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FULL-LENGTH RESEARCH ARTICLE

Specific *Glycoprotein E* (*gE*) Gene Based Nested Polymerase Chain Reaction Assay for Detection of Marek's Disease Virus in Chickens

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Abstract
Poultry industry is one of the prime agricultural sectors that not only contributes to Global economy but also supports livelihood sustainability of the poultry farmers. However, this sector often faces severe economic losses due to various infectious diseases. Marek's disease (MD) is one of the common viral diseases of poultry, caused by Marek's disease virus (MDV). In spite of routine vaccination, events of vaccine failure as well as disease outbreaks are often seen in the field conditions. Several methods including polymerase chain reaction (PCR) based techniques are available for diagnosis of MD. Nested PCR (nPCR) is one of the highly sensitive and specific techniques used for disease diagnosis. Present study reports a highly sensitive *glycoprotein E* (*gE*) gene based nPCR for MDV detection. The outer set of primers amplified 567 base pair (bp), while inner set of primers amplified 230 bp of the of the MDV genome. The minimum detection limit was 17.6 picogram of genomic material in clinical sample. It was also shown as highly specific for the detection of field MDV and did not amplify one of the commercial vaccine strains, mostly used for vaccination against MD in chickens i.e., Turkey Herpes virus (HVT) FC 126 strain. It also did not amplify the fowl pox virus (FPV) genome. The applicability of the technique was assessed with the field tissue (liver) samples (n = 22), comprising of eleven (n = 11) samples collected during post-mortem examination of birds suspected of MD and eleven (n = 11) samples from apparently healthy birds collected from commercial retail poultry outlets. It was found positive in eight out of eleven clinical samples in nPCR; while, all samples from healthy birds were tested negative. The developed assay was found highly sensitive and specific. The developed nPCR technique could be used for diagnosis of MD infection caused by the field strains in the vaccinated as well as non-vaccinated poultry flocks.

Keywords Poultry · Diagnostics · Marek's disease · Glycoprotein *E* (*gE*) · Sensitivity and specificity

Introduction

Global poultry sector is one of the major agricultural sectors around the world. The poultry sector is not only important in terms of production of meat and eggs but also, in terms of employment of manpower and strengthening poultry farmer's income and livelihood. However, the poultry sector is often challenged by various non-infectious as well as infectious diseases [7]. Among the infectious diseases, various viral diseases are constantly posing risk to the poultry industry. Marek's disease (MD), caused by Marek's disease virus (MDV) i.e., *Gallid alphaherpesvirus2* species, a member of the *Herpesviridae* family, is one of the threats to the poultry industry [22]. Affected birds show the symptoms that include depression, stunted growth, swollen feather follicles, paralysis, and often death. It is characterized by immunosuppression, neoplastic transformation of CD4 + T cells i.e., T cell lymphomas of the visceral organs [14], cutaneous form with nodular lesions at the base of the feather follicles, enlargement of the sciatic nerve (unilateral or bilateral) and brachial nerves

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resulting from lymphoblast infiltration, neurological disorders, greying of the iris caused due to lymphoblastoid cell infiltration and polyneuritis or inflammation of the visceral organs and major nerves in chicken [3]. MDV is a highly cell associated virus that captures the host cell machinery for its replication [4]. Based on pathogenicity and virulence, MDV is classified under serotypes 1, 2, and 3. Out of these MDV serotypes, serotype 1 is categorized as a virulent strain, while serotypes 2 and 3 are mild or vaccinal strains. Serotype 1 MDV strain is further classified into four pathotypes based on their pathogenicity. These pathotypes are mild (m), virulent (v), very virulent (vv), and very virulent plus (vv +) MDVs [10]. MDV possesses unique short (U_S) and unique long (U_L) genomic regions. The genome contains many glycoprotein encoding genes those express various glycoproteins. Certain glycoproteins like *gB*, *gC*, *gD* and *gE* genes are the most conserved regions among the MDV strains [21]. The *gE* and *gI* as well as UL49 proteins have been seen to play important role in the replication of the virus [15]. The virus most certainly enters the susceptible birds via the respiratory route, when the bird inhales the virus-contaminated air in the poultry house. Horizontal transmission is the major route of transmission of this highly contagious disease [14] in the poultry flocks. Commercial vaccines are available for vaccination against MD in chickens. Despite widespread vaccination, MD incidences have been reported even in vaccinated flocks [10]. Such incidences may be due to events of vaccination failure, inadequate immunity status in the poultry flock or circulation of variant forms of viral strains than that of vaccinal strains. Many diagnostic techniques are available for detection of the pathogen in the affected poultry flocks. Considering the economic importance of poultry sector and the ill effects of MD, rapid yet accurate diagnosis of the disease is essential, especially, at field level. Here, development of a highly sensitive and specific nested polymerase chain reaction (nPCR), targeting the conserved *gE* region of the MDV genome for the detection of MD is reported.

Materials and Methods

Sample Collection

A total of twenty-two (n = 22) liver tissue samples of chickens were collected comprising of eleven (n = 11) liver tissue samples of chickens, collected during post-mortem examination of the birds suspected to be died of MD infection and eleven (n = 11) liver tissue samples from apparently healthy birds. The samples were transported over ice to laboratory for processing.

Briefly, the clinical samples (n = 11) were collected during post-mortem examination round the year including winter (December–January; n = 4), spring (March–April; n = 3), summer (May–July; n = 1) and autumn (October; n = 3) periods of the year from various age group of birds i.e., younger to adult ones. The samples were assessed for determining the applicability of the technique. Apart from clinical samples, liver tissue samples (n = 11) from apparently healthy birds were also collected from commercial retail poultry outlets/poultry-meat (chicken) vendors and included in the study during assessment of the applicability of the developed technique. Appropriate, biosafety measures were undertaken during collection and transportation of the clinical samples. The samples were stored at − 80 °C till further processing.

Sample Processing and DNA Extraction

The liver tissue samples were triturated with sterile 1X phosphate buffer saline (PBS) and tissue suspension was prepared with due biosafety precautionary measures. The DNA was extracted as per the protocol described earlier [16]. The concentration and purity of extracted DNA was checked in Nanodrop 1000 spectrophotometer (ThermoScientific, USA). The extracted DNA was stored at − 20 °C till further use.

Sequence Retrieval and Analysis

A total of fifty-seven complete coding domain sequence (CDS) of *gE* gene of 1494 bp from various countries around the world were retrieved from the National Centre for Biotechnology Information (NCBI) GenBank database including the NCBI reference sequence of *gE* gene complete CDS (Accession no. NC_002229.3). The details of the retrieved sequences were shown in table (Supplementary File). The retrieved sequences were analysed using BioEdit sequence alignment editor tool [8].

Primer Designing

Two sets of primers (outer and inner sets) were designed for the development of nPCR assay. The outer and inner sets primers targeting the *gE* gene were designed based on a highly conserved/consensus region of the analysed *gE* genes to amplify 567 bp genomic region as outer product followed by 230 bp of genomic region within the outer genomic region, respectively. Both sets of primers are shown in Table 1. The primers were corresponding to 181st–200th nucleotide positions for outer forward, 728–747th for outer reverse primers, while 451st–470th for inner forward and 661st–680th for inner reverse primers (Fig. 1). The positions of primers were corresponding to

Table 1 Sequence of primers used for nested nPCR

Primer	Sequence	Product Size (bp)
gE-Outer-Forward	5′-GAAACGGCAACAGGCTATGT-3′	567
gE-Outer-Reverse	5′-CTCTGGTTCTTTGGGGTGAA-3′	
gE-Inner-Forward	5′-AGGACTGCAAAGCTGGATGT-3′	220
gE-Inner-Reverse	5′-CGGAGGAACAACACCCTAAA-3′	

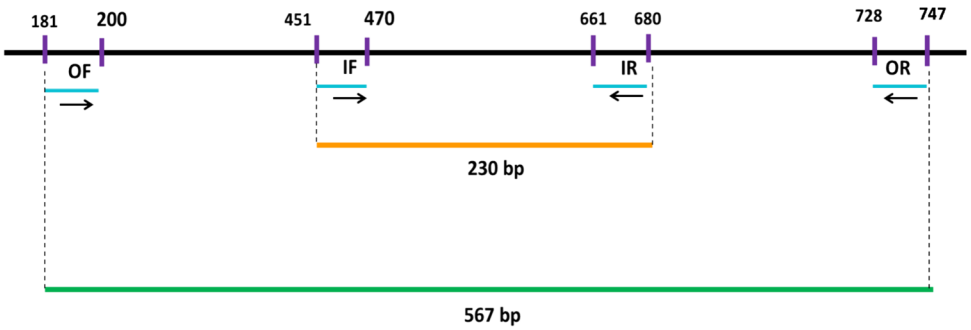

Fig. 1 Primer designing strategy of nPCR. The position of primers were corresponding to the NC_002229.3 reference *gE* gene complete CDS (1494 bp, 162389–163882th nucleotide position). OF and OR denote outer forward and outer reverse primers respectively, while IF and IR denote respectively, the inner forward and inner reverse primers

the NC_002229.3 reference *gE* gene complete CDS of 1494 bp (162389–163882th nucleotides). The primers were specific to MDV and did not align with the HVT sequence (AF291866.1: 136045–137052th nucleotides).

Nested PCR (nPCR) Standardization

First Round Amplification

The first round of amplification of nPCR (hereafter also referred as conventional PCR) was carried out using the outer set primers. The amplification was carried out in the thermal cycler i.e., mastercycler nexus gradient (Eppendorf, Germany). A total 20 µl volume of PCR reaction mixture was comprised of 10 µl of 2 × Dream *Taq* Master Mix (1 × final concentration), 1 µl each of outer forward and reverse primers of 10 pmol/microlitre (pm/µl) concentration (final concentration: 0.5 pm/µl), and 2 µl of DNA template. A non-template reaction was also run with each reaction to rule out any non-specific amplification. The amplification was standardized first at an annealing temperature gradient of 2 °C increment i.e., 52 °C to 58 °C. The first round of amplification conditions was standardized which included an initial denaturation at 94 °C for 5 min; followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, extension at 72 °C for 45 s; and a final elongation for 7 min at 72 °C. For standardization, a 567 bp amplified genomic region of *gE* gene was sequenced (NCBI GenBank accession number PP955194) and used as known positive control.

During the standardization process of conventional PCR, the amplified PCR products were separated in ethidium bromide (final concentration: 0.5 µg/ml) stained 1.5% agarose gel at 80 V. The separated PCR products were visualized under ultraviolet (UV) light and recorded in Gel documentation system (G Box, Syngene, UK).

Amplification with Inner Set of Primers

Inner primers as shown in Table 1 were used for standardization of second round of amplification. Initially, the inner primers were used to amplify the target genomic region of 230 bp directly in the genomic DNA instead of amplified PCR product of first round. The amplification condition as well as composition of reaction mixture were same as shown in "First Round Amplification" section; only the outer set of primers were replaced with the inner set of primers. Gradient PCR with annealing temperatures of 2 °C increment i.e., 52 °C, 54 °C, 56 °C and 58 °C was used for standardization of inner set of primers similar to outer ones. The temperature depicting the sharp and specific band was chosen for standardization. The amplified products were separated in agarose gel and visualized as shown in previous section.

Adoption of Nested Reactions (nPCR)

Following standardization of amplification with outer set of primers and inner set of primers separately in template DNA, the nPCR was optimized. The nested set of reaction was developed by incorporating amplified PCR product of

first round of amplification using outer set of primers directly as template for amplification by inner set of primers (nested primers) in place of template DNA. The second round amplification was run first by gradient PCR with annealing temperatures of 2 °C increment starting from 52 °C to 60 °C taking the amplified PCR product of first round of amplification directly. The reaction components and standardized amplification conditions were the same as described in previous sections.

Minimum Detection Limit (Sensitivity) of nPCR

The sensitivity of the developed technique was evaluated as follows. The known MDV positive DNA template was tenfold diluted serially, spanning from 1765 nanogram/µl (ng/µl) to 0.176 attogram/µl (ag/µl). The diluted genomic DNA templates were assayed for positivity (amplification positive) by the nPCR. During the assessment, the reactions were set from higher dilutions (concentration with lower genomic DNA) to lower dilutions (concentration with higher genomic DNA).

Specificity of nPCR

The specificity of the nPCR for detection of MDV was evaluated by taking the known positive DNA samples of other avian viruses. The nPCR was tested with Turkey Herpes virus (HVT) FC 126 strain DNA prepared from commercially available vaccine as well as fowl pox virus (FPV) genome and was found negative for both of the templates. The concentration of the DNA templates were kept in equivalent concentration to that of positive control template. The PCR product of sequenced MDV DNA was used as positive control whereas, nuclease-free water was added in place of DNA template as non-template control. Before performing specificity run, the FPV genome was confirmed with amplification of 560 bp genomic region using the primers and PCR amplification conditions as described [2].

Applicability of nPCR with Tissue Samples

The clinical tissue samples (n = 11) as well as the tissue samples (n = 11) collected from the healthy birds were examined for assessing the applicability of the developed technique. The genomic DNA extracted from liver samples of chickens were used as template for outer set of primers, while first round amplified PCR products were used in second round of amplification of nPCR. The concentrations of genomic DNA in all the clinical samples to be used as templates were brought to a similar range of concentration by dilution. The nPCR was performed on all the clinical samples as well as samples from healthy birds as known negative samples. The sequenced PCR product (NCBI GenBank accession number PP955194) was used as positive control and nuclease-free water was added in place of template DNA as non-template control.

Results

Standardization of Amplification with Outer and Inner Set of Primers Separately

Amplifications of MDV genome were standardized by gradient PCRs with the outer set of primers (Fig. 2) and inner set of primers (Fig. 3) separately on the genomic DNA template. The annealing temperature of 58 °C was selected for both set of primers those could be used further for nesting of reactions by nPCR.

Standardization of nPCR

The nPCR was standardized following a gradient PCR (Fig. 4) by taking amplified PCR product of first round of amplification with outer primers directly without any purification step as template for second round of amplification with inner primers. The annealing temperature of 58 °C was found suitable for nesting of the reaction (by inner primers). The purpose of gradient PCR with inner primers in nesting condition was to ensure the behaviour of the nested primers with the direct genomic DNA template as shown in Fig. 3 and with the first round amplified as well as unpurified PCR product as template as shown in Fig. 4. The bands observed at ~ 230 bp were corresponding to the nPCR condition, whereas, faint bands observed at ~ 296 bp and ~ 567 bp in few lanes were corresponding to semi-nested condition with inner forward

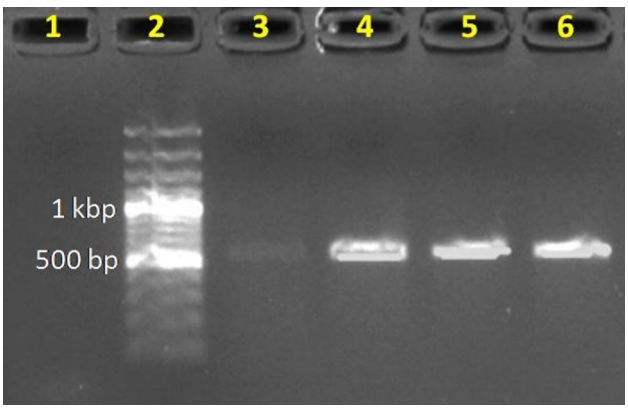

Fig. 2 First round of amplification with outer set of primers of nested PCR. Lane 1: No template control (NTC); Lane 2: 100 bp DNA Ladder; Lane 3–6: Gradient temperatures (52 °C, 54 °C, 56 °C and 58 °C) showing amplifications at ~ 567 bp

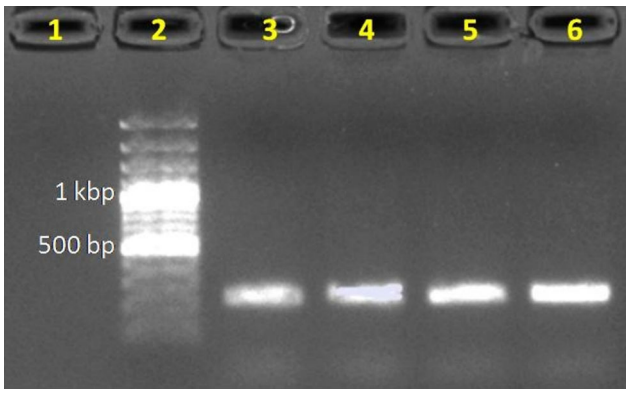

Fig. 3 Amplification with inner set of primers with genomic DNA template. Lane 1: No template control (NTC); Lane 2: 100 bp DNA Ladder; Lane 3–6: Gradient temperatures (52 °C, 54 °C, 56 °C and 58 °C) showing amplifications at ~ 230 bp

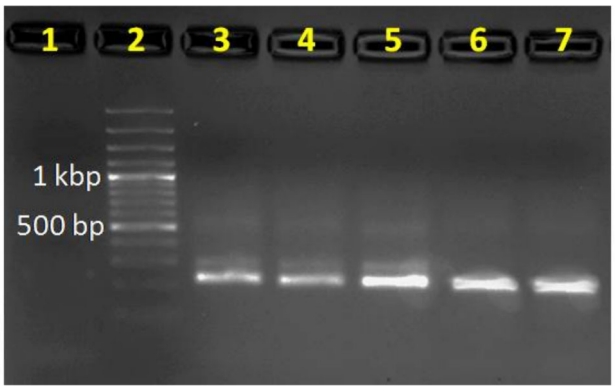

Fig. 4 Gradient nPCR: Amplification by inner set of primers of nested PCR with the amplified PCR product of outer pair of primers as template. Lane 1: No template control (NTC); Lane 2: 100 bp DNA Ladder; Lane 3–7: Gradient temperatures (52 °C, 54 °C, 56 °C and 58 °C) showing amplifications at ~ 230 bp. The faint bands observed at ~ 567 bp in lanes 3–5 correspond to the outer PCR amplified products. Similarly, the faint bands observed at ~ 296 bp in lane 3–5 correspond to the semi-nested PCR products generated by inner forward and residual outer reverse primers

and residual outer reverse primers and residual amplified PCR products of outer set of primers respectively. The PCR product of first round of amplification was sequenced (NCBI GenBank accession number PP955194) to know the specificity of the amplification.

Sensitivity

On assessment of the sensitivity of nPCR, the minimum detection limit was defined as the lowest dilution in the tenfold series that produced amplification. The minimum detection limit found was 176 picogram/µl of genomic DNA template for conventional PCR (amplification with first set of primers only) (Fig. 5), while it was 17.6 pg/µl for the nPCR (Fig. 6). It was clear from the results that nesting of reactions increased the minimum detection limit and sensitivity of the testing.

Specificity

The designed primers of nPCR specifically detected the MDV genome, while no amplification bands could be observed for other homologous host viruses tested i.e., HVT and FPV genomes (Fig. 7). This demonstrated the specificity of the nested PCR assay for MDV detection.

Assessment with Field Tissue Samples

On comparative assessment with the conventional PCR (first round of amplification only), it was only the three out of 11 clinical tissue samples; those could be detected positive for MDV genome by the conventional PCR (Supplementary File, Fig. 1a). However, out of these same 11 clinical tissue samples, the MDV genome could be detected in eight samples as positive through nPCR (Supplementary File, Fig. 2a). Hence, false negative amplifications were overcome with incorporation of nested condition in the nPCR. Similarly, false positive amplification was ruled out as there was no amplification on the eleven tissue samples collected from healthy birds.

Discussion

MD is one of the lymphotropic viral diseases, which affect poultry birds. MD continues to pose a threat to the poultry industry due to increased incidences worldwide. The occurrence of MD outbreaks could be attributed to the possible evolution/genetic variation of virulent strains, improper vaccine handling, vaccination failure, compromised biosecurity measures etc. [10]. It results in morbidity and mortality in the affected poultry flock, apart from the economic losses incurred thereby. Hence, it is crucial to develop specific and sensitive diagnostic techniques to curb the problem of MD in poultry flocks for an early diagnosis to undertake effective controlling measures. The diagnosis of MD infection includes observation of signs and symptoms, mortality as well as vaccination history of the affected flock etc. Tentatively, field diagnosis is based on observation of symptoms like depression, loss of weight, flaccid neck, and paralysis of wing and leg. Splenomegaly, hepatomegaly, renomegaly etc. were also reported in MD infected flocks [1]. The histopathological examination of affected organs shows marked cellular polymorphism, with the presence of lymphoblasts, lymphocytes, fibroblasts, and infiltration of tumour cells which are arranged in circumscribed or diffused form. Necrosis and destruction of lymphoid cells are observed in the thymus and bursa of

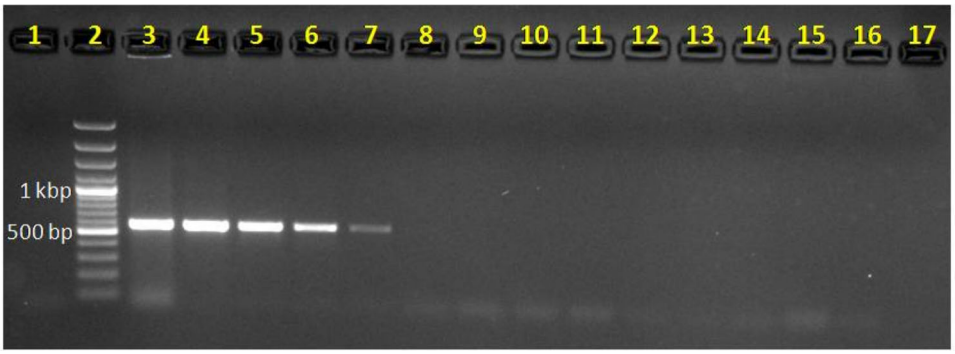

Fig. 5 Sensitivity of outer pair of primers with genomic DNA as template. Lane 1: No template control (NTC); Lane 2: 100 bp DNA Ladder; Lane 3: 1765 ng/µl; Lane 4: 176.5 ng/µl; Lane 5: 17.6 ng/µl; Lane 6: 1.76 ng/µl; Lane 7: 176 pg/µl; Lane 8: 17.6 pg/µl; Lane 9: 1.766 pg/µl; Lane 10: 176 fg/µl; Lane 11: 17.6 fg/µl; Lane 12: 1.7 fg/µl; Lane 13: 0.17 fg/µl; Lane 14: 176 ag/µl; Lane 15: 17.6 ag/µl; Lane 16: 1.76 ag/µl; Lane 17: 0.176 ag/µl. Amplification was observed up to lane 7 i.e., 176 pg/µl of genomic DNA template

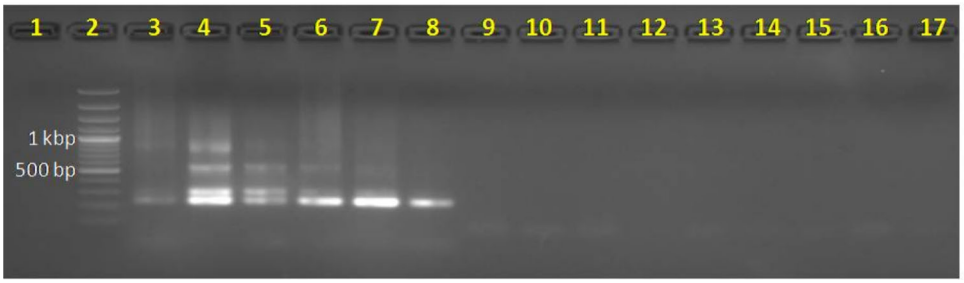

Fig. 6 Sensitivity of nPCR. Lane 1: No template control (NTC); Lane 2: 100 bp DNA Ladder; Lane 3: 1765 ng/µl; Lane 4: 176.5 ng/µl; Lane 5: 17.6 ng/µl; Lane 6: 1.76 ng/µl; Lane 7: 176 pg/µl; Lane 8: 17.6 pg/µl; Lane 9: 1.766 pg/µl; Lane 10: 176 fg/µl; Lane 11: 17.6 fg/µl; Lane 12: 1.7 fg/µl; Lane 13: 0.17 fg/µl; Lane 14: 176 ag/µl; Lane 15: 17.6 ag/µl; Lane 16: 1.76 ag/µl; Lane 17: 0.176 ag/µl. Amplification was observed up to lane 8 i.e., 17.6 pg/µl of genomic DNA template

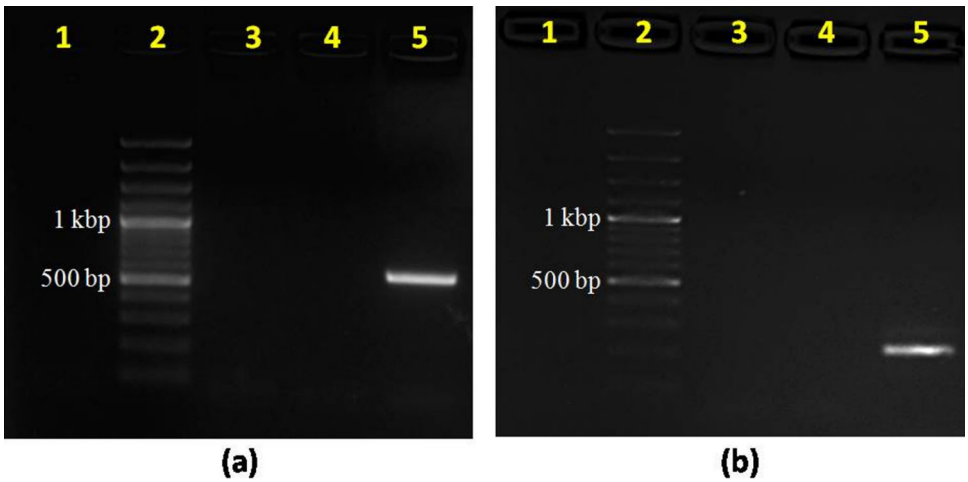

Fig. 7 Specificity assessment: **a** Specificity assessment using outer set of primers; **b** Specificity assessment of nPCR. Lane 1: No template control (NTC); Lane 2: 100 bp DNA Ladder; Lane 3: FPV DNA; Lane 4: HVT DNA; Lane 5: MDV DNA. Amplifications were observed in lane 5 of both the figures (**a**) and (**b**)

Fabricius. Apart from histopathological examination, the immunohistochemistry is also reported as promising in MD infection. Moderate to severe reactivity for MDV antigens was observed by immunohistochemical staining in the cytoplasm of the infiltrating neoplastic cells in organs like liver, heart, kidney, proventriculus, spleen, pancreas, and thymus, while mild positive reactivity in the hepatocytes, myocardial fibres, tubular epithelial cells of the kidney, and epithelial cells of the intestine [20]. Other techniques include serological diagnosis, cultivation of the virus in egg embryos and different cell lines etc. [23]. However, all these methods discussed above are time consuming. Hence, molecular diagnostic tools are very essential not only

because of their ease of diagnosis but also for the rapidity and accuracy of the results.

Polymerase chain reaction (PCR) based molecular techniques are promising in the rapid diagnosis of the disease condition in affected population. Among the various formats of PCR, the nPCR is one of the molecular methods that can be employed for an accurate yet rapid diagnosis of the ailment. In general, nesting of amplification, i.e., amplification of a genomic region within an already amplified region increases the specificity as well as sensitivity of the detection process, as a principle of nPCR. Certain nPCR have been reported for the detection of MDV [6, 11, 12, and 18]. Glycoproteins like gB [5], gD [12] etc. have been targeted for the detection of MD infection in chickens. In the present report, two sets of primers were designed from the consensus region of various *gE* gene sequences available around the world, for amplification of 567 bp and 230 bp genomic regions of the *gE* gene, as outer and nested amplification products, respectively.

Interestingly, the disease has been reported from natural hosts other than chickens. In one of the much earlier reports, the genomic DNA sequence of avian oncogenic herpesvirus i.e., MDV was detected in human sera [12]. MD infection with clinical signs and symptoms as well as mortality was reported from commercial turkeys [25]. Even, the events of pathogen spill-over to wild hosts (wild birds) have been reported e.g., wild geese [17], red-crowned cranes [13].

Despite routine immunoprophylaxis being used in day-old chicks in poultry farms, incidences of MD have been observed [9, 19, and 24]. Even the genetic variants of MDV, circulating in MD vaccinated flocks (vaccinated with bivalent MDV Rispens CVI988 and HVT FC-126 strains vaccine) have been reported [24]. The nPCR technique based on *meq* gene was reported for differentiation of highly virulent strains to that of MDV vaccine strain, CVI988 in experimentally infected chickens [18]. Meanwhile, the present report could detect the MD infection in the field samples caused by the circulating field strain(s), however, the developed technique could not detect one of the non-pathogenic vaccine strains commonly used in vaccination against MD in chickens, i.e., HVT FC-126 vaccine strain. It enables the technique to be specific for circulating field strains. Similarly, differential diagnosis of MDV infection to that of FPV could be achieved as the reported technique did not show any amplification with the FPV genome. Furthermore, the developed nPCR technique could detect as few as 17.6 picograms/µl concentration of genomic DNA from clinical tissue (liver) samples, which is ten times more sensitive than the conventional PCR (first round of amplification only). Therefore, the nPCR technique developed in this study could be used to detect birds suspected of MD infection at much earlier stages of infection with lower viral loads.

In conclusion, a sensitive, specific, and reliable nPCR diagnostic assay based on *gE* gene was developed for detection of MD infection in poultry. The developed assay will help poultry farmers for an accurate and early diagnosis of MD infection for undertaking necessary preventive measures at farm level for upliftment of livelihood sustainability and farmer's income.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s40003-024-00777-9.

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Author Contributions Designing of the experiment was done by AM, MM, GK, and CSM; sample collection was done by MB; conduct of experiment was done by MB, AM, MM; result interpretation was done by MB, AM, MM, CSM, and YSM; manuscript writing was done by MB, AM; manuscript editing, reading and approval were done by MB, AM, MM, GK, CSM, and YSM.

Declarations

Conflict of interest Authors declare that there are no potential financial or non-financial competing interests.

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