

## COMPARISON OF POLYMERASE CHAIN REACTION AND PRECIPITATION TEST IN DETECTION OF MAREK'S DISEASE IN VACCINATED POULTRY FLOCKS OF HARYANA

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Received: 27.02.2013; Accepted: 05.12.2013

### ABSTRACT

Marek's disease (MD), a lymphoproliferative disease, remains one of the economically most important diseases of chickens. The samples obtained from 10 poultry flocks affected with MD during June 2005 to March 2006 from different geographic locations of Haryana were tested by precipitation test and polymerase chain reaction (PCR) assay. Fifty percent samples were found positive for the presence of MD virus (MDV) by precipitation test. The feather follicle tips obtained from affected birds in all the ten poultry flocks proved positive for MDV serotype-1 in PCR assay using primers derived from ICP4 gene of MDV serotype-1. These ten samples were negative for MDV serotype-3 when tested using primers from US3 gene. The PCR had higher positivity than the precipitation test. The study indicates the circulation of virulent MDV serotype-1 in Haryana state and this calls for a review of the vaccination strategy so as to reduce the incidence of Marek's disease.

**Key words:** Marek's disease, precipitation test, polymerase chain reaction

Marek's disease (MD) caused by *Gallid herpesvirus-2* (MDV serotype-1), is a lymphoproliferative disease of chickens which is characterized by number of conditions such as visceral lymphomatosis, unilateral or bilateral enlargement of nerves due to infiltration of lymphoblasts, particularly sciatic and brachial nerve and graying of iris (ocular form). Huge economic losses are incurred due to MD because of cost of vaccination together with continuing losses due to the disease. The annual losses due to Marek's disease prior to introduction of the vaccination in 1970 were estimated to be \$150 million in U.S. and \$40 million in U.K. (Fenner *et al.*, 1993). The annual losses of more than 1 billion US dollars world over have been estimated (Nair, 2005). The vaccination can prevent tumor formation but the generation of infectious virus is not prevented. The virus is shed in the environment as dander from feather follicles. For this reason, this disease remains one of the most economically important diseases of poultry in countries where intensive rearing of poultry is practiced. Polymerase chain reaction (PCR) is a highly sensitive test in detecting MDV (Silva, 1992; Davidson *et al.*, 1995).

Marek's disease viral antigen can be detected in

the feather tips by the agar gel precipitation test (AGPT, OIE, 2004; Kamaldeep *et al.*, 2007; Palanivel *et al.*, 2007) which is a simple and sensitive technique (Marquardt, 1972). Keeping this in view, the study is envisaged with the objective of comparison of PCR and AGPT in the detection of MDV.

### MATERIALS AND METHODS

**Collection of Tissue Specimens:** Tips of feathers were picked up from chickens clinically affected with MD and were stored at -20°C until their use. The feather tips collected from affected birds of a flock were pooled to represent one sample. Thus a total of 10 samples (A to J) were obtained from flocks affected with MD during June 2005 to March 2006 from different geographic locations of Haryana (Kamaldeep *et al.*, 2007). Herpes Virus of Turkey (HVT), a commercial vaccine, was used as a positive control.

**Precipitation Test:** Single radial immunodiffusion method was used to demonstrate precipitation test using feather follicles obtained from the affected birds and were kept at -20°C without any preservative till use. Convalescent MD-specific serum was kindly provided by Dr. D.S. Shivdekar, Indovax Pvt. Ltd., Hisar. The release of MDV from feather follicles was studied following the method

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of Marquardt (1972) with suitable modifications (Kamaldeep *et al.*, 2007).

**Extraction of DNA:** DNA from feather follicles of MDV infected birds was extracted using phenol:chloroform extraction method. From each sample, 1 cm of the proximal shaft of four feathers of different sizes was shaken overnight at 55°C in 200 µl proteinase K mixture (400 µg/ml proteinase K in appropriate buffer: 50mM ethylenediamine tetraacetic acid, 5% sodium dodecyl sulphate, 100mM Tris-HCl; pH 8.0). Twenty microlitre of 5M sodium acetate (pH 5.2) and 200 µl phenol/chloroform/isoamyl alcohol (25:24:1) were added, and the mixture was centrifuged at 13000g for 15 minutes. The hydrophilic phase was transferred to 200 µl isopropanol. The pellet was washed in cold (-20°C) 70% ethanol for 10 minutes, dried, resuspended in 100 µl sterile purified nuclease-free water and left at room temperature for at least 30 min before storing at -20°C until used in PCR assay.

**PCR Assay:** Two sets of primer pair were used to detect the presence of MDV in feather tips. For the amplification of MDV serotype-1 primers derived from ICP4 gene were used (Handberg *et al.*, 2001) and the primer sequences were: F: 5' GGATCGCCCACCACGATTACTACC3' and R: 5'ACTGCCTCACACAACCTCATCTCC3'. The second pair of primers was for Herpes Virus of Turkey (MDV serotype-3) and these primers were derived from US3 gene. The forward primer (5'ATGGAAGTAGATGTTGAGTCTTCG3') corresponded to nucleotides 4222-4245 and the reverse primer (5'CGATATACACGCAT TGCCATACAC3') to nucleotides 4704-4727 of the previously published MDV strain (accession no. X68653). Both the primer pairs (MDV serotype-1 and HVT) were got synthesized from Sigma-Aldrich Chemicals Pvt. Ltd., New Delhi in freeze-dried, desalted form; reconstituted in nuclease free water to 200 pmol/µl stock solution. Both the primer pairs were used in a single reaction.

**Amplification:** The reactant concentrations for 50 µl found optimum were: 10 µl DNA, 1 µl of each primer containing 20 pmol, pair 1 and 2, *Taq* DNA polymerase 3 U, 5 µl of 10X PCR buffer containing 1.5 mM MgCl<sub>2</sub>, 1 µl of 10 mM dNTPs. The amplification was carried out in a thermalcycler (Biometra) and the reaction conditions were: primary denaturation at 94°C for 3 min, 34 cycles of denaturation at 94°C for 40 sec, annealing at 58°C for 40

sec and extension at 72°C for 1 min followed by an extension step at 72°C for 5 min. The amplified PCR products were electrophoresed in 1% agarose gel containing ethidium bromide (0.5 µg/ml).

## RESULTS AND DISCUSSION

**Precipitation Test:** A white precipitating ring around feather tips was observed in agarose gel in samples A, B, E, G and I indicating positive reaction whereas no precipitation was observed in samples C, D, F, H and J. Thus a total of five samples were positive for the presence of MDV by this test.

**PCR:** A band of expected size of about 318-319 bp was observed in all the 10 samples (A to J) by PCR with ICP4 primers indicating all the samples to be positive for MDV serotype-1. However, amplification of HVT was not observed in these 10 samples although all the MD-affected flocks were vaccinated with HVT. A 505 bp size band was observed in HVT vaccine alone i.e. without any tissue DNA used as a positive control (Fig. 1).

The present study utilizes one-time data (June 2005 to March 2006) regarding the occurrence of Marek's disease in ten poultry flocks in six districts of Haryana. These poultry flocks had been vaccinated with HVT alone or bivalent (HVT+SB1) vaccine (Kamaldeep *et al.*, 2007). Occurrence of Marek's disease in vaccinated poultry flocks (breeders/commercial layers) calls for investigations into vaccination failures. In a previous study, Narang *et al.* (2003) also reported the occurrence of MD in vaccinated layer flocks in Haryana.

In the present study, proximal tip of feather follicles

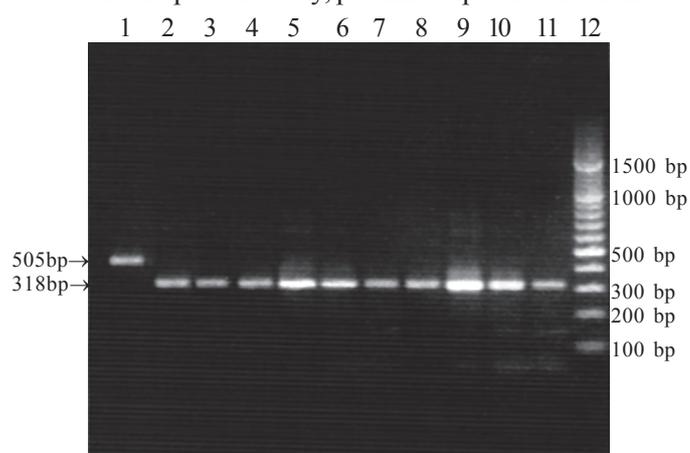


Fig 1. PCR analysis of field samples.

Lane 1: HVT vaccine; Lanes 2-11 (field samples A to J); Lane 12: 100 bp DNA marker

was utilized for extraction of MDV-specific DNA for amplification by PCR. All the ten poultry flocks were positive for the presence of MDV as determined by PCR assay. Handberg *et al.* (2001) detected MDV serotype-1 by PCR in feather tip extracts at the rates varying from 60% to 100%. Raja *et al.* (2009) also reported the presence of MDV serotype-1 in poultry flocks from three states of India.

In the PCR assay, the primers used were derived from ICP4 gene of genomic DNA of virulent GA strain of MDV serotype-1 (accession no. M75729) which corresponded to nucleotide numbers 6404-6427 and 6699-6722 of internal short repeat of ICP4 gene of *Gallid herpesvirus-1* (accession no. U17705). A band of MDV serotype-1 of expected size of 319 bp was observed on amplification from DNA extracts of feather follicles obtained from eight breeder flocks ranging from 8-22 weeks of age and two layer flocks of 24 weeks of age. Although, DNA from the HVT vaccine virus was amplified giving a band of expected size (505 bp) in the PCR assay but the same was not detectable from feather tips of MDV infected birds that were vaccinated with HVT alone or HVT plus SB1 vaccine. The expected size is in accordance with published sequences for MDV-1 (accession nos. M75729 and AF243438) and for HVT (accession no. X68653).

It may be concluded from the study that virulent MDV serotype-1 is widespread in Haryana state and this calls for a review of the vaccination strategy so as to reduce the incidence of Marek's disease. Polymerase chain reaction can be used as a potential tool for routine monitoring of flocks for early exposure to MDV as reported by Young and Gravel (1996). Moreover, PCR could be used to determine how vaccine level in feather correlates with protection against challenge and for identifying optimal timing, vaccine delivery route and optimal vaccination regimes for different breeds of chicks

as said by Baigent *et al.* (2006).

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