyzed by 1.5% agarose gel electrophoresis (Fig. 2). The ladder-like pattern of amplified products was observed on the gel under UV light. It is due to formation of a mixture of stem-loop DNA with various stem lengths. Cauliflower-like structures with multiple loops were formed by annealing between alternative inverted repeats of the target sequence in the similar strand. In ESE Quant Tube

Table 1: List of names and sequences of all 6 LAMP primers (Wang *et al*., 2011)

Primer name	Sequence (5´-3´)
F3	CCTTCTGATGCCAACAACCG
B3	TAGCTGGCCGGTAGGTTC
FIP	CCTGTGTCCCCCACAGTCAAATTCCGGACGACACCCTAGA
BIP	CCTGGCTCAATTGTGGGTGCTCGTCAGGAGCATCTGATCGA
F loop	TCTGACCTGAGAGTGTGCTTC
B loop	CACTACACACTGCAGAGCAATG

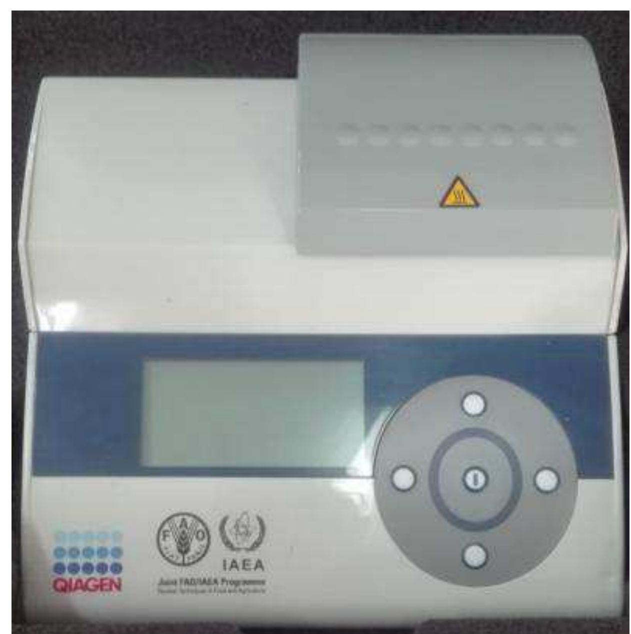

Fig. 1: ESE Quant Tube scanner. Instrument used for amplification of field samples in RT-LAMP

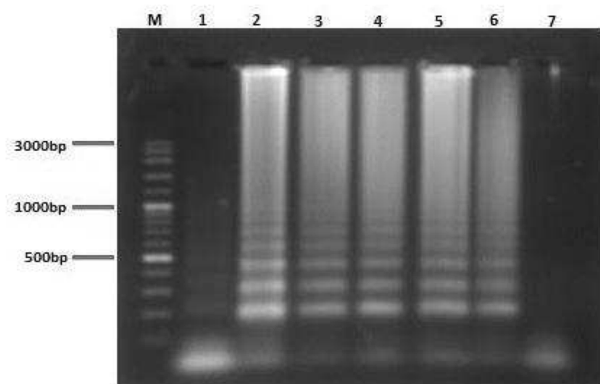

Fig. 2: Gel electrophoresis (1.5% agarose) of RT-LAMP products of 10-fold serial dilutions of isolated RNA samples of IBDV. M: 100 bp DNA ladder. Lane 1: Negative control (FMDV RNA sample), Lane 2: Positive control (original isolated IBDV RNA sample) contained RNA-35.71 ng/µL, Lanes 3-7: 10-Fold serial dilutions of isolated IBDV RNA as 10^{-1} dilution contained RNA-3.57 ng/µL, 10^{-2} dilution contained RNA-357 pg/µL, 10^{-3} dilution contained RNA-35.7 pg/µL, 10^{-4} dilution contained RNA-3.57 pg/µL, 10^{-5} dilution contained RNA-357 fg/µL (negative dilution)

scanner the assay was considered as positive if the amplification curve is generated within 40 min. For gel electrophoresis, 10 µL of inactivated amplified product of reaction was used for separation on 1.5% agarose gel, stained with Ethidium Bromide, and photographed under a UV transilluminator.

Results

Detection of IBDV in clinical sample

RT-PCR was applied to diagnose IBD in field isolates. From a total of 76 samples, collected from different regions, 56 were detected positive for IBDV (Table 2). RT-LAMP boosted 63 specimens from stock. For better results, target samples were amplified at different temperatures. The slight change in temperature improves the results.

To evaluate the diagnostic ability of RT-LAMP assay, supernatant of ground specimen can be used directly with this technique rather than RNA extraction from clinical samples as used for RT-PCR. Both RT-LAMP assay and conventional RT-PCR were applied for detection of IBDV in these samples. All the samples which were detected by conventional RT-PCR were also positive by RT-LAMP assay as shown in Table 2. These results indicate that RT-LAMP assay can replace conventional-RT-PCR. The LAMP mix, IBDV LAMP primers and extracted RNA of selected sample were mixed homogeneously in aliquot. The prepared samples were placed in each well of tube scanner (Fig. 1). The concentrated purified RNA, different dilutions along with positive and negative control, RT-LAMP reaction was carried out at 58°C for less than 1 h, the rising curves within 35-40 min were diagnosed IBDV positive birds. The results that appeared in graphical form were shown on the screen of monitor/laptop Fig. 3. The amplified products were inactivated at 80°C for 5 min. The same mixture was loaded in the wells of 1.5% agarose gel. Under UV light, elongated product showed a ladder like pattern (Fig. 2). The reaction containing sample of FMDV (negative control) showed no products. These results suggest that the LAMP assay with specifically designed primers is highly specific and convenient for detection of IBDV.

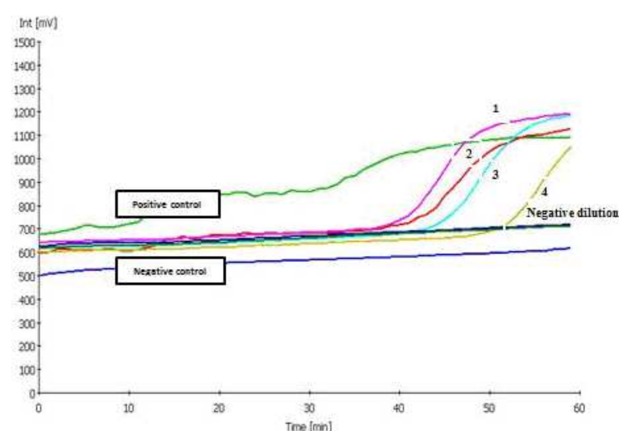

Fig. 3: Pattern of differences in amplification curves obtained during RT-LAMP reaction of 10-fold serial dilutions of isolated RNA samples of IBDV. Green: **Positive control** (original isolated IBDV RNA sample); Pink, red, light blue, light green, black: 10-Fold serial dilutions of isolated IBDV RNA as 10^{-1} (**1**), 10^{-2} (**2**), 10^{-3} (**3**), 10^{-4} (**4**), 10^{-5} **(negative dilution**); Dark blue: **Negative control** (FMDV RNA sample)

For estimation of specificity of the test, 76 samples were processed with both techniques and found that 9.2% more samples were detected positive with RT-LAMP. It shows that six sets of primers, amplifies more specifically than the pair of primers used in RT-PCR. It is also noted that there is no cross reaction with other RNA viruses. Foot and Mouth Disease Virus (FMDV), containing single strand of RNA is amplified to check cross reactivity with the same set of primers. No amplified product is detected in the first well of agarose gel (Fig. 2). It indicates that RT-LAMP is applicable to a wide range of IBD viruses. It can be strongly suggested for the broader applications of RT-LAMP. Among all, 13 samples (17.1%) were found negative with both nucleic acid detecting techniques.

Table 2: Detection of clinical samples through RT-PCR and RT-LAMP

Type of sample	No of samples	Conventional RT-PCR		RT-LAMP assay	
		Positive	Negative	Positive	Negative
Supernatant of raw sample (bursa)	76	56	20	63*	13

* 9.2% more samples were amplified by RT-LAMP

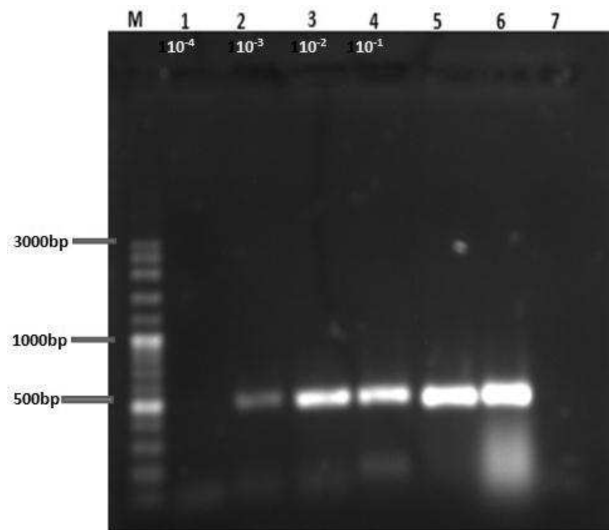

Fig. 4: Gel electrophoresis (1.5% agarose) of RT-PCR products of 10-fold serial dilutions of isolated RNA samples of IBDV. M: 100 bp DNA ladder. Lanes 1-4: 10-Fold serial dilutions of isolated IBDV-RNA as 10^{-4} (negative dilution) dilution contained RNA-3.57 pg/µL, 10^{-3} dilution contained RNA-35.7 pg/µL, 10^{-2} dilution contained RNA-357 pg/µL, 10^{-1} dilution contained RNA-3.57 ng/µL, Lane 5: Original isolated IBDV RNA sample contained RNA-35.71 ng/µL, Lane 6: Positive control, and Lane 7: Negative control (FMDV RNA sample)

In terms of the sensitivity, when the RT-LAMP was performed with a 10-fold dilution series of viral RNA from 10^{-1} to 10^{-6}, the IBDV viral RNA were detected from dilution 10^{-1} to 10^{-4}. However, when the one step RT-PCR was performed with the same dilution, the viral RNA was detected from 10^{-1} to 10^{-3} as shown in gel electrophoresis of RT-PCR products given in Figs. 2, 3 and 4, indicating that RT-LAMP is more sensitive than RT-PCR.

Specificity of RT-PCR and RT-LAMP techniques

In RT-PCR, single pair of primer is used for amplification against targeted region. It amplified the amplicon specifically. On the other hand, six primers were used to amplify 6-8 regions in RT-LAMP so it provides more specificity to the given field isolate. The cross reactivity of the IBDV primers was evaluated with other available common animal pathogens. Foot and Mouth Disease (FMD) sample was selected to check cross reactivity. RNA was extracted from FMD sample with standard protocol. It was also elaborated with IBDV primers in a LAMP reaction. It showed no cross reactivity. The FMDV template containing LAMP reaction mixture also considered as negative control under the same conditions.

Sensitivity of RT-PCR and RT-LAMP diagnostic tools

For evaluation of sensitivity of both molecular techniques, a positive sample (KY000833-IBDV-NIAB-PUN-PAK-037-2016) was selected from the list. It was successfully amplified by RT-PCR and RT-LAMP. RNA standards were prepared by 10-fold serial dilution from 10^{-1} to 10^{-6} and used as templates for one step RT-PCR and RT-LAMP. RNA in all dilutions was quantified by nano drop spectrophotometer. The lowest amount of RNA detectable under the conditions described above in RT-PCR and RT-LAMP was defined as the detection limit of both the techniques. The lowest detectable limit by RT-PCR is 10^{-3} whereas 10^{-4} is traceable quantity by other technique. Therefore, RT-LAMP is 10 times more sensitive (Figs. 3 and 4).

A graph was plotted between time consumed for amplification and pure/diluted RNA of field virus. It is observed that concentration of RNA/DNA is inversely proportional to time for positivity. If the quantity of viral genome is higher than it will take less time to raise the curve to show positivity (Fig. 5).

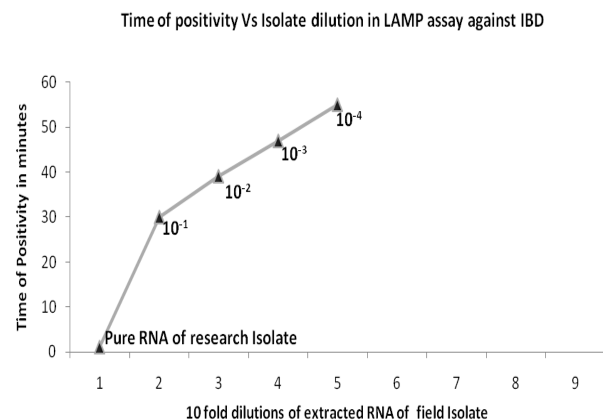

Fig. 5: Graph depicts the amplification of RNA/DNA (quantity) inversely proportional to time to shows its positivity

Phylogenetic analysis of IBDV isolate

The product of positive sample (IBDV-NIAB-PUN-PAK-037-2016) through both diagnostic methods is further processed for genetic sequencing. The product amplified through RT-PCR is re-amplified and recovered DNA is purified by gel electrophoresis with the help of kit (Biobasic, USA) protocol as described by the manufacturer. The prepared samples were sent to a third party (Macrogen, Inc. is a South Korean Public Biotechnology Company) for sequencing. Nucleotide sequence was assembled and edited using BioEdit software, version 7.0.0. Sequences originated from field isolates were edited with the “EditSeq” program of Lasergene software (DNASTAR, Madison, USA). Nucleotide sequence of gene was submitted to GenBank for registration. Accession number, KY000833 is allotted to the field isolate. The reported sequence was aligned by the Clustal W method with already reported putative sequences. Phylogenetic tree was constructed in MEGA6 using the neighbor-joining (NJ) method with the Kimura two-parameter evolutionary model (Kimura, 1980; Tamura *et al*., 2011; Tamura *et al*., 2013). Topologically reliability of the phylogenetic tree was inferred by the bootstrap method with 1000 replicates. A phylogenetic tree was plotted with tested isolate to determine the genetic relationship with reported sequences of local

available vaccinal strains, Pakistani isolates and circulating vvIBDVs of other countries. It is observed that recovered vvIBDV shows maximum similarity with Indian IBD viruses. Common vvIBDVs are found in Indo-Pak. It showed the emergence of a new wild type which was not incorporated in field vaccines. In Fig. 6 there is development of independent clade of strains of field vaccines.

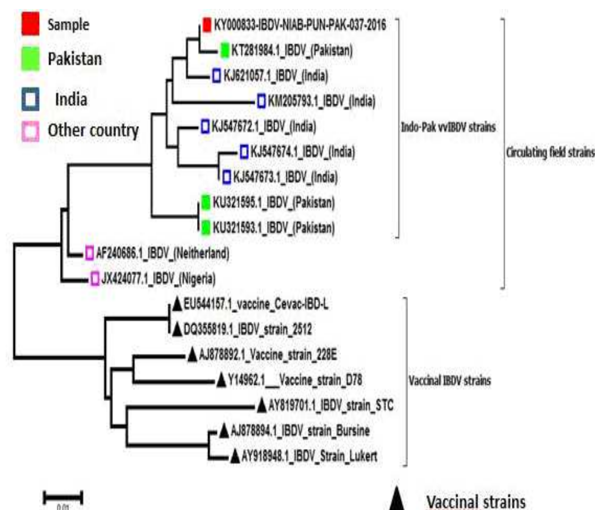

Fig. 6: Phylogenetic tree calculated by the NJ method based on the hypervariable domain of the *VP2*, *capsid* gene of IBDV serotype 1 strains using nucleotide alignment created in Clustal W, MEGA 6. Research sample and other isolates bearing following accession numbers: KY000833-IBDV-NIAB-PUN-PAK-037-2016; KT281984.1 (Pakistan); KU321595.1 (Pakistan); KU321593.1 (Pakistan); AY819701.1 (IBDV strain STC); AJ878892.1 (Vaccine strain 228E); Y14962.1 (strain D78); EU544157.1 (Cevac-IBD-L); DQ355819.1 (IBDV strain 2512); AJ878894.1 (strain Bursine); AY918948.1 (strain Lukert); KJ547672.1 IBDV (India); KJ547674.1 IBDV (India); KJ547673.1 IBDV (India); KJ621057.1 IBDV (India); KM205793.1 IBDV (India); JX424077.1 IBDV (Nigeria); AF240686.1 IBDV (Neitherland)

Discussion

In this study LAMP is compared with RT-PCR as a more sensitive and rapid assay for clinical diagnosis of IBDV. All samples are collected from field outbreaks on the basis of clinical signs and PM conduction. From a total of 76 samples RT-LAMP detects 63 samples positive for IBDV. From these 63 samples, 56 samples were the same samples which were detected as IBDV positive by conventional RT-PCR. However samples which are considered IBDV negative by RT-PCR are detected positive through RT-LAMP (Mansour *et al.*, 2015). It is the advanced technique which amplifies minute quantity (3.57 pg/µL) of viral nucleic acid in clinical samples to detectable level with high specificity and no cross reactivity (Dhama *et al.*, 2014). The RT-PCR detected up to 35.7 pg/µL viral genome whereas RT-LAMP detection level is 3.57 pg/µL.

It was found that RT-LAMP is considered a simple, specific, sensitive and adaptable assay as compared to RT-PCR for rapid IBDV identification in clinical samples. This method can easily be used for the diagnosis of IBDV at farm level. It amplifies the viral genome with more sensitivity and specificity as 2-3 pairs of primers are used against more conserved targeted regions for amplification (Nagamine *et al.*, 2002). There is no need of thermo cycler and other heavy instruments. Only a small easily portable instrument (ESE Quant Tube scanner) can perform the complete procedure for amplification. This technique has the unique feature that it may use complementary DNA directly for amplification or RNA of concerned virus. In case of RNA viruses, by adding only RT enzyme, a few copies can be amplified into millions successfully. There was no need of fluorescent dye i-e SYBR Green I, a fluorescent dsDNA intercalating dye used for visual detection, change in color of mixture (Parida *et al.*, 2008) Table 3.

Comparative evaluation in terms of sensitivity of RT-LAMP with one step RT-PCR was analyzed by preparing 10-fold serial dilution of known positive IBDV-RNA sample (Wang *et al.*, 2011) RNA was quantified in each dilution by nano drop. RT-LAMP detected IBDV upto 10^{-4} of dilution of RNA which is one serial dilution more than detected by RT-PCR. It indicates that RT-LAMP is 10 times more sensitive as compared to RT-PCR. It is confirmed by gel-based electrophoresis with both diagnostic tools. In RT-LAMP the amplified products gives ladder like band pattern (Fig. 2) which co-relates with the number of rising positive curves in the graphical presentation Fig. 3.

The negative detected field samples in RT-PCR may have less amount of genetic material (RNA/DNA) which is not in detectable limits or the quantity of viral genome may be lost during the extraction process. The amount of

Table 3: Comparison of LAMP (used in routine), RT-PCR and LAMP (performed in lab)

Characteristics	LAMP (used in routine)	RT-PCR	LAMP (performed in lab)
Amplification time	1 h	3-4 h	Less than 1 h
One step technique	Yes	No (multiple steps involved)	Yes
Specific and sensitive technique	Yes	Yes	Yes
Sensitive than RT-PCR	100 times	-	10 times
Can amplify without template extraction	Yes	No	Yes
Simple/low cost amplification tool	Yes	No	Yes
Portable to Farm level	Yes	No	Yes
Required florescent dye/UV light	Yes	Yes	No
Declaration of positive results	Turbidity or showing color by adding fluorescent dye	Band on 1-2% agarose gel in electrophoresis apparatus	* Observing the rising pattern of graph curves

* The results can be viewed directly from the graph as shown on the screen of monitor (Fig. 2). This graph advances towards an end point at x-axis after completion of each cycle. The weight of tube scanner is less than 1 kg

extracted RNA (IBDV) of field isolate was not enough to initiate the RT-PCR reaction. Different extraction protocols and kits should be tried for improving the extraction efficacy to solve this critical issue. There might be mutation in the field isolate at primer binding sites, resulting in mismatch at available binding site of genetic sequence. The single change in nucleotide at the binding site of primer affects the amplification of template (Kwok *et al*., 1990). In LAMP there are more primer pairs working at amplification regions so slight mutation does not affect the reaction. Routine turbidity in the aliquot is an indication of amplification so there is no need for verification by gel electrophoresis. By plotting standard curve the quantity of amplified product or time required for amplification can be determined (Mori *et al*., 2004) (Fig. 5). For viral detection, conventional RT-PCR is the best diagnostic approach but it has the disadvantage that it is purely a laboratory-based diagnostic technique. For analysis of field samples RT-LAMP method is more convenient than RT-PCR and can be done either by using a fluorescence staining reagent such as SYBR Green I or by just observing the graphical peaks (this study). Therefore, a well-equipped laboratory, well-trained staff, and multiple reaction steps are not needed, which makes the RT-LAMP assay more suitable to large-scale field investigations. During transportation of suspected IBD samples, the IBDV concentration decreases rapidly in unfavorable environmental conditions. It is double stranded RNA virus and affected greatly by temperature fluctuation (Johnson, 1990). If the sample is shipped or transported from far away regions to lab for evaluation of field isolate, the titre of virus may reach below the detectable level. The presence of subclinical (sc) IBDV (Del-E strain) results in major economic losses to the poultry industry (Jackwood *et al*., 2016). Therefore, early diagnosis should be performed with more sensitive and specific diagnostic techniques (Elankumaran *et al*., 2002). Moreover, this technique can be applied widely for other infectious diseases by designing specific primers.

Phylogenetic tree is built with the help of sequences available in GenBank which shows more similarities with research isolate. The vaccinal strains are also incorporated to evaluate the declared isolate. The constructed tree using NJ method (Saitou and Nei, 1987) showed that vvIBDVs matches 99% with Pakistani and Indian circulating IBD viruses.

In developing countries the resources are quite limited for new developmental projects. The friendly use of LAMP technique allows adaptability to field conditions and works well in progressive countries with resource-limited laboratories. However, this diagnostic tool is still underutilized in the field of veterinary diagnostics despite its huge capabilities (Mansour *et al*., 2015).

Prevention of disease is the primary goal in animal husbandry and it is becoming harder due to continuous genetic variability in already existing pathogens or emergence of new diseases and pathogens day by day. Rapid diagnosis is the only way for in time treatment. In summary, RT-LAMP is highly specific, very efficient and more sensitive than RT-PCR for the identification of IBDV and it is more convenient and cost effective than conventional RT-PCR. Due to less weight of ESE Quant Tube scanner, it is easily portable along with laptop by a single operator in field for diagnosis of prevalent diseases.

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