Sequence analysis of Meq oncogene among Indian isolates of Marek's disease herpesvirus

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ABSTRACT

Marek's disease (MD), caused by Marek's disease virus (MDV), is a highly contagious neoplastic disease of chicken that can be prevented by vaccination. However, in recent years many cases of vaccine failure have been reported worldwide as chickens develop symptoms of MD in spite of proper vaccination. Distinct polymorphism and point mutations in Meq gene of MDV have been reported to be associated with virulence and oncogenicity. The present study was carried out with the objective to isolate and characterize field isolates of MDV on the basis of Meq gene. Twenty five samples of suspected cases of MD were collected and processed for virus isolation in duck embryo fibroblast (DEF) primary culture where 28% (7 of 25) samples showed characteristic cytopathic effects of MDV in the form of plaques and syncytia. Additional evidence of presence of MDV in these samples was confirmed by PCR. To analyze diversity in all seven isolates of MDV, a polymorphism study was carried out by cloning and sequencing of full length of Meq gene (1020 bp). Sequence homology of 7 isolates with 23 reference strains showed 98.10–99.40% similarity in nucleotide and 95.90–98.50% similarity in amino acid sequences. Six isolates revealed 5 repeat sequences of 4 prolines (PPPP) whereas, one isolate revealed only 4 repeats. In phylogenetic analysis, these isolates formed a separate cluster showing close relatedness to the Chinese isolates. The study indicates a high mutation rate in field isolates of MDV that may be probable cause of vaccination failure.

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1. Introduction

Marek's disease is an infectious poultry disease caused by cell associated alphaherpesvirus, Marek's disease virus (MDV) (Calnek and Witter, 1997). MDV belongs to subfamily *Alphaherpesvirinae*, genus *Mardivirus* and species: *Gallid herpesvirus 2*, *Gallid herpesvirus 3* and *Meleagrid herpesvirus 1* as reported in ICTV (Davison, 2010). These species of MDV group are classified under three serotypes - 1, 2 and 3 – on the basis of their pathogenicity and virulence (OIE, 2010). Marek's disease virus serotype 1 (MDV1) is categorized as virulent strain where as serotype 2 (MDV2) and serotype 3, herpesvirus of turkeys (HVT) are mild and vaccinal strains, respectively (Islam and Walkden-Brown, 2007; Malkinson et al., 1992). The disease is associated with several distinct pathological syndromes, of which the lymphoproliferative syndromes are the most frequent and are of most practical significance (Lobago and Woldemeskel, 2004). The disease can occur at 3–4 weeks of age or later but is most common between 12 and 30 weeks of age (OIE, 2010). Although clinical disease is not always apparent in infected flocks, till chickens become persistently infected with MD virus (MDV) resulting in the decreased growth rate and egg production (Fabricant and Fabricant, 1999). Tentative diagnosis is made based on the neurological symptoms like partial or complete paralysis of the legs and wings, skin leukosis, depression and death. Lymphoid lymphomas, gross or microscopic lesions are associated with atrophy of organs such as thymus, bursa and spleen which is further continued by virus isolation, demonstration of viral antigen or antibodies and detection of the viral nucleic acid (Churchill and Biggs, 1967).

The incidence of MD is quite variable in commercial flocks and depends on various parameters like strain and dose of virus, age at exposure, maternal antibody, host gender and genetics, other concurrent diseases, and several environmental factors including stress. In India reports of MD outbreaks from Orissa, Punjab, Assam, Uttar Pradesh, Andhra Pradesh, Arunachal Pradesh and Tripura, Gujarat appeared periodically and the annual loss accounts for Rs. 4 crores (Sudhakar and Nair, 2013). Marek's disease (MD) incidence induced by Indian MDV strains was found to be 57.5% and 25% in monovalent and bivalent vaccine inoculated birds, respectively compared to uninoculated control (100%) in a protection test (Suresh et al., 2015).

MD may be prevented by vaccination in ovo or at hatch with monovalent or multivalent live virus vaccines of various types (Witter, 1998). Vaccination does not prevent the birds from infection with the field MDV and their transmission. However, it does reduce the amount of virus shed in dander, hence reduces the horizontal spread of the disease and thus reduce the economic losses. The field isolates of MDV-1 have

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tended to increase in virulence and cause MD even in vaccinated chickens (Woźniakowski et al., 2011). The number of MD cases has increased in vaccinated chickens during the last few years due to adenovirus infections, which commonly exist in poultry flocks, can be one of the reasons for vaccination failure (Niczyporuk et al., 2013). Thus, MD still remains a serious problem in individual flocks or in selected geographic areas despite of proper vaccination (Shamblin et al., 2004). Distinct polymorphism and point mutations in *Meq* gene have been reported to correlate with MDV virulence and oncogenicity (Shamblin et al., 2004; Woźniakowski et al., 2011; Zhang et al., 2011; Gong et al., 2013). Among different genes of virus, the main MDV encoded *Meq* gene which bears resemblance to Jun/Fos family of transcriptional activators is found to have oncogenic expression and is responsible for transformation of T-lymphocyte (Lupiani et al., 2004). The *Meq* gene is mainly found in serotype-1 strains of MDV in long and short forms (Spatz and Silva, 2007) and is related to virulence (Shamblin et al., 2004). Live attenuated herpesvirus of turkey (HVT) vaccine based on attenuated FC 126 (HVT) vaccine strain, deficient of various monogenic genes, one of them is Meq gene (Afonso et al., 2001). Renz et al. (2012) showed that the number of repeat sequences of four prolines (PPPP repeats) in the *Meq* gene (overall range 2 to 8) was strongly associated with virulence across all the isolates they studied, with the most pathogenic isolates having the fewest number of repeats. Therefore, the present study was carried out with the objective to isolate and characterize field isolates of Marek's disease virus on the basis of *Meq* gene.

2. Materials and methods

2.1. Sample collection

Liver and spleen samples (25 each) from vaccinated White Leg Horn (WLH) and Rhode Island Red (RIR) broilers of different age groups, were collected in sterile containers having 50% phosphate buffered glycerin (standard buffer used for collection of samples for virus isolation) from five different flocks of a single poultry farm (See Table 1). All the birds were vaccinated at day one with the live MD-FC 126 vaccine (Hester Biosciences Ltd.) having cell associated HVT strain FC 126. These broilers without any specific clinical symptoms suspected to have died of Marek's disease (MD)/lymphoid leukosis (LL) were brought for postmortem to the Department of Veterinary Pathology, College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab, India during the year 2012. The poultry farm had a previous history of nearly 0.5–1.0% annual mortality due to Marek's disease, confirmed by postmortem analysis, although the live birds did not show any MDV specific clinical symptoms.

2.2. Sample processing

Liver and spleen samples were washed twice in sterile PBS, cut into small pieces, triturated in the sterile pestle and mortar using sterilized sand and then mixed with phosphate buffered saline (PBS) for preparing a 10% tissue suspension from each sample. The tissue suspensions were transferred into 15 ml tubes and centrifuged at 3000 ×*g* for 10 min at 4 °C. The supernatant was aspirated into new tubes and after addition of 2–3 drops of antibiotic (antibiotic antimycotic solution 100× liquid endotoxin tested, HIMEDIA) it was incubated at 37 °C for 35–45 min. The inoculums were then used for infecting the duck embryo fibroblast (DEF) cell monolayers or stored at −80 °C for future use. The second fractions were stored in liquid nitrogen for back up.

2.3. Isolation of MDV in DEF cells

Primary culture of duck embryo fibroblast (DEF) cells was prepared from 11-day old duck embryos, grown in 6 well cell culture plates using MEM supplemented with 10% fetal bovine serum (FBS, Gibco) and antibiotic antimycotic solution (100× liquid endotoxin tested, HIMEDIA;

having penicillin 100 units/ml, streptomycin 0.10 μg/ml and amphotericin B 0.25 μg/ml of medium) and incubated at 37 °C in presence of 5% CO_2. Complete DEF monolayers were observed within 24–48 h of culture which were then infected with the inoculums prepared from the field samples keeping one uninoculated well as cell control per plate. Around 500 μl inoculum and 300 μl maintenance medium (MM) were added to each well of a 6 well plate following removal of the growth medium and the plates were incubated at 37 °C for 60 to 90 min for virus adsorption. Then the infected monolayers were washed twice with MM, added 2 ml of MM per well and the plates were incubated for 12 to 15 days for observing visible cytopathic effects (CPE) during which MM was changed at 3 days interval for 2–3 times. Irrespective of the CPE observed, the infected cultures were harvested 12–15 days post inoculation, subjected to 3 cycles of trypsinization and then stored in liquid nitrogen. All the 25 samples (DEF passaged) were given three subsequent passages in the DEF monolayers.

2.4. Detection of MDV by polymerase chain reaction

Genomic DNA was extracted from all 25 DEF passaged Marek's disease (MD)/lymphoid leukosis (LL) suspected samples by using standard laboratory protocol (Sambrook, 1989). The published primer sequences M1 (forward) 5′-TACTTCCTATATAGATTGAGACGT-3′ and M2 (reverse) 5′-GAGATCCTCGTA AGGTGTAATATA-3′ were used for diagnosis of MDV in the genomic DNA extracted from the DEF passaged samples (Davidson and Borenshtein, 1999). The PCR products were analyzed by gel electrophoresis at 100 V for 40 min in 1% agarose gel prepared in 0.5× TBE having 0.5 μg/ml ethidium bromide and the amplicon size was determined by comparing with the 100 bp Plus DNA Ladder (Fermentas) loaded along with the samples.

2.5. PCR amplification of the complete MDV Meq gene

New primers sequences Meq-F: 5′-ATCCGCTAGCAGAGATGGCTCA GGAGCC-3′ and Meq-R: 5′-GATCACCCGGGGTCTCCCGTCACC-3′ were designed manually for PCR amplification of the complete *Meq* gene (amplicon size 1040 bp) following analysis of GenBank available MDV *Meq* gene sequences. The genomic DNA samples detected as MDV positive by diagnostic PCR were used as template for PCR amplification of *Meq* gene. The PCR reaction mixture of 50 μl volume was prepared containing 5 μl of 10× *Pfx* platinum buffer (Invitrogen), 5 μl of 10× PCR Enhancer, 1.5 μl of 50 mM magnesium sulphate, 1 μl each of Meq-F (20 pmol) and Meq-R (20 pmol) primers, 1 μl of dNTPs mix (10 mM), 0.5 μl of platinum *Pfx* DNA polymerase (hot start, 2.5 U/μl), 5–10 μl of genomic DNA (~1.0 μg) and the rest volume nuclease free water. Cycling conditions standardized for PCR amplification included one cycle of initial denaturation at 94 °C for 6 min followed by 5 step up cycles each of denaturation at 94 °C for 45 s, annealing at 56 °C + 1 °C/cycle for 45 s and extension at 72 °C for 2 min, followed by 30 cycles each of denaturation (94 °C for 45 s), annealing (61 °C for 45 s) and extension (72 °C for 2 min), and a final extension at 72 °C for 10 min.

2.6. Cloning and sequencing of the MDV Meq gene

The PCR amplified MDV *Meq* gene was purified by gel extraction method using Pure Link Quick Gel extraction and PCR Purification Combo Kit (Invitrogen) and then cloned in pJET1.2/blunt vector (Thermo Scientific) according to manufacturer's instructions. The selected clones were grown overnight in Luria Bertani (LB) broth in a shaker incubator kept at 37 °C and the plasmids were prepared by alkaline lysis method (Sambrook, 1989). The presence of *Meq* gene insert and its orientation in the purified recombinant plasmid was confirmed by PCR and restriction enzyme (*Pvu* I, *Bgl* II and *Bsp* HI, Fermentas) analysis as per standard protocol. One of the recombinant plasmids (clones) carrying *Meq* gene from each isolate, confirmed in PCR and RE analysis, was then got sequenced by commercial outsourcing.

2.7. Analysis of the MDV Meq gene sequences of the isolates

The obtained *Meq* gene sequences were assembled following online Blast analysis, using DNASTAR (Lasergene 7) Software and manually edited. Sequence data containing obscurities were substantiated by resequencing additional recombinant plasmids. The *Meq* gene nucleotide sequence (1020 bp) and the deduced amino acid sequence (339aa) of seven Ludhiana isolates (LDH 1758, LDH 2003, LDH 2483, LDH 2700, LDH 2929 and LDH 3262) were compared with 23 reference MDVs for their similarity. Among these 23 reference strains, one strain was vaccine strain (CV 1988/Rispens), three from Tamil Nadu (India), ten strains were from USA, six from China, two from Australia and one from Russia. Out of the 23 reference strains 13 strains were well characterized with respect to their virulence and pathotype (Table 2). A phylogenetic tree was constructed based on the *Meq* gene amino acid sequences of the seven Ludhiana isolates and 23 MDV reference strains contained within the GenBank database using the maximum parsimony method of Mega 5.20 software. The topological accuracy of the tree was estimated by 1000 bootstrap re sampling.

3. Results

3.1. Virus isolation

Primary culture of duck embryo fibroblasts (DEF) was prepared in 6 well cell culture plate where a confluent monolayer was observed within 24–48 h [Fig. 1(a)]. The viral inoculums prepared from liver and spleen tissues of 25 MD suspected birds when used to infect the confluent monolayer of DEF, the MDV specific clear and distinct cytopathic effects (CPE) were observed in 28.00% (7 out of 25) samples within 9–11 days post inoculation (dpi) on second passage onwards [Fig. 1(c)]. The DEF cells started to detach and degenerate 12–13 days onward which was clearly visible on 15 dpi [Fig. 1(d)]. An early CPE was seen as small rounded cells reflecting light waves [Fig. 1(b)]. Later on these cells formed foci and syncytias that detached from the wall of cell culture flask causing formation of plaques [Fig. 1(b), (c) and (d)].

Table 1
History of MDV samples collected from 5 different flocks of a single farm.

S. no.	Type and age of birds	Lab identification no.	Vaccination	Clinical symptoms
1	Broiler 12–18 weeks	2697	HVT (FC 126)	MD/LL suspected
2	Broiler 12–18 weeks	2233	HVT (FC 126)	MD/LL suspected
3	WLH adult	708	HVT (FC 126)	MD suspected
4	RIR adult	1597	HVT (FC 126)	MD suspected
5	Broiler 12–18 weeks	1758	HVT (FC 126)	MD suspected
6	Broiler 12–18 weeks	2003	HVT (FC 126)	MD suspected
7	Broiler adult	2483	HVT (FC 126)	MD/LL suspected
8	Broiler adult	2552	HVT (FC 126)	MD/LL suspected
9	Broiler 12–18 weeks	2546	HVT (FC 126)	MD/LL suspected
10	Broiler 12–18 weeks	3079	HVT (FC 126)	MD/LL suspected
11	RIR 1–6 weeks	2025	HVT (FC 126)	MD suspected
12	Broiler 12–18 weeks	2547	HVT (FC 126)	MD/LL suspected
13	Broiler 12–18 weeks	2614	HVT (FC 126)	MD suspected
14	Broiler 18–24 weeks	2700	HVT (FC 126)	MD/LL suspected
15	Broiler 18–24 weeks	2929	HVT (FC 126)	MD suspected
16	Broiler 12–18 weeks	3262	HVT (FC 126)	MD suspected
17	Broiler 12–18 weeks	3077	HVT (FC 126)	MD suspected
18	Broiler 12–18 weeks	3087	HVT (FC 126)	MD suspected
19	Broiler 12–18 weeks	3088	HVT (FC 126)	MD/LL suspected
20	WLH adult	3265	HVT (FC 126)	MD suspected
21	Broiler 12–18 weeks	3302	HVT (FC 126)	MD suspected
22	Broiler adult	3992	HVT (FC 126)	MD suspected
23	BV-300 adult	3977	HVT (FC 126)	MD/LL suspected
24	Adult WLH	3989	HVT (FC 126)	MD/LL suspected
25	Adult RIR	4433	HVT (FC 126)	MD/LL suspected

LL – lymphoid leukosis; MD - Marek's disease.

Table 2
*Meq* gene from different MDV isolates analyzed.

MDV strain/sample	Number of PPPP repeats	Virulence	Origin	Accession number
LDH 1758	5	N/A	India (North)	KF895029.1
LDH 2003	5	N/A	India (North)	KF895030.1
LDH 2483	5	N/A	India (North)	KF895031.1
LDH 2614	5	N/A	India (North)	KF895032.1
LDH 2700	5	N/A	India (North)	KF895033.1
LDH 2929	4	N/A	India (North)	KF895034.1
LDH 3262	5	N/A	India (North)	KF895035.1
tn-n1	5	N/A	India (South)	HM749324.1
tn-n2	4	N/A	India (South)	HM749325.1
tn-n3	4	N/A	India (South)	HM749326.1
FT158	5	vvMDV	Australia	EF523771.1
MPF57	5	vMDV	Australia	EF523774.1
LCD (SC07II)	4	N/A	China	HQ658611.1
LDH (JL07I)	3	N/A	China	HQ658614.1
LHC4 (LN08IV)	3	N/A	China	HQ658618.1
LHC5 (LN08V)	3	N/A	China	HQ658619.1
WSO3	3	N/A	China	HQ638152.1
LSY2 (LN071)	3	N/A	China	HQ658625.1
L strain	2	vv + MDV	USA	AY362717.1
567 strain	4	v + MDV	USA	AY362709.1
595 strain	2	vv + MDV	USA	AY362715.1
GA strain	5	vMDV	USA	M89471.1
CU-2 strain	7	mMDV	USA	AY362708.1
JM strain	7	vMDV	USA	HM488348.1
Md5 strain	4	vvMDV	USA	AF243438.1
N strain	2	vv + MDV	USA	AY362718.1
RB1B	5	vvMDV	USA	AY243332.1
TK strain	2	vv + MDV	USA	AY362721.1
3004 strain	7	attMDV	USA	EU032468.1
CVI988	4	attMDV	Commercial vaccine	AF493555.1

3.2. PCR detection of MDV and amplification of complete Meq gene

PCR could detect MDV specific product of ~434 bp size in 28% (7 out of 25) samples confirming the presence of MDV in the DEF passaged samples (Fig. 2). The same MDV positive DNA samples were used for PCR amplification of the complete *Meq* gene with the help of newly designed primers and proof reading (platinum Pfx) DNA polymerase (Invitrogen). The PCR could successfully amplify a ~1040 bp *Meq* gene product from all the seven Ludhiana isolates (LDH 1758, LDH 2003, LDH 2483, LDH 2700, LDH 2929 and LDH 3262) which were also detected positive in MDV diagnostic PCR (Fig. 3). PCR amplified *Meq* gene products were purified and cloned into pJET/1.2 blunt vectors. Presence of the *Meq* gene insert in the plasmid was confirmed by PCR and RE digestion. Plasmids were prepared from the positive clones and PCR was again carried out to confirm the presence of *Meq* gene insert in the purified recombinant plasmids where ~1040 bp product could be amplified from all the seven samples. Presence of the exact sized insert was further confirmed by RE digestion.

3.3. Sequence analysis of isolates

The GenBank obtained accession numbers for sequences of Meq gene (LDH 1758, LDH 2003, LDH 2483, LDH 2614, LDH 2700, LDH 2929, LDH 3262) of seven Ludhiana isolates are from KF895029.1–KF895035.1 also given in Table 2. Comparisons of the *Meq* gene nucleotide sequences of Ludhiana MDV isolates revealed a nucleotide similarity of 98.9–99.8% although all the isolates were obtained from a single poultry farm over a period of six months. The highest nucleotide similarity between the LDH 1758 and LDH 2483 was 99.80% where as the lowest of 98.90% was found between LDH 2003 and LDH 2929 MDV isolates. The derived amino acid sequence similarity within the 7 Ludhiana isolates was between 98.20 and 100% where the highest similarity
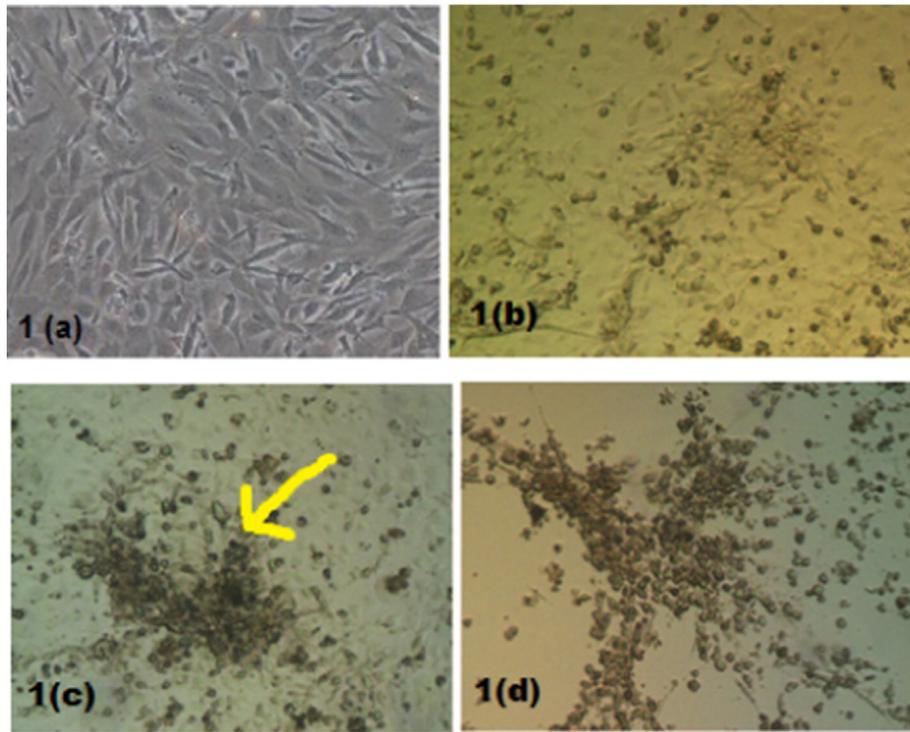
Fig. 1. (a) Primary culture of duck embryo fibroblast (DEF) cells, (b) cells started showing CPE of MDV at 7 dpi, (c) CPE at 11 dpi, (d) CPE at 15 dpi.

(100%) was found between the LDH 1758 and LDH 2483 and the lowest (98.20%) between the LDH 2003 and LDH 2929.

The sequence analysis of all the seven Ludhiana isolates revealed nucleotide substitution at twenty one and amino acid substitution at ten places for all the isolates as compared with each other.

### 3.4. MDV Meq gene sequence analysis

Comparisons of the *Meq* gene nucleotide sequences of Ludhiana isolates with that of 23 reference strains revealed the nucleotide similarity of Ludhiana isolates with those 23 reference strains between 98.10 and 99.40%. Ludhiana *Meq* sequences had the highest similarity of 99.40% with LSY (LN07I) and LCD (SC07II) strain of China, CVI 988/Rispens vaccine strain and CU-2 strain of USA whereas the lowest similarity was found with the L strain of USA at 98.10% as calculated by the MegaAlign programme of DNASTAR Lasergene using Gonnet 250 algorithm (Fig. 5).

The derived amino acid Meq sequences of Ludhiana MDV isolates were 339aa long whereas Australian, two USA and one Russian isolates showed 57aa sequence insertion in the Meq protein. The Meq amino acid sequence comparisons of the Ludhiana isolates with that of 23 reference strains revealed a similarity between 95.90 and 98.50% (Fig. 5).

Ludhiana Meq sequences had the highest amino acid similarity with the LCD (SC07II), LHC5 (LN08V), WS03 and LSY2 (LN07I) strains of China and, CVI 988 vaccine and CU-2 strains of USA at 98.50%. The lowest similarity of Ludhiana isolates (LDH 2003) with that of 23 reference MDVs was 95.90% with the TK strain of USA. Also, according to Tian et al. (2011), similarity of Meq gene of China and other countries is 95.0–98.8%.

Meq aa sequence of the six Ludhiana isolates (LDH 1758, LDH 2003, LDH 2483, LDH 2614, LDH 2700, LDH 3262) revealed presence of five repeat sequences of four prolines (PPPP repeats) and one isolate (LDH 2929) showed four repeats indicating their virulence potential.

A phylogenetic tree was constructed based on the *Meq* gene amino acid sequences of the 7 Ludhiana isolates and 23 MDV reference strains which revealed five major clusters (Fig. 4). All the 7 Ludhiana isolates formed a separate cluster (I) and found to be closely related with the Chinese isolates [LCD(SC07II), WS03, LSY2(LN07I), LDH(JL07I), LHC4(LN08IV) and LHC5(LN08V)] along with one USA strain 567 which formed a separate cluster (II) as compared to the other reference strains. The third cluster (III) comprised of Tamil Nadu strains (TN-n1, TN-n2 and Tn-n3), USA mild virulent (CU-2), USA virulent (GA) very virulent (vv) strains (595, Md-5 & RB1B), and three very virulent plus
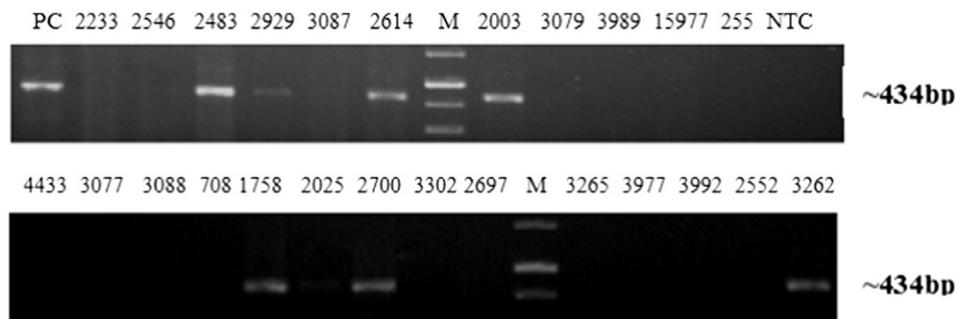
Fig. 2. PCR detection of MDV with an amplicon size of ~434 bp. Lane 1: positive control, 2: 2233, 3: 2546, 4: 2483, 5: 2929, 6: 3087, 7: 2614, M: 1 kb plus GeneRuler DNA Ladder, Thermo, 8: 2003, 9: 3079, 10: 3989, 11: 15,977, 12: 255, 13: NTC, 14: 4433, 15: 3077, 16: 3088, 17: 708, 18: 1758, 19: 2025, 20: 2700, 21: 3302, 22: 2697, M: 1 kb plus GeneRuler DNA Ladder, Thermo, 23: 3265, 24: 3977, 25: 3992, 27: 2552, 28: 3262.
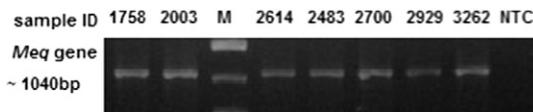
Fig. 3. Meq gene amplification of positive samples.

(vv +) strains (L, N & TK). The fourth cluster (IV) comprised of Australian strains (MPF57 and FT158), Russian vaccine strain 3004 and USA strain JM and the fifth cluster (V) is comprised of CVI988/Rispens which represents the amino acid sequence of Meq protein of the named CVI988/Rispens strain was highly divergent. Thus this strain formed a separate branch (Fig. 4).

4. Discussion

In the past 40 years, the virulence of MDVs has been increasing gradually, possibly caused by the selective pressure of vaccination (Dudnikova et al., 2007; Woźniakowski and Samorek-Salamonowicz, 2014). Moreover, early infection of the birds with some viruses like chicken infectious anaemia virus, infectious bursal disease virus and fowl adenovirus strains which cause long-lasting immunosuppression can decrease the efficiency of the Marek's disease vaccine. Polymorphism, point mutations and the number of repeat sequences of four prolines (PPPP repeats) in the Meq gene (overall range 2 to 8) have been reported to correlate with MDV virulence and oncogenicity (Shamblin

et al., 2004; Renz et al., 2012). In spite of proper vaccination, MD has frequently being reported from different parts of India. In this study, liver and spleen tissue samples from 25 MD/LL suspected birds collected within a period of six months from a single farm when processed for virus isolation in DEF primary culture, 7 samples showed characteristic CPE of MDV in the form of plaques and syncytia. Several workers have successfully isolated Marek's disease virus (MDV) serotype 1 in DEF cells from the field samples (Shamblin et al., 2004; Zhang et al., 2011). Presence of MDV in these 7 samples was confirmed by PCR using specific diagnostic primers and the complete Meq oncogene (~1040 bps) was successfully amplified cloned and then sequenced from all the 7 Ludhiana isolates. Many other workers have amplified MDV Meq gene with a variable product length of 814–1062 bp (Chang et al., 2002; Shamblin et al., 2004; Shi et al., 2008; Woźniakowski et al., 2011; Zhang et al., 2011).

Compared with 23 reference strains, 7 Ludhiana isolates had the highest Meq nucleotide sequence similarity with LSY (LN07I), LCD (SC07II), CVI 988/Rispens vaccine strain and mCU-2 strain at 99.40% whereas the lowest similarity was found with the vv + L strain at 98.10%. Interestingly, the nucleotide similarity among the 7 Ludhiana isolates varied from 98.90–99.80% due to point mutation at various places throughout the gene length. Although, all the seven Ludhiana isolates were obtained from a single poultry farm over a period of six months the Meq gene sequence variation might be due to high mutation capability of the MDV field isolates. The reasons of this high mutation rate of MDV may be antibody pressure of the host against the virus
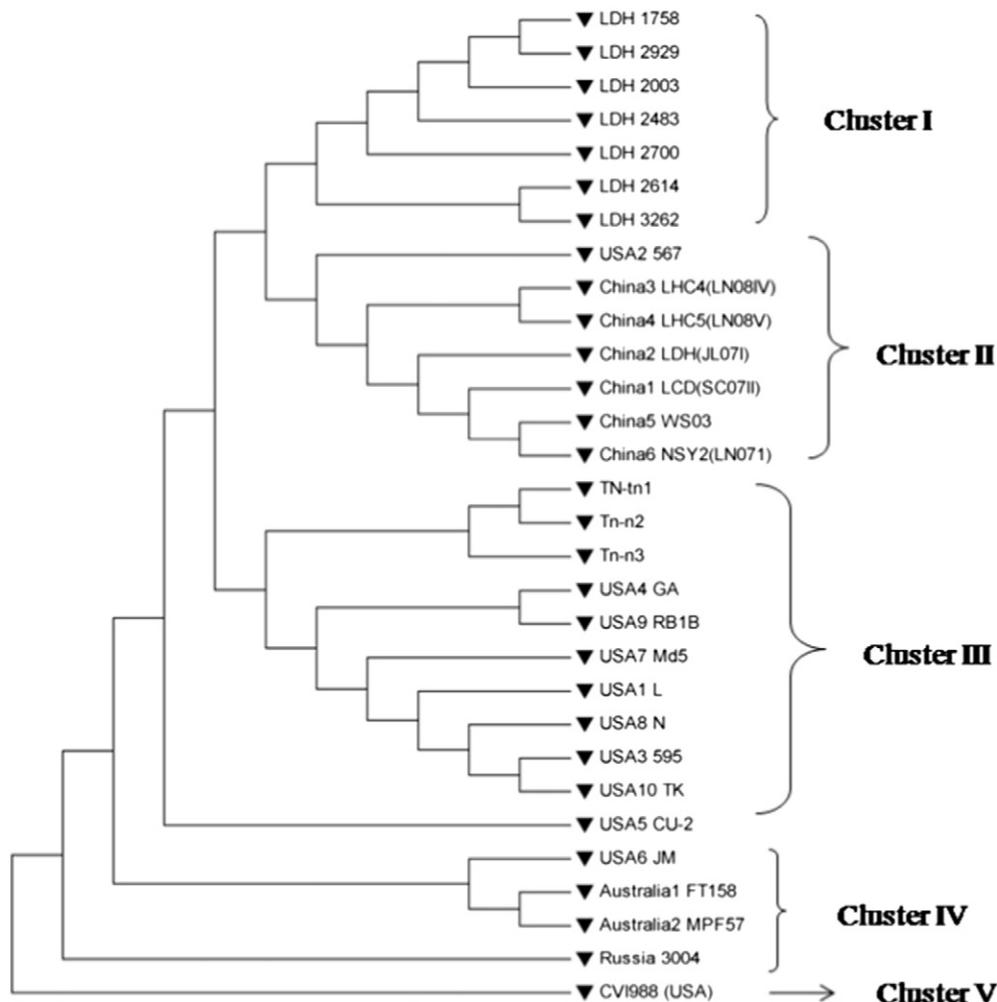

Fig. 4. The tree shown was generated with Mega 5.0 using Meq protein sequences of different MDV isolates. The tree was generated using maximum parsimony method with 1000 bootstrap value.

Percent Identity

Divergence \ Identity	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30		Strain
1	■	98.8	100.0	99.4	99.1	99.4	99.7	97.4	97.6	98.2	97.6	97.6	98.5	98.2	98.2	98.5	98.5	97.1	98.5	98.2	97.1	98.5	98.5	98.2	97.6	96.8	98.5	97.1	98.2	98.5	1	LDH 1758.
2	1.2	■	98.8	98.2	97.9	98.2	98.5	96.8	96.5	97.1	96.5	96.5	97.4	97.1	97.1	97.4	97.4	95.9	97.4	97.1	95.9	97.4	97.3	97.1	96.5	95.6	97.4	95.9	97.1	97.3	2	LDH 2003.
3	0.0	1.2	■	99.4	99.1	99.4	99.7	97.4	97.6	98.2	97.6	97.6	98.5	98.2	98.2	98.5	98.5	97.1	98.5	98.2	97.1	98.5	98.5	98.2	97.6	96.8	98.5	97.1	98.2	98.5	3	LDH 2483.
4	0.6	1.8	0.6	■	98.5	98.8	99.1	96.8	97.1	97.6	97.1	97.1	97.9	97.6	97.6	97.9	97.9	96.5	97.9	97.6	96.5	97.9	97.9	97.6	97.1	96.2	97.9	96.5	97.6	97.9	4	LDH 2614
5	0.9	2.1	0.9	1.5	■	98.5	98.8	96.5	96.8	97.4	96.8	96.8	97.6	97.4	97.4	97.6	97.6	96.2	97.6	97.3	96.8	97.6	97.6	97.3	96.8	95.9	97.6	96.2	97.3	97.6	5	LDH 2700.
6	0.6	1.8	0.6	1.2	1.5	■	99.1	96.8	97.1	97.6	97.1	97.1	97.9	97.6	97.6	97.9	97.9	96.5	97.9	97.6	96.5	97.9	97.9	97.6	97.1	96.2	97.9	96.5	97.6	97.9	6	LDH 2929.
7	0.3	1.5	0.3	0.9	1.2	0.9	■	97.1	97.4	97.9	97.3	97.3	98.2	97.9	97.9	98.2	98.2	96.8	98.2	97.9	96.8	98.2	97.9	97.9	97.4	96.5	98.2	96.8	97.9	98.2	7	LDH 3262.
8	2.7	3.3	2.7	3.3	3.6	3.3	3.0	■	98.5	98.5	97.4	97.4	97.1	97.4	97.4	97.1	97.1	97.4	97.4	98.8	98.2	97.6	97.9	97.1	98.8	97.4	97.9	98.2	97.9	98.2	8	tn-n1.
9	2.4	3.6	2.4	3.0	3.3	3.0	2.7	1.5	■	99.4	97.9	97.9	97.4	97.6	97.6	97.4	97.4	97.6	97.6	99.1	98.8	98.2	98.2	97.4	99.1	97.6	98.5	98.8	98.5	98.8	9	tn-n2 gene.
10	1.8	3.0	1.8	2.4	2.7	2.4	2.1	1.5	0.6	■	98.5	98.5	97.9	98.2	98.2	97.9	97.9	98.2	98.2	99.7	99.4	98.8	98.8	97.9	99.7	98.2	99.1	99.4	99.1	99.4	10	tn-n3 India.
11	2.4	3.6	2.4	3.0	3.3	3.0	2.7	2.7	2.1	1.5	■	99.7	98.5	98.8	98.8	98.5	98.5	97.6	98.5	98.5	98.7	98.2	97.9	98.2	97.3	98.5	97.6	98.2	98.8	98.2	11	FT158 Australia.
12	2.4	3.6	2.4	3.0	3.3	3.0	2.7	2.7	2.1	1.5	0.3	■	98.5	98.8	98.8	98.5	98.5	97.6	98.5	98.2	97.6	98.7	99.0	98.2	97.3	98.5	97.6	98.5	98.8	98.8	12	MPF57 Australia.
13	1.5	2.7	1.5	2.1	2.4	2.1	1.8	3.0	2.7	2.1	1.5	1.5	■	99.4	100.0	100.0	97.6	100.0	100.0	97.6	98.2	97.9	97.9	97.4	98.2	97.6	97.9	98.2	97.9	98.2	13	LCD(SC07II) China.
14	1.8	3.0	1.8	2.4	2.7	2.4	2.1	2.7	2.4	1.8	1.2	1.2	0.6	■	99.4	99.4	99.4	97.9	99.4	98.8	97.9	98.5	98.2	97.9	97.9	97.4	98.2	97.9	98.2	98.2	14	LDH(JL07I) China.
15	1.8	3.0	1.8	2.4	2.7	2.4	2.1	2.7	2.4	1.8	1.2	1.2	0.9	0.6	■	99.1	97.6	99.1	98.8	97.6	98.5	98.5	98.2	98.2	97.4	98.2	97.6	97.9	98.5	98.5	15	LHC4(LN08IV) China.
16	1.5	2.7	1.5	2.1	2.4	2.1	1.8	3.0	2.7	2.1	1.5	1.5	0.0	0.6	0.9	■	100.0	97.6	100.0	98.5	97.6	98.2	97.9	97.9	97.9	97.4	98.2	97.6	97.9	98.2	16	LHC5(LN08V) China.
17	1.5	2.7	1.5	2.1	2.4	2.1	1.8	3.0	2.7	2.1	1.5	1.5	0.0	0.6	0.9	0.0	■	97.6	100.0	98.5	97.6	98.2	98.2	97.9	97.9	97.4	98.2	97.6	97.9	98.2	17	WSO3 China.
18	3.0	4.2	3.0	3.6	3.9	3.6	3.3	2.7	2.4	1.8	2.4	2.4	2.4	2.1	2.4	2.4	2.4	■	97.6	98.2	100.0	98.2	97.9	97.3	98.2	99.7	98.5	100.0	97.6	97.9	18	Lstrain USA.
19	1.5	2.7	1.5	2.1	2.4	2.1	1.8	3.0	2.7	2.1	1.5	1.5	0.0	0.6	0.9	0.0	0.0	2.4	■	98.5	97.6	98.2	98.2	97.9	97.9	97.4	98.2	97.6	97.9	98.2	19	LSY2(LN07I) USA.
20	1.8	3.0	1.8	2.4	2.7	2.4	2.1	1.2	1.2	0.6	1.5	1.5	1.8	1.5	1.5	1.8	1.5	1.8	1.5	■	98.2	98.5	98.5	98.5	97.6	98.2	98.2	98.5	98.2	98.5	20	567 USA.
21	3.0	4.2	3.0	3.6	3.9	3.6	3.3	2.7	2.4	1.8	2.4	2.4	2.4	2.1	2.1	2.4	2.1	0.0	2.4	1.8	■	98.5	97.9	97.3	98.2	99.7	98.2	100.0	97.6	97.9	21	595 USA.
22	1.5	2.7	1.5	2.1	2.4	2.1	1.8	1.2	0.9	0.3	1.5	1.8	1.5	1.8	1.8	1.5	1.8	2.4	1.5	1.5	1.5	■	99.4	98.8	99.1	98.2	100.0	98.5	99.1	99.4	22	GA USA.
23	1.5	2.7	1.5	2.1	2.4	2.1	1.8	1.8	1.2	0.6	1.5	1.3	1.5	1.5	1.5	1.8	1.8	2.1	1.8	1.5	2.1	0.6	■	99.2	98.5	97.6	99.4	98.8	99.7	100.0	23	CU-2 USA
24	1.8	3.0	1.8	2.4	2.7	2.4	2.1	2.4	1.8	1.2	2.1	1.0	2.1	1.8	2.1	2.7	2.1	2.4	2.7	2.1	2.7	1.2	0.8	■	97.9	97.1	98.8	97.3	99.0	99.1	24	JM USA.
25	2.4	3.6	2.4	3.0	3.3	3.0	2.7	2.1	1.8	1.2	1.8	1.8	1.8	2.1	1.8	1.8	2.1	2.1	1.8	0.9	1.5	2.1	2.1		■	97.9	99.1	98.2	98.2	98.5	25	Md5 USA.
26	3.3	4.6	3.3	3.9	4.2	3.9	3.6	2.7	2.1	2.7	2.7	2.7	2.4	2.7	2.7	2.7	2.7	0.3	2.7	2.1	0.3	1.8	2.4	3.0	2.1	■	98.2	99.7	97.3	97.6	26	N strain USA.
27	1.5	2.7	1.5	2.1	2.4	2.1	1.8	1.2	0.9	0.3	1.5	1.5	1.5	1.8	1.5	1.8	0.0	2.4	0.0	1.2	0.9	1.8					■	98.5	99.1	99.4	27	RB1B USA.
28	3.0	4.2	3.0	3.6	3.9	3.6	3.3	2.7	2.4	1.8	2.4	2.4	2.1	2.4	2.4	2.4	0.0	2.4	1.8	0.0	1.5	2.1	2.7	1.8	0.3	1.5		■	97.6	97.9	28	TK Strain USA.
29	1.8	3.0	1.8	2.4	2.7	2.4	2.1	1.8	1.5	2.1	1.8	1.5	2.1	1.8	1.5	2.1	2.1	2.4	2.1	1.8	2.4	2.1	2.7	0.9	2.4				■	99.7	29	3004strain-Russia.
30	1.5	2.7	1.5	2.1	2.4	2.1	1.8	1.8	1.2	0.6	1.2	1.2	1.8	1.5	1.5	1.8	1.8	2.1	1.8	1.5	2.1	0.6	0.0	0.9	1.5	2.4	0.6	2.1	0.3	■	30	CVI988 vaccine strain.
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30		

Fig. 5. Sequence distances of deduced amino acid sequences prepared by using Gonnet 250 algorithm of MegaAlign programme of DNASTAR Lasergene software following clustal W analysis.

because of wide spread vaccination with HVT or it could be repeated exposure of the birds of different age groups and circulation of the virus among various generations in the poultry farm. The other possibility is that these might have occurred during the serial passage of the MDV isolates in DEF primary culture, which is however, less likely.

Several point mutations have been shown in 13 MDV Polish strains which were correlated with the MDV virulence (Woźniakowski et al., 2011). Likewise, distinct polymorphism and point mutations have been shown in the gene encoding C terminal sequences of the Meq protein C terminus of the encoded Meq protein (Shamblin et al., 2004).

Furthermore, Meq protein sequence of all the six Ludhiana isolates (LDH 1758, LDH 2003, LDH 2483, LDH 2614, LDH 2700 and LDH 3262) revealed presence of five repeat sequences of four prolines (PPPP repeats) and one isolate (LDH 2929) showed four repeats indicating its virulence potential. It has been established that the number of repeat sequences of four prolines (PPPP repeats) in the *Meq* gene (overall range 2 to 8) was strongly associated with virulence across all isolates, with the most pathogenic isolates having the fewest number of repeats (Renz et al., 2012).

On phylogenetic analysis, the 7 Ludhiana isolates and 23 MDV reference strains revealed five major clusters. All the Ludhiana isolates formed a separate cluster and found to be closely related with the Chinese isolates along with one USA strain 567 which formed a separate cluster as compared to the other reference strains.

The Meq protein amino acid sequence similarity (95.90–98.50%) of the seven Ludhiana isolates was found to be more than the *Meq* gene nucleotide sequence similarity (98.90–99.80%) (Fig. 5) which further confirmed the higher mutation rate of the MDV field strains. Similarly, high point mutation rate and higher polymorphism in Meq protein coding sequences have been reported by several workers which was also correlated with the virulence and transformation efficiency of the MDVs (Gong et al., 2013; Renz et al., 2012; Shamblin et al., 2004; Woźniakowski and Samorek-Salamonowicz, 2014; Zhang et al., 2011).

In conclusion, out of 25 MDV suspected samples virus could be detected and isolated from 7 samples only (28.00%). Meq amino acid sequence variation was found to be more as compared to the nucleotide sequences among the Ludhiana MDV isolates obtained from a single farm. Six MDV field isolates showed 5 repeat sequences of 4 prolines (PPPP) and one isolate showed 4 such repeats indicating their virulence potential. Though, the virulence of a specific isolate of MDV strain is not likely to be exclusively determined only by mutations in a single gene of the virus, the outcome of this research propose that sequencing of the Meq gene and amino acid alignment to determine the number of four proline repeats will provide a useful indication of the probable pathogenicity of the isolate.

Further detailed molecular epidemiological studies of the MDV isolates from different regions are required which will help in choosing a better immunization protocol so as to prevent the MDV infection in field.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.mgene.2016.07.009.

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