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Development and Standardization of Polymerase Spiral Reaction for Detection of Marek's Disease Virus

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ABSTRACT

Marek's disease (MD) is an infectious malignant-cell lymphoma proliferative disease caused by Marek's disease virus (MDV). As MD has a significant impact on the poultry industry, numerous molecular diagnostic techniques have been developed for the detection of MDV. Recently, polymerase spiral reaction (PSR) has been emerging as a molecular technique to diagnose infection with high sensitivity and specificity. In this study, a PSR diagnostic was standardized for the detection of MDV infection. For PSR standardization purposes, the highly conserved glycoprotein E (gE) gene of MDV-1 was cloned into pJET1.2 plasmid and the recombinant plasmid was used as a template for standardization of PSR. Evaluation of the sensitivity of PSR indicated that it can detect up to one target molecule. The PSR and real-time polymerase chain reaction (RT-PCR) have similar sensitivity batter than PCR-based sensitivity for detection of MDV-1. While evaluation of the specificity indicated PSR, RT-PCR, and PCR-based diagnostics have similar specificity for MDV-1 diagnosis.

HIGHLIGHTS

- PSR, and RT-PCR based diagnostic have similar sensitivity batter than PCR-based sensitivity for the detection of MDV-1.
- PSR, RT-PCR, and PCR based diagnostics have similar specificity for MDV-1 diagnosis.

Keywords: Marek's disease virus (MDV-1) , polymerase spiral reaction (PSR), sensitivity, and specificity

Worldwide, tremendous growth in the poultry industry has been achieved in the past few decades. Fast growth in this sector resulted in a significant concentration of poultry in some geographic areas (Torres *et al*., 2019). India ranks fourth for broiler and third for egg production in the world. Currently, with annual growth rates of 8.51 percent for broiler and 7.52 percent for egg production, and the poultry sector is one of the fastest-growing industries in the segment agriculture industry (Kolluri *et al*., 2021).

The poultry sector has been challenged by non-infectious and infectious diseases (Desta, 2021). Infectious diseases are broadly three types i.e. parasitic, bacterial, and viral. Many viral infections are zoonotic like avian influenza (AI) as well as variant strains of nonzoonotic like infectious laryngotracheitis (ILT), Newcastle disease (ND), Infectious bronchitis (IB), Marek's disease virus (MD), *etc* have been reported in poultry (Remignon, 2018). The MD caused by Marek's disease virus (MDV), a member of the *Herpesviridae* family is one of the big constraints on this industry (Torres *et al*., 2019). The MD in poultry is close to other neoplastic conditions like lymphatic leukosis caused by the Avian leucosis virus (ALV). When MDV pathogenesis was recognized in the late 1960s, it became possible to distinguish between MDV and ALV-induced neoplasia (Osterrieder *et al*., 2006).

Considering the economic significance of MD in the poultry industry, numerous molecular diagnostic techniques have been developed for the detection of MDV in poultry,

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including polymerase chain reaction (PCR), loop-mediated isothermal amplification (LAMP), and real-time PCR (RT-qPCR) (Woźniakowski and Samorek-Salamonowicz, 2014). However, there are a few drawbacks to PCR and RT-qPCR, such as labor-intensive, pricey equipment and lengthy, onerous laboratory processes (Ganguli *et al*., 2020). The choice of a correct and appropriate target for amplification, which should be a highly conserved area for a specific strain of microbe, is limited by LAMP-based diagnosis. Additionally, LAMP needs 4-6 primers to focus on less than ten primers in a narrow target sequence for high sensitivity and specificity. This makes it challenging to create precise primers for the LAMP assay (Wong *et al*., 2018). In light of the aforementioned limitations of many existing molecular diagnostic tests, the polymerase spiral reaction (PSR) can be a good alternative as a molecular diagnostic test for MDV. In light of this, this research was planned on the development and standardization of PSR-based diagnostics for MDV detection.

MATERIAL AND METHODS

Identification of conserved regions of MDV-1 genome

The complete genome of MDV-1 (*Gallid alphaherpesvirus* 2) reported from China was retrieved in FASTA format from GenBank, NCBI (Accession Number: MW531728). The retrieved sequence was analyzed by the nucleotide Basic Local Alignment Search Tool (BLASTn) to the identification of the conserved regions of the MDV-1 genome. Subsequently, gE (US8) gene (accession number ON698671), one of the most conserved regions of MDV-1 encoding glycoprotein E (partial) was used for BLASTn analysis to identify the most conserved regions for primer designing.

Cloning gE gene into pJET1.2 vector

Partial gE gene was amplified by using forward primer (5'- TTCGCTCGAGTCCATTAAACAAATAGAATC GCTAAG-3') and reverse primer (5'- AGCCGG ATCCTC AGTGGTATAAATCTAAGCGTTTC-3'). The reaction mixture (20 µl) was having 8.5 µl nuclease-free water (NFW), 10.0 µl 2X Master Mix (KAPA HiFi HotStart ReadyMix), 0.5 µl forward primer, 0.5 µl reverse primer, and 0.5µl MDV-1 genomic DNA. The PCR conditions having initial denaturation (95°C for 4 min) followed by 25 cycles of denaturation (98°C for 45 sec), annealing (55°C for 30 sec), and extension (72°C for 60 sec), and a final extension (72°C for 7 min). The amplified PCR product was confirmed by electrophoresis in agarose gel (1%) prepared in 1X TAE followed by visualization using UV Trans-illuminator.

The PCR product gene (~1180 bp size) was purified using Gene JET Gel Extraction Kit. Purified DNA was ligated into pJET1.2 vector (100ng/ul) using T4 ligase. The ligated product was transformed into competent *E.coli* (DH5α) cells. The transformed cells were spread on Luria-Bertani (LB) agar plate having 50 µl/ml ampicillin. The plate was incubated overnight at 37°C. One colony was selected from and incubated in the 5 ml LB broth (with 50 µl/ml ampicillin) for 16-18 hrs at 37°C in a shaker incubator (200 rpm). Plasmid from bacterial culture was isolated using GeneJET Plasmid Miniprep kit. Isolated plasmid (recombinant gE-pJET1.2) was confirmed by PCR and also by *Sal*I/*Hind*III restriction double digestion.

Primers designed for PCR/ real-time PCR (RT-PCR) and PSR

The primer pair for PCR/ RT-PCR was manually designed with the help of Oligo Explorer software. The primers were analyzed using the OligoAnalyzer Tool of online available Integrated DNA Technologies (IDT). The PCR primers with extra stuffer sequences at the 5' end of oligonucleotides were used as PSR.

Optimization of temperature and time for diagnostic PSR

PSR reactions using PSR primers were performed at 60º, 62º, 64º, 65º, 66º, 68º, and 70ºC (for 60 minutes) for optimization of PSR temperature. Reaction mixtures (25 µl) were having 8.0 µl NFW, 2.5 µl Buffer (10X), MgSO4 (100mM), 1.5 µl forward primer, 1.5 µl reverse primer, 1.5 µl dNTPs (10 mM), 4.0 µl Betaine, 1.0 µl Bst II DNA polymerase (8 U/ µl) and 1.0µl gE-pJET1.2 plasmid (10^5 molecule/µl). The amplified products were checked by agarose gel (2.5%) electrophoresis and also by visual detection by adding 1 µl 1:10 diluted SYBR Green I (10,000 × concentrated in DMSO).

For optimization of the duration of PSR, PSRs were performed for 30 min, 45 min, 60 min, 75 min, and 90 min at 65º C. Amplified products were checked by agarose gel (2.5%) electrophoresis and also by visual detection by adding 1 µl 1:10 diluted SYBR Green I (10,000 × concentrated in DMSO).

Sensitivity of diagnostic PSR

For estimation of sensitivity, several PSRs with different copy numbers of recombinant gE-pJET1.2 plasmid (as template) were performed. The molecular weight of gE-pJET1.2 plasmid was estimated using the online available Sequence Manipulation Suite tool (https://www.bioinformatics.org/sms2/dna_mw.html). The nanodrop spectrophotometer measured the concentration of gE-pJET1.2 plasmid in 'µg/ml'. The µg/ml was converted into 'molecule/µl' unit for recombinant plasmid. Subsequently, gE-pJET1.2 plasmid was diluted to seven different concentrations i.e. 10^5, 10^4, 10^3, 10^2, 10^1, 10^0, and 10^{-1} molecule/µl. One µl each of tenfold serially diluted gE-pJET1.2 plasmid DNA having 10^5, 10^4,10^3, 10^2, 10^1, 10^0 (1), and 10^{-1} molecules were used as templates in PSR reactions at 65º C for 60 min. The amplified products were checked by agarose gel (2.5%) electrophoresis and also by visual detection by adding 1 µl 1:10 diluted SYBR Green I (10,000 × concentrated in DMSO).

Specificity of PSR

The specificity of PSR was evaluated by using gE-pJET1.2 plasmid (10^5 molecule/µl), NTC, and also genomic DNA of fowl pox virus, ILTV, and MDV-1. One µl of each of above mentioned nucleic acids was used in PSR. The amplified products were checked by agarose gel (2.5%) electrophoresis and also by visual detection by adding 1 µl 1:10 diluted SYBR Green I (10,000 × concentrated in DMSO).

Sensitivity of RT-PCR, and PCR

For evaluation of sensitivity, one µl each of tenfold serially diluted DNA having 10^5, 10^4,10^3, $10^{2,}$ $10^{1,}$ 10^0 (1), and 10^{-1} molecules were used as templates in RT-PCR reactions. Reaction mixtures (25 µl) were having 9.5 µl NFW, SYBR Green master mix (2X), 1.0 µl forward primer (2.5 pmol/µl), 1.0µl reverse primer (2.5 pmol/µl), and 1.0µl template. For each dilution of DNA and NTC, triplicate real-time PCR experiments were carried out. The RT-PCR conditions having initial denaturation (95°C for 5 min) followed by 40 cycles of denaturation (95°C for 5 sec), annealing and extension (55°C for 30 sec), one cycle of melting curve analysis (Ramp from 65 to 95°C). The data acquisition was carried out during the annealing and extension step of RT-PCR. The result of RT-PCR was analyzed by the linear regression curve method using GraphPad Prism software for efficiency calculation as described by Svec *et al* (2015) using the following formula:

$$Efficiency\ (\%) = (10^{-1/slope} - 1) \times 100$$

The sensitivity of PCR was evaluated by using seven different concentrations of gE-pJET1.2 plasmid as templates. One µl of seven different concentrations i.e. 10^5, 10^4, 10^3, 10^2, 10^1, 10^0 and 10^{-1} molecule/µl, and also NTC were used in PCR reactions. The reaction mixtures (25 µl) were having 19.5 µl NFW, 2.5 µl 10X buffer, 1.0 µl 25mM $MgCl_2$, 0.5 µl forward primer, 0.5 µl reverse primer, 1.0 µl 10 mM dNTPs, 0.5µl *Taq* polymerase, and 0.5 µl template. The PCR conditions having initial denaturation (95°C for 3 min) followed by 35 cycles of denaturation (94°C for 30 sec), annealing (55°C for 30 sec), and extension (72°C for 30 sec), and a final extension (72°C for 7 min).

Specificity of RT-PCR, and PCR

The specificity of RT-PCR and PCR were evaluated by using gE-pJET1.2 plasmid (10^5 molecule/µl), NTC, and also genomic DNA of fowlpox virus, ILTV, and MDV-1 as template. One µl of each of above mentioned nucleic acids was used in RT-PCR and PCR.

RESULTS

Identification of conserved regions of MDV-1

Conserved regions of the MDV-1 genome were identified by BLASTn tools available at NCBI using *Gallid alphaherpesvirus*-2 (MDV-1) complete genome (Accession Number: MW531728; China) as a reference sequence. The BLASTn result indicated more than 99.6 % identity with other 46 complete genome sequences of MDV-1 isolates. The graphic summary of BLASTn result indicated that most regions including the glycoprotein E

(gE) gene (between 161454 and 162947 bp) of MDV-1 genome has high alignment score (Fig. 1).

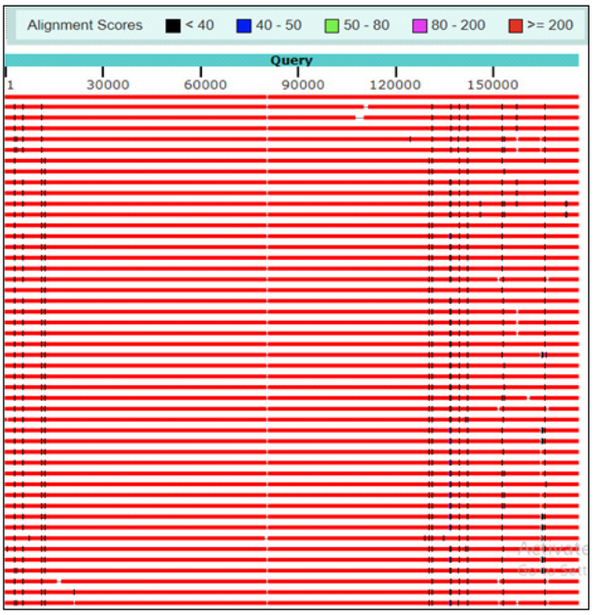


Fig. 1: Graphic summary of BLASTn analysis MDV-1 complete genome

Cloning of the gE gene of MDV-1

Partial gE gene (-1180 bp) was amplified using high-fidelity DNA polymerase and the amplified product was cloned into pJET1.2 vector. The recombinant (gE-pJET1.2) plasmid was confirmed by PCR. A specific band of PCR product of approximately ~l.2 Kbp size in agarose gel electrophoresis confirmed recombinant plasmid (Fig. 2A). Subsequently, confirmation of recombinant plasmid was performed by *Sal*I and *Hind*III restriction enzymes digestion. Two specific bands of 638 bp and 3521 bp size in agarose gel confirmed the recombinant plasmid (Fig. 2B).

The recombinant plasmid was isolated using GeneJET Plasmid Miniprep kit having 141 ng/µl concentration and 1.81 $OD_{260/280}$. The molecular weight (1284.55 KDa) and concentration (141ng/µl) of gE-pJET1.2 plasmid were used for estimation in the unit "molecule/µl'. Manual calculation indicated that plasmid stock had 6.61×10^{10} molecule/µl. Subsequently, the stock plasmid was diluted to achieve the concentration of 10^5, 10^4, 10^3, 10^2, 10^1, 10^0, and 10^{-1} molecule/µl.

Primer designing

Based upon bioinformatics (BLASTn) analysis, a conserved region of 171 bp size in the gE gene (Table 1) was selected for primers design. Primers were designed manually using Oligo Explorer software and analyzed by Oligo Analyzer Tool of online available Integrated DNA Technologies (IDT) (Table 2).

Table 1: Nucleotide sequence of gE gene (16-186; partial) for designing of primers (the lower case indicated regions where primer bind)

5'gaatcgctaagtctgaatggagTTCCGAATATATTCCTATCTACGAAAGCAAGTAACAAGTT
GGAGATACTAAATGCTAGCCTACAAAATGCGGGTATCTACATTCGGTATTCTAGA
AATGGGACGAGGACTGCAAAGCTGGATGTTGTTGTGgttggcgttttgggtcaa3'

Table 2: Primers used for PCR /real-time PCR

Primer	Forward	Reverse
Sequence	5'- GAA TCG CTA AGT CTG AAT GGA G -3'	5'- TTG ACC CAA AAC GCC AAC -3'
Complement	5'- CTC CAT TCA GAC TTA GCG ATT C -3'	5'- GTT GGC GTT TTG GGT CAA -3'
Length (Position within gE gene)	22 (16-37)	18 (169-186)
GC content	45.50%	50%
Melt temp	53.1 ºC	54 ºC
Molecular weight	6823.5 g/mole	5421.6 g/mole
Extinction coefficient	223800 L/(mole.cm)	172100 L/(mole•cm)
nmole/OD260:	4.47	5.81
µg/OD260:	30.49	31.5

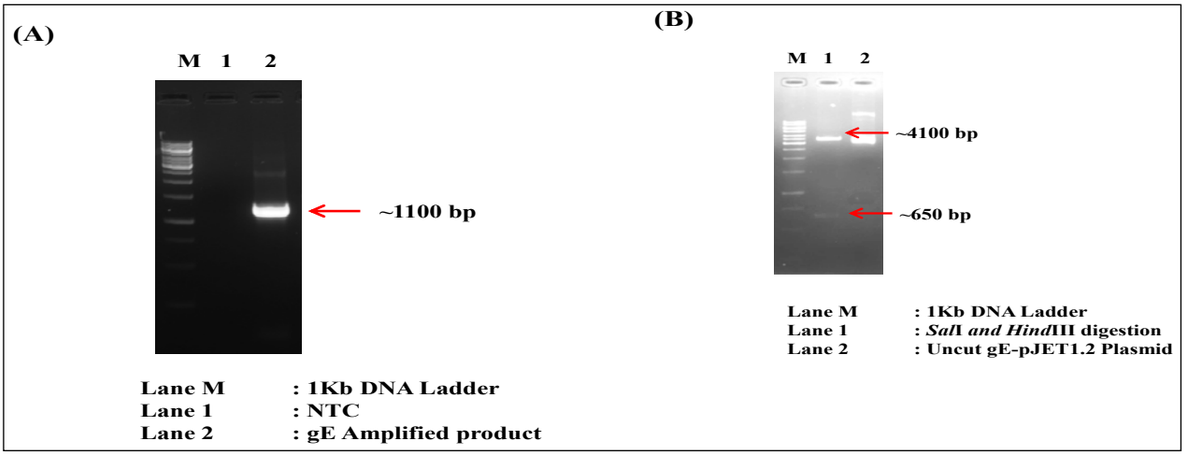


Fig. 2: Confirmation of recombinant gE-pJET1.2 plasmid by PCR (A) and RE digestion

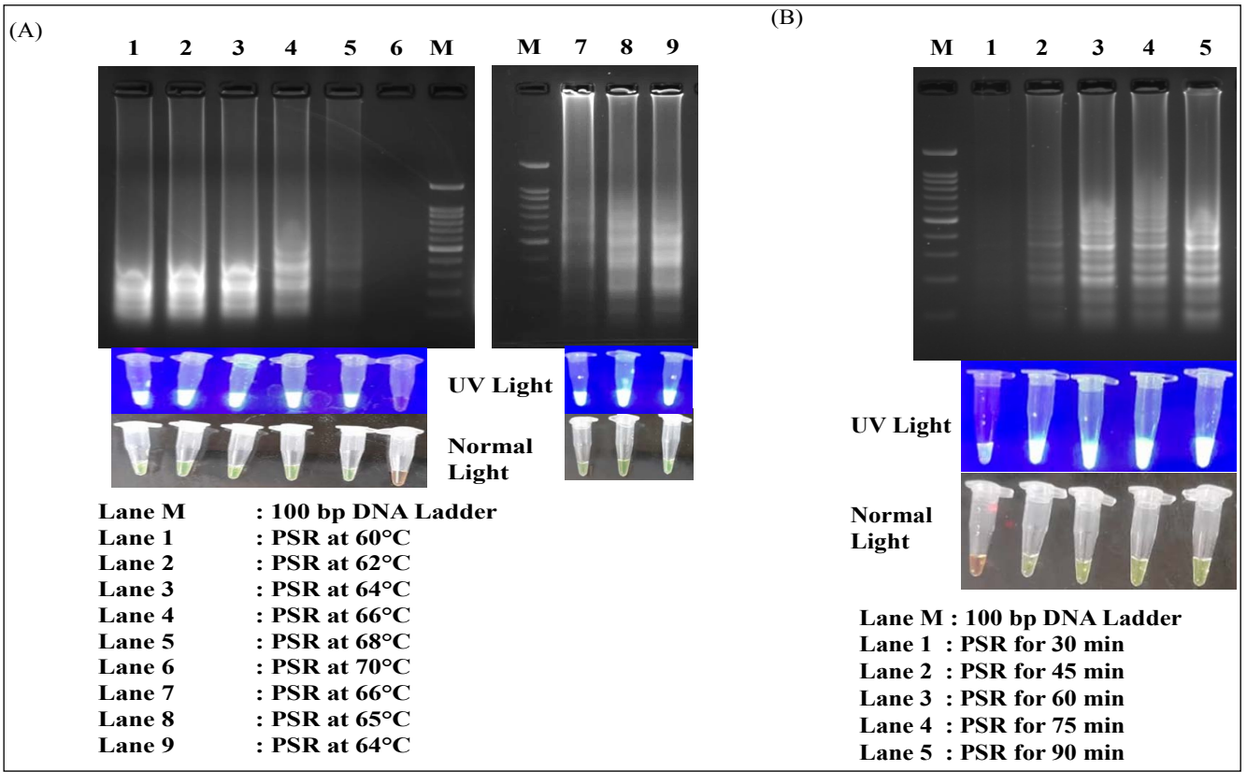


Fig. 3: Optimization of PSR reaction temperature **(A)**, and time **(B)**

Optimization of reaction temperature and time of PSR

The reaction temperature for PSR was standardized by keeping P'SR reactions at six different temperatures i.e. 60°, 62°, 64°, 66°, 68°, and 70°C for 60 min. The reaction at 64°C was having brightest DNA bands while at 66°C the most distinct laddering pattern bands were observed in agarose gel (Fig. 3A). Subsequently, another gradient PSR at 64°, 65°, and 66 °C was carried out. The PSR at 65 °C was having the brightest and most distinct DNA laddering bands (Fig. 3A).

The reaction time for PSR was optimized by keeping PSR reactions for 30 min, 45 min, 60 min, 75 min, and 90 min at 65°C. The PSR reaction of 30 min was not having any band or laddering bands while the reaction of 45 min was having light laddering bands. Reactions kept for 60 min 75min, and 90 min were having distinct DNA laddering bands (Fig. 3B).

PSR Sensitivity

The sensitivity of PSR was evaluated by using 10^5, 10^4, 10^3, 10^2, 10^1, 10^0, and 10^{-1} molecules of recombinant plasmid as template, and also NTC was also kept to rule out the non-specific result. The agarose gel electrophoresis image indicated laddering bands of PSR in which 10^5, 10^4, 10^3, 10^2, 10^1, and 10^0 copy number gE-pJET1.2 plasmid were used as template, while no specific laddering bands of DNA were observed in 10^{-1} molecule template molecule and NTC in agarose gel (Fig. 4).

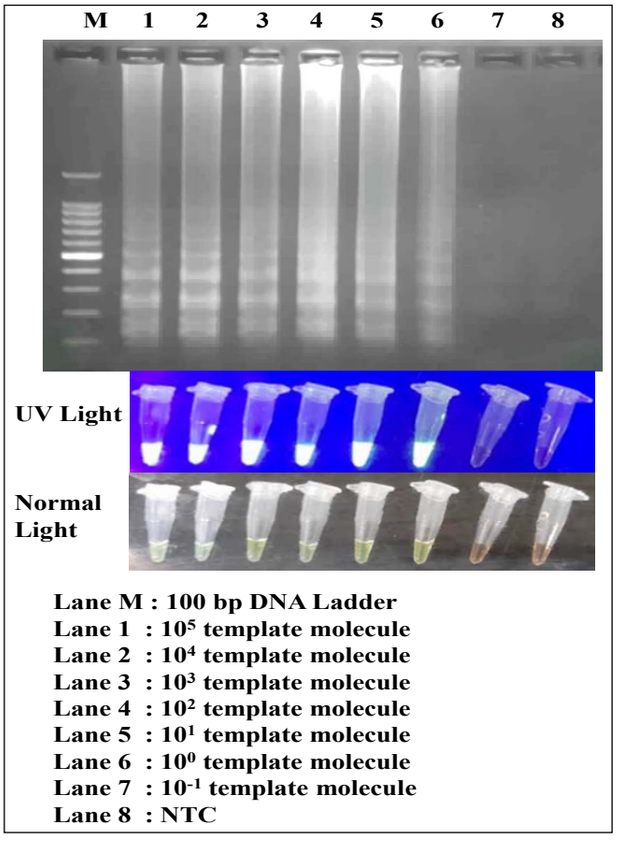


Fig. 4: PSR sensitivity

PSR Specificity

The PSR specificity was evaluated by using recombinant plasmid as positive control DNA and genomic DNA from MDV-l, FPV, and ILTV were used as test samples. The NTC reaction was also performed in PSR to rule out non-specific amplification. Agarose gel electrophoresis indicated laddering bands of DNA in PSR reactions with positive control and MDV-1 DNA as template. PSR reactions with FPV and ILTV DNA and also NTC were not having any band (Fig. 5).

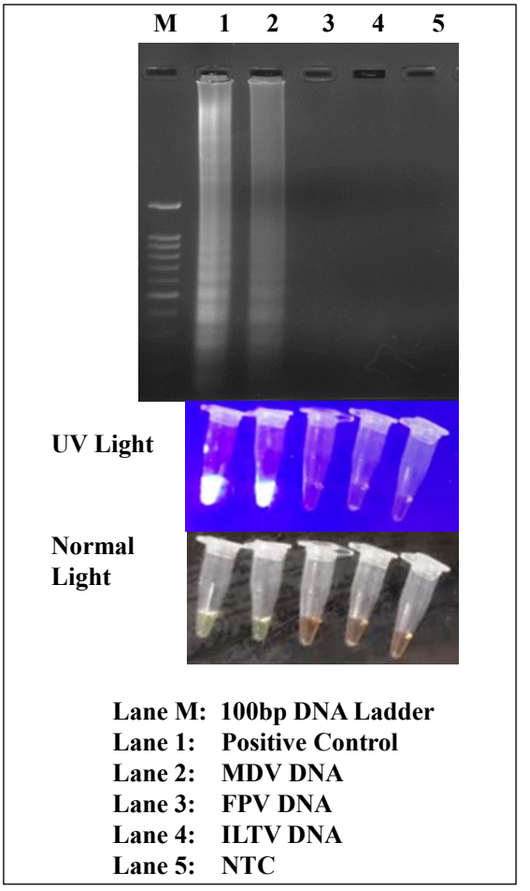


Fig. 5: PSR specificity

Sensitivity of RT-PCR and PCR

The sensitivity of real-time PCR was evaluated by using 10^5, 10^4, 10^3, 10^2, 10^1, 10^0, and 10^{-1} molecules of recombinant plasmid (gE-pJET1.2) as template and also

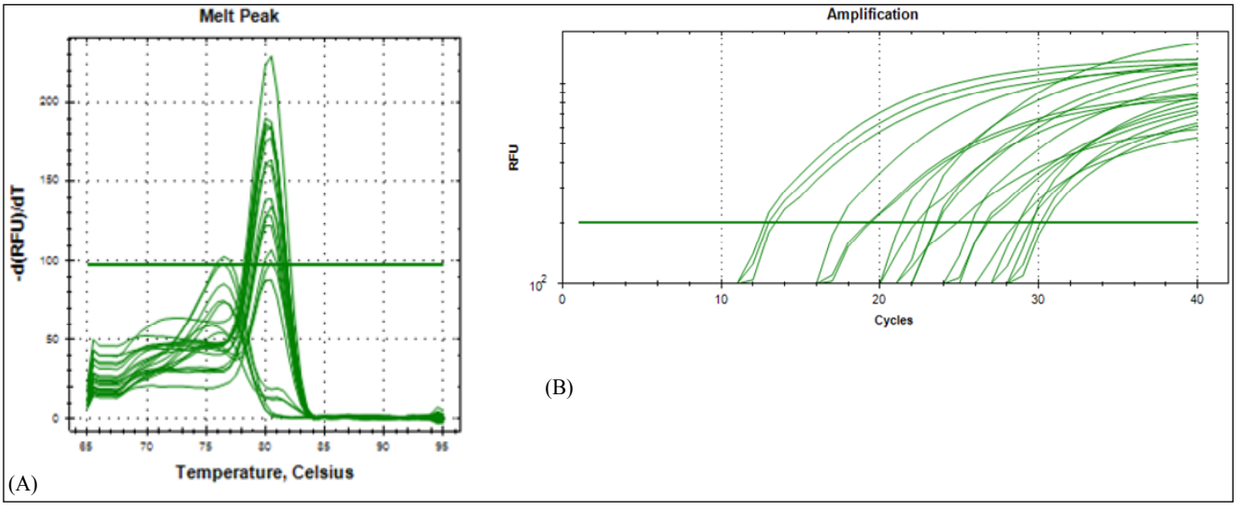


Fig. 6: Sentitivity plot of real-time PCR melting curve analysis **(A)**, and amplification plot **(B)**

NTC to rule out the non-specific result. The melting curve analysis indicated specific amplification in 10^5, 10^4, 10^3, 10^2, 10^1, and 10^0 gE gene DNA molecule reactions, while no specific amplification was observed in 10^{-1} molecule and NTC (Fig.6A). Using C_t values (Fig. 6B and Table 3) and copy numbers, a graph was plotted using GraphPad Prism software (Fig. 7). The slope of graph was = -2.943 + 0.2129 (Mean + Standard Deviation) [P value = < 0.0001]. The efficiency of RT-PCR was calculated as described by Svec *et al.* (2015). In this study, the efficiency of real-time PCR was 118.6%.

Table 3: C_t values of real-time PCR using different copy number of target DNA

DNA Molecules	C_t			Mean	Standard Deviation
10^5	12.70	12.97	13.37	13.01	0.275
10^4	17.37	19.25	19.38	18.67	0.918
10^3	22.25	24.83	21.31	22.80	1.488
10^2	22.68	23.53	23.63	23.28	0.426
10^1	25.74	26.62	26.76	26.37	0.451
10^0	28.59	29.40	28.69	28.89	0.361
10^{-1}	29.47	30.10	30.36	29.98	0.375
NTC	29.52	30.08	30.40	30.00	0.363

The sensitivity of PCR-based diagnostic was also evaluated. It was observed that PCR reactions with 10^5, 10^4, and 10^3 template molecules were having a strong single specific band in agarose gel while with 10^2, and 10^1 template molecules, faint/weak intensity (Fig. 8) Mos of times PCR with 10^0 template molecules were negative for any band.

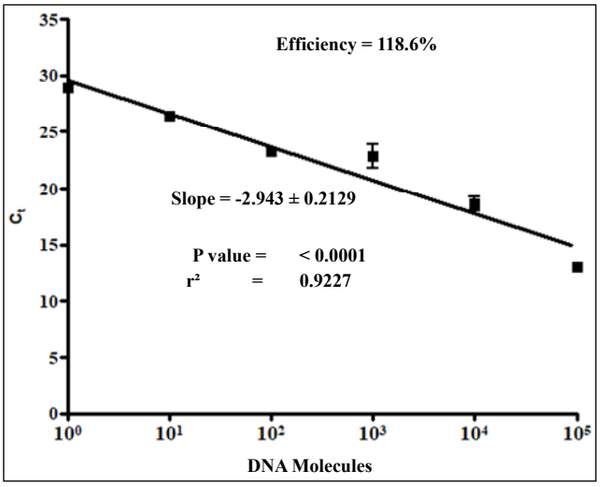


Fig. 7: Real-time PCR efficiency graph

Specificity of RT-PCR and PCR

The RT-PCR specificity was evaluated by using recombinant plasmid as positive control DNA and genomic DNA from MDV-l, FPV, and ILTV were used as test samples. The melting curve analysis indicated

specific amplification in positive control plasmid and MDV-I genomic DNA reactions, while no amplification was observed in FPV, ILTV, and NTC reactions (Fig. 9A). The C_t values of real-time PCR amplification curve also indicated specific amplification in positive control and MDV-I genomic DNA reactions (Fig. 9B, and Table 4).

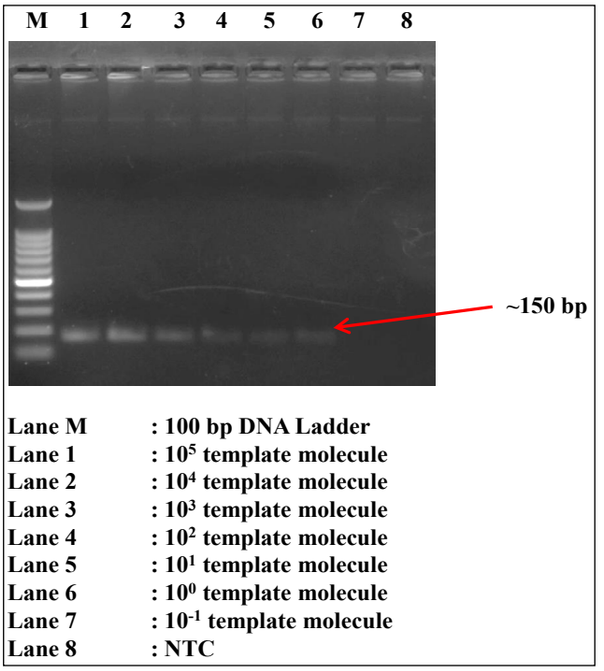


Fig. 8: PCR sensitivity

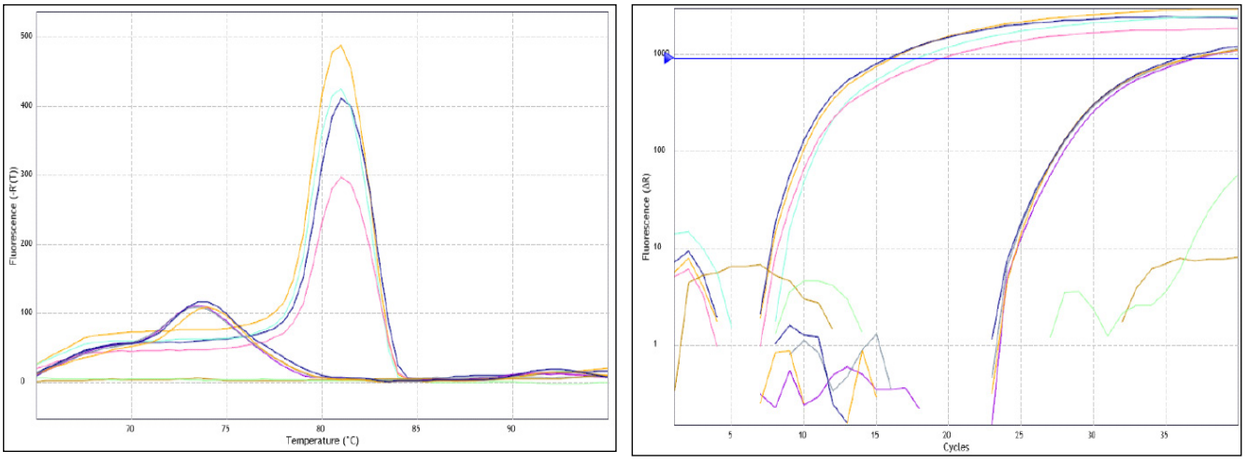
Table 4: C_t values of real-time PCR for specificity

DNA Molecules		C_t
Positive Control	16.26	15.99
MDV-1	18.12	19.84
FPV	37.62	37.26
ILTV	37.20	36.37
NTC	Nil	Nil

The PCR specificity was also carried using recombinant plasmid as positive control DNA and genomic DNA from MDV-l, FPV, and ILTV were used as test samples. Agarose gel electrophoresis indicated specific bands in PCR reactions with positive control and MDV-1 DNA as template. PCR reactions with FPV and ILTV DNA and also NTC were not having any band (Fig. 10 and Table 6).

Comparison of sensitivity and specificity of PSR, RT-PCR, and PCR

Among PSR, RT-PCR, and PCR-based diagnostic for MDV-1, the PCR has the lowest sensitivity and can detect efficiently up to 10 molecules of target DNA. The PSR and RT-PCR have equal sensitivity, and both diagnostics can detect efficiently up to a single molecule of target DNA (Table 5). PSR, RT-PCR, and PCR-based diagnostic for the target gene were having equal specificity as the product for only MDV-I specific DNA was amplified (Table 6).



(A) **(B)**

Fig. 9: Specificity plot of real-time PCR melting curve analysis **(A)**, and amplification plot **(B)**

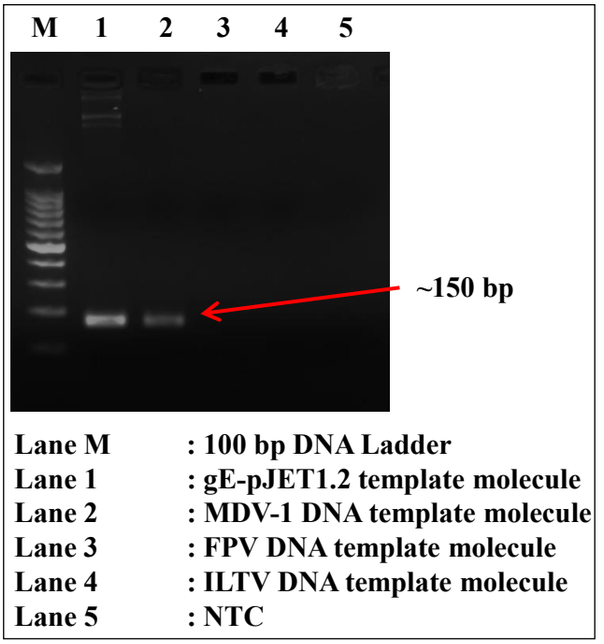


Fig. 10: PCR specificity

Table 5: Comparison of sensitivity of PCR, real-time PCR and PSR

	Number of template molecule							
Test	10^5	10^4	10^3	10^2	10^1	10^0	10^{-1}	NTC
PCR	++	++	++	+	+	±	–	–
Real-time PCR	++	++	++	++	++	++	–	–
PSR	++	++	++	++	++	++	–	–

Table 6: Comparison of specificity of PCR, real-time PCR and PSR

Test	Positive Control	MDV-1 genomic DNA	FPV	ILTV	NTC
PCR	++	++	–	–	–
Real-time PCR	++	++	–	–	–
PSR	++	++	–	–	–

DISCUSSION

Currently, the poultry industry has been growing at the rate of 7.52% and 8.5% in broiler and egg production, respectively, as compared to 2.9% for agricultural growth, in India. As per the estimation of the All-India Poultry Breeders Association, USD 17.31 billion annually contributed to the Indian economy and provides direct and indirect employment to 50 million people in the poultry sector. In the poultry sector, the broiler and layer segment constitutes about 65.3 and 34.7% with a monthly turnover of 400 million chicks and 8,400 million eggs, respectively. Around 1 million farmers are engaged in poultry farming activities with 85% of them having less than 2 hectares of land or are landless. Poultry in India has emerged as the most dynamic and diversified subsector with an annual production of 851.8 million birds. Globally, India has the third rank in egg-producing and fourth rank in broiler-producing (Kolluri *et al*., 2021).

The poultry sector has been challenged by several infectious diseases (Desta, 2021) including Marek's disease (MD). The MD caused by Marek's disease virus (MDV), a member of the *Herpesviridae* family, is a constraint on this industry (Torres *et al*., 2019). Gupta *et al.* (2016) compared the *Meq* gene of MDV isolates from Ludhiana. Among Ludhiana MDV isolates (MDV samples collected from the same farm within six months period) were having 98.9–99.8% similarity in nucleotide sequences. A similar kind of study by Molouki *et al.* (2022) indicated only 0.1–0.8% genetic dissimilarity among the *meq* gene of MDV of Iranian strains. The *meq* sequences comparison among MDV indicated that it has 98.9–99.8% similarity at the nucleotide level (Gupta *et al*., 2016) while at amino acids levels it has 99.5%–100% similarity (Kannaki *et al*., 2021). To date, the work on MDV-1 complete genome nucleotide similarity analysis has not been carried out. BLASTn analysis indicated various isolates of MDV-1 have more than 99.6 % nucleotide identity in their complete genome and the gE gene of MDV-1 is highly conserved. As the gE gene is highly conserved in MDV-1, it was cloned into pJET1.2 vector and recombinant plasmid (gE-pJET1.2) was used as template for the development and standardization of molecular diagnostic.

Isothermal nucleic acid amplification techniques have emerged as a possible substitute for PCR-based techniques because it produces effective amplification at the same temperature without the need of a thermocycler (Li and Macdonald, 2015). The first report on isothermal nucleic acid amplification assays appeared about 30 years ago with good sensitivity and promising specificity, criteria for developing a perfect diagnostic test in an environment

with restricted resources (Kosack *et al*., 2017). Several isothermal amplification techniques including Loop-Mediated Isothermal Amplification (LAMP) (Mansour *et al*., 2015), Recombinase Polymerase Amplification (RPA) (Tamer *et al*., 2022), Nucleic Acid Sequences Based Amplification (NASBA) (Lau *et al*., 2004), Polymerase Spiral Reaction (PSR) (Tomar *et al*., 2020), *etc* have been used for viral disease diagnosis. LAMP is the most often used isothermal amplification technique (Aslan *et al*., 2019). The choice of a correct and appropriate target for amplification, which should be a highly conserved area for a specific strain of microbe, is limited by LAMP-based diagnosis. Additionally, LAMP needs 4-6 primers to focus on less than ten primers in a narrow target sequence for high sensitivity and specificity. This makes it challenging to create precise primers for the LAMP assay (Wong *et al*., 2018).

The Polymerase Spiral Reaction (PSR) has been used as a unique isothermal nucleic acid amplification technique with high sensitivity and specificity since 2015. A Bst DNA polymerase and two primers designed to specifically target the gene sequence are used in the PSR approach. The forward and reverse primer sequences are complementary to their respective target nucleic acid sequences at their 3' ends, but they are reversed to one another at their 5' ends. The PSR reaction at an isothermal temperature between 61 and 65 °C produces complex spiral shape amplified DNA molecules (Liu *et al*., 2015).

Since 2015, PSR has been developed for the detection of several viral pathogens including West Nile Virus (Tomar *et al*., 2020), SARS-CoV-2 (Maiti *et al*., 2022), Canine parvovirus-2 (CPV-2) (Gupta *et al*., 2017), Bovine Herpesvirus 1 (Malla *et al*., 2018), *etc*. The PSR reaction standardized in this study was having an optimum temperature of 65 °C for the one-hour duration. The PSR developed was highly sensitive and could detect up to one copy number of target nucleic acid molecules and is as good as RT-PCR in terms of sensitivity. Moreover, PSR developed in this study was highly specific and detect only positive recombinant plasmid and MDV-1 genomic DNA. The specificity of PSR was equivalent to the specificity of RT-PCR, and PCR. The visualization of PSR products were carried out by agarose gel electrophoresis (laddering pattering for positive result and SYBE Green based colour change of reactioin sample (green fluorescent colour for positive result). The PSR developed and standerized can be useful for diagnosis of MDV-1 infection of field samples with high sensitivity and specificity.

CONCLUSION

The PSR diagnostic was developed in this study for the detection of MDV-1 nucleic acid with high sensitivity and specificity. This PSR-based diagnostic can be useful for the diagnosis of MDV-1 infection of field samples.

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