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Molecular Method of Marek's Disease Virus Serotype 2 Detection using Polymerase Chain Reaction in Chicken

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ABSTRACT

Background: Marek's disease is an oncogenic virus that produces malignant lymphomas in chickens. In this study Marek's disease virus serotype 2 identification using polymerase chain reaction with unique gene.

Methods: DNA isolated from MDV infected feather follicles, liver, spleen and commercially available serotype 2 vaccines (SB 1 and HVT combined bivalent, HVT monovalent) by phenol-chloroform DNA isolation method. Serotype 2 specific primers were designed by using primer 3.0 software targeting Long Open Reading Frame (LORF) gene-specific to serotype 2 SB 1 strain. DNA template from vaccines showed amplification of the LORF gene. PCR parameters were optimized and the analysis of sensitivity and specificity of primers was carried out.

Result: MDV serotype 2 with an optimized annealing temperature of 53°C with primer concentration of 3 pmol/µl. LORF gene of SB1 of MDV did not cross-react with HVT and MDV 1 strains and also with other oncogenic viruses of Avian Leucosis Virus (ALV) and Reticuloendotheliosis Virus (REV). The sensitivity was analyzed based on the different DNA concentrations and amplification up to 0.5 ng/µl. Screening of field samples of 260 along with three MDV serotype 2 vaccine SB1 strain samples revealed that all the filed samples did not show positivity for MDV serotype 2 whereas in the vaccine strain samples of MDV serotype 2 vaccine SB1 showed positivity by amplification at 270bp and these results indicated that MDV serotype 2 SB1 strain did not cause an outbreak in chicken and might be inferred that no incidence of spontaneous Lymphoid Leucosis-like lymphomas with augmentation property of MDV serotype 2. This serotype 2 specific assay was used to detect Marek's disease caused by serotype 2 which is commonly used as a vaccine.

Key words: MDV serotype 2 SB1 strain, PCR.

INTRODUCTION

Marek's disease virus is an alpha herpesvirus belonging to the genus Mardi virus produces malignant lymphomas in chicken few weeks after infection (Calnek and Witter, 1997; OIE, 2017) Marek's disease virus is under herpesvirus group having three serotypes namely Gallid herpes virus II (serotype1 and also known as Mareks disease virus), Gallid herpesvirus III (serotype2) and Meleagrid herpes virus I or herpes virus of Turkey bird (HVT) (serotype 3). Serotypes 2, 3 include all the naturally avirulent strains, some of which are used as vaccines. Antigenically related HVT strains are also used as a vaccine against MD (OIE, 2017).

The MDV infection is detected in inoculated susceptible cells and the cultures are incubated to find the viral plaque appearance and also by immunofluorescent assay (Lee *et al*., 1983).

At present the diagnostic techniques to detect MDV 1 and 3 are by polymerase chain reaction (Becker *et al*., 1992; Silva, 1992, Zhu *et al*., 1992, Bumstead *et al*., 1997; Handberg *et al*., 2001, Sadeghi *et al*., 2006) in suspected field samples. The vaccination against Marek's disease virus is currently available by using serotype 2 strain SB 1 or HVT or a mixture of both (Witter and Lee, 1984). In Asia, the serotypes are used as a vaccine candidate against Marek's disease such as serotypes 2 and 3 (SB1 and HVT) except in Japan, Europe, USA, South America using cv1988+HVT strains (Baigent *et al*., 2006). Both ALV subgroup E (ALV-E) and MD Serotype 2 are having an important role in the enhancement of spontaneous Lymphoid Leucosis-like tumors in susceptible chickens (Mays *et al*., 2019). *In ovo* vaccination using MD serotype 2 augment

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the incidence of spontaneous Lymphoid Leucosis-like lymphomas in transgenic chicken line (ALVA6) chickens (Cao *et al*., 2014). In this regard, separate development of polymerase chain reaction for serotype 2 is very much needed in India by using unique region of MDV serotype 2 with the designed primers to amplify the Long Open Reading Frame (LORF) gene of MDV serotype 2 SB1 strain.

MATERIALS AND METHODS

Vaccine purchase

The commercially available serotype 2 vaccines (SB1+HVT combined bivalent) and serotype 3 vaccine (HVT only monovalent), were purchased from Zoetis Company, USA and utilized in this study.

MDV and other strains

The field MDV suspected birds feather follicles, liver and spleen were collected from Namakkal District, Tamil Nadu andhra Pradesh, Maharashtra and Puducherry, India. Avian Leucosis Virus and Reticuloendotheliosis Virus were collected from Animal Biotechnology Department, Madras Veterinary College, Chennai.

Isolation of DNA

The DNA was extracted using a DNA extraction kit (QIAGEN, USA) from the cell-associated vaccine (serotype 2/3), lyophilized vaccines (serotype 3) as per the protocol given in the kit. The DNA was isolated from MDV infected chicken feather follicles, liver and spleen by phenol-chloroform DNA isolation method (Handberg *et al*., 2001). The purity and concentration of DNA measured using Nanodrop one (Thermo fisher scientific company, USA) and stored at-20°C.

MDV serotype I specific PCR

Chicken feather follicles, liver and spleen were screened for MDV serotype I using previously reported primers (Sathish *et al.,* 2012).

Primer designing for PCR

Serotype-II specific primers were designed using primer3.0 software (*frodo.wi.mitedu/primer3/input.htm)* targeting the LORF gene (Table 1) which is specific to the serotype 2 SB 1 strain.

PCR optimization

DNAs isolated from vaccines were used as templates for optimizing the PCR parameters such as melting temperature (Tm value) from 53°C to 61°C and primer concentration (0.1pmol/µl to 10 pmol/µl.)

PCR conditions

DNA from vaccines were subjected to PCR with the LORF primers followed by the reaction conditions as initial denaturation at 94°C for 1 min, 35 cycles of denaturation at 94°C for 1min, annealing at 59°C for 1min and extension at 72°C for 1 min and final extension at 72°C for10 mins. PCR products were checked in 1.5% agarose gel electrophoresis.

Specificity of primers

LORF gene-specific primers of SB-1 strain were cross-checked with HVT and MDV-1strains and the other oncogenic viruses of ALV and REV under the same PCR conditions.

Sensitivity of primers

The PCR was carried out with different DNA concentrations (0.5 ng/µl to 30 ng/µl) of vaccines to detect the sensitivity of the assay.

Sequence analysis

The positive PCR products were purified and sequenced by Eurofins Company. The sequences were aligned and showed 99% homology with MDV serotype II by BLAST analysis (Fig 1).

RESULTS AND DISCUSSION

Optimization of PCR

The DNA template isolated from vaccines for PCR optimization with PCR primers is shown in the amplification of the LORF gene (270 bp) for MDV serotype 2 at an optimized annealing temperature of 53°C (Fig 2) with a Primer concentration of 3 pmol/µl (Fig 3).

Specificity of PCR primers for serotype 2

The LORF gene of SB1strains specific primers was cross-checked with HVT and Serotype I strains and the results revealed that these primers did not cross-react with HVT and Serotype I strains (Fig 4). Further, it was not amplified with other oncogenic viruses ALV and REV (Fig 5).

Sensitivity of primers

Sensitivity was analyzed based on the different DNA concentrations and showed amplification up to 0.5 ng/µl for the PCR assay (Fig 6).

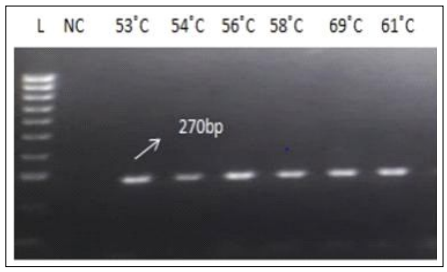

Fig 1: Optimization of Tm value.

Table 1: Serotype-II specific primers.

SB1 strain specificity	Primer set	Product size
LORF	Fp:5′GACGTGGTGCTTGATGACAG3′ Rp: 5′TGAGAGGTCGTCATCCCTTC3′	270 bp.

The screening of field samples from various parts of the country revealed that feather follicles, liver and spleen, did not show positivity in the developed PCR assay whereas MDV serotype vaccine SB1 strain samples showed positivity as amplification at 270 bp in 1.5% agarose gel electrophoresis (Table 2).

Marek's disease virus is having three serotypes serotype 1, (oncogenic), serotype 2 and 3 (non-oncogenic). The polymerase chain reaction for the MDV detection method is simple and results are detected within a day (Sadeghi *et al*., 2006). PCR is a direct detection assay of Marek's disease virus as per the earlier report of Silva (1992). The suspected antigen of Marek's disease virus is cross-reacting with others serotypes and MDV (Rispens *et al*., 1972; Hirai *et al*., 1986). Sadeghi *et al.* (2006) developed a polymerase chain reaction for detection and differentiation of MDV 1 and HVT strain. Sathish *et al.* (2012) reported the multiplex PCR assay for the detection of different avian oncogenic viruses (MDV, ALV, REV).

A perusal of literature, the MDV serotype 2 is having the property to augment the incidence of spontaneous Lymphoid Leucosis-like lymphomas in commercial chickens (Mays *et al*., 2019) and transgenic chicken line (ALVA6) chickens (Cao *et al*., 2014). Serotype 2 specific PCR is not available to diagnose MDV serotype 2 in India and this study has given the information of specific PCR of serotype 2 with amplification at 270 bp size which is unique to the LORF gene of MDV serotype 2 SB1 strain. The forward primer and reverse primer pair used in the study were given amplification at 270 bp that encodes the LORF gene of SB1.

The developed assay for detection of serotype 2 primers as given specific amplification of the LORF gene of serotype 2 SB1 strain without any cross-reaction with another serotype of MDV 1 and 3 indicated that specificity to serotype 2 only. Screening of field samples of 260 along with three MDV serotype 2 vaccine SB1 strain samples showed that no positivity was observed in the 260 filed samples but in the vaccine strain samples of MDV serotype 2 vaccine SB1 showed positivity by amplification at 270 bp. In the present study, the specific PCR for serotype 2 of MDV give an idea about disease outbreaks from the screened field samples that did not occur due to MDV serotype 2 and it could be occurred due to vaccine failure or virulence strains of serotype 1. In addition to that, the study indicated that MDV serotype 2 SB1 strain did not cause an outbreak in chicken and might be informed that there is no incidence of spontaneous Lymphoid Leucosis-like lymphomas with augmentation property of MDV serotype 2 (Cao *et al*., 2014; Mays *et al*., 2019).

The PCR for serotype 2 MDV detection is useful to find the Marek's disease virus caused by MDV 2 or not. This assay could be employed to differentiate Marek's disease bivalent vaccine (SB1/HVT) from the monovalent vaccine (HVT). This assay is useful to detect Marek's disease caused

Table 2: Screening of field samples.

Sample type	Total	Positivity	Serotype I specific PCR
Feather follicles	240	0/240	51/240
Liver	10	0/10	4/10
Spleen	10	0/10	5/10
Vaccine	3	3/3	0/3

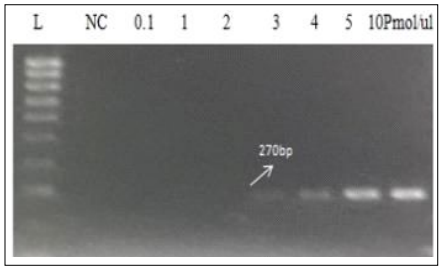

Fig 2: Optimization of primer concentration.

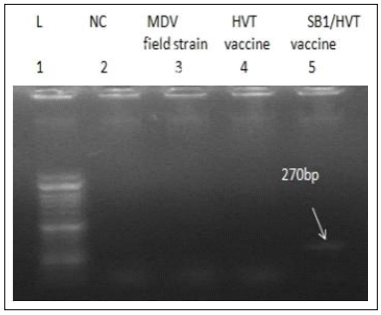

Fig 3: Cross reactivity of PCR primers with MDV field strains and HVT vaccine.

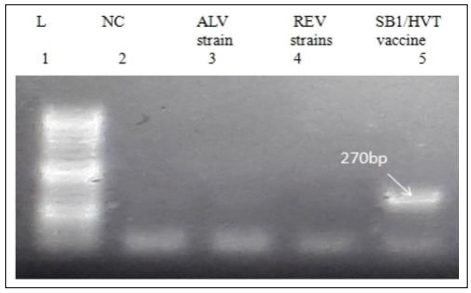

Fig 4: Cross reactivity of PCR primers with ALV and REV.

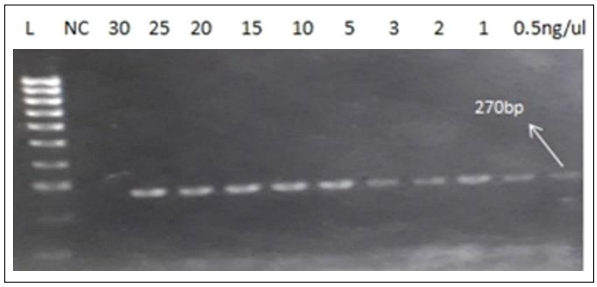

Fig 5: Sensitivity of PCR primers.

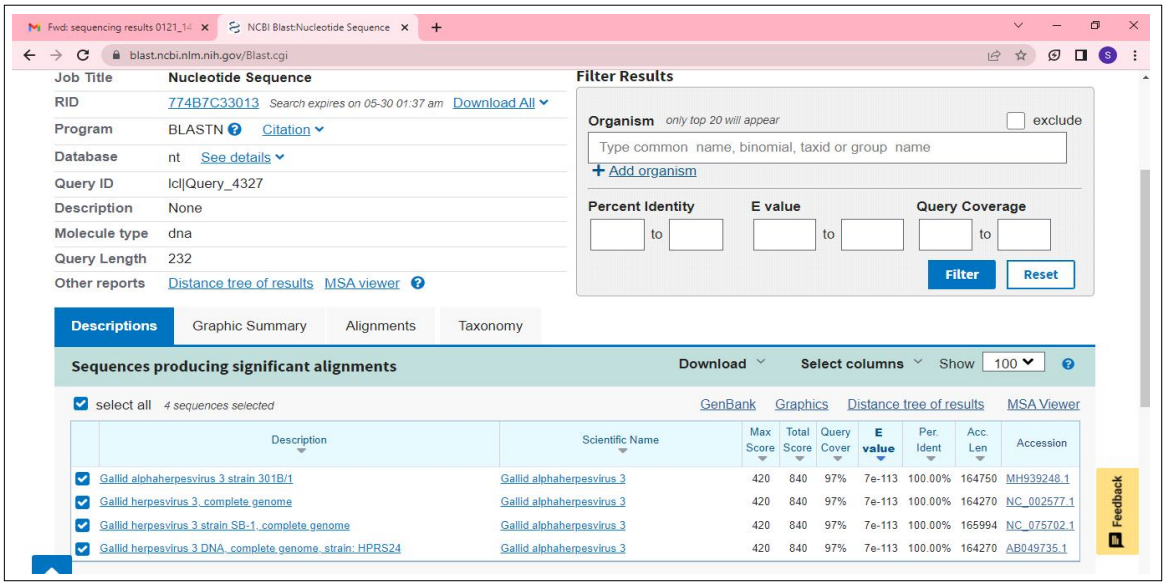

Fig 6: Blast analysis of sequencing results.

by serotype 2, which is commonly used as a vaccine strain of MDV.

CONCLUSION

In the present study, MDV serotype 2 specific PCR was developed and screening of field suspected MDV samples did not show positivity to MDV serotype 2 while vaccine samples showed positivity for MDV serotype 2.

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Conflict of interest

The authors declare no conflict of interest.

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