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Molecular detection and phylogenetic analysis of Marek's disease virus virulence-associated genes from vaccinated flocks in southern India reveals circulation of virulent MDV genotype

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Abstract

Marek's disease (MD) is a re-emerging viral disease of chickens and a serious economic threat to the poultry industry worldwide. Continuous surveillance with molecular investigation is essential to monitor the emergence of virulent Marek's disease virus (MDV) strains and to devise any appropriate vaccination strategy and implement bio-security programmes. In the present study, we investigated the cases of MD outbreaks in vaccinated poultry flocks. The MD outbreak was confirmed through necropsy (mainly visceral tumours), histopathology and viral gene specific PCR. The pathotypes of the field MDV strains were assessed by molecular analysis of three virulence-associated genes, *meq*, *pp38* and *vIL-8*. The Meq sequence of the field strains analyzed in this study lacked the 59 aa unique to mild strains, indicating that they are potentially virulent strains. Mutation at position 71 and the presence of five proline rich repeats in the transactivation domain, both associated with virulence were observed in these strains; however, the signature sequences specific to very virulent plus strains were absent. Phylogenetic analysis of *meq* oncogene sequences revealed clustering of the field strains with North Indian strains and with a very virulent plus ATE 2539 strain from Hungary. Analyses of pp38 protein at positions 107 and 109 and vIL-8 protein at positions 4 and 31 showed signatures of virulence. Sequence and phylogenetic analysis of oncogene and virulence-associated genes of field MDVs from vaccinated flock indicated that these strains possessed molecular features of virulent strains.

KEYWORDS
Marek's disease virus (MDV), *meq*, oncogene, pathotype, phylogenetics, virulence-associated genes

1 | INTRODUCTION

Marek's disease (MD) causes huge economic impact by directly causing significant levels of mortality due to visceral lymphomas. In addition, the sub-clinical immunosuppression induced by virulent strains also cause production loss indirectly (Gimeno, 2014). The Marek's disease virus (MDV), classified as gallid alphaherpes virus 2 (GaHV-2), belongs to the genus *Mardivirus* of the sub-family *Alphaherpesvirinae*, family *Herpesviridae* (International Committee on Taxonomy of Viruses, 2017). MDV has been divided into three serotypes as serotype 1, 2 and

3 (Bulow & Biggs, 1975). Of these three serotypes, MDV-1 includes all oncogenic strains, whereas serotypes 2 and 3 include mildly virulent non-oncogenic strains and avirulent strains, respectively (Witter et al., 2005). The virulence of the MDV-1 has been shifted from mild (m) to virulent (v), very virulent (vv) and very virulent plus (vv+) over a period due to mutations and positive selection (Gimeno & Schat, 2018; Witter, 1997).

Although the disease is effectively controlled by vaccination and bio-security measures, outbreaks due to emerging virulent strains were observed in recent times worldwide including India (Nair, 2018). In India, both monovalent (serotype 3/ HVT) and bivalent (serotype 2 SB1/ SB303 and serotype 3/ HVT) vaccines are currently used in commercial layers and breeder flock. Despite intensive vaccination, outbreaks in vaccinated flocks are noticed in different parts of dense poultry rearing zones (Gupta et al., 2016; Prathiba et al., 2018; Puro et al., 2018; Suresh et al., 2015). Various factors are involved in the MD outbreaks in vaccinated flocks including faulty vaccination procedure, mycotoxin in feed and concurrent immunosuppressive diseases.

Although in vivo pathogenicity studies are mandatory to ascertain the pathotype of emerging MDV strains (Dudnikova et al., 2007; Witter et al., 2005), the assays are tedious and complex. On the other hand, molecular analysis of certain marker genes specifically *Meq* oncogene has been proved valuable by recent studies (Mescolini et al., 2019). The unique oncogene and virulence-associated genes, namely Marek's EcoRI-Q encoded protein-*Meq* (Jones et al., 1992), phosphoprotein-*pp38* (Z. Cui et al., 1999) and *vIL*-8 (viral interleukin-8) (Parcells et al., 2001) are associated with oncogenicity and pathogenicity. Their potential as pathotype markers has been demonstrated successfully by recent workers in emerging field strains from several countries, namely China (Tian et al., 2011), the United States (Padhi & Parcells, 2016), Colombia (Lopez-Osorio et al., 2017), Japan (Abd-Ellatieff et al., 2018) and Italy (Mescolini et al., 2019).

In the present study, we investigated the Indian MDV field strains from MD visceral tumours occurring in vaccinated poultry breeder flocks and analyzed three virulence-associated genes, namely *meq oncogene*, *pp38* and *vIL-8* of 17 MDVs to ascertain their virulence pathotype.

2 | MATERIALS AND METHODS

2.1 | Sample collection

Tissue samples were collected between 2017 and 2018 from 13 flocks of different layer/broiler parent lines/breeds maintained at ICAR-Directorate of Poultry Research (DPR), Hyderabad, India. The parent flocks were used in the production of layer/broiler chickens or dual type-coloured varieties suitable for backyard poultry rearing. All the flocks were vaccinated with HVT @ 0.2 ml/chick with 1500 PFU/dose by subcutaneous route on day old at in-house hatchery. Deceased birds from different flocks presented for necropsy showed MD specific lesions such as enlargement of liver, spleen and visceral tumours. All tumour-associated organs including liver, spleen, kidney, proventriculus and sciatic nerve were collected from MD suspected birds in 10% neutral buffered formalin for histopathological examination. Another set was collected and kept frozen at −20°C until use for DNA extraction and subsequent use in PCRs. Sample details of the present study are provided in Table S1.

2.2 | Histopathological examination

The tissues in 10% neutral buffered formalin were processed for histopathological tissue section and preparation of slides as per the standard protocol. The tissue sections were stained by H and E stain and visualized under light microscope.

2.3 | Genomic DNA extraction

Tissue samples were used for DNA extraction by standard procedure. Briefly, the samples were lysed in lysis buffer, extracted with phenol-chloroform-isoamyl alcohol (25:24:1) twice, followed by precipitation of DNA with absolute isopropanol, washed with 70% ethanol, dried at room temperature and resuspended in nuclease-free water.

2.4 | Screening of samples for MDV by PCR

The samples were screened for MDV using extracted genomic DNA by PCR. PCR targeting BamH1-H region 132-bp tandom repeats by using M1 and M2 primers described previously by Davidson and Borenstein (1999) was employed (Table S2). Briefly, the PCR amplification was carried out using 2 μl of template DNA in a total volume of 25 μl reaction volume containing 2.5 μl of 10× Taq buffer with $MgCl_2$, 1 μl of each primer (20 pmol), 0.5 μl (10 mMol) of dNTPs and 0.25 μl (5 U/ml) of Taq DNA polymerase and nuclease-free water. The PCR conditions were as follows: 94°C for 4 min, 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min and final elongation at 72°C for 10 min. The presence of 434 bp product after amplification in the samples was considered MD positive, and these samples were used for further amplification of MDV virulence-associated genes.

2.5 | PCR amplification of MDV virulence-associated genes and sequencing

Out of 120 MDV suspected tissue samples investigated from 13 flocks, 17 samples from five flocks were found to be MDV positive by 132-bp repeat PCR. MDV positive samples were used for the amplification of MDV oncogene and virulence-associated genes, *meq*, *vIL-8* and *pp38*. The primer sequences used are provided in Table S2. The PCR amplifications of *meq* and *vIL-8* genes were carried out as previously described with slight modifications (Tian et al., 2011). The PCR amplification was carried out using 4 μl of template DNA in a total volume of 50 μl reaction volume containing 5 μl of 10× Taq buffer with $MgCl_2$,

TABLE 1 Details of nucleotide sequences of Meq, pp38 and vIL-8 of field Marek's disease viruses (MDVs) submitted to GenBank

	Virulence-associated genes		
MDV strains	*Meq*	*pp38*	*vIL-8*
GaHV-2/India/Ck/001/17	–	MK497159	
GaHV-2/India/Ck/002/18	MK388077	MK497160	MT925954
GaHV-2/India/Ck/003/18	MK388078	MK497161	MT925955
GaHV-2/India/Ck/005/18	–	MK497162	–
GaHV-2/India/Ck/006/18	MK388079	MK497163	MT925956
GaHV-2/India/Ck/007/18	MK388080	–	MT925957
GaHV-2/India/Ck/008/18	MK388081	–	MT925958
GaHV-2/India/Ck/009/18	MK388082	–	MT925959
GaHV-2/India/Ck/011/18	MK388083	–	MT925960
GaHV-2/India/Ck/012/18	MK388084	–	MT925961
GaHV-2/India/Ck/013/18	MK388085	MK497164	MT925962
GaHV-2/India/Ck/015/18	MK388086	–	MT925963
GaHV-2/India/Ck/016/18	MK388087	MK497165	MT925964
GaHV-2/India/Ck/017/18	–	MK497166	–
GaHV-2/India/Ck/018/18	MK388088	MK497167	MT925965
GaHV-2/India/Ck/019/18	MK388089	MK497168	MT925966
GaHV-2/India/Ck/020/18	–	MK497169	–

–: Not available.

2 μl of each primer (20 pmol), 1 μl (10 mMol) of dNTPs and 0.5 μl (5 U/ml) of Taq DNA polymerase and nuclease-free water. The PCR conditions for *meq* and vIL-8 were as follows: 94°C for 4 min, 35 cycles of 94°C for 1 min, 56°C for 1 min, 72°C for 1 min and final elongation at 72°C for 10 min. PCR amplification of *pp38* gene was carried out as per the method of Zhuang et al. (2015). The amplified products were checked in 1.5% agarose gel and PCR product was purified. The products were sequenced by Sanger sequencing. The obtained sequences were edited and aligned in EditSeq and MegAlign programme of DNASTAR software. The sequences of the field MDV strains were submitted to NCBI database (Table 1).

2.6 | Sequence and phylogenetic analysis

The nucleotide gene sequences of *meq*, *pp38* and *vIL-8* of all MDV strains were subjected to blast analysis by using NCBI BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and compared with other MDV strains available in the public domain. A total of 97 sequences including 54 *meq*, 25 *pp38* and 18 *vIL-8* reference gene sequences of MDV of different pathotypes were retrieved for analysis (Table S3). The sequences along with those of the present study were aligned by CLUSTAL W and annotated using MEGA X version 10.0.5 (www.megasoftware.net) (Kumar et al., 2018). The best-fit model was selected by comparing the Bayesian information criterion (BIC) scores. Phylogenetic trees were constructed separately for each of three genes by using the Neighbour-joining method with 1000 bootstrap replicates based on the Tamura-Nei model (Tamura & Nei, 1993). All positions containing gaps and missing data were eliminated.

3 | RESULTS

3.1 | Gross lesions and histopathology

Grossly, in most of the cases presented for necropsy, discrete greyish white nodules of various sizes were observed predominantly in liver and spleen and less commonly in heart, lungs, proventriculus and mesentery. Enlargement of liver and spleen were invariably present. None of the MD suspected carcasses showed enlargement or loss of striation in sciatic nerve. Histopathological examination of liver and spleen showed extensive infiltration with pleomorphic cells and lymphocytes (Figure S1).

3.2 | Screening of samples by PCR

Out of 120 MD suspected tissue samples investigated from 13 flocks, 17 samples from five flocks were found to be MD positive by 132-bp repeat PCR and histopathology. The age of the flocks during MD outbreak ranged from 14 to 65 weeks. The outbreak occurred in seven coloured broiler breeder, six Aseel – (native chicken) breeder parent, three White Leghorn layer breeder and one Vanaraja, backyard dual purpose variety.

3.3 | Sequencing of virulence-associated genes and sequence analysis

At least one virulence-associated gene was amplified and sequenced for each of the 17 MDV positive samples, a total of 13 *meq*, 11 *pp38* and 13 *vIL-8* gene sequences were sequenced. The obtained sequences were deposited in GenBank with accession numbers. The details are given in Table 1.

The *meq* sequences analysis of the current field MDV strains showed 99.8%–100% and 99.5%–100% similarity at nucleotide and amino acid levels, respectively. All strains showed a *meq* gene length of 1020 bp encoding for Meq protein comprising 339 aa. In the present study, five proline rich repeats (PPPPs) were observed at positions 152-155, 175-178, 191-194, 216-219 and 232-235 in all these strains. The amino acid mutations in the Meq displayed similarity at several positions including 71, 77, 80, 88, 93, 119, 139, 153, 176, 180, 217, 277, 283, 320 and 326. Two mutations associated with MDV virulence were observed in all 13 Meq sequences at positions 71 (Alanine) and 80 (Tyrosine). Four distinctive substitutions including one in the basic region and three in the leucine zipper domain were observed in all sequences except one (MK388077) as follows: 80 Y (Tyrosine; D80Y), 88 T (threonine; A88T), 93 R (arginine; Q93R) and 139 A (alanine; T139A) (Table 2).

As for *pp38* gene, the identity ranged from 98.6% to 99.9% and 98.6% to 100% at nucleotide and amino acid levels, respectively among

TABLE 2 Amino acid substitutions in the Meq protein of field Marek's disease virus (MDV) strains compared with reference strains

Strain	Pathotype	Meq length (aa)	Number of PPPP repeats	Amino acid substitutions														
				Basic region		Leucine zipper					Transactivation domain-Proline rich repeats							
				71	77	80	88	93	119	139	153 PPPP	176 PPPP	180	217/276[a]	277/336	283/342	320/379	326/385
CVI988	Attenuated	398	7	S	E	D	A	Q	C	T	P	P	T	P	L	A	I	I
CU-2	Attenuated	398	7	S	E	D	A	Q	C	T	P	P	T	P	L	A	I	T
JM/102W	Virulent	399	7	A	E	D	A	Q	C	T	P	P	T	P	L	A	I	T
Md5	Very virulent	339	4	A	K	D	A	Q	C	T	P	P	T	A	L	A	T	T
648A	Veryvirulent plus	339	2	A	K	D	A	Q	R	T	Q	A	A	A	P	V	I	T
GaHV2/India/Ck/002/18		339	5	A	E	D	T	Q	C	A	P	P	T	P	L	A	I	T
GaHV2/India/Ck/003/18		339	5	A	E	Y	T	R	C	A	P	P	T	P	L	A	I	T
GaHV2/India/Ck/006/18		339	5	A	E	Y	T	R	C	A	P	P	T	P	L	A	I	T
GaHV2/India/Ck/007/18		339	5	A	E	Y	T	R	C	A	P	P	T	P	L	A	I	T
GaHV2/India/Ck/008/18		339	5	A	E	Y	T	R	C	A	P	P	T	P	L	A	I	T
GaHV2/India/Ck/009/18		339	5	A	E	Y	T	R	C	A	P	P	T	P	L	A	I	T
GaHV2/India/Ck/011/18		339	5	A	E	Y	T	R	C	A	P	P	T	P	L	A	I	T
GaHV2/India/Ck/012/18		339	5	A	E	Y	T	R	C	A	P	P	T	P	L	A	I	T
GaHV2/India/Ck/013/18		339	5	A	E	Y	T	R	C	A	P	P	T	P	L	A	I	T
GaHV2/India/Ck/015/18		339	5	A	E	Y	T	R	C	A	P	P	T	P	L	A	I	T
GaHV2/India/Ck/016/18		339	5	A	E	Y	T	R	C	A	P	P	T	P	L	A	I	T
GaHV2/India/Ck/018/18		339	5	A	E	Y	T	R	C	A	P	P	T	P	L	A	I	T
GaHV2/India/Ck/019/18		339	5	A	E	Y	T	R	C	A	P	P	T	P	L	A	I	T

Note: Distinctive substitutions in Indian strains are framed.
[a]Position according to 398 aa long Meq isoform.

the field MDV sequences. Eleven sequences of this study were compared with five reference MDVs at the amino acid level. The analysis revealed mutation at amino acid position 109 (glutamic acid→glycine). All the 11 sequences had G320A mutation at the nucleotide level and conserved glutamine at position 107 in the amino acid sequences (Table 3).

vIL-8 comprises 134 amino acids encoded by 682 nucleotides from three exons (1..64,240..375,474..678). The homology of the nucleotide and deduced amino acid sequences of 13 sequences were 99.5%–100% and 100%, respectively. The complete nucleotide and amino acid sequences of five reference MDVs were compared with the sequences of the present study. All the 13 sequences had two mutations at nucleotide position 11 (T →C) and 92 (A→G) resulting in amino acid substitutions at positions 4 (leucine to serine; L4S) and 31 (aspartate to glycine; D31G) (Table 4).

3.4 | Phylogenetic analysis

Phylogenetic tree of *meq* gene sequences with other reference sequences revealed that all the current strains (except one –002 MDV strain) clustered together with earlier reported Indian strains from northern part of the country and with vv+ ATE 2539 strain from Hungary (Figure 1). One MDV strain (MK388077) of the present study clustered closely with two Italian strains (MK139661 and MK139672). Two Indian MDV strains TNN1 (HM749324) and TNN2 (HM749325) reported earlier from southern India clustered away from present strains.

The phylogenetic tree of the *pp38* gene showed clustering of the current 11 MDV field strains with other Indian strains from northern and southern parts of the country. They also clustered with two Chinese strains DY04 (HQ638161) and XJ03 (HQ638175). The other cluster included vaccine strains, m, v and vv strains (Figure 2).

The phylogenetic tree of the *vIL*-8 gene showed that all 13 sequences of the present study clustered close to vv and vv+ strains from the United States and one virulent strain from China. The sequences of other Indian strains from southern part also fell on the same cluster. Mild and vaccine MDV strains formed a separate cluster (Figure 3).

4 | DISCUSSION

MD is one of the re-emerging viral diseases of poultry causing a serious economic threat to the poultry industry (Schat & Nair, 2013). Although vaccines provide strong immunity and are highly effective against

TABLE 3 Amino acid substitutions in the pp38 protein of field Marek's disease virus (MDV) strains compared with reference strains

Strain	Pathotype	Amino acid position	
		107	109
CVI988	Attenuated	R	G
CU-2	Attenuated	Q	G
MD70/13	Virulent	Q	G
Md5	Very virulent	Q	E
GaHV2/India/Ck/001/17	Field strains of this study	Q	E
GaHV2/India/Ck/002/18		Q	E
GaHV2/India/Ck/003/18		Q	G
GaHV2/India/Ck/005/18		Q	G
GaHV2/India/Ck/006/18		Q	E
GaHV2/India/Ck/013/18		Q	G
GaHV2/India/Ck/016/18		Q	E
GaHV2/India/Ck/017/18		Q	G
GaHV2/India/Ck/018/18		Q	E
GaHV2/India/Ck/019/18		Q	G
GaHV2/India/Ck/020/18		Q	G

TABLE 4 Amino acid substitutions in the vIL-8 protein of field Marek's disease virus (MDV) strains compared with reference strains

Strain	Pathotype	vIL-8 (aa)	Amino acid position	
			4	31
CVI988	Attenuated	121	L	D
CU-2	Attenuated	134	L	D
JM/102W	Virulent	134	L	D
Md5	Very virulent	134	L	D
648A	Very virulent plus	134	L	D
GaHV2/India/Ck/002/18	Field strains of this study	134	S	G
GaHV2/India/Ck/003/18		134	S	G
GaHV2/India/Ck/006/18		134	S	G
GaHV2/India/Ck/007/18		134	S	G
GaHV2/India/Ck/008/18		134	S	G
GaHV2/India/Ck/009/18		134	S	G
GaHV2/India/Ck/011/18		134	S	G
GaHV2/India/Ck/012/18		134	S	G
GaHV2/India/Ck/013/18		134	S	G
GaHV2/India/Ck/015/18		134	S	G
GaHV2/India/Ck/016/18		134	S	G
GaHV2/India/Ck/018/18		134	S	G
GaHV2/India/Ck/019/18		134	S	G

clinical disease, outbreaks in vaccinated flocks are not uncommon. More virulent and pathogenic MDVs have evolved in recent times worldwide including India. The disease has shifted to visceral lymphomas rather than neural form in recent outbreaks (Kannaki et al., 2020). Moreover, the sub-clinical infection of young chicks with immunosuppressive viruses such as chicken infectious anaemia virus (CIAV), infectious bursal disease virus (IBDV) and fowl adeno-virus (FAV) also contribute to MD outbreaks in vaccinated flocks, because

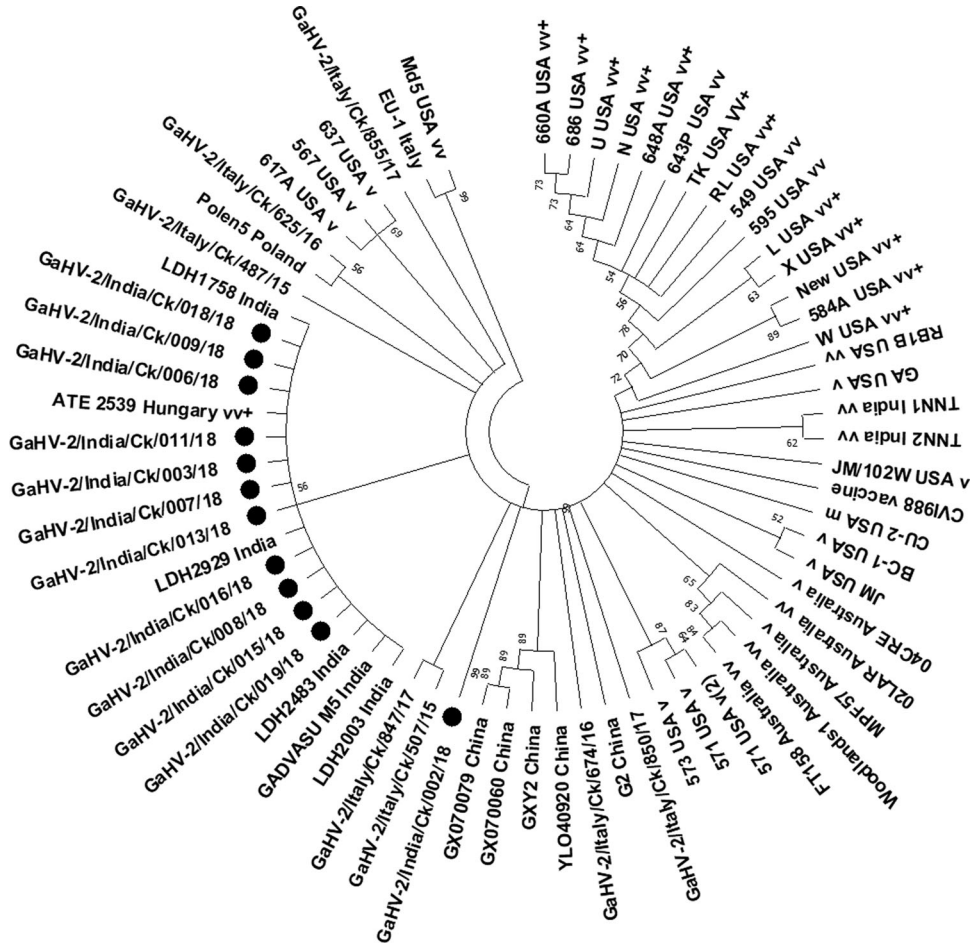

FIGURE 1 Phylogenetic tree of *Meq* gene nucleotide sequences of 13 field Marek's disease virus (MDV) strains and the 54 reference sequences retrieved from NCBI database. Phylogenetic tree was constructed with neighbour-joining method with 1000 bootstrap replicates using the Tamura-Nei model in MEGA 10.0.5 software. Strains of this study were highlighted (•)

immunosuppression inhibits the development of immunity after MD vaccination. Some of the unique genes of serotype I MDVs are associated with viral oncogenicity and pathogenicity. In the present study, we investigated the three genes associated with oncogenicity and virulence, namely *meq*, *pp*38 and *vIL*-8 of 17 MDV field strains from MD visceral tumours occurred in vaccinated poultry breeder flocks.

The nucleotide similarity of the present MDV strains ranged from 98.5% to 100% for all three virulence-associated genes due to point mutation at various positions. Although all strains are from the same location, the variation may be attributed to high mutation capacity of the field MDVs. Different age groups and genetic background, production type (layer breeder, broiler breeder and native breed), other vaccines and vaccine immunity could be other factors that might have contributed to the emergence of virulent MDV strains.

Meq gene of vv and vv+ MDV pathotypes encodes for 339 amino acids containing an N-terminal basic leucine zipper (bZIP) transactivation domain and C-terminal proline rich trans-repression domain. Dimerization and activation of the transactivation domain contribute to cell transformation and subsequent tumourigenesis (Liu et al., 1999; Levy et al., 2003). Meq of mild or vaccine strains such as CVI988, CU-2 or JM have 59 aa insertion in the C-terminal region known to have suppression effect on the Meq expression and oncogenicity (Chang et al., 2002; Shamblin et al., 2004). Meq proteins of all field MDVs investigated here are of 339 aa length encoded by 1020 bp coding sequence, indicating that they are potentially virulent strains. Moreover, the amino acid changes at positions 71 and 77 are considered as a feature of high virulence MDVs (Shamblin et al., 2004). Mutation at position 71 was observed in all current field strains. However, mutation at position 77 was not present similar to Chinese and other field strains (Tian et al., 2011). Mutation at position 77 seems to be a feature of virulent strains of the United States (Tian et al., 2011). The three amino acid substitutions at positions 119, 153 and 156, unique to vv+ strains such as 584A and 648A, were not present in the current strains, indicating that they have not yet evolved as vv+. However, some unique mutations at positions 80 (D80Y), 88 (A88T), 93 (Q93R) and 139 (T139A) were observed in the field strains. Unique amino acid substitutions in *meq* gene have been recently reported in MDV field strains circulating in Italy (Mescolini et al., 2019), China (Yu et al., 2013), Colombia (Lopez-Osorio et al., 2017) and Turkey (Ozan et al., 2021). Geographically restricted independent evolution of field strains was observed in vaccinated flocks. In the present study also, three unique amino acid substitutions are observed in the field MDV strains. These unique

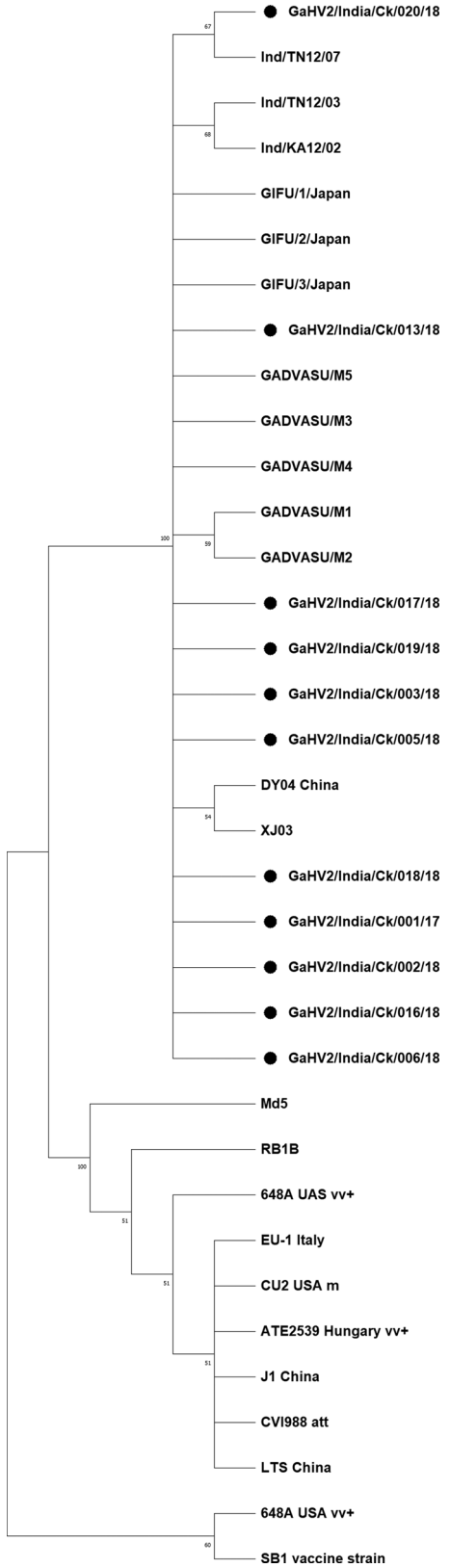

FIGURE 2 Phylogenetic tree of *pp38* gene nucleotide sequences of 11 field Marek's disease virus (MDV) strains and the 25 reference sequences retrieved from NCBI database. Phylogenetic tree was constructed with neighbour-joining method with 1000 bootstrap replicates using the Tamura-Nei model in MEGA 10.0.5 software. Strains of this study were highlighted (•)

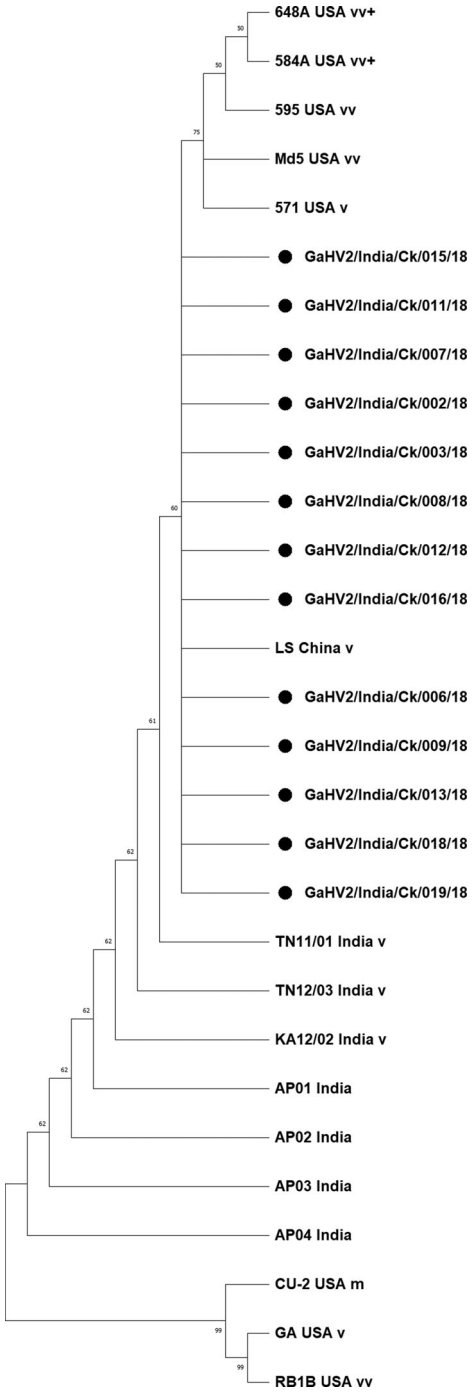

FIGURE 3 Phylogenetic tree of *vIL-8* gene nucleotide sequences of 13 field Marek's disease virus (MDV) strains and the 18 reference sequences retrieved from NCBI database. Phylogenetic tree was constructed with Neighbour-Joining method with 1000 bootstrap replicates using the Tamura-Nei model in MEGA 10.0.5 software. Strains of this study were highlighted (•)

mutations are also observed in few other field strains reported from different regions of this country. Previous studies show the regularity in the mutations of *meq* genes of MDVs from outbreaks of increased virulence (Zhang et al., 2011). Although GaHV-2 is a DNA virus, the *meq* gene of GaHV-2 has high mutation frequency of approximately 10^{-4}

substitutions per year like that of RNA viruses (Padhi & Parcells, 2016). High mutation frequency combined with positive selection in vaccinated flocks could drive the emergence of highly virulent pathotypes. However, it needs to be ascertained whether these contribute to a continuing evolutionary drift leading to increased virulence of MDV strains circulating in India.

The Number of proline repeats is strongly associated with the level of MDV virulence (Renz et al., 2012; Shamblin et al., 2004). Negative correlation exists between their number and the virulence. The number of four-proline repeats ranges between 2 and 10 in all MDV strains. Generally, v to vv MDV strains have been shown to possess five 'PPPP' repeats in *meq* gene (Abdallah et al., 2018; Mescolini et al., 2019). All the current strains had five repeats. Earlier reported MDV strains from India with v to vv pathotype also possessed four to five 'PPPP' repeats (Kalyani et al., 2010; Prathibha et al., 2018). Phylogenetic analysis revealed that current strains clustered with Indian strains from northern India and with vv+ ATE 2539 strain from Hungary. Surprisingly, the current strains clustered away from earlier vv strains from southern India reported a decade ago (TNN1 and TNN2).

The *pp38* gene encodes a 38 kDa phosphoprotein that has a role in cell transformation and virus reactivation from latency (Gimeno et al., 2005). Minor variations were observed in amino acid substitutions among the field strains. Glutamine at position 107 which is equivalent to the mutation at nucleotide position 320 is conserved among virulent MDVs, while CVI988 vaccine strain has arginine instead of glutamine. At nucleotide level, while CVI 988 has G at position 320, all virulent strains have conserved A at this position. All the current MDV strains also have conserved G320A mutation resulting in glutamine. The mutation at 107 is considered a reliable biomarker for differentiating field strains from CVI988 vaccine (Baigent et al., 2016). Glutamate at position 109 present in vv and vv+ strains was observed in five out of 11 sequences investigated. Both these positions, namely 107 and 109 can be considered as virulence indicators as observed earlier in the virulent strains of the United States and China (Shamblin et al., 2004; Tian et al., 2011).

The *vIL-8* gene consists of three exons and encodes a 134-aa length peptide that is expressed during cytolytic infection and shows closest homology to mammalian and avian IL-8 (X. Cui et al., 2004). vIL-8 is considered important for the switch of infection from cytolytic to latent infection (Schat & Xing, 2000). This gene was shown to be highly conserved among different pathotypes; hence, change in virulence can be strongly speculated with any mutations within the gene. The two-point mutations at positions 4 (L4S) and 31 (D31G), initially considered as unique to Chinese MDV strains (Tian et al., 2011), are observed in the current study as well. These mutations were observed in other field strains from Japan and India (Abd-Ellatieff et al., 2018; Prathibha et al., 2018). The phylogenetic tree of the *vIL-8* gene revealed the clustering of present strains with vv and vv+ strains from the United States and China.

The present study supports the argument that the vaccination pressure imposed the genetic drift of emerging strains towards increased virulence (Nair, 2018; Padhi & Parcells, 2016; Wozniakowski & Salamonowicz, 2014). Sequence and phylogenetic analyses of virulence-associated genes from field MDVs from vaccinated flocks indicate that these strains possess molecular features of virulent strains. Although vv+ strains are not yet reported in Indian poultry flocks and were not observed in the current investigation, the rate of evolution of *meq* gene in recent times by various reports suggests the rapid evolution in MDV strains towards high pathogenicity. *Meq* gene followed by *vIL-8* and *pp38* genes could be of additional value in deciphering the pathotype of MDV strains. Molecular characterization of virulence-associated genes would be the most rapid, reliable and affordable method to indicate the virulence of field strains; however, in vivo pathotyping assays need to be performed in parallel for confirming the pathotypes. In conclusion, continuous surveillance is essential to monitor the emergence of virulent MDV strains and to devise appropriate vaccination strategy and bio-security programmes.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICS STATEMENT

This study did not involve any animal subjects. For handling the samples at ICAR-DPR appropriate bio-safety measures were followed as per the Institute biosafety guidelines.

AUTHOR CONTRIBUTIONS

T. R. Kannaki Conceived the study and collected the samples. E. Priyanka and Y. Nishitha carried out PCR work and sequencing. T. R. Kannaki and Madhuri Subbiah carried out sequence analysis and phylogenetic analysis. T. R. Kannaki prepared the manuscript and Santosh Haunshi and Madhuri Subbiah critically reviewed the manuscript. All authors have read and approved the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the supplementary material of this article (Tables S1–S3).

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REFERENCES

Abdallah, F., Hassanin, O., Attar, E., Ali, H., Megahed, M., & Nair, V. (2018). Marek's disease virus in Egypt: Historical overview and current research based on the major MDV-encoded oncogene Meq. *Hosts and Viruses*, *5*, 35–43.

Abd-Ellatieff, H. A., Abou Rawash, A. A., Ellakany, H. F., Goda, W. M., Suzuki, T., & Yanai, T. (2018). Molecular characterization and phylogenetic analysis of a virulent Marek's disease virus field strain in broiler chickens in Japan. *Avian Pathology*, *47*(1), 47–57.

Baigent, S. J., Nair, V. K., & Le Galludec, H. (2016). Real-time PCR for differential quantification of CVI988 vaccine virus and virulent strains of Marek's disease virus. *Journal of Virological Methods*, *233*, 23–36.

Bulow, V. V., & Biggs, P. M. (1975). Differentiation between strains of Marek's disease virus and turkey herpes virus by immunofluorescence assays. *Avian Pathology*, *4*, 133–146.

Chang, K. S., Ohashi, K., & Onuma, M. (2002). Suppression of transcription activity of the MEQ protein of oncogenic Marek's disease virus serotype 1 (MDV-1) by L-MEQ of non-oncogenic MDV-1. *The Journal of Veterinary Medical Science*, *64*(12), 1091–1095.

Cui, X., Lee, L. F., Reed, W. M., Kung, H. J., & Reddy, S. M. (2004). Marek's disease virus-encoded vIL-8 gene is involved in early cytolytic infection but dispensable for establishment of latency. *Journal of Virology*, *78*(9), 4753–4760.

Cui, Z., Qin, A., Lee, L. F., Wu, P., & Kung, H. J. (1999). Construction and characterization of a H19 epitope point mutant of MDV CVI988/Rispens strain. *Acta Virologica*, *43*(2/3), 169–173.

Davidson, I., & Borenstein, R. (1999). Multiple infection of chickens and turkeys with avian oncogenic viruses: Prevalence and molecular analysis. *Acta Virologica*, *43*(2–3), 136–142.

Dudnikova, E., Norkina, S., Vlasov, A., Slobodchuk, A., Lee, L. F., & Witter, R. L. (2007). Evaluation of Marek's disease field isolatesby the "best fit" pathotyping assay. *Avian Pathology*, *36*, 135–143.

Gimeno, I. M. (2014). Marek's disease and differential diagnosis with other tumor viral diseases of poultry. *Encyclopedia of agriculture and food systems* (pp. 156–171). Academic Press.

Gimeno, I. M., & Schat, K. A. (2018). Virus-induced immunosuppression in chickens. *Avian Diseases*, *62*, 272–285.

Gimeno, I. M., R. L. Witter, A. M. Fadly, & R. F. Silva. (2005). Novel criteria for the diagnosis of Marek's disease virus-induced lymphomas. *Avian Pathology*, *34*, 332–340.

Gupta, M., Deka, D., & Ramneek. (2016). Sequence analysis of Meq oncogene among Indian isolates of Marek's disease herpesvirus. *Meta Gene*, *9*, 230–236.

International Committee on Taxonomy of Viruses (2017). *Master species list 2017 [WWW Document]. ICTV*. https://talk.ictvonline.org/files/master-species-lists/m/msl/7185

Jones, D., Lee, L., Liu, J. L., Kung, H. J., & Tillotson, J. K. (1992). Marek's disease virus encodes a basic-leucine zipper gene resembling the fos/jun oncogenes that is highly expressed in lymphoblastoid tumors. *Proceedings of the National Academy of Sciences*, *89*, 4042–4046.

Kalyani, I. H., Tajpara, M. M., Jhala, M. K., Bhanderi, B. B., Nayak, J. B., & Purohit, J. H. (2010). Characterization of the ICP4 gene in pathogenic Marek's disease virus of poultry in Gujarat, India, using PCR and sequencing. *Veterinarski Arhiv Journal*, *80*, 683–692.

Kannaki, T. R., & Gowthaman, V. (2020). Marek's disease: Time to review the emerging threat in Indian poultry. *World's Poultry Science Journal*, *76*(1), 91–99.

Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution*, *35*(6), 1547–1549.

Levy, A. M., Gilad, O., Xia, L., Izumiya, Y., Choi, J., & Tsalenko, A. (2005). Marek's disease virus: Meq transforms chicken cells via the v-Jun transcriptional cascade: A converging transforming pathway for avian oncoviruses. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(41), 14831–1486.

Liu, J. L., Lin, S. F., Xia, L., Brunovskis, P., Li, D., Davidson, I., & Kung, H. J. (1999). Meq and V-IL8: Cellular genes in disguise? *Acta Virologica*, *43*, 94–101.

López-Osorio, S., Piedrahita, D., Espinal-Restrepo, M. A., Ramírez-Nieto, G. C., Nair, V., Williams, S. M., Baigent, S., Ventura-Polite, C., Aranzazu-Taborda, D. A., & Chaparro-Gutiérrez, J. J. (2017). Molecular characterization of Marek's disease virus in a poultry layer farm from Colombia. *Poultry Science*, *96*(6), 1598–1608.

Mescolini, G., Lupini, C., Felice, V., Guerrini, A., Silveira, F., Cecchinato, M., & Catelli, E. (2019). Molecular characterization of the meq gene of Marek's disease viruses detected in unvaccinated backyard chickens reveals the circulation of low- and high-virulence strains. *Poultry Science*, *98*(8), 3130–3137

Nair, V. (2018). Spotlight on avian pathology: Marek's disease. *Avian Pathology*, *47*, 440–442.

Ozan, E., Müftüoğlu, B., Sahindokuyucu, I., Kuruçay, H., Inal, S., Kuruca, N., Elhag, A., Karaca, E., Tamer, C., Gumusova, S., Albayrak, H., & Barry, G., Gulbahar, M. Y., & Yazici, Z. (2021). Marek's disease virus in vaccinated poultry flocks in Turkey: Its first isolation with molecular characterization. *Archives of Virology*, *166*. 1–11.

Padhi, A., & Parcells, M. S. (2016). Positive selection drives rapid evolution of the meq oncogene of Marek's disease virus. *Plos One*, *11*(9), e0162180.

Parcells, M. S., Lin, S. F., Dienglewicz, R. L., Majerciak, V., Robinson, D. R., Chen, H. C., Wu, Z., Dubyak, G. R., Brunovskis, P., & Hunt, H. D. (2001). Marek's disease virus (MDV) encodes an interleukin-8 homolog (vIL-8): Characterization of the vIL-8 protein and a vIL-8 deletion mutant MDV. *Journal of Virology*, *75*, 5159–5173.

Prathibha, Y., Sreedevi, B., Vinod Kumar, N., & Srilatha, C. (2018). Molecular characterization and phylogenetic analysis of oncogenes from virulent serotype-1 Marek's disease virus in India. *Acta Virologica*, *62*(3), 277–286.

Puro, K. U., Bhattacharjee, U., Baruah, S., Sen, A., Das, S., Ghatak, S., Doley, S., Sanjukta, R., & Shakuntala, I. (2018). Characterization of Marek's disease virus and phylogenetic analyses of meq gene from an outbreak in poultry in Meghalaya of Northeast India. *Virus disease*, *29*(2), 167–172.

Renz, K. G., Cooke, J., Clarke, N., Cheetham, B. F., Hussain, Z., Fakhrul Islam, A. F. M., & Walkden-Brown, S. W. (2012). Pathotyping of Australian isolates of Marek's disease virus and association of pathogenicity with meq gene polymorphism. *Avian Pathology*, *41*(2), 161–176.

Schat, K. A., & Nair, V. (2013). Neoplastic diseases: Marek's disease. In D. E. Swayne, J. R. Glisson, L. R. McDougald, L. K. Nolan, D. L. Suarez, & V. L. Nair (Eds.), *Diseases of Poultry* (13th ed. pp. 515–552). Wiley-Blackwell Hoboken

Schat, K. A., & Xing, Z. (2000). Specific and nonspecific immune responses to Marek's disease virus. *Development and Comparative Immunology*, *24*(2–3), 201–221.

Shamblin, C. E., Greene, N., Arumugaswami, V., Dienglewicz, R. L., & Parcells, M. S. (2004). Comparative analysis of Marek's disease virus (MDV) glycoprotein-, lytic antigen pp38- and transformation antigen Meq-encoding genes: Association of meq mutations with MDVs of high virulence. *Veterinary Microbiology*, *102*(3–4), 147–167.

Suresh, P., Johnson Rajeswar, J., Sukumar, K., Harikrishnan, T. J., & Srinivasan, P. (2015). Pathotyping of recent Indian field isolates of Marek's disease virus serotype 1. *Acta Virologica*, *59*(2), 156–165.

Tamura, K., & Nei, M. (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution*, *10*(3), 512–526.

Tian, M., Zhao, Y., Lin, Y., Zou, N., Liu, C., Liu, P., & Huang, Y. (2011). Comparative analysis of oncogenic genes revealed unique evolutionary features of field Marek's disease virus prevalent in recent years in China. *Virology Journal*, *8*, 121.

Witter, R. L. (1997). Increased virulence of Marek's disease virus field isolates. *Avian Diseases*, *41*(1), 149.

Witter, R. L., Calnek, B. W., Buscaglia, C., Gimeno, I. M., & Schat, K. A. (2005). Classification of Marek's disease viruses according to pathotype: Philosophy and methodology. *Avian Pathology*, *34*, 75–90.

Woźniakowski, G., & Samorek-Salamonowicz, E. (2014). Molecular evolution of Marek's disease virus (MDV) field strains in a 40-year time period. *Avian Diseases*, *58*(4), 550–557.

Yu, Z.-H., Man, T., Lou, J., Wang, X.-W., Ding, K., Yu, L.-L., & Liu, J.-X. (2013). Molecular characteristics and evolutionary analysis of field Marek's disease virus prevalent in vaccinated chicken flocks in recent years in China. *Virus Genes*, *47*, 282–291.

Zhang, Y. P., Liu, C. J., Zhang, F., Shi, W., & Li, J. (2011). Sequence analysis of the meq gene in the predominant Marek's disease virus strains isolated in China during 2006–2008. *Virus Genes*, *43*(3), 353–357.

Zhuang, X., Zou, H., & Shi, H. (2015). Outbreak of Marek's disease in a vaccinated broiler breeding flock during its peak egg-laying period in China. *BMC Veterinary Research*, *11*, 157.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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