Isolation and molecular characterization of a virulent Marek's Disease virus serotype-1 from Andhra Pradesh, India

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Isolation,
Characterization,
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India.

Summary
Marek's disease (MD) is one of the most significant neoplastic diseases of poultry caused by Marek's disease virus (MDV), an oncogenic avian herpesvirus which is responsible for great economic losses to the poultry industry worldwide. MD is being manifested as an acute disease with lymphomas in multiple visceral organs. In the present study, an outbreak of MD was investigated in one of the poultry farms from Andhra Pradesh, India. The gross lesions in the affected birds included lymphomas in different visceral organs like liver, spleen, proventriculus, heart and ovaries. Histopathology revealed presence of uniform lymphoblastoid cell infiltration typical of MD. The isolation of the virus was carried out in duck embryo fibroblast cells. After three blind passages, the cell cultures revealed plaque formation typical of MDV. Further confirmation of the virus was carried out by PCR targeting 132 bp repeats of serotype-1 MDV and the oncogenes Meq and vIL-8 were amplified and sequenced. The nucleotide and phylogenetic analysis of the virus confirmed the virus as virulent serotype- 1 MDV. The present outbreak suggests the need for change in the vaccination regimen of MD vaccination with appropriate serotype- 1 MD vaccines in Indian poultry flocks as the HVT and bivalent vaccines are unable to protect the flocks against virulent MDV.

Introduction

Marek's disease (MD) is one of the most significant neoplastic diseases of poultry caused by Marek's disease virus (MDV) responsible for great economic losses to the poultry industry worldwide. MDV is an oncogenic avian herpesvirus classified as Gallid alphaherpesvirus 2, genus *Mardivirus*, family *Herpesviridae*, subfamily *Alphaherpesvirinae*. MDV is divided into three serotypes MDV-1, MDV-2 and the antigenically related Meleagrid alphaherpesvirus-1 known as serotype three (Herpes virus of turkey) (Dunn *et al.* 2014, ICTV 2018). Only strains of serotype 1 are capable of causing disease, while MDV-2 and MDV-3 strains are avirulent (Calnek, 2001). The disease was earlier described as paralytic/classical disease which is characterized by spastic paralysis of limbs with gross enlargement of peripheral nerves. But in the recent outbreaks, MD has been characterised by an acute disease with lymphomas in multiple visceral organs.

Initially, chickens were protected efficiently by vaccination with Herpesvirus of turkey (HVT). The disease was well controlled by wide spread use of commercially available HVT vaccines. Later on, bivalent vaccines consisting of HVT and serotype-2 herpesvirus were introduced to control the Marek's disease. Lately, even after vaccination with bivalent vaccines, outbreaks of MD have been reported in chickens of 21-40 weeks of age with high mortality rates (Sudhakar and Nair 2013). The increased evidence of MD in vaccinated poultry flocks could be attributed to the continuing evolution of more virulent MDV strains despite wide spread vaccination (Biggs and Nair 2012, Prathiba *et al.* 2018, Torres *et al.* 2019). There was gradual emergence of virulent pathotypes of MDV ranging from mild (m) to virulent (v), very virulent (vv) and very virulent plus (vv+) in the field in different countries of the world (Witter *et al.* 2005, Singh *et al.* 2012, Torres *et al.* 2019, Bertzbach *et al.* 2020). In the present study, an outbreak of Marek's disease in an organized poultry farm from Andhra Pradesh, India was investigated. Isolation of Marek's disease virus and

further molecular characterization of oncogenes was carried out.

Materials and methods

Collection of samples

In the present study, 18 tissue samples from postmortem cases and 2 blood samples from MD suspected live birds were collected from an organized poultry farm of around 1,000 birds in Andhra Pradesh, India. The flock consisted of 6 month old layer birds presenting high mortality rate. The history of vaccination of zero day chicks with bivalent vaccine (HVT and SB-1) at hatcheries and sudden mortality with no clinical signs but enlargement of different visceral organs was reported. Heparinized blood samples were collected in sterile vacutainers from live birds in MD suspected flocks and were transported to the laboratory under refrigerated conditions. Different organs including liver, spleen, kidneys, ovaries, heart, sciatic nerve, proventriculus with visible lymphomatosis and enlargement were collected in sterile sample containers from dead birds during postmortem for virological and molecular examination. Part of the representative organs were also stored in 10% formal saline for histopathological examination.

Extraction of DNA from tissues, blood and cell cultures

The extraction of DNA was done from tissues and Buffy coat cells using Alkaline lysis-Phenol-Chloroform method (traditional method) and QIAamp® DNA Blood Mini kit (Kit method), respectively. The extraction of DNA from duck embryonated fibroblasts (DEF) cell cultures was done using QIAamp® DNA Mini kit (QIAGEN) method as per the manufacturer's instructions. The absorbance of the DNA at wavelengths 260 nm and 280 nm was measured using Nanodrop. The viral DNA extracted from MDV infected tissue showed A260/A280 ratio of 1.8 to 2.

Oligonucleotide primers

The oligonucleotide primers which were used in the present study were procured from Eurofins Genomics India Pvt Ltd (Bangalore). The primer sequences reported by Tian and colleagues (Tian *et al.* 2011) in MDV amplification were used in the present study. As the primers of 132 bp repeat region identifies serotype-1 specific MDV's, it was considered as diagnostic primer for the detection of MD which can differentiate serotype-1 field MDV strains from vaccine strains. The length of one copy of 132 bp repeat is 182 bp long, containing one 132 bp repeat and 50 nucleotides from primers, the length of two copies of 132 bp repeats is 314 bp, and the length of three copies of 132 bp repeats is 446 bp. Meq and vIL-8 genes of Marek's disease virus were amplified which are responsible for the oncogenecity and pathogenecity of Marek's disease virus. The amplicon length of Meq and vIL-8 were 1,081 and 887 bps, respectively. The details of the primers used are shown in Table I.

Standardization of PCR targeting 132 bp repeat region

A positive MD DNA sample that was obtained from Directorate of Poultry Research, Rajendranagar, Hyderabad was used for standardization of PCR. The optimum conditions for PCR of 132 bp repeat region were as follows: initial denaturation at 94 °C for 4 min, 35 cycles of denaturation at 94 °C for 1 min, annealing at 56 °C for 1 min, elongation at 72 °C for 1 min, final elongation at 72 °C for 10 min and hold at 4 °C for 5 min.

Isolation of MDV in duck embryo fibroblast cell cultures

Isolation of the Marek's disease virus was attempted as per the standard method of OIE (OIE 2010). Briefly, buffy coat cells were suspended in minimum essential medium and freeze thawed for three times in order to lyse the cell membrane. Then, centrifugation was performed and the virus inoculum was made ready by filtering the supernatant with 0.45 µm syringe filter. 0.5 ml of the inoculum was inoculated on to 70% confluent DEF grown in 25 cm^2 tissue culture flask and incubated at 37 °C for one hour. After one hour of adsorption, the cells were rinsed and 8 ml of maintenance medium were added. An uninoculated DEF monolayer was maintained as a control. Both the inoculated and uninoculated cultures were subsequently incubated at 37 °C in an incubator with 5% (v/v) CO_2 atmosphere for 3-5 days. Serial passages were made on DEF monolayers and, at

Table I. *Primers used in PCR for detecting Marek's disease virus.*

Gene	Primer sequence	Length (Nucleotides)	GC Content (%)
MD-132 – F	5' ATG CGA TGA AAG TGC TAT GGA G 3'	22	45.5
MD-132 – R	5' ATC CCT ATG AGA AAG CGC TTG A 3'	22	45.5
Meq – F	5' GGC ACG GTA CAG GTG TAA AGA G 3'	22	54.5
Meq – R	5' GCA TAG ACG ATG TGC TGC TGA G 3'	22	54.5
vIL-8 – F	5' GAG ACC CAA TAA CAG GGA AAT C 3'	22	45.5
vIL-8 – R	5' TAG ACC GTA TCC CTG CTC CAT C 3'	22	54.5

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every passage, infected DEFs were checked for cytopathic effect and were preserved at - 80 °C for further passages.

Standardization of PCR for oncogenes (Meq and vIL8)

The PCR for amplification of oncogenes i.e. Meq and vIL-8 was standardized as per the method described by Tian and colleagues (Tian *et al.* 2011). The optimum conditions for PCR of Meq and vIL8 genes were as follows: initial denaturation at 94 °C for 4 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 59.4 °C for 1 min, elongation at 72 °C for 1 min and final elongation at 72 °C for 10 min and hold at 4 °C for 5 min.

Sequence analysis of oncogenes

PCR products of 132 bp repeat region, Meq and vIL-8 genes were sent for sequencing to Genomics Corp – Xcelris (Ahmedabad) (genomics.corp@ xcelrislabs.com). The sequences obtained were submitted to the Genbank via Sequin software. The nucleotide sequences obtained were analyzed with Genbank sequences by National Center for Biotechnology Information – Basic Local Alignment Search Tool (NCBI – BLAST) (www.ncbi.nlm.nih.gov/blastn) to check for homology. The nucleotide sequence of Meq gene of AP-01 MDV was compared with the sequences of 26 reference and other MDV strains (Table II) using MEGA 10.0 software. The nucleotide sequence of vIL-8 gene of AP-01 MDV was compared with the sequences of 14 reference and other MDV strains (Table III).

Phylogenetic analysis of the MDV isolate

A phylogenetic tree of Meq and vIL-8 genes was constructed with the aligned sequences of reference MDV strains and AP-01 MDV isolate using MEGA 10.0 software by Neighbour joining method using Tajima nei model.

Table II. *Marek's disease virus (MDV) reference strains of Meq gene published in GenBank.*

S. No.	MDV strains	Virulence	Geographic origin	Accession number
1	CU-2	Mild	USA	DQ534538
2	CVI988	Commercial vaccine	Netherland	DQ534538
3	BC-1	Virulent	USA	AY362707
4	JM	Virulent	USA	AY243331
5	567	Virulent	USA	AY362709
6	571	Virulent	USA	AY362710
7	573	Virulent	USA	AY362711
8	617A	Virulent	USA	AY362712
9	GA	Virulent	USA	M89471
10	Md-5	Very virulent	USA	AF243438
11	RB-1B	Very virulent	USA	AY243332
12	549	Very virulent	USA	AY362714
13	643P	Very virulent	USA	AY362716
14	595	Very virulent	USA	AY362715
15	L	Very virulent plus	USA	AY362717
16	N	Very virulent plus	USA	AY362718
17	New	Very virulent plus	USA	AY362719
18	W	Very virulent plus	USA	AY362723
19	X	Very virulent plus	USA	AY362724
20	648A	Very virulent plus	China	AF493558
21	584A	Very virulent plus	USA	DQ534532
22	Tn-n1	Very virulent	Tamil Nadu	HM749324
23	Tn-n2	Very virulent	Tamil Nadu	HM749325
24	Ind/KA12/02	Isolate	Tamil Nadu	KP342383
25	Ind/TN11/01	Isolate	Tamil Nadu	KP342384
26	Ind/TN12/03	Isolate	Tamil Nadu	KP342385

Results

Clinical signs and gross lesions

Birds with signs of paleness of combs and wattles, feather loss at neck region with enlargement of feather follicles were suspected for MD. From these MD suspected birds, blood samples were collected and buffy coat was separated. Grossly, in majority of the MD cases, the liver, spleen, proventriculus and kidneys were most commonly affected. Sciatic nerve, ovaries, heart, mesentery and lungs were, less commonly,

Table III. *Marek's disease virus (MDV) reference strains of vIL-8 gene published in GenBank.*

S. No	MDV strains	Virulence	Geographic origin	Accession number
1	CU-2	Mild	USA	EU499381
2	CVI988	Commercial vaccine	Netherland	DQ534538
3	LS	Virulent	China	HQ638183
4	LMS	Virulent	China	HQ658622
5	571	Virulent	USA	DQ534531
6	Ind/TN11/01	Virulent	Tamil Nadu	KP644421
7	Ind/TN12/03	Virulent	Tamil Nadu	KP644420
8	Ind/KA12/02	Virulent	Tamil Nadu	KP644422
9	GA	Virulent	USA	AF147806
10	Md5	Very virulent	USA	AF489275
11	RB1B	Very virulent	USA	EF523390
12	595	Very virulent	USA	DQ534533
13	648A	Very virulent plus	USA	DQ534534
14	584A	Very virulent plus	USA	DQ534532

affected. All these organs showed enlargement with discrete greyish white nodules of various sizes.

In all observed cases, liver was enlarged several imes beyond its normal size with multifocal greysh white nodules of 2.5 in diameter (Figure 1). Multifocal nodules were observed in lungs (Figure 2). Enlargement of the organ upto the size of a small egg and numerous nodules/ lymphomas of the size of a pipoint to 1 mm distributed throughout the surface of the spleen were noticed in the majority of the samples (Figure 3). In proventriculus, the wall was thickened and in few birds, ulceration and congestion of proventricular mucosa were noticed (Figure 5). White multi focal nodules of 1 mm in size were observed in the heart (Figure 6). In sciatic nerve, edematous lesion with loss of cross striations was observed in few birds (Figure 4).

Histopathological changes

The histopathological examination of liver showed extensive proliferation of pleomorphic lymphoid cells in between hepatic cords resulting in loss of hepatic architecture and replacement of hepatic parenchyma by infiltration of pleomorphic cells (Figure 7). Massive proliferation of lymphoblast cells and infiltration of pleomorphic cells were noticed in the spleen (Figure 11). This led to lack of differentiation between red and white pulp and also lack of differentiation between splenic corpuscles and germinal centers. In the proventriculus, infiltration of pleomorphic cells into interstitial connective tissue with cystic dilatation and atrophy of proventricular glands was observed (Figure 10). In the kidney, diffuse proliferation and infiltration of lymphocytes, plasma cells and macrophages in the interstitial connective tissue and partial to complete loss of renal architecture i.e. renal tubules and glomerulus were noticed (Figure 9). Ovary revealed

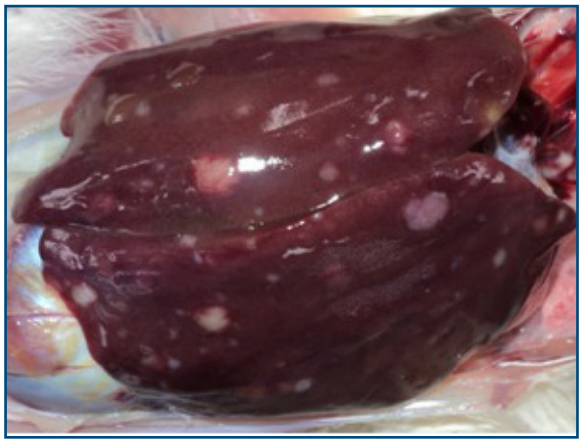

Figure 1. *Enlarged liver with multifocal whitish nodules of a Marek's disease virus infected chick.*

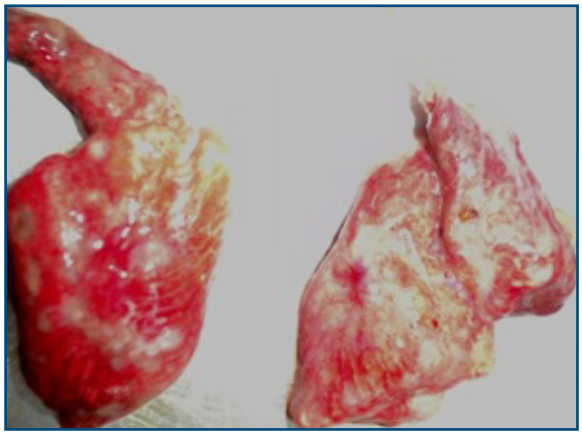

Figure 2. *Multifocal nodules in lungs of a Marek's disease virus infected chick.*

Figure 3. *Spleen showing greyish white multifocal nodules of a Marek's disease virus infected chick.*

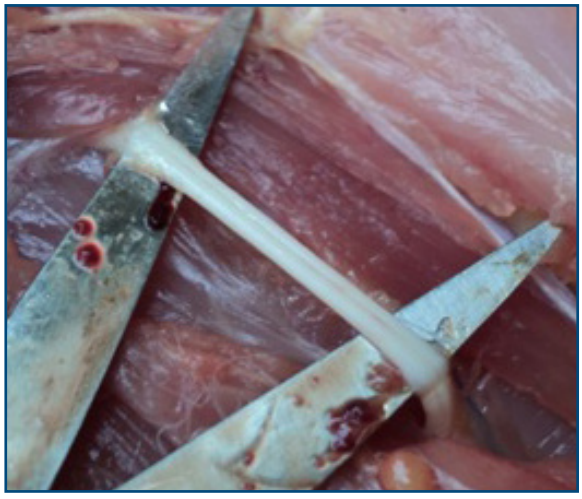

Figure 4. *Sciatic nerve showing edema and loss of cross striations of a Marek's disease virus infected chick.*

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extensive infiltration of stromal tissue around graffian follicle and corpus luteum obliterating normal parenchyma of ovary (Figure 8).

PCR targeting 132 bp repeat region of serotype-1 MDV

DNA isolated from different tissue and blood samples was subjected to PCR targeting 132 bp repeat region as mentioned earlier. Out of 18 tissue and 2 blood samples tested, all were positive for serotype-1 specific MD yielding a 314 bp product in PCR amplification which indicates presence of two copies of 132 bp tandem repeats (264 bases) along with 50 bases of primers (Figure 12).

Isolation of Marek's disease virus in duck embryo fibroblast cell cultures

Buffy coat lysate was inoculated into DEF monolayer and serial passages were made. After five passages in DEF, MDV positive samples showed cytopathic effect. The CPE included appearance of round refractile cells and after incubation for three days

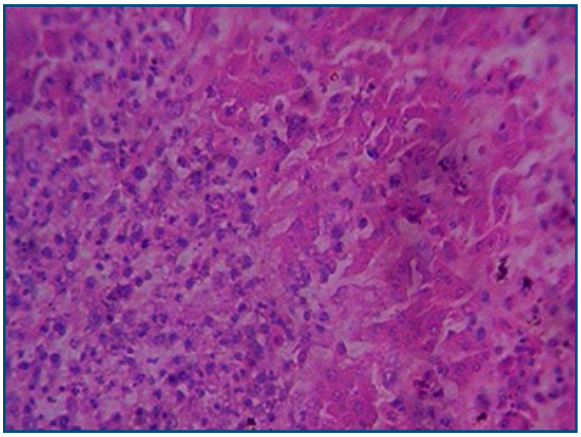

Figure 7. *Liver of a Marek's disease virus infected chick showing proliferation of pleomorphic lymphoid cells with loss of hepatic architecture H & E x280.*

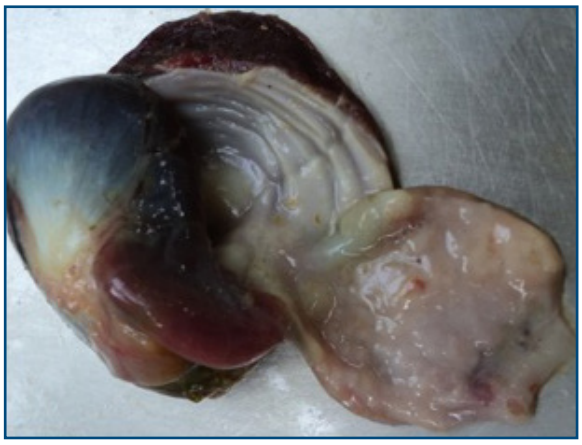

Figure 5. *Thickened pro-ventricular wall of a Marek's disease virus infected chick with ulceration of mucosa.*

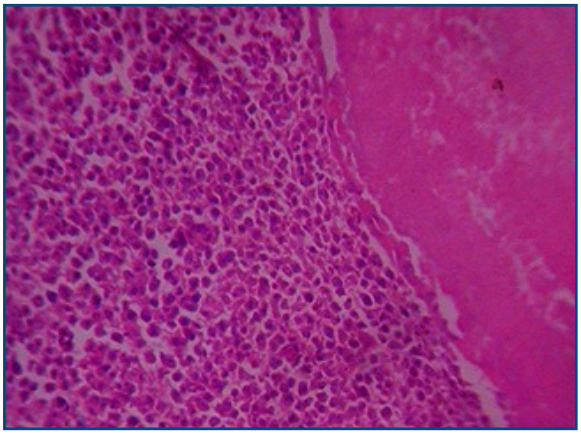

Figure 8. *Ovary of a Marek's disease virus infected chick with pleomorphic cell infiltration with obliteration of ovarian parenchyma H & E x280.*

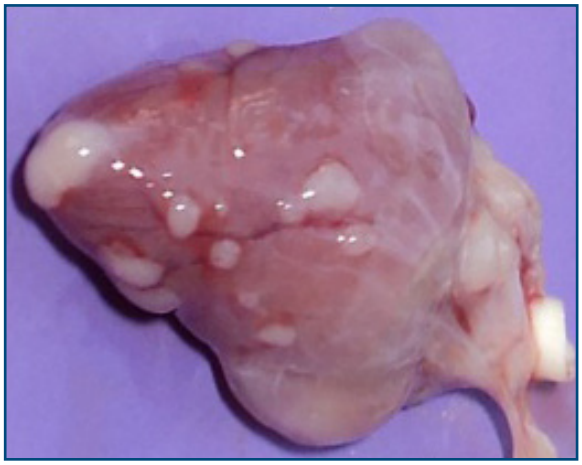

Figure 6. *Multifocal greyish white nodules over the epicardial surface of heart of a Marek's disease virus infected chick.*

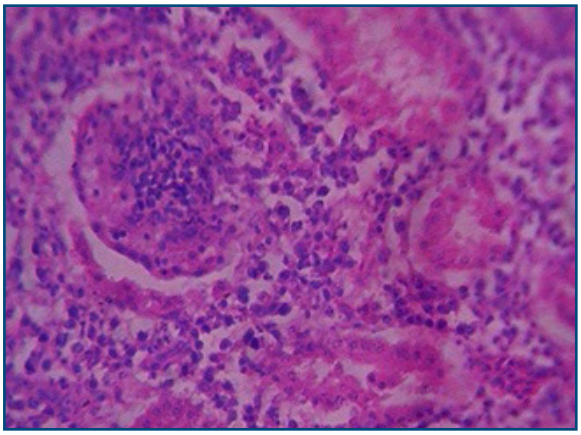

Figure 9. *Kidney of a Marek's disease virus infected chick showing infiltration of lymphoblasts with obliteration of renal tubules H & E x280.*

post infection, each plaque was surrounded by the additional refractile cells at periphery with formation of syncytia. Further incubation showed development of clear areas in the centre of the plaques six days post infection (Figures 13 to 15).

Confirmation of MDV serotype-1 from cell cultures

The cells were then freeze thawed three times and the cell culture fluids were harvested from cells showing characteristic CPE specific to MDV. DNA extraction was performed using QIAamp® DNA Mini Kit (QIAGEN). PCR was performed using primers targeting 132 bp repeat region of MDV. The sample showed a 314 bp PCR product confirming the presence of MD serotype 1 virus in the cell culture fluids. Appropriate negative controls were incorporated in the test. The isolate was designated as AP-01 MDV.

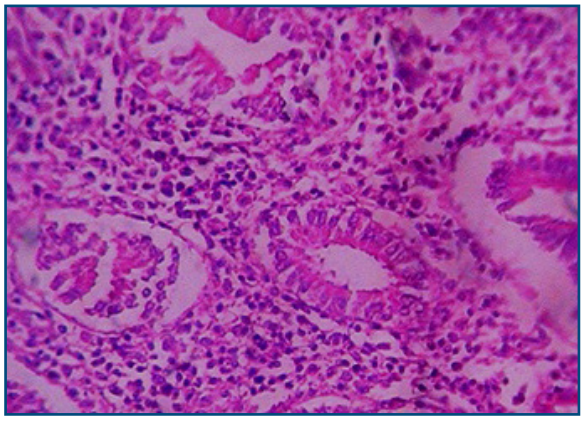

Figure 10. *Proventriculus of a Marek's disease virus infected chick with infiltration of lymphoblasts in between proventricular glands H & E x280.*

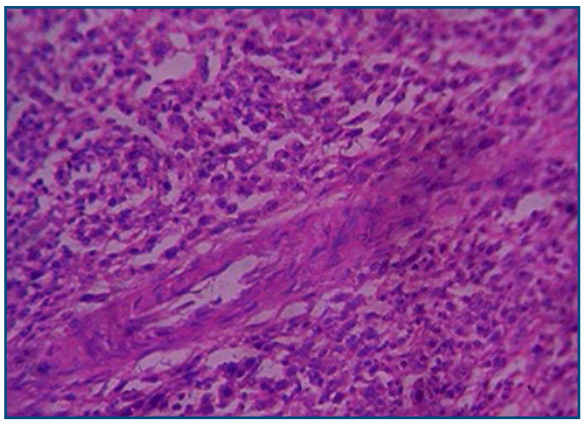

Figure 11. *Spleen of a Marek's disease virus infected chick showing pleomorphic lymphoid cell infiltration H & E x280.*

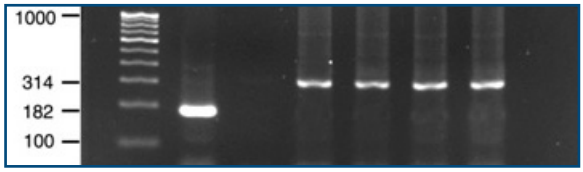

Figure 12. *Screening of Marek's disease suspected field samples using PCR for 132 bp repeat region.* Lane M = Molecular weight marker (100 bp); Lane 1 = Positive control; Lane 2 = Negative control; Lane 3 to 6 = Field samples positive for Marek's disease virus serotype-1.

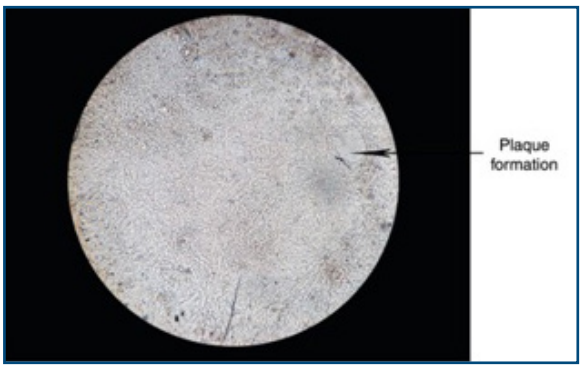

Figure 13. *Duck embryo fibroblast cell monolayer – Normal.*

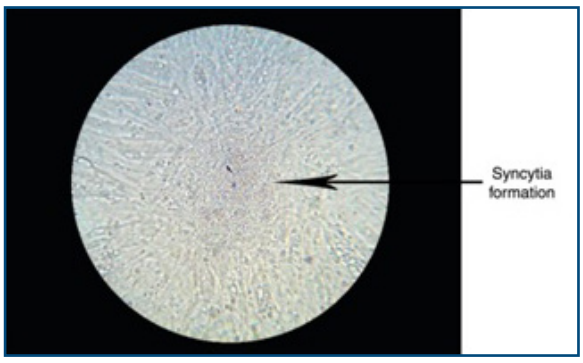

Figure 14. *Duck embryo fibroblast cell monolayer infected with Marek's Disease virus (MDV) serotype-1 showing syncytia formation on day 3-PI.*

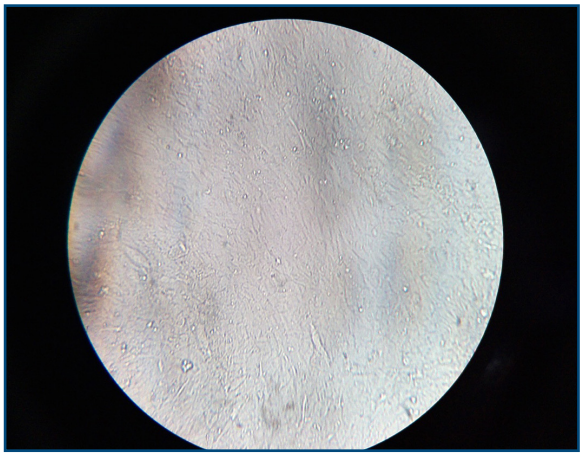

Figure 15. *Duck embryo fibroblast cell monolayer infected with Marek's Disease virus (MDV) serotype-1 showing plaque formation with zone of clearance on day 7-PI.*

PCR and sequence analysis of oncogenes (Meq and vIL-8) from Ap-01 MDV

The DNA obtained from AP-01 isolate was further subjected to PCR for amplification of oncogenes (Meq and vIL-8). The DNA samples were found to be positive for the presence of oncogenes (Meq and vIL-8) and yielded amplicons of 1,081 bp and 887 bp, respectively (Figures 16 and 17). The purified PCR products of Meq and vIL-8 were sent for sequencing to Genomics corp – Xcelris, Ahmedabad. A total of 989 nucleotides of Meq and 821 nucleotides of vIL-8 were read. The obtained nucleotide sequences of Meq and vIL-8 genes were verified by NCBI-BLAST for homology analysis. The nucleotide sequences showed homology with Meq and vIL-8 genes of different strains of serotype-1 specific MDV strains. The nucleotide sequences of Meq and vIL-8 genes were submitted to Genbank database. The accession number for Meq gene is KT246100 and vIL-8 is KT272874.

Comparison of AP-01 MDV nucleotide sequence of Meq gene with Genbank sequences

Comparison of the nucleotide sequence of Meq gene of AP-01 MDV showed from 84.5 to 99.6% homology with other sequences of MDV. The sequences of AP01 had the highest homology with RB1B (99.6%) and GA (99.5%) and had the lowest homology with CVI988 strain (84.5%). AP-01 showed nucleotide mutations at positions 229 (A→G), 238 (G→T), 415 (A→G), 626 (T→C) of 1,020 Meq gene in comparison with RB1B (very virulent) and GA (virulent) strains. AP01 nucleotide sequence showed variations at three positions 262 (A→G), 278 (G→A) and 626 (T→C) with closely related Tamil Nadu MDV isolates.

Comparison of AP-01 MDV nucleotide sequence of vIL-8 gene with Genbank sequences

Comparison of the nucleotide sequence of vIL-8 gene of AP-01 MDV showed from 99.44 to 99.87% homology with other sequences of MDV. The sequence of AP-01 had the highest homology (99.87%) with LS and LMS virulent strains and had the lowest homology (99.44%) with CVI988 strain. When AP-01 MDV nucleotide sequence of vIL-8 gene was compared with the reference strain LMS, one variation at position 106 (T→C) was shown. Nucleotide sequences of vIL-8 gene of AP-01 MDV showed 100% similarity with sequences of field isolates of Tamil Nadu (Ind/TN11/01 and Ind/TN12/03) and Karnataka (Ind/KA12/02).

Comparison of Ap-01 MDV amino acid sequences of oncogenes with Genbank sequences

The total amino acid length of Meq protein was 339 amino acids and the total length of vIL-8 protein was 134 amino acids. Comparison of Meq and vIL-8 gene amino acid sequences of AP-01 MDV was done with those of reference strains and field isolates of Tamil Nadu and Karnataka.

The amino acid sequence of Meq gene of AP- 01 was neither mild type nor very virulent plus MDV. There was a mutation at position 71 (S→A) in the Meq gene which is associated with higher virulence in the MDV strains. Compared to closely related strains RB-1B (vv) and GA (v), AP01 showed variations in 77 (K to E), 80 (D to Y), 139 (T to A), 209 (L to P) amino acid positions. Compared to closely related strains 571 and 573 (v), AP01 showed variations in 115 (A to V), 139 (T to A), 176 (H to P), 209 (L to P) amino acid

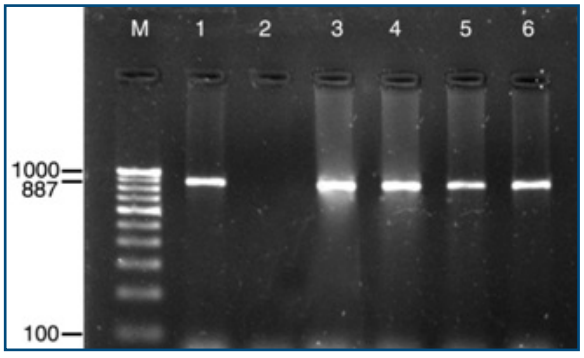

Figure 16. *Screening of Marek's disease suspected field samples using PCR for 132 bp repeat region.* Lane M = Molecular weight marker (100 bp); Lane 1 = Positive control; Lane 2 = Negative control; Lane 3 to 6 = Field samples positive for Marek's disease virus serotype-1.

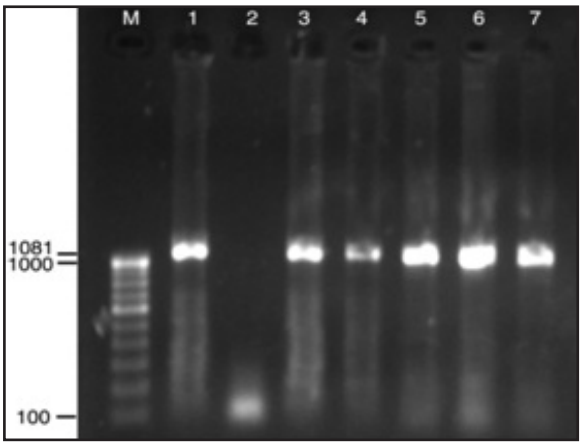

Figure 17. *Screening of Marek's disease suspected field samples using PCR for 132 bp repeat region.* Lane M = Molecular weight marker (100 bp); Lane 1 = Positive control; Lane 2 = Negative control; Lane 3 to 6 = Field samples positive for Marek's disease virus serotype-1.

positions. The deduced amino acid sequences of Meq protein of AP01 MDV showed three variations in the amino acid sequence at positions 88 (T→A), 93 (R→Q) and 209 (L→P) compared with field isolates of Tamil Nadu (Ind/TN11/01 and Ind/TN12/03) and Karnataka (Ind/KA12/02) (Table IV).

Alignment analysis of the deduced amino acid sequence of vIL-8 gene of AP01MDV and 14 published MDVs was performed. Comparison of amino acid sequence of vIL-8 protein of AP01 with that of reference strains like CU-2, GA, RB1B, 648A, 584A, Md5, 595, 571 and CVI988 showed that mutations exists at position 4 (L→S) and 31 (D→G). The amino acid mutations at position 4 and 31 were similar to the virulent reference strains LMS and LS. The amino acid sequence was 100% identical with field isolates of Tamil Nadu (Ind/TN11/01 and Ind/TN12/03) and Karnataka (Ind/KA12/02) (Table V).

Phylogenetic analysis of Meq and vIL-8 genes of MDV

The phylogenetic analysis of Meq gene of the AP-01 MDV nucleotide sequence with different reference and other strains in the Genbank (n = 26) formed a cluster with 571, 573 virulent and other MDV strains from Tamil Nadu and Karnataka as shown in the Figure 18. The phylogenetic analysis of vIL8 gene of the AP-01 MDV nucleotide sequence with different reference and other strains in the Genbank (n = 14) formed a cluster with LS and LMS virulent MDV strains and those of Tamil Nadu and Karnataka as shown in the Figure 19.

Discussion

MD almost devastated the poultry industry in the 1960s but the disease was brought under control after introduction of live vaccine containing HVT. Subsequently, severe cases caused by more virulent strains of MDV which could not be controlled by using HVT vaccination occurred. Hence, bivalent vaccines consisting of HVT and attenuated serotype-2 were developed and used in the field. The widespread use of vaccines against Marek's Disease has been suggested to have led to the evolution

Table IV. *Aminoacid substitutions in the Meq protein of Marek's Disease virus serotype-1 (MDV-1) field strains with reference strains.*

S. No	Virulence & field samples	Strain name	Amino acid substitutions in the Meq protein of MDV-1 field strains and reference strains																
			71	77	80	88	93	115	119	139	153	176	180	209/ 268[a]	217/ 276[a]	277/ 336[a]	283/ 342[a]	320/ 379[a]	326/ 385[a]
1	Vv	Strain RB1B	A	K	D	A	Q	V	C	T	P	P	T	L	P	L	A	I	T
2	V	Strain GA	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
3	V	Strain 567	.	E	Y	.	.	.	R	.	.	.	.	.	A	.	.	.	.
4	V	Strain 617A	.	E	Y	.	.	.	R	.	.	.	.	.	A	.	.	.	.
5, 6	V	Strain 571, 573	.	E	Y	.	.	A	.	.	.	H	.	.	.	.	.	.	.
7	Field sample	AP01 Meq	.	E	Y	.	.	.	.	A	.	.	.	P	.	.	.	.	.
8	TN Isolate	Ind/TN11/01	.	E	Y	T	R	.	.	A	.	.	.	.	.	.	.	.	.
9	TN Isolate	Ind/TN12/03	.	E	Y	T	R	.	.	A	.	.	.	.	.	.	.	.	.
10	KA Isolate	Ind/KA12/02	.	E	Y	T	R	.	.	A	.	.	.	.	.	.	.	.	.
11	Vv	Isolate Tn-n1	.	.	.	.	.	.	.	.	.	.	.	P	.	.	.	.	.
12	Vv	Isolate Tn-n2	.	.	.	.	.	.	.	.	.	.	.	P	.	.	.	.	.
13	V	Strain BC-1a	S	A	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.
14	V	Strain JMa	S	A	.	.	R	A	.	.	.	.	.	.	.	.	.	.	.
15	M	Strain CU-2a	S	E	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
16	Vaccine	Strain CVI988a	S	E	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I
17	Vv	Strain Md5	.	.	.	.	.	.	.	.	.	.	.	.	A	.	V	T	.
18	Vv+	Strain W	.	.	.	.	.	.	.	.	.	.	.	.	A	.	V	T	.
19	Vv	Strain 643P	.	.	.	.	.	.	R	.	Q	A	A	.	A	F	.	.	.
20, 21	Vv+	Strain New, 584A	.	.	.	.	.	.	R	.	Q	A	.	.	A	.	V	T	.
22, 23	Vv	Strain 549, 595	.	.	.	.	.	.	R	.	Q	A	A	.	A	.	.	.	.
24, 25	Vv+	Strain L,X	.	.	.	.	.	.	R	.	Q	A	A	.	A	.	.	.	.
26	Vv+	Strain 648A	S	E	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.
27	Vv+	Strain N	.	.	.	.	.	.	R	.	Q	A	A	.	A	P	.	.	.

[a] Denote strains containing 59 amino acid proline rich repeat amplification

Table V. *Amino acid substitutions in the vIL-8 protein of Marek's Disease virus serotype-1 (MDV-1) field and reference strains.*

S.No	Virulence and Field samples	Strain Name	4	31	67	81
1	V	Strain LMS	S	G	M	E
2	V	Strain LS	.	.	I	G
3	Field sample	AP01 vIL-8	.	.	I	.
4	Vv	Ind/KA12/02	.	.	I	.
5	V	Ind/TN11/01	.	.	I	.
6	V	Ind/TN12/02	.	.	I	.
7	V	Strain 571	L	D	I	.
8	V	Strain GA	L	D	I	.
9	Vv	Strain RB1B	L	D	I	.
10	Vv	Strain Md5	L	D	I	.
11	Vv	Strain 595	L	D	I	.
12	Vv+	Strain 584A	L	D	I	.
13	Vv+	Strain 648A	L	D	I	.
14	M	Strain CU-2	L	D	I	.
15	Vaccine	Strain CVI988	L	D	I	.

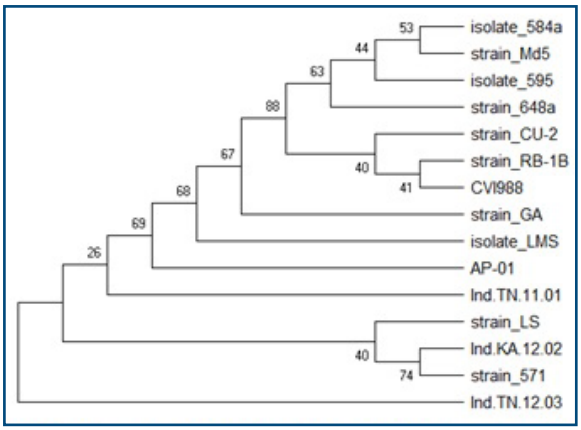

Figure 18. *Phylogenetic analysis of Meq gene nucleotide sequence of AP-01 with other reference sequences.* The phylogenetic tree was constructed using the MEGA version 10.0 by the neighbor joining method with 1,000 bootstrap replicates using Tamura nei model.

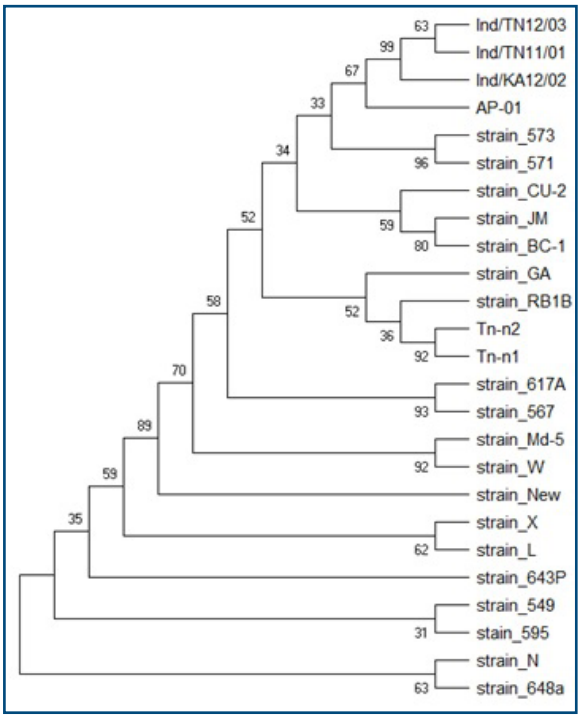

Figure 19. *Phylogenetic analysis of vIL8 gene nucleotide sequence of AP-01 with other reference sequences.* The phylogenetic tree was constructed using the MEGA version 10.0 by the neighbor joining method with 1000 bootstrap replicates using Tamura nei model.

of field viruses with greater virulence. A number of pathotypes classified as vMDV, vvMDV, and vv+MDV have in fact been isolated (Witter 2001, Witter *et al.* 2005). These more virulent strains could overwhelm the protection conferred by currently available vaccines (Witter 1997). These virulent strains of MDV are resulting in production of lymphomas in different visceral organs.

In the present study, 18 tissue samples from postmortem cases and 2 blood samples from MD suspected live birds were collected from an organized poultry farm, Tirupati, Andhra Pradesh. Some of the birds were found dead without showing clinical signs. Some birds were found depressed with paleness of combs and wattles, feather loss at neck region with feather follicle enlargement. Lately, clinical picture of MD is slowly changing from the classical form of Marek's disease characterised by birds showing nervous symptoms with unilateral paralysis with typical posture of one leg stretched forward and other leg backward. In the current outbreak, the birds were apparently normal without any clinical manifestation as the disease progression is more towards the formation of lymphomas rather than the classical nervous manifestation. Similar observations were also reported by other researchers where the involvement of oncogenic more virulent serotype-1 MDV strains was reported (Okwor and Eze 2011, Sudhakar and Nair 2013, Puro *et al.* 2018). The postmortem examination of the MD suspected birds in the present investigation revealed the involvement of liver, spleen, proventriculus and kidneys in majority of the cases. Organs like sciatic nerve, ovaries, heart, mesentry and lungs were also found involved in some of the birds.

PCR targeting 132 bp repeat region was standardized and all the samples yielded a 314 bp product in PCR indicating the presence of two copies of 132 bp tandem repeats (Doosti and Golshan 2011, Tian *et al.* 2011). It has been reported that no comparable 132 bp repeat region exists in either serotype-2 or HVT (Silva *et al.* 2004). The results of sequencing confirmed the presence of pathogenic serotype-1

MDV strains in the field samples tested in the present study. Hence, PCR targeting 132 bp repeat region can be used for specific detection of serotype-1 MDV strains and can differentiate the pathogenic MDV strains from that of vaccine strains.

Attempts for the isolation of the MDV was carried out in DEF cell cultures. After three subsequent blind passages, a characteristic CPE of serotype-1 MD virus was observed in the infected cell cultures. After three day of inoculation, the monolayers showed the appearance of round refractile cells with formation of syncytia. After further 3 days of incubation clear areas developed in the centre of plaques. The CPE was more pronounced and clear in further passages (5^{th} to 7^{th}). Similar CPE was reported in DEF monolayers by Gopal and colleagues (Gopal *et al.* 2012) and Gong and colleagues (Gong *et al.* 2013). The presence of serotype-1 MDV in the cell culture fluid was confirmed by extraction of DNA and PCR for 132 bp repeat region.

The genome of serotype-1 MDVs contains several oncogenes which are important in viral pathogenesis and in the clinical manifestation of Marek's disease viruses in the field outbreaks. In the present outbreak, affected birds showed the presence of lymphomas in different visceral organs. Hence, further studies were carried out for characterization of viral oncogenes. The nucleotide sequence of Meq gene of AP01 had the highest homology with RB1B (99.6%) and GA (99.5%) strains and had the lowest homology with CVI988 strain (84.5%). The nucleotide sequence of vIL8 gene of AP01 showed the highest homology (99.87%) with the LS and LMS virulent strains and the lowest homology (99.44%) with CVI988 strain. It was 100% identical with the sequences of Ind/TN11/01, Ind/KA12/02 and Ind/TN12/03.

The deduced amino acid sequences of Meq and vIL-8 genes of AP-01 were compared with those of the reference and other previously reported MDV strains. The amino acid variations are mostly located at nine positions i.e. 71, 77, 80, 115, 119, 139, 153, 176 and 217 (Tian *et al.* 2011, Gong *et al.* 2013, Sathish *et al.* 2014).

Low virulence MDV strains including CU-2, BC-1, JM and CVI988 showed 59 amino acid insertions with proline-rich repeats in the deduced amino acid sequences of Meq gene. The amino acid sequences of AP-01 MDV were of 339 amino acids without the 59 amino acid proline rich insertion indicating that these strains belong to more virulent MDV strains. In AP-01, the amino acid positions 71 and 77 showed the presence of Alanine and Glutamate, respectively. The amino acid mutations at positions 80 (Aspartate→Tyrosine), 139 (Threonine→Alanine) were also observed. The mutation in positions 80, 115, 139, 176 of Meq protein could be used as virulent genetic characteristic of the circulating MDVs in China (Zhang *et al.* 2013). The deduced amino acid sequences of Meq and vIL8 genes of AP01are hence comparable to virulent/very virulent MDVs.

In the very virulent plus MDVs, there was an amino acid mutation at position 119 (C→R). Higher virulence (vv and vv+) MDVs had point mutations at the positions 153 (P→Q), 176 (P→A) and 217 (P→A) which were not present in the amino acid sequences of AP-01in the present study.

The alignment analysis of the deduced amino acid sequences of vIL-8 gene of AP-01 was similar to the LMS and LS (virulent) reference strains and other virulent MDVs reported from India. The present study showed that Meq is the most important oncogene which shows variable mutations in MDVs of different virulence. The Meq gene is present only among MDV oncogenic strains. The strongest association with the observed increased virulence is the polymorphisms identified in the major oncoprotein and transcription factor Meq. Despite the rather low evolutionary rate of double-stranded DNA viruses (Firth *et al.* 2010, Trimpert *et al.* 2019), it has been reported that the meq gene is evolving at a much faster rate than most genes in double-stranded DNA viruses (Padhi and Parcells 2019). The vaccine strains i.e. HVT and MDV serotype-2 lack Meq gene, it is therefore likely that the changes in Meq gene might have occurred as a result of functional selection for their effects on MDV and/or cellular gene expression. Hence, it can be concluded that mutations in the putative oncogene Meq appear to be correlated with increased MDV virulence. Similar results were recorded by Kumar and colleagues (Kumar *et al.* 2012) and Suresh (Suresh 2013).

In the present study, the samples were collected from MD outbreaks of chicken which received HVT and SB-1 bivalent MD vaccines. Based on the nucleotide, amino acid, phylogenetic analysis and the vaccination status of the affected flocks, the MDV strain obtained in the present outbreak of Andhra Pradesh could be designated as virulent/ very virulent MDV. The control strategies for MDV in poultry flocks includes the use of live vaccines and maintenance of good hygiene and management in the poultry farms. Initially, the HVT live vaccine was developed and administered for several years in India. As the protection conferred by this vaccine was not optimum, a bivalent vaccine including HVT and an avirulent serotype 2 MDV was developed. As MDV strains evolved becoming more virulent, bivalent vaccines were not anymore capable of protecting poultry flocks. In most of the European countries, USA and China, more efficacious serotype-1 MD vaccine with CVI988/Rispens strain is being successfully used (Kumar *et al.* 2012, Dunn and Gimeno 2013). The increasing virulence of MDV may pose a threat to the standard MD prevention

strategy, progressively reducing the success of vaccine protection, especially for programs based on HVT strain vaccines. Research and continuing surveys may provide answers regarding the epidemiology of MD, the evolving virulence of circulating MDV strains, and might enable determining the best fit vaccination protocols and strategy (Bertzbach *et al.* 2020).

In India, we are still practicing single or bivalent vaccines and serotype-1 MD viruses are not yet used for vaccination in the field. In the context of emergence of more virulent MD viruses in the southern parts of India and failure of present MD vaccines, it is appropriate to recommend usage of more efficient serotype-1 MDV vaccine strains in the poultry flocks to reduce the Marek's disease incidence in the country.

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