

## EPIDEMIOLOGY OF MAREK'S DISEASE IN COMMERCIAL LAYER FLOCKS

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Received: October 13, 2016; Accepted: October 27, 2016

**Abstract:** *A cross sectional study was carried out to assess the epidemiological measures of disease occurrence and casual association of Marek's disease (MD) in 12 different commercial layer flocks. Twenty representative blood and feather tip samples were collected from 12 different MD outbreak flocks. All the samples from each flock were pooled together and subjected to PCR using serotype 1 specific primer (BamH1/BamH2) for confirmation of MD. An epidemiological measures of casual association between MD and identified risk factors such as presence of aflatoxin in feed (> 0.02 ppm), inadequate down time (<12 weeks) and vaccination status were assessed by relative risk and odd's ratio. Marek's disease virus (MDV) was detected in feather follicle of nine flocks and four flocks in buffy coat of clinically affected birds by PCR. The morbidity and mortality rates were ranged from 2.01 to 9.15 and 1.03 to 7.6%, respectively. The aflatoxin level in the feed (> 0.02 ppm), type of vaccine and administration of booster dose had positive influence on occurrence of MD compared with inadequate down time.*

**Key words:** Marek's disease, Chickens

### INTRODUCTION

Marek's disease (MD) is a lymphoproliferative disorder of chickens characterized by oncogenic transformation of T cells that infiltrate into lymphoid tissues, peripheral nerves and visceral organs resulting in a complex pathogenesis that usually leads to death of the affected birds [1]. It is an economically important disease affecting poultry health worldwide [2], causing about \$1-2 billion annual economic loss to this industry [3]. The disease is caused by Marek's disease virus (MDV) and is classified as an alphaherpes virus belongs to the family Herpesviridae, subfamily alphaherpesvirinae and genus Mardivirus (Marek's disease like viruses) [4]. This shares

biological characteristic with gammaherpes viruses. Three serotypes were identified based on virulence and antigenic properties, such as serotype 1 (gallid herpes virus - 2) is oncogenic, serotype 2 (gallid herpes virus - 3) and serotype 3 (melegrid herpes virus -1) which is non-oncogenic in nature. The MDV serotypes are 50-80% similar at DNA sequence level [5]. Some strains of serotype 2 and 3 used as vaccine strain to protect birds against MD. Within serotype 1 MDV, four pathotypes are recognized such as mild (m), virulent (v), very virulent (vv) and very virulent plus (vv+) [6].

Following introduction of vaccination in 1970s, the problems of MD morbidity and mortality receded,

but vaccine breaks occurred due to continuing evolution of virulence and emergence of more virulent MDV pathotype [2]. The important factor is air borne transmission of virus from feather follicle and dander [7], due to poor sanitation of poultry house environment, inadequate down time, over stocking, immunosuppression, stress and co-infection with other diseases, which favours the spread of disease [8]. Recently, a high morbidity and mortality with suggestive of lesions of MD have been reported from several farms in India [9]. The objective of this study is to investigate the Marek's disease outbreaks in commercial layer flocks by PCR and associated risk factors by relative risk and odds ratio.

## MATERIALS AND METHODS

**Collection of clinical samples:** Twenty representative blood and feather tip samples were collected from 12 different Marek's disease outbreak commercial layer flocks located in and around Namakkal district of Tamil Nadu, India. All the samples from each flock were pooled together and considered as a single sample. The buffy coat was separated from blood by using Hisep LSM as per manufacturer's procedure. The cells were resuspended in a Basal Medium Eagle containing 25ml of foetal bovine serum and 10ml of dimethyl sulphoxide. The feather tip and purified cell suspension was stored at -20 °C until use.

**Data collection:** A cross sectional study was carried out in MD outbreak commercial layer flocks to assess the casual association of Marek's disease with associated risk factors. A detailed survey was undertaken using the questionnaires. Epidemiological data such as flock strength, number of birds affected and died, testing of aflatoxin level in feed (estimated after confirmation of MD), interval between two batches and vaccination status (type of vaccine and administration of booster dose).

**Extraction of DNA from feather tip:** Extraction of DNA was carried out as per method prescribed by Handberg et al. [10] with slight modification of RPM. The Proximal shaft (about one cm length) of feather tips were mixed with 50 µg of proteinase K

in 200 µl of proteinase K buffer and kept overnight at 55°C. Twenty microliter of 5M sodium acetate (pH 5.2) and 200 µl of phenol: chloroform: isoamyl alcohol mixture (25:24:1) was added and mixed well for 10 min. The contents were centrifuged under refrigeration at 12000 rpm for 15 min. Hydrophilic phase was transferred to 200 µl of isopropanol, mixed well and then centrifuged for 15 min at 12000 rpm under 4°C. Supernatant was removed and the DNA pellet was washed twice with cold (4°C) ethanol for 10 min by vortexing and centrifuged at 12000 rpm for 15 min under refrigerated condition. The ethanol was removed and air dried the DNA pellet for 15-30 min and dissolved in 50 µl sterile triple glass distilled water and left at room temperature for 30 minutes.

**Extraction of DNA from buffy coat:** Purified lymphocyte cell suspension was thawed at room temperature and cells were harvested by centrifugation at 1000 rpm for 10 min and supernatant was removed. Cell sediment was added with 50 mg of proteinase K and 200 ml of proteinase K buffer and the suspension was incubated at 55 °C for overnight. The DNA extraction procedure for buffy coat was similar to that of feather follicle.

**Polymerase chain reaction (PCR):** All the processed samples were subjected to PCR using serotype 1 specific primers. The primers were selected as suggested by Becker et al.[11], and PCR was carried out as per method prescribed by Handberg et al. [10]. The sequence of the primers were as follows:

BAMH1(F) TACTTCCTATATAGATTGAGACGT  
BAMH1(R) GAGATCCTCGTAAGGTGTAATATA

The PCR was performed with 100 p mol BamH1 (FP), 100 p mol BamH2 (RP), 12.50 µl of 2 x PCR Master mix, 7.50 µl DNase, RNase free water and 3 µl of DNA template. After mixing the contents, the DNA amplification was carried out by initial denaturation at 94 °C for 1 min followed by applying 31 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min with final extension at 72 °C for 10 min. The samples were analyzed with 1 % agarose gel electrophoresis using ethidium bromide.

$$\text{Morbidity rate (\%)} = \frac{\text{Number of birds manifesting the disease}}{\text{Number of birds at risk}} \times 100$$

$$\text{Mortality rate (\%)} = \frac{\text{Number of birds dying of the disease}}{\text{Number of birds at risk}} \times 100$$

**Data analysis:** Generally, the epidemiological measures of disease occurrence such as morbidity and mortality rates were calculated as per the reported method reported by Thrushfield [12] as follows

Epidemiological factors such as relative risk and odd's ratio were used to determine the association between diseases and hypothesized causal factors [12].

Group	Disease present	Disease absent	Total
Factor exposed	a	b	a+b
Factor not exposed	c	d	c+d
Total	a+c	b+d	a+b+c+d

Odds that a case is exposed divided by odds that a control is exposed as

$$\text{Relative risk} = \frac{\text{Incidence in birds exposed to risk factors}}{\text{Incidence in birds not exposed to risk factors}} = \frac{a/a+b}{c/c+d}$$

$$\text{Odds ratio} = \frac{ad}{bc}$$

## RESULTS AND DISCUSSION

The PCR was performed in feather tip and buffy coat by using serotype 1 specific primer Bam H1/BamH2 flanking 132 bp tandem repeats produced an expected band size of 434 bp (Fig.1). Out of twelve flocks, Marek's disease outbreaks were detected in feather follicle of nine flocks and four flocks in buffy coat of clinical affected birds. The PCR was capable of detecting MDV antigen in the feather tips and buffy coat of clinically affected birds. This observation was in agreement with the finding of earlier workers [10,11,14]. They detected MDV-1 antigen in feather follicle and buffy coat by PCR.

In the present study, the recovery of MDV antigen in feather tip was high compared to buffy coat. Hence, feather tips might be ideal sample for screening of MDV antigen in affected commercial layer flocks. This finding was in agreement with Handberg et al. [10] who proved that the PCR was a convenient tool for monitoring MDV in the poultry population and feather tips were the most convenient and quite sensitive samples.

Vaccination schedule and other epidemiological data were recorded in twelve different outbreak flocks under this study are shown in Table 1. The morbidity and mortality rates were ranges from 2.01 to 9.15 and 1.03 to 7.6 % respectively, were recorded in white leghorn breeds between 16 to 76 weeks age group.

In this study, cumulative mortality rate was less than 5% and this was in accordance with published report of Schat and Nair [7]. MD occurs in white leghorn breeds between 16 - 76 weeks age group of birds in this study, whereas the previous report showed MD commonly occur in 3 to 4 weeks old chicken and gradually builds to a peak between 12 and 30 weeks of age [15].

The epidemiological measures of casual association between disease and factors are shown in Table 2. Morbidity rate is taken for calculation of relative risk and odds ratio. The relative risk (RR) and odds ratio (OR) is > 1.0 indicates a positive influence, whereas <1.0 indicates negative influence on occurrence of disease. The aflatoxin level in feed samples were > 0.02 ppm. The relative risk and odds ratio were > 1.0 for presence of aflatoxin level in feed (> 0.02 ppm or 20 ppb), type of vaccine and administration of booster dose. This indicates that presence of high level of aflatoxin in feed, type of vaccine and administration of booster dose had positive influence on occurrence of MD. For inadequate down time, the relative risk and odds ratio was < 1.0 and it showed that it doesn't have influence on occurrence of MD.

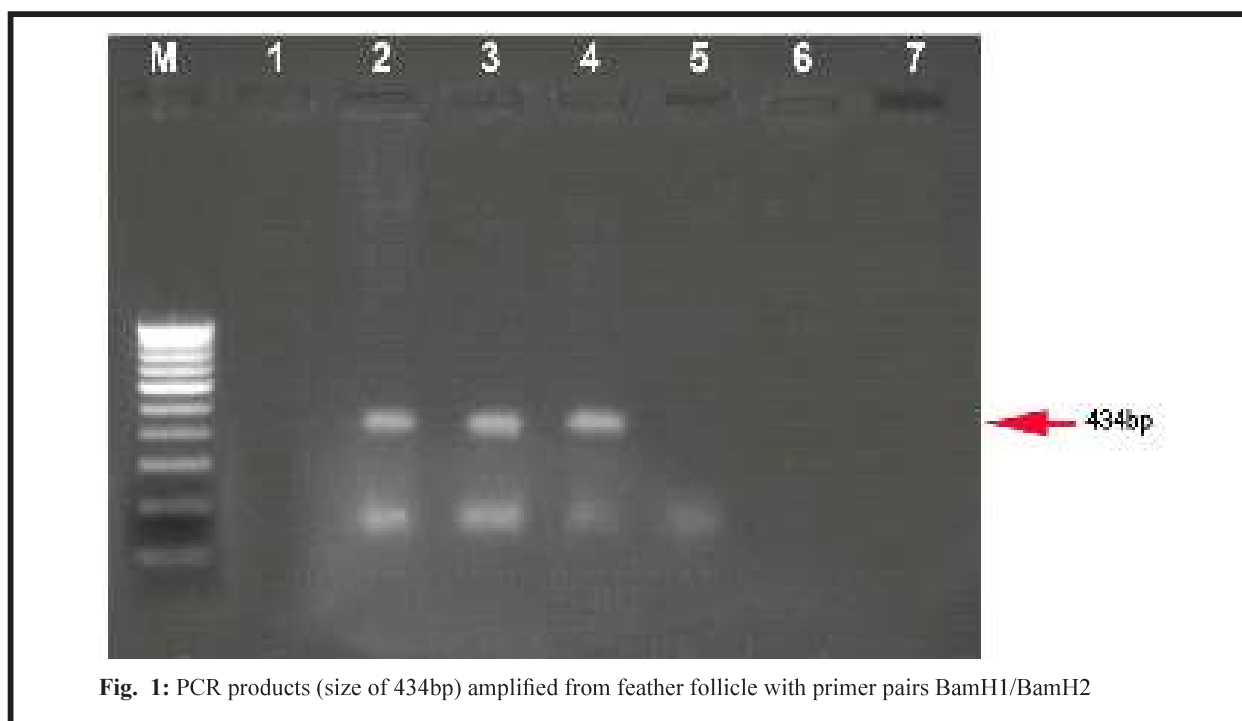
The presence of high level (>0.02 ppm) of aflatoxin in feed (permissible level 0.02 ppm or 20 ppb) [15] was casually associated with Marek's disease outbreak. Which might be due to possible fungal contamination (aflatoxin) of feed during storage. Batra et al. [17] also reported that the frequency and severity of gross and microscopic lesion of MD were significantly higher in those birds, which had been vaccinated with HVT and challenged with MDV when fed with aflatoxin B1 @ 0.5 ppm in the feed than in those given normal feed. Likewise, Monson et al. [18] discovered that Aflatoxin B1 (AFB1) consumption decreases

**Table 1:** Epidemiological measures of disease occurrence in MD affected flocks. + Presence of factor, - Absence of factor

Flock No.	Flock strength	Number of birds affected	Number of birds died	Morbidity rate (%)	Mortality rate (%)	Vaccination		Epidemiological factors	
						Type of vaccine on day old chicks	Booster dose within one weeks of primary vaccination	Aflatoxin in feed(>0.02ppm)	Down time (<12 weeks)
1.	10000	330	206	3.30	2.06	HVT	-	-	+
2.	10000	392	157	3.92	1.57	HVT	-	-	+
3.	6000	209	124	3.48	2.06	HVT	-	-	+
4.	8000	497	331	6.21	4.14	HVT+SB1	HVT+SB1	+	+
5.	25000	958	504	3.83	2.02	HVT+SB1	HVT+SB1	-	-
6.	2000	183	152	9.15	7.60	HVT	-	+	+
7.	6000	305	180	5.08	3.00	HVT	-	+	+
8.	10000	554	223	5.54	2.23	HVT+SB1	HVT+SB1	+	+
9.	8000	161	83	2.01	1.03	HVT	-	-	-
10.	4000	140	66	3.50	1.65	HVT	-	-	+
11.	2500	78	49	3.12	1.96	HVT	-	-	+
12.	5000	270	152	5.40	3.04	HVT+SB1	HVT+SB1	+	+

**Table 2:** Epidemiological measures of casual association between disease and factors

Epidemiological factors	Relative risk (RR)	Odds ratio (OR)
Alfatoxin level in feed (> 0.02 ppm or 20ppb)	1.68	1:73
Inadequate down time (< 12 wks)	0.73	0.72
Type of vaccine and booster dose	1.28	1.29



**Fig. 1:** PCR products (size of 434bp) amplified from feather follicle with primer pairs BamH1/BamH2

resistance to viral pathogens, including infectious bronchitis virus, infectious bursal disease virus, Marek’s disease virus and Newcastle disease virus.

The administration of bivalent vaccine (HVT+MDV 2) instead of monovalent vaccine (HVT) at hatchery had influence on occurrence of MD outbreak. This

was in accordance with the suggestion of Witter and Lee [19].

### CONCLUSION

The feather follicle was the ideal choice of sample for screening of Marek's disease (MD) compared to buffy coat and the presence of high level of aflatoxin in feed, type of vaccine and administration of booster dose had positive influence on occurrence of MD compared with inadequate down time. Further study required for identification of strains involved in MD outbreak flocks.

### ACKNOWLEDGEMENTS

The authors are thankful to Department of Veterinary Epidemiology and Preventive Medicine, Department of Veterinary Microbiology, Veterinary College and Research Institute, Namakkal for providing facilities to carry out research work. The authors also express their gratitude to Tamil Nadu Veterinary and Animal Sciences University, Chennai, Tamil Nadu, India for providing necessary facilities during the study and thanks to B.V. Rao Poultry Research foundation for financial assistance.

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