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Molecular characterization and phylogenetic analysis of oncogenes from virulent serotype-1 Marek's disease virus in India

Y. PRATHIBHA, B. SREEDEVI, N. VINOD KUMAR, CH. SRILATHA

Department of Veterinary Microbiology, College of Veterinary Science, Sri Venkateswara Veterinary University, Tirupati – 517502, Andhra Pradesh, India

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Summary. – Marek's disease (MD) is one of the most important neoplastic diseases of poultry caused by Marek's disease virus (MDV), an oncogenic avian herpes virus, which is responsible for great economic losses to the poultry industry worldwide. Inspite of the usage of HVT and bivalent vaccines in the poultry flocks, MD continues to be a major threat to the poultry industry in India. In the present study, MD outbreaks were reported in poultry farms from different regions of Andhra Pradesh, India. The postmortem examination of dead birds showed presence of lymphomas in different visceral organs suggestive of virulent oncogenic MDVs. Histopathological examination revealed infiltration of pleomorphic lymphoblastoid cells typical of MD. The blood and tissue samples were collected and PCR was standardized targeting a 132 bp tandem repeat region specific for serotype-1 MD viruses. Further, the characterization of the oncogenes i.e. Meq and viral interleukin 8 (vIL-8) was carried out by PCR and nucleotide sequencing. The sequence analysis of Meq gene of different clinical cases from India revealed >99 % homology with RB1B (very virulent) and GA (virulent) strains and that of vIL-8 gene showed >99 % identity with virulent strains LS and LMS. Phylogenetic analysis of oncogenes was carried out with other available sequences in the GenBank. Finally, we conclude that MDV strains obtained in the present outbreaks in India could be designated as virulent or very virulent pathotypes based on nucleotide, amino acid and phylogenetic analysis of the viruses.

Keywords: Marek's disease; virulent MDV; PCR; oncogenes; poultry; India

Introduction

Marek's disease virus (MDV) is an oncogenic avian herpes virus, which is responsible for great economic losses to the poultry industry worldwide. MDV belongs to the family *Herpesviridae*, the subfamily *Alphaherpesvirinae*, and the genus *Mardivirus* (Marek's disease like viruses). MDV-Herpes virus group has been divided into three serotypes based on their biological properties, by using the type specific monoclonal antibodies, as serotype-1, -2 and -3 (Bulow and Biggs, 1975). Serotype-1 includes oncogenic MDV, serotype 2 includes non-oncogenic MDV and serotype-3 includes herpes virus of turkey (HVT) (Witter *et al*., 2005). All three serotypes share common antigens.

Initially, chickens were protected efficiently by vaccination with Herpes virus of turkey and MD was well controlled by widespread use of commercially available HVT vaccines. Later on, bivalent vaccines consisting of HVT and serotype-2 herpes virus (SB-1) were introduced to control the Marek's disease in India. Of late, even after vaccination with bivalent vaccines, outbreaks of MD are being reported in chickens of 21–40 weeks age group with high mortality rates. Earlier, the clinical manifestation of MD was seen as paralytic/classical form, but now it is more towards the oncogenic MD with tumor formation in different visceral organs. The increased evidence of MD in vaccinated poultry flocks could probably be attributed to the continuing evolution of more virulent MDV strains despite widespread vaccination (Biggs and Nair, 2012). There was gradual emergence of virulent

E-mail: yprathiba@gmail.com; phone: +919182322765.

Abbreviations: MD = Marek's disease; MDV = MD virus; HVT = herpes virus of Turkey; Meq = Marek's Eco-RI Q; vIL-8 = viral interleukin 8

pathotypes of MD ranging from mild (m) MDV to virulent (v), very virulent (vv) and very virulent plus (vv+) MDV's in the field outbreaks in different countries of the world (Witter, 1997).

The molecular mechanism for MDV pathogenicity and oncogenecity remain largely unknown. Moreover, virus-encoded factors (oncogenes) contributing to the enhanced virulence of MDV field strains remain uncharacterized. Several genes unique for MDVs have been identified. Among these genes, Meq and vIL-8 were reported to have the greatest possibility to be associated with viral oncogenecity and pathogenicity. Therefore, molecular characterization and sequence analysis of these oncogenes (Meq and vIL-8) can help to decipher the molecular characteristics of field MDVs (Tian *et al*., 2011) and further evolutionary changes that are occurring in the virus in developing virulence can be understood. In the present study, we report the incidence of virulent serotype-1 MDV strains and their phylogenetic relationship with other MDVs from Andhra Pradesh, India.

Materials and Methods

Samples. Severe MD outbreaks were experienced in different poultry flocks of Andhra Pradesh, India, despite bivalent vaccination with HVT and SB-1 strains. A total of 27 blood samples from live suspected birds and 84 tissue samples from post mortem cases were collected from MD outbreaks of different regions. Different organs including liver, spleen, kidneys, ovaries, heart, sciatic nerve and proventriculus with visible lymphomatosis and enlargement were collected in sterile sample containers and stored at -20°C until further use and part of representative organs were also stored in 10% formal saline for histopathological examination.

Histopathological examination. Representative tissue samples showing lymphomatosis were collected in 10% formal saline and processed by paraffin embedding procedure (Culling, 1974). The paraffin-embedded tissues were sectioned at 4 µm thickness and stained by routine haematoxylin and eosin staining procedure. The stained sections were examined for the histological changes.

DNA extraction. Buffy coat was separated from heparinized blood samples by density gradient centrifugation using Lymphoprep (Sambrook and Russell, 2001). The extraction of DNA from buffy coat was done using QIAamp® DNA Blood Mini Kit (QIAGEN) following manufacturer's instructions.

The extraction of DNA from tissues was done using alkaline lysis method. In brief, the sliced tissue samples were treated with 400 µl of tissue lysis buffer (0.5 mol/l Tris HCl, 0.5 mol/l EDTA, 2% SDS) and 200 µg proteinase K/ml and incubated at 37°C overnight. Then an equal volume of phenol : chloroform : isoamylalcohol (25 :24 :1 v/v) was added to the lysate and centrifuged. The aqueous phase was separated and precipitation of DNA was done with double volume 100% isopropyl alcohol with 15 min of incubation, followed by a washing step with 70% isopropyl alcohol. Finally, the pellet was air dried and suspended in 400 µl of sterile nuclease free water and stored at -20 °C until used.

MDV detection by PCR amplification of 132 bp tandem repeats. As the primers of 132 bp repeat region identifies serotype-1-specific MDVs, it was considered as diagnostic primer for the detection of MDV that can differentiate serotype-1 field MDV strains from vaccine strains. The sequences of the primers used for PCR targeting 132 bp repeat region are shown in Table 1.

PCR amplification of 132 bp repeat region was carried out using 2 µl DNA as template in a total volume of 25 µl containing 2.5 µl 10x Taq DNA polymerase buffer, 1.5 µl of 25 mmol/l magnesium chloride, 0.5 µl of 10 mmol/l dNTPs, 1 µl of Taq DNA polymerase, 1 µl of 10 pmol/l of each of the two primers, and 15.5 µl of nuclease free water. The optimum conditions for this amplification 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.

PCR amplification of oncogenes. Amplification of full-length Meq and vIL-8 genes was carried out using specific primers (Table 1).

The PCR reaction mix consisted of 2.5 µl DNA as template in a total volume of 25 µl containing 2.5 µl 10x Taq DNA polymerase buffer, 1 µl of 25 mmol/l magnesium chloride, 1 µl of 10 mmol/l dNTPs, 1 µl of Taq DNA polymerase, 1 µl of 10 pmol/l of each of the two primers, and 14.5 µl of nuclease free water. The optimum conditions for PCR of Meq and vIL-8 genes were as follows: Initial denaturation at 94°C for 4 min, 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, final elongation at 72°C for 10 min and hold at 4°C for 5 min. The amplified PCR products were separated on 1% agarose gels and visualized using Gel documentation (Alpha Innotech) after staining

Table 1. Primers used for PCR targeting 132 bp repeat region, Meq and vIL-8 genes

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

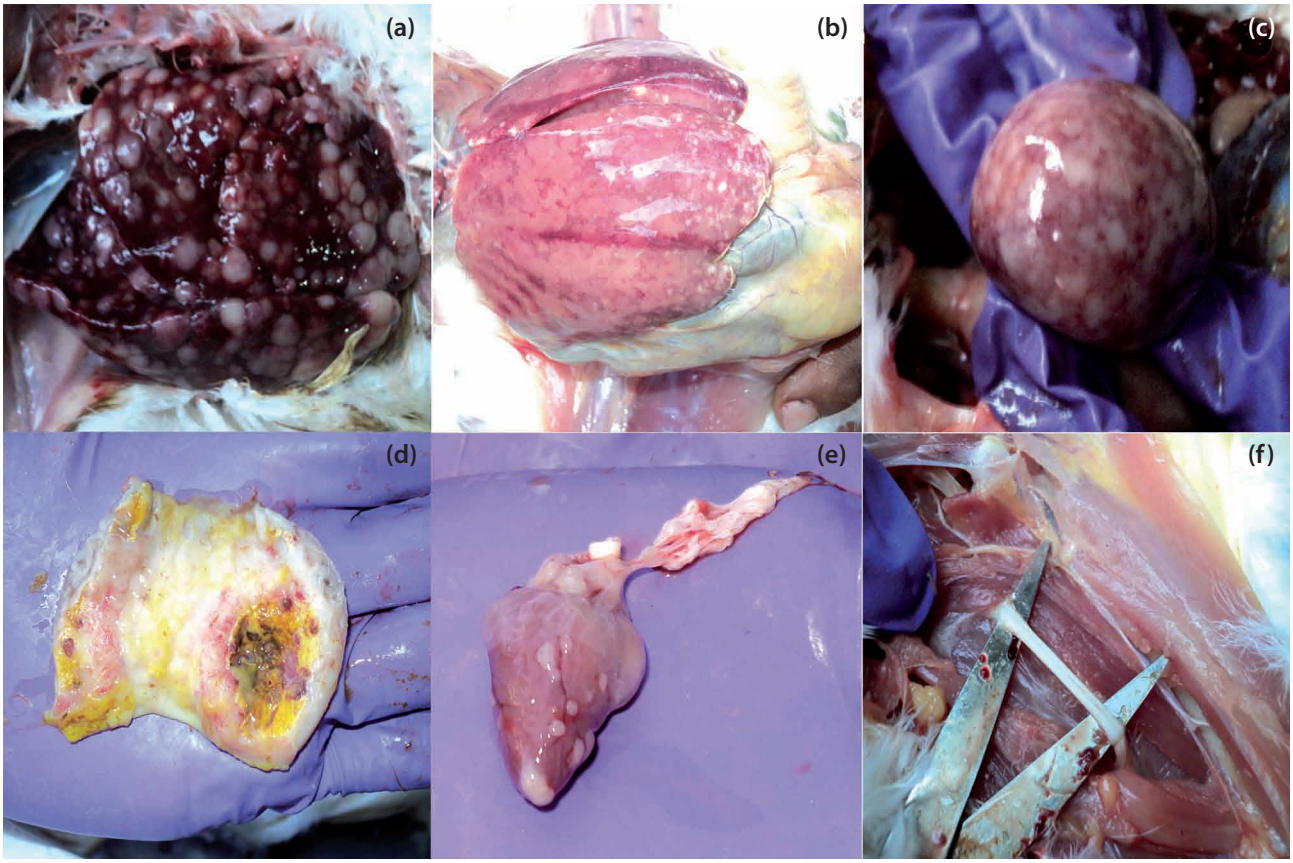

Fig. 1

Gross lesions of organs in MD affected birds

(a) Enlarged liver with multifocal whitish nodules of 4–5 mm in diameter; **(b)** Enlarged liver with multifocal grayish white nodules of pinpoint to 4 mm in size; **(c)** Spleen showing greyish white multi focal nodules; **(d)** Thickened proventricular wall with ulceration of proventricular mucosa; **(e)** Multifocal grayish white nodules of 0.5–1 mm in diameter over the epicardial surface of heart; **(f)** Sciatic nerve showing edema and loss of cross striations.

with ethidium bromide. The desired PCR products from agarose gels were purified using QIAquick® Gel extraction kit (QIAGEN) as per the manufacturer's instructions.

Sequencing of PCR products. Representative samples from different regions were selected and the purified PCR products of Meq and vIL-8 genes were sent for sequencing at Genomics corp- Xcelris, Ahmedabad, India using gene-specific primers.

Sequence analysis of oncogenes. The obtained nucleotide sequences and the deduced amino acid sequences of Meq and vIL-8 genes of MDV were analyzed and compared with other reference sequences from GenBank database using MEGA 6.0 software.

Phylogenetic analysis of Meq and vIL-8 genes. Phylogenetic analysis was carried out using MEGA 6.0 software by Neighbor joining method using Tajima nei model with 1000 boot strap replications.

Results

Clinical signs, gross lesions and histopathology

Birds showed signs of paleness of combs and wattles, feather loss at neck region with enlargement of feather follicles. Grossly, in majority of the MD cases, the liver, spleen, proventriculus and kidneys were affected. Less commonly, sciatic nerve, ovaries, heart, mesentry and lungs were affected. All organs showed enlargement with discrete grayish white nodules of various sizes (Fig. 1). Organs like liver, spleen, kidneys, ovaries and proventriculus collected from dead birds in 10% formal saline were subjected to histopathological examination. The results showed extensive infiltration of pleomorphic cells with lymphocytes, plasma cells and macrophages (Fig. 2).

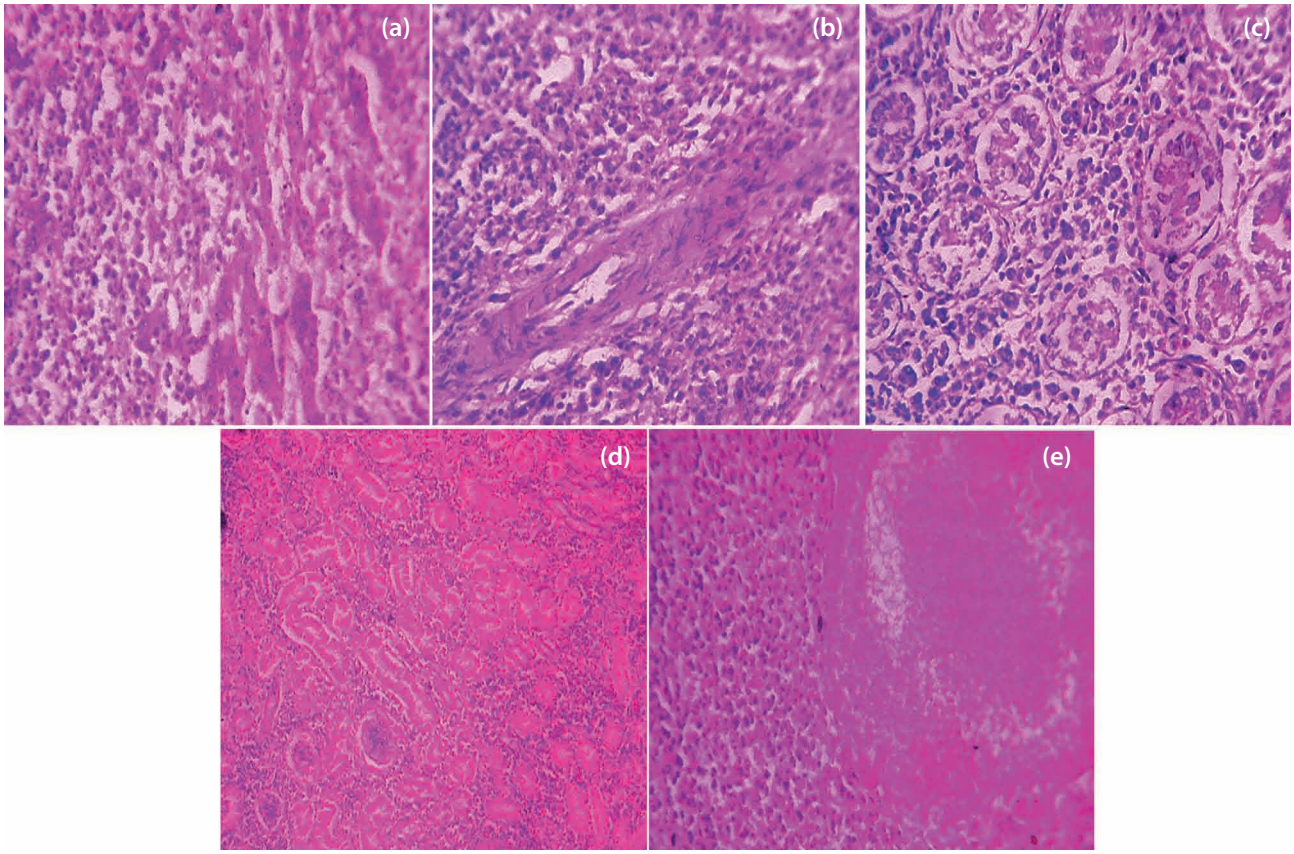

Fig. 2

Histopathological examination of tissue sections in MD affected birds

(a) Section of liver showing extensive proliferation of pleomorphic lymphoid cells in between hepatic cords resulted in loss of hepatic architecture and replacement of hepatic parenchyma, H&E x280; **(b)** Spleen section showing pleomorphic lymphoid cell infiltration, H&E x280; **(c)** Proventriculus showing extensive infiltration of pleomorphic lymphoblast cells in between proventricular glands, H&E x280; **(d)** Kidney section showing extensive infiltration of lymphoblast cells, into interstitial connective tissue and obliteration of renal tubules, H&E x280; **(e)** Ovary showing extensive pleomorphic cell infiltration leading to obliteration of ovarian parenchyma, H&E x280.

PCR targeting 132 bp repeat region

Standardization of PCR targeting 132 bp repeat region was done using a positive MD DNA sample, which was obtained from Project Directorate on Poultry, Hyderabad, India. A 35-cycle PCR with annealing temperature of 56°C and 1.5 mmol/l concentration of magnesium chloride was found to be optimum for the amplification of 182 bp product size with a single copy of 132 bp repeat region. Out of 84 tissue and 27 blood samples collected, all the tissue samples and 20 blood samples were found to be positive for serotype-1-specific MD yielding a 314 bp product in PCR amplification (Fig. 3a).

PCR targeting oncogene

The DNA samples, which were positive for 132 bp repeat region, were further used for amplification of oncogenes. Standardization of PCR targeting oncogenes (Meq and vIL-8) was done by keeping a 35-cycle PCR reaction with annealing temperature of 59.4°C. The concentration of magnesium chloride at 2.0 mmol/l was found to be optimum for the amplification of 1081 bp product of Meq gene and 887 bp product of vIL-8 gene. All 84 tissue samples and 20 blood samples were found to be positive for the presence of viral oncogenes (Fig. 3b and 3c). The purified PCR product of four positive samples of Meq and vIL-8 genes from each region of Andhra Pradesh, India, were sent for sequencing to Genomics corp- Xcelris, Ahmedabad.

Nucleotide sequence analysis of oncogenes (Meq and vIL-8)

The obtained nucleotide sequences of Meq and vIL-8 genes from four different regions of Andhra Pradesh (Tirupati, Guntur, Chittoor, Tanuku designated as AP01, AP02, AP03 and AP04) were verified by NCBI-BLAST and sequences

Table 2. Details of nucleotide sequences of Meq and vIL-8 genes of different field samples from Andhra Pradesh submitted to GenBank

S. No.	Code of the sequence	Geographic origin	Acc. No.
1.	AP01 Meq	Tirupati	KT246100
2.	AP02 Meq	Guntur	KT246101
3.	AP03 Meq	Chittoor	KT246102
4.	AP04 Meq	Tanuku	KT246103
5.	AP01 vIL-8	Tirupati	KT272874
6.	AP02 vIL-8	Guntur	KT272875
7.	AP03 vIL-8	Chittoor	KT272876
8.	AP04 vIL-8	Tanuku	KT272877

showed homology with Meq and vIL-8 genes of different serotype-1-specific MDV strains. The accession numbers for oncogenes from different regions AP01, AP02, AP03 and AP04 were KT246100, KT246101, KT246102, KT246103 for Meq gene and KT272874, KT272875, KT272876, KT272877 for vIL-8 gene, respectively (Table 2).

Table 3. MDV reference strains of Meq gene published in GenBank

S. No.	MDV strains	Virulence	Geographic origin	Acc. No.
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

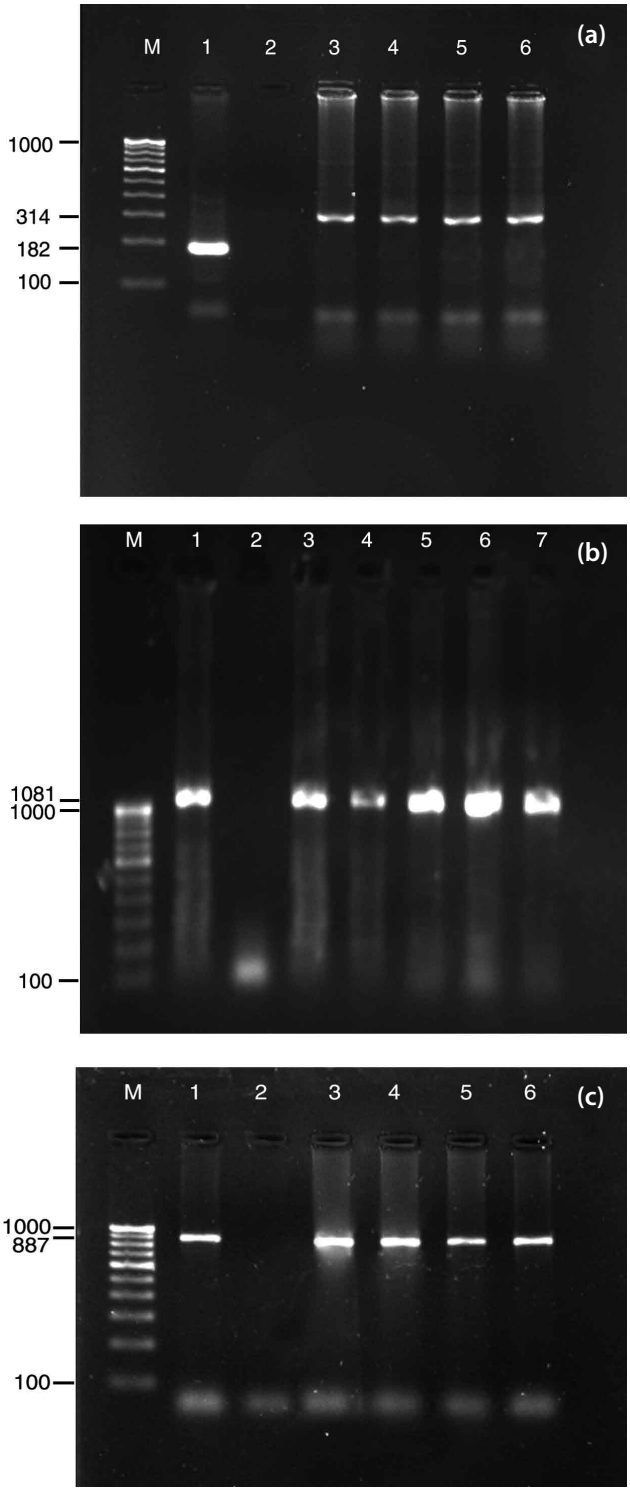

Fig. 3

PCR amplification of 132 bp repeat region, Meq and vIL-8 genes in MD affected flocks

(a) Screening of MD-suspected field samples using PCR for 132 bp repeat region; **(b)** PCR amplification of Meq gene of different MD-positive field samples; **(c)** PCR amplification of vIL-8 gene of different MD-positive field samples.

Table 4. Sequences of vIL-8 genes MDV reference strains published in GenBank

S. No.	MDV strains	Virulence	Geographic origin	Acc. No.
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

The representative nucleotide sequences (26) of Meq gene of different pathotypes such as mild (m), virulent (v), very virulent (vv) and very virulent plus (vv+) MDVs were selected from GenBank (Table 3), were compared with field MDV sequences using MEGA 6.0 software and the results showed 84.5 to 99.6 % homology. The nucleotide sequences of AP01, AP03 and AP02, AP04 had the highest homology of 99.6 and 99.5 % with strains RB1B and GA, and had the lowest homology of 84.5 and 84.4 % with CVI988 strain, respectively.

Comparison of four field MDV nucleotide sequences of Meq gene with reference strains RB1B (very virulent) and GA (virulent) showed variations at four positions in sequences of AP01 and AP03 and five variations in sequences of AP02 and AP04. AP01 showed mutations at positions 229 (A→G), 238 (G→T), 415 (A→G), 626 (T→C); whereas AP03 showed mutations at 229 (A→G), 238 (G→T), 278 (A→G), 415 (A→G) of 1020 nucleotides of the Meq gene. Nucleotide mutations at positions 229 (A→G), 238 (G→T),

Table 5. Amino acid substitutions in the Meq protein of MDV-1 field strains compared 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	Field sample	AP02 Meq	.	E	Y	T	R	.	.	A	.	.	.	.	.	.	.	.	.
9	Field sample	AP03 Meq	.	E	Y	.	R	.	.	A	.	.	.	.	.	.	.	.	.
10	Field sample	AP04 Meq	.	E	Y	T	R	.	.	A	.	.	.	.	.	.	.	.	.
11	TN Isolate	Ind/TN11/01	.	E	Y	T	R	.	.	A	.	.	.	.	.	.	.	.	.
12	TN Isolate	Ind/TN12/03	.	E	Y	T	R	.	.	A	.	.	.	.	.	.	.	.	.
13	KA Isolate	Ind/KA12/02	.	E	Y	T	R	.	.	A	.	.	.	.	.	.	.	.	.
14	Vv	Isolate Tn-n1	.	.	.	.	.	.	.	.	.	.	.	P	.	.	.	.	.
15	Vv	Isolate Tn-n2	.	.	.	.	.	.	.	.	.	.	.	P	.	.	.	.	.
16	V	Strain BC-1[a]	S	A	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.
17	V	Strain JM[a]	S	A	.	.	R	A	.	.	.	.	.	.	.	.	.	.	.
18	M	Strain CU-2[a]	S	E	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
19	Vaccine	Strain CVI988[a]	S	E	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I
20	Vv	Strain Md5	.	.	.	.	.	.	.	.	.	.	.	.	A	.	V	T	.
21	Vv+	Strain W	.	.	.	.	.	.	.	.	.	.	.	.	A	.	V	T	.
22	Vv	Strain 643P	.	.	.	.	.	.	R	.	Q	A	A	.	A	F	.	.	.
23,24	Vv+	Strain New, 584A	.	.	.	.	.	.	R	.	Q	A	.	.	A	.	V	T	.
25,26	Vv	Strain 549, 595	.	.	.	.	.	.	R	.	Q	A	A	.	A	.	.	.	.
27,28	Vv+	Strain L,X	.	.	.	.	.	.	R	.	Q	A	A	.	A	.	.	.	.
29	Vv+	Strain 648A	S	E	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.
30	Vv+	Strain N	.	.	.	.	.	.	R	.	Q	A	A	.	A	P	.	.	.

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

262 (G→A), 278 (A→G), 415 (A→G) of Meq gene were seen in AP02 and AP04.

Comparison of nucleotide sequences of Meq gene of four field MDVs (AP01, AP02, AP03 and AP04) with sequences of other Indian field isolates (Ind/TN11/01 and Ind/KA12/02) revealed that the sequences are 100% identical with AP02 and AP04. The nucleotide sequence of AP03 showed one variation at position 262 (A→G) and AP01 nucleotide sequence showed variations at three positions 262 (A→G), 278 (G→A) and 626 (T→C).

Comparison of the nucleotide sequences of vIL-8 gene of field samples with the sequences of 14 reference strains (Table 4) showed 99.44 to 99.87% nucleotide homology. The sequences of four field samples had the highest homology of 99.87% with virulent strains LS and LMS and had the lowest homology of 99.44% with CVI988 strain. Comparison of four field MDV nucleotide sequences of vIL-8 gene with reference strain LMS showed one variation at position 106 (T→C) in all the sequences. Also one nucleotide deletion in the sequence of AP02 was seen at position 815 instead of base T. Comparison of nucleotide sequences of vIL-8 gene of four field MDVs (AP01, AP02, AP03 and AP04) with sequences of other Indian field isolates (Ind/TN11/01, Ind/TN12/03 and Ind/KA12/02) showed 100% similarity.

Table 6. Amino acid substitutions in the vIL-8 protein of MDV-1 field and reference strains

S. No.	Virulence and field samples	Strain name	Aminoacid substitutions in the vIL-8 protein of MDV 1 field and reference strains			
			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	Field sample	AP02 vIL-8	.	.	I	.
5	Field sample	AP03 vIL-8	.	.	I	.
6	Field sample	AP04 vIL-8	.	.	I	.
7	Vv	Ind/KA12/02	.	.	I	.
8	V	Ind/TN11/01	.	.	I	.
9	V	Ind/TN12/02	.	.	I	.
10	V	Strain 571	L	D	I	.
11	V	Strain GA	L	D	I	.
12	Vv	Strain RB1B	L	D	I	.
13	Vv	Strain Md5	L	D	I	.
14	Vv	Strain 595	L	D	I	.
15	Vv +	Strain 584A	L	D	I	.
16	Vv +	Strain 648A	L	D	I	.
17	m	Strain CU-2	L	D	I	.
18	Vaccine	Strain CVI988	L	D	I	.

Amino acid sequence analysis of oncogenes (Meq and vIL-8)

The total 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 amino acid sequences of four representative field samples from different regions (Tirupati, Guntur, Chittoor, Tanuku) was done with those of reference strains and other Indian field isolates.

Alignment analysis of the deduced amino acid sequences of Meq gene of four field samples and 26 published MDVs was performed using MEGA 6.0 Software. The amino acid mutation in the Meq protein of MDVs displayed regularity at nine positions, including 71, 77, 80, 115, 119, 139, 153, 176 and 217. Low virulence MDV strains including CU-2, BC-1, JM and CVI988 showed 59 amino acid insertion with proline-rich repeats in the deduced amino acid sequences of the Meq protein. In very virulent plus (vv+) 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 field samples. The amino acid sequences of the Meq protein of the field strains were neither mild type nor very virulent plus MDVs. There was a mutation at position 71 (S→A) in the Meq protein of the field strains, which is associated with higher virulence in the MDV strains (Table 5).

Compared to closely related strains 571 and 573 (v), AP01, AP02, AP03 and AP04 showed variations at amino acid positions 209; 88, 93, 115, 139; 93, 115, 139 and 88, 93, 115, 139, respectively. Minimum amino acid variations were present in all four field amino acid sequences of Meq in comparison with the deduced amino acid sequences of virulent strains 571 and 573. Hence, the field MDV strains obtained in the present study could be categorized into virulent pathotypes.

Comparison of the deduced amino acid sequences of Meq protein of four field MDVs (AP01, AP02, AP03 and AP04) with other Indian field isolates (Ind/TN11/01 and Ind/KA12/02) showed no variations in the amino acid sequences of AP02 and AP04. There were three variations in the amino acid sequence of AP01 at positions 88 (T→A), 93 (R→Q) and 209 (L→P) and one variation in the amino acid sequence of AP03 at position 88 (T→A).

Amino acid sequence analysis of vIL-8 gene of four field samples (AP01, AP02, AP03 and AP04) and 14 published MDVs was performed (Table 6). Comparison of amino acid sequences of vIL-8 protein with that of reference strains like CU-2, GA, RB1B, 648A, 584A, Md5, 595, 571 and CVI988 showed that mutations exist at position 4 (L→S) and 31 (D→G). The amino acid mutations at positions 4 and 31 were similar to the virulent reference strains LMS and LS. In comparison with that of the other Indian field isolates, the field MDV strains showed no variations in these sequences.

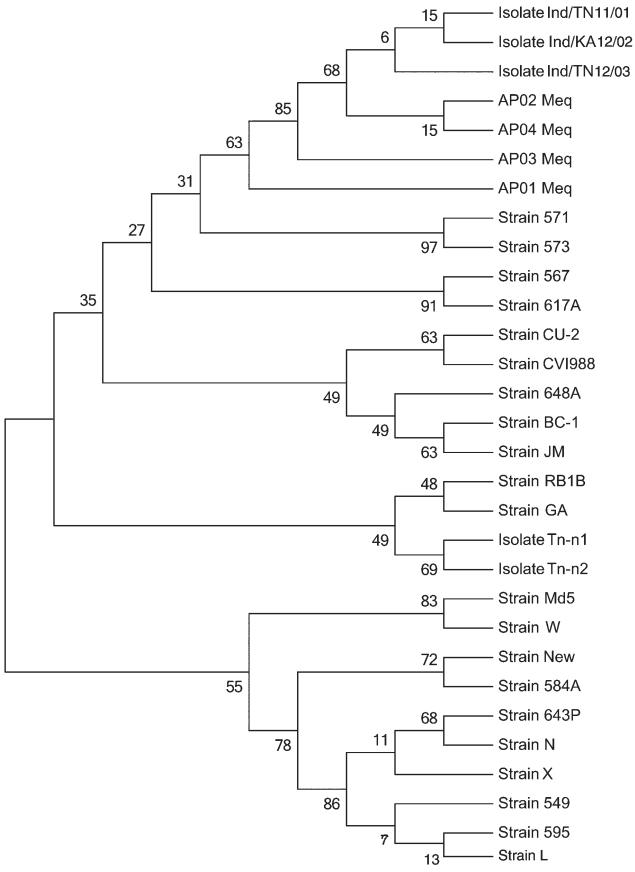

Fig. 4

Phylogenetic analysis of Meq gene nucleotide sequences of four field MDV strains with other reference sequences available in NCBI database

The phylogenetic tree was constructed using the MEGA version 6.0 by the neighbor-joining method with 1000 bootstrap replicates using Tamura nei model.

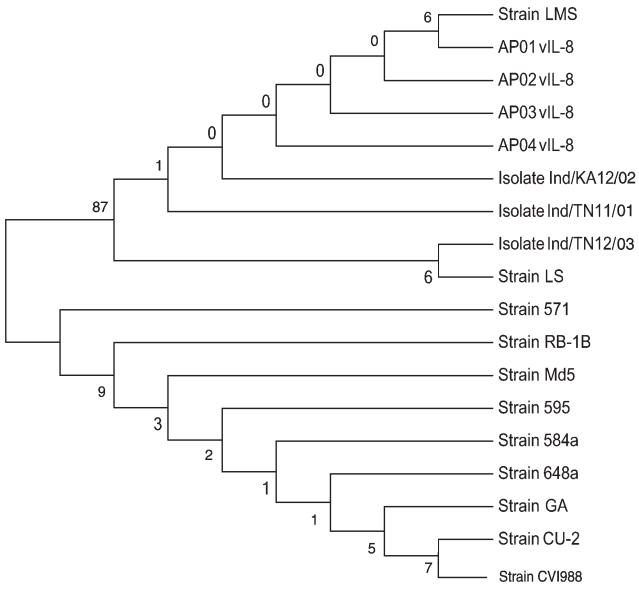

Fig. 5

Phylogenetic analysis of vIL-8 gene nucleotide sequences of four field MDV strains with other reference sequences available in NCBI database

The phylogenetic tree was constructed using the MEGA version 6.0 by the neighbor-joining method with 1000 bootstrap replicates using Tamura nei model.

Phylogenetic analysis of oncogenes

The phylogenetic analysis of Meq gene of the field MDV nucleotide sequences with different reference strains formed a cluster with virulent MDV strains 571, 573 and other Indian MDV strains (Fig. 4).

The phylogenetic analysis of vIL8 gene of the field MDV nucleotide sequences with different reference strains showed that they formed a cluster with virulent strains LS and LMS and other Indian MDV strains (Fig. 5).

Discussion

Marek's disease is one of the most important diseases of poultry and is responsible for great economic losses to the poultry industry worldwide. In India, poultry farmers were practicing HVT vaccination for the control of MDV and more recently bivalent vaccines are being used. Sporadic outbreaks of MD have been reported recently even after vaccination with bivalent vaccines. In Indian poultry flocks, in the past few years, there appears to be a significant change in the clinical manifestation of MD from classical/paralytic form to an acute lymphomatosis resulting in tumor formation in affected birds. The probable reasons could be the increased virulence of MDV, which led to the emergence of more virulent serotype-1 MDV strains. The genome of serotype-1 MDVs contains several oncogenes, which are important in viral pathogenesis and oncogenesis (Tulman *et al.*, 2000). Of these, Meq (Jones *et al.*, 1992), vIL-8 (Parcells *et al.*, 2001) and pp38 (Cui *et al.*, 1991) genes are most important in viral oncogenesis of serotype-1 MDVs (Wozniakowski *et al.*, 2010; Tian *et al.*, 2011). Mutations in nucleotide sequences of Meq and vIL-8 genes are most important in evolution of serotype-1 MDVs from less virulent to most virulent forms that result in high mortality rates with infiltration of lymphoblastoid cells in different visceral organs (Silva, 1992; Shamblin *et al.*, 2004; Tian *et al.*, 2011; Kumar *et al.*, 2012; Gong *et al.*, 2013).

In the present study, characterization of viral oncogenes of MDV from the poultry flocks was carried out. For this, primers were synthesized to specifically amplify 132 bp repeat region in MDV genome. Amplification of 132 bp re-

peat region can distinguish pathogenic and non-pathogenic MDVs (Becker *et al.*, 1992; Silva, 1992). The clinical samples from the MD outbreaks yielded a 314 bp product in PCR, which indicates the presence of two copies of 132 bp tandem repeats. All pathogenic serotype-1 MDVs contain two or three copies of 132 bp tandem repeats in their genome (Becker *et al.*, 1992; Silva *et al.*, 2004; Doosti and Golshan, 2011). The samples, which were detected as positive for 132 bp repeat region, were used for amplification of oncogenes. Sequence analysis of Meq and vIL-8 genes of MDV was done. Maximum sequence homology of Meq gene showed 99.6 and 99.5% was with RB-1B (very virulent) and GA (virulent) strains. The sequences of vIL-8 gene of field strains had the highest homology of 99.87% with virulent strains LS and LMS.

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 oncogenic MDV strains. 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. The phylogenetic analysis of the nucleotide sequences of Meq and vIL-8 genes revealed that the field samples grouped with virulent MDV strains.

In conclusion, the present study demonstrates that based on the nucleotide, amino acid and phylogenetic analysis of oncogenes, the MDV strains obtained in the outbreaks of Andhra Pradesh, India could be designated as virulent MDVs. However, in India we are still practicing HVT or bivalent vaccines, and serotype-1 MD viruses are not yet used for vaccination in the field conditions. 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 in order to control MD in the country.

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