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Development of TaqMan probe- based RT-qPCR assays for detection of BoHV-1 latency in Bovine

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Bovine herpesvirus-1 (BoHV-1) is a major pathogen that affects cattle and buffaloes all over the world and is responsible for mid-to-late abortions, genital disease, respiratory disease, infectious pustular balano-posthitis, infected pustular vulvovaginitis, and infectious bovine rhinotracheitis. It is a member of the family Herpesviridae, genus Varicellovirus and subfamily Alpha herpesvirinae (Ostler and Jones 2023). In ganglionic neurons of the peripheral nervous system, BoHV-1 develops lifelong latency after acute infection. The development of latency is a distinctive trait of the virus. The only gene locus that is highly expressed in latently infected neurons is the latency-related (*LR*) gene locus, which also encodes a number of proteins and at least two micro-RNAs (Brock *et al*. 2020). Latent viruses produce latency-related proteins that prevent apoptosis in latently infected cells (Mweene *et al*. 1996, Ostler and Jones 2023).

During latency, only LR-RNA is highly expressed (Inman *et al*. 2004). *In situ* hybridization localizes LR-RNA to the nucleus of the latently infected neurons (Kutish *et al*. 1990). The *LR* gene is translated antisense to the bICP0 mRNA and is 2 kbp in length (980 bp of promoter and 1180 bp of transcribed region) (Kutish *et al*. 1990). The *LR* gene sequence consists of two open reading frames (ORFs) (ORF1 and 2) and two reading frames that lack an initiating ATG (RF-B and C) (Ostler and Jones 2023).

Most of the time, the virus-carrying animals go unreported, continue to be quiet shedders of the pathogen, and serve as potent sources of infection for other farm animals. As a result, the disease continues to spread unchecked throughout the ecosystem and severely impacts livestock (Office International des Epizooties (OIE) 2004). The illness often does not pose a life-threatening threat, but additional bacterial infections could complicate the scenario.

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Trigeminal ganglia (TG) are the main location of latency for BoHV-1 (Rock *et al*. 1987) making it challenging to detect latency in live animals. However, it was noted that peripheral blood lymphocytes and tonsillar lymphoid cells also exhibited latency (Mweene *et al*. 1996). To the best of our knowledge, no research work has been done to correlate the sero-prevalence and latency in PBMC's. Therefore, keeping in view the emerging nature of the disease and its economic importance, impact on livestock and peculiar expression pattern of virus, present work was designed for detection of BoHV-1 latency in bovines using rapid TaqMan based Real-time PCR.

Primers and probes: For designing primers and probes, LAT specific ORF-1 and miRNA (bhv1-miR-B5) were selected using multiple sequence alignment of published sequences found in the NCBI GeneBank database. Primer3 and the miRNA Design Tool, were used to create the primers and probes for the LAT-specific ORF-1 and miRNA, respectively (Table 1 and 2).

Synthetic positive control: ORF-1 and microRNA sequences were synthesized, cloned into a pUC57 vector, and commercially obtained. This recombinant plasmid served as a positive control. Following transformation into E. coli (DH5 alpha cells), plasmid extraction utilized the QIAprep® Spin Miniprep Kit (Qiagen) following manufacturer's protocol. Plasmid DNA concentration and quality were assessed using a spectrophotometer.

Optimization of TaqMan assay using ORF-1 and miRNA specific RT-qPCR primers: Primer optimization involved a checkerboard approach, varying the forward primer against a consistent range of 0.1 M to 0.7 M for the reverse primer and probe. The combination yielding the lowest Ct value determined the final primer concentrations for the RT-qPCR assay. Probe concentration for primer assessment was set at 0.125 μM. Conversely, for miRNA-specific RT-qPCR, the manufacturer-supplied, pre-optimized primer and probe combination was used, eliminating the need for further optimization.

The PCR plates were placed in the real time PCR detection system Step One plus ™ real time PCR thermal cycler with following optimised programme parameters of

Table 1. ORF-1 specific primers and probe

	Sequence (5'-3')	Reference gene	Amplicon size
LAT-ORF1-F	TGCTGACATACTGTCTTTCCGC	NC_001847.1	171 bp
LAT-ORF1-R	CTTGGTCGGAGTCAGAAGAGTC		
LAT-ORF-1-P	FAM-CGCCTCGACTCAACCTCCGCGTCCGTCT-MGB		

Table 2. miRNA specific primers and probe

mirRNA specific	Sequence (5'-3')	Amplicon size
Stem-loop	GTTGGCTCTGGTGCAGGGTCCGAGGTATTC GCACCAGAGCCAAC GAGTCG	
Universal reverse primer	GTGCAGGGTCCGAGGT	73 bp
Forward primer	TTGTTCTCGGAGGGCTCG	
Probe	UPL Probe 21 (reporter :FAM and quencher:MGB)	

50°C for 2min, 95°C for 10 min followed by 40 cycles of 95°Cfor 0.15 min and 60°C for 1.0 min.The results were analysed using 2.2 version software and the real time PCR products were analysed by agarose gel electrophoresis.

Analytical sensitivity and efficiency: The standard curve method assessed analytical sensitivity and limit of detection (LOD) for ORF-1 and miRNA-specific RT-qPCR. Ct values were plotted against initial DNA copies using a 10-fold dilution series (10^{-2} to 10^{-10}). The StepOne Plus™ Softwarev2.3 generated the standard curve, and RT-qPCR efficiency was calculated using the formula: PCR efficiency E % = (10^{-1} /slope – 1) × 100.

Screening of field samples: In EDTA vials, 50 peripheral blood samples were collected from BoHV-1 seropositive animals in Hisar, Haryana. Peripheral blood mononuclear cells (PBMC) were isolated from all samples, and total RNA and miRNA were extracted using TRIZOL and ReliaPrep miRNA cell and tissue miniprep system (Promega) following the provided protocols. All the isolated RNA and miRNA were tested using developed RT-qPCR assays followed by sensitivity comparison using conventional PCR.

RESULTS AND DISCUSSION

Optimization of RT-qPCR assays: First of all, primers and probes concentration were standardized. Out of all different combinations used, 0.5 µM concentration of forward and reverse primers of LAT-ORF-1 and 0.2 µM concentration of probe was observed as optimum.

Agarose gel analysis of RT-qPCR amplified product: The performance of primers and probes used for the detection of LAT specific ORF-1 and miRNA was checked by analysing the RT-qPCR amplified product on 2% agarose gel, a visible band was seen at 171 bp (Fig. 1) and 73 bp respectively (Fig. 2).

Analytical sensitivity and efficiency of the RT-qPCR assays: The sensitivity evaluation of the assay was done using ten-fold serial dilution of positive control (10^{-2} to 10^{-10}). The template was detected upto seventh dilution in both LAT specific ORF-1 and miRNA specific RT-qPCR assays. The copy number calculation was done using the quantified DNA concentration according to the following formula:

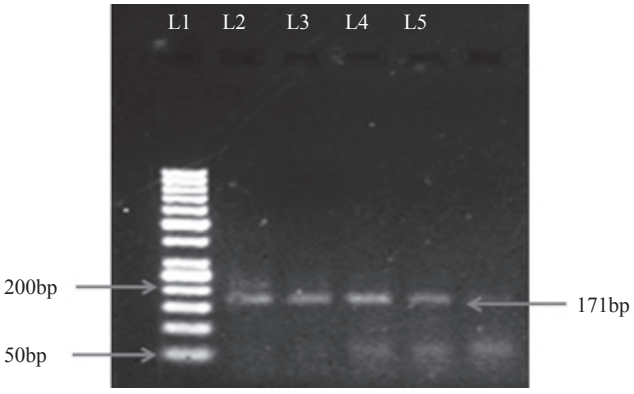

Fig. 1. RT-qPCR amplified product of LAT-ORF-1 (171bp) analysed on 2% agarose gel. (L1-50bp ladder, L2-L5: positive control).

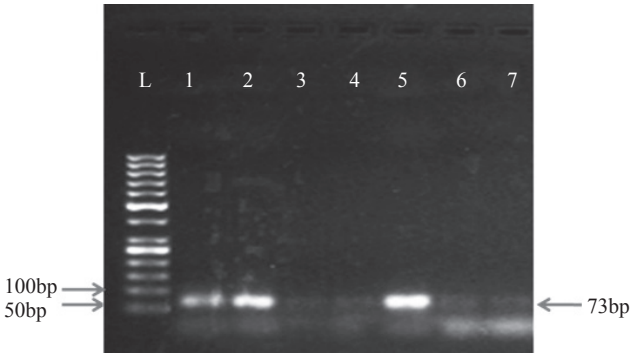

Fig. 2: RT-qPCR amplified product of miRNA (73bp) analysed on 2% agarose gel (L-50bp ladder, L1-L7: positive control).

Copy number = A × No. / Length in base pair × 10^9 × 650.

Where, A, DNA concentration in ng/ µl, No., Avogadro's number (6.022 × 10^{23}); Length, amplicon size. Therefore, the limit of detection (LOD) was found to be 460 copies in ORF-1-RT-qPCR and 170 copies in miRNA-RT-qPCR assay.

The efficiency for the ORF-1specific RT-qPCR was computed. A slope of -3.673 and a linear correlation (R^2) of 0.985 was found across various dilution ranges. By using obtained Ct values and slope, the amplification efficiency was found to be 93.5%. Similarly in the miRNA specific

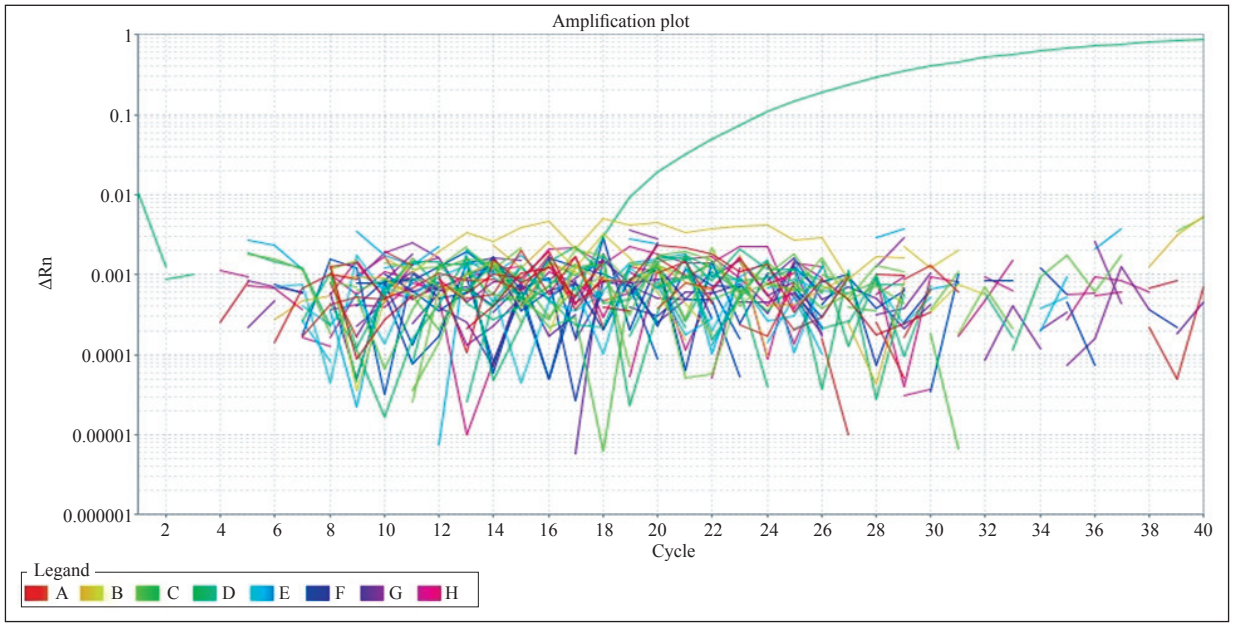

Fig. 3. Amplification plot of sero-positive PBMC samples using ORF-1 specificRT-qPCR.

RT-qPCR assay, slope was observed as -3.372 with the correlation coefficient (R^2) of 0.99. By using obtained Ct values and slope, the amplification efficiency was found to be 97.93%. The log amplification plot and standard curve were obtained based on the Ct value (Supplementary Fig. 1 and 2).

By observing all parameters, it was found that the present study was in accordance with the minimum information for publication of real-time PCR experiments (MIQE) guidelines, according to which the slope should be between -3.1 to -3.6 with 90-110% efficiency and an R^2 value of 0.99.

Evaluation of developed assays: A total of 50 sero-positive samples from male animals were analysed using developed ORF-1 and miRNA specific TaqMan probe based RT-qPCR assays. It was seen that none of the sero-positive samples were found positive in RT-qPCR assays specific to miRNA (bhv1-miR-B5) and ORF-1 transcript (Fig. 3 and Fig. 4). It might be due to the absence of BoHV-1 latency in PBMC of seropositive animals or the expression of LAT specific RNA or miRNA were beyond the LOD of developed assays, which is in agreement with previous study which reported that only a few leukocytes may contain BoHV-1from infected cattle and BoHV-1 can be transported via the bloodstream to target organs and confirm the tendency of the virus to

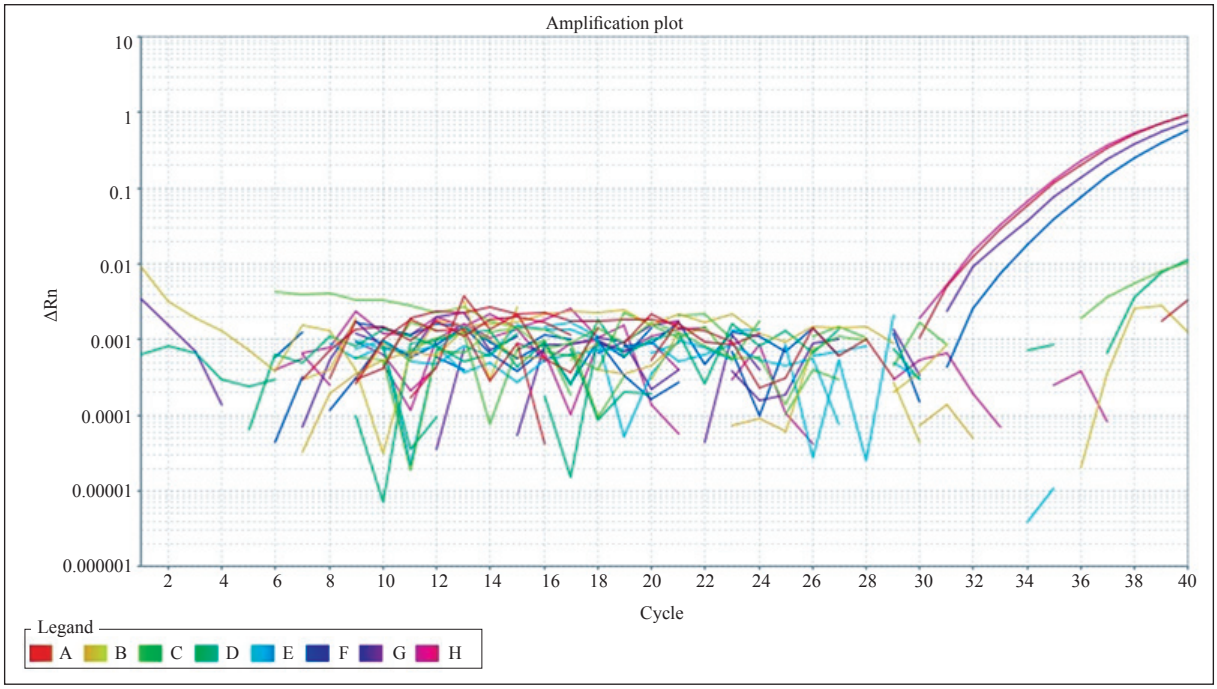

Fig. 4. Amplification plot of sero-positive samples generated in miRNA specific RT-qPCR.

accumulate in various lymphoid tissues of acutely infected animals (Tikoo *et al*. 1995). Additionally, a nested PCR study reported that the LR transcript is not abundantly expressed in the tonsils of latently infected calves or that only a small population of cells was persistently or latently infected (Winkler *et al*. 2000). However, other viral transcripts, but not the LR transcript, may be abundantly expressed in these tissues.

Further, it was reported that *LR* gene promoted tonsil tissue growth. However, following DEX therapy, the LR mutant was unable to reactivate from latency *in vivo*, leaving the tonsils with a permanent or latent infection (Perez *et al*. 2005). Sensitive PCR experiments proved that LR mutant DNA was present in the tonsils of acutely and latently infected calves, even though the LR mutant was not consistently found in the tonsils by *in situ* hybridization.

The major problem with BoHV-1 infections is the carrier status that develops in the animals, therefore the presence of antibodies in an animal may not always mean that it is actively infected. Because of this significant flaw in serum-based assays, the virus or its antigen must be detected in order to classify any animal as having the BoHV-1 virus (Anonymous 2008). The presence latency in tonsillar lymphoid cells and peripheral blood lymphoycts was reported by Mweene *et al.* 1996.

Due to latency, an animal once infected generates antibodies and remains infected for life; every time the animal is subjected to stress conditions (disease/change in weather/change in feed/place etc.); it excretes the virus possibly potentiating subsequent antibody formation (Chandranaik *et al*. 2013).As a result, an animal that tests positive for antibodies has the virus and will continue to do so whenever the right stimuli are present.

SUMMARY

Bovine herpesvirus-1 is highly contagious virus of cattle and buffaloes all over the world. It establishes lifelong latency in ganglionic neurons of the peripheral nervous system. Since, trigeminal ganglia are the main sites of latency, therefore, it is challenging to detect BoHV-1 in latently infected live animals. No research work has been done to correlate the sero-prevalence and latency in peripheral blood mononuclear cells (PBMC). The present study was designed to detect BoHV-1 latency related transcript or microRNA in peripheral blood mononuclear cells of sero-positive animals. The highly sensitive RT-qPCR assays based on TaqMan chemistry have been developed for the detection of transcripts of BoHV-1 latency. The limit of detection (LOD) of the assays for ORF-1 specific RT-qPCR and miRNA specific RT-qPCR was 460 copies and 117 copies respectively. The efficiency of the developed assays was 93.5% for ORF-1 and 97.93% for miRNA transcript. None of the PBMC samples of seropositive animals found positive for ORF-1 and miRNA transcripts in developed assays.The absence of latency specific transcripts in PBMC might be due to very low expression i.e. beyond the LOD of newly developed assays or absence of latency in PBMC of seropositive animal. However, further studies are required to establish the fact. To the best of authors knowledge, this is the first report of latency-specific RT-qPCR assay development and its application in PBMC of BoHV-1 seropositive cattle.

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