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Genetic characterization of re-emerging Marek's disease virus in Japanese quails in Tamil Nadu

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Abstract
Marek's disease is an important viral disease of poultry characterized by visceral lymphomas in poultry, although mortality has been significantly reduced by vaccination. However, the recent past has witnessed an emergence of more virulent strains of Marek's disease virus (MDV) in other avian species like quails, turkeys in addition to commercial poultry in India and other countries. The present study involved molecular detection and characterization of major virulence determinants of MDV from an unvaccinated flock of Japanese quails in Tamil Nadu. The Meq oncogene sequences, with 98.6% identity matrix, revealed various amino acid substitutions and interruptions in the PPPP motifs, suggesting very virulent nature of isolate. However, vIL8 sequences showed 99.87% identity with reference database. This study has provided the first Indian quail-MDV-vIL8 sequence and third quail-MDV-Meq sequence, to be accessioned in GenBank. Phylogenetic analysis of Indian quail isolate revealed *Meq* sequences clustering with Nigerian MDV isolate, while v*IL-8* sequences clustered with the recent Chinese and North Indian MDV isolates.

Keywords: Marek's disease virus, Japanese quail, oncogene, virulence, sequence analysis

1. Introduction
Marek's disease (MD) is a lymphoproliferative and neuropathic disease affecting chickens and occasionally turkeys, quails and geese. Ever since the first report of the disease as 'Fowl paralysis' in 1907 by Jozsef Marek, the virus has gained importance due to its oncogenic potential. The etiological agent is Marek's disease virus (MDV), an Alphaherpesvirus of the *Genus: Mardivirus*, also known as *Gallid alphaherpesvirus-2* (GaHV-2) or MDV serotype 1. The *Mardivirus* genus encompasses three species (serotypes), with Serotype 1 comprising all known pathogenic strains that vary in their pathogenicity, including some attenuated strains. Serotypes 2 and 3 consist of naturally avirulent strains isolated from chickens and turkeys Non-oncogenic herpes virus of turkey (HVT), MDV serotype-2 (MDV-2) or attenuated MDV-1 vaccines are frequently used for the control of the disease in chickens [1].
The disease is characterised by a neurological form causing paralysis of legs and wings and/or the lymphoproliferative form resulting in multicentric lymphoma affecting various organs such as the liver, gonads, spleen, kidneys, lungs, proventriculus, heart [2]. The visceral form of MD had been a common manifestation since long [3] in commercial poultry, despite vaccinations at hatch. Chicken is the predominant natural host for MDV, although quails [4, 5] and turkeys [6] get infected when raised in proximity to chicken farms as reported from many countries. Experimental infections have demonstrated the susceptibility of quails to MDV although varied serological manifestations prevailed in quails [7]. The existing MD vaccines for chicken have significantly reduced disease outbreaks, but ongoing virus evolution continues to pose a threat to India's major poultry regions with the emergence of more virulent strains and immunosuppression [8] in various species of poultry. The disease had been reported in native breeds of unvaccinated chicken [9] including Japanese quails [10] in various parts of India.
The MDV genome consists of a double-stranded linear DNA molecule, with a similar genetic composition in all strains of serotype-1 MDVs.

The field infections in chicken due to MDV serotype-1 are classified into four pathotypes based on the lesions and mortality rate as-mild (m), virulent (v), very virulent (vv), and very virulent plus (vv+) MDV strains [2]. This serotype-1, with an oncogenic history, in many virulent strains, is characterised predominantly by the BamH1-H region of the viral genome flanking the 132bp repeat region [11], *Meq* oncogene and/or other virulence-associated genes including, *vIL8, UL15, UL49, pp38, and pp24* [2]. The *Meq* gene is the predominant virulent gene responsible for the T-cell lymphomas in poultry. The *pp38* gene encodes a lytic antigen associated with enhanced pathogenesis and very often expressed during the transformation processes. The *vIL-8* gene is conserved across different MDV pathotypes, whose mutations contribute to the varying virulence patterns of the disease. The close linkage between the *Meq, pp38, vIL-8* genes and the pathogenic properties of MDV have been well-established by the earlier researchers, with varying severity of disease caused by the MDV isolates. This has resulted in sporadic occurrences of the disease in many places, even in vaccinated chicken [12, 13].

Nowadays, the human consumption of eggs and meat of chicken and other avian species like Japanese quail, turkey and ducks are on an increasing trend due to its affordability and awareness of their nutritive potentials. The absence of a definite vaccination regimen for all poultry species in the rural backyard sector has paved way for the emergence of virulent viruses like MDV in birds other than chicken too. This is a report of such an incidence of GaHV-2 in rural backyard Japanese quail flock of Tamil Nadu in South India, emphasising the need for potential vaccines against Marek's disease for all domesticated poultry species.

2 Materials and Methods

2.1 Sample collection

A poultry farm in the outskirts of Chennai district of Tamil Nadu having about 3500 Japanese quails of varying age groups, had reported diarrhoea and mortality of about 3-4 quail chicks (six weeks old) per day for a brief period. The geographical location had a poultry farm, housing 15,000 chicken (broilers) in the near vicinity of 2-3 kms distance. Two to three days after the onset of clinical signs in chicks, a few adult quails also started showing similar clinical signs and the mortality slowly increased in a period of 7 days. There were no neurological signs in the ailing birds, and the observed clinical signs were much generalised. Postmortem examination of the dead birds was done to harvest appropriate organ samples showing abnormal growths.

2.2 Sample processing and DNA extraction

Representative pooled organ samples with lesions, from each group of ailing chicks and adult birds were pooled and processed for DNA extraction and virus isolation. The tissue samples were homogenised using sterile mortar and pestle with sterile PBS containing 2X concentration of antibiotic and antimycotic solution to prepare a 10% (w/v) suspension. The tissue suspension was then centrifuged at 3,000 rpm for 10 mins at 4°C. The clear supernatant was transferred to a sterile microcentrifuge tube and treated with 1X antibiotic and antimycotic solution for about 30 mins at 37 ˚C. The supernatant was filtered through 0.45µm syringe filter (Whatman) and stored for virus isolation studies also.

DNA extraction from the suspected tissue suspensions was done by alkaline lysis method. Approximately 200µl of virus-suspected sample was treated with twice the volume of lysis buffer (0.5M Tris HCl, 0.5 M EDTA, 2% SDS) initially followed by addition of 200µg proteinase K/ml. The genomic DNA was extracted by treating the lysate with phenol-chloroform-isoamyl alcohol (25:24:1 v/v), precipitated with absolute isopropanol (preferably overnight at-20ºC), washed using 70% ethanol, air-dried at room temperature and resuspended using nuclease-free water. The purity and concentration of DNA was estimated in Nanodrop and the DNA was stored at-20˚C until use.

2.3 Screening of samples for MDV, Avian leukosis virus (ALV) and Reticuloendotheliosis virus (REV) by PCR

The genomic DNA extracted from the tissue tumors were subjected to diagnostic PCR for avian oncogenic viruses like MDV, ALV and REV, using virus-specific primers. The MDV-PCR utilized specific primers to target the *BamH1-H region* 132-bp tandem repeats [14] while ALV-PCR and REV-PCR were directed against *pol* and *LTR* regions of the viruses respectively [15]. The amplification was carried out in a 25µl reaction volume using 2µl of template DNA, 2X Red dye master mix (Ampliqon®, Denmark), 1µl of each primer (10pmol), and nuclease-free water, in C1000 Biorad® thermal cycler. The details of the primers and the PCR cycling conditions used in the detection of the avian oncogenic viruses are provided in Table 1.

Table 1: Primers and cycling conditions used for diagnostic PCR of MDV, LLV, REV and amplification of MDV-Meq and vIL8 genes in the study

Target virus /gene	Primer Name	Primer sequence	Amplicon size (bp)	PCR cycling conditions
MDV-132bp Tandem repeat	MDV-F MDV-R	5'-TACTTCCTATATAGATTGAGACGT-3' 5'-GAGATCCTCGTAAGGTGTAATATA-3'	434	94˚ºC: 4 min, 35 x(94˚ºC :1 min, 58.6 ºC:1 min,72˚ºC:1 min), 72˚ºC: 10 min
REV-LTR gene	REV-F REV-R	5'-ACACATTGTTGTGACGTGCG-3' 5'-ATCCCTACCCCACCCAGTAG-3'	128	94˚ºC: 3 min, 35 x(94˚ºC :1 min, 60ºC:1 min,72˚ºC:1 min), 72˚ºC: 5 min
ALV - pol gene	ALV-F ALV-R	5'-TACTAAGCTACGCGCTTCGG-3' 5'-ATTTTCGCAACAAACGCGGA-3'	429	94˚ºC: 3 min, 35 x(94˚ºC :1 min, 60ºC:1 min,72˚ºC:1 min), 72˚ºC: 5 min
MDV-Meq gene	Meq-F Meq-R	5'-GGCACGGTACAGGTGTAAAGAG-3' 5'-GCATAGACGATGTGCTGCTGAG-3'	1081	94ºC:4 min, 35 x(94ºC:1 min, 61ºC : 1min, 72ºC : 1 min), 72ºC : 5 min
MDV-vIL8 gene	vIL8-F vIL8-F	5'-GAGACCCAATAACAGGGAAATC-3' 5'-TAGACCGTATCCCTGCTCCATC-3'	887	94ºC:4 min, 35 x(94ºC:1 min, 59.5ºC : 1min, 72ºC : 1 min), 72ºC : 5 min

After the PCR amplification, 5µl of PCR products were analysed by running on 1.5% (w/v) agarose gel stained with ethidium bromide. One Kb plus DNA ladder was used to determine the size of double stranded DNA on agarose gel. The image was captured using gel documentation system (Syngene®, Switzerland). Samples that showed the presence of a 434 bp product after amplification were considered MDV-positive and were used for virus isolation.

2.4 Virus isolation

Isolation of MDV from the suspected tissue samples was carried out as per standard isolation procedure [1] with some

modifications. The quail embryonic fibroblast (QEF) cells from 8-10 days old quail embryos were used for the primary isolation of MDV [7] from the PCR-positive tissue samples and incubated at 37°C with 5% CO_2 along with suitable cell controls. The tissue culture supernatant was harvested after a period of 4-5 days by repeated freeze-thaw and subjected to atleast five serial secondary passages in chicken embryonic fibroblast (CEF) cells under similar incubation conditions for 5-7 days. At every passage, the infected cells were observed for cytopathic effects. Also, the tissue culture supernatants from every passage were used to extract DNA and screened for MDV by the 132bp specific PCR.

2.5 PCR amplification of MDV virulence-associated genes

The virus-infected tissue culture supernatant confirmed by the diagnostic PCR was subjected to PCR amplification of two predominantly associated genes with virulence namely *Meq* and *vIL8* [16, 17] with modifications. The PCR amplification was carried out in a 25µl reaction volume using 2µl of template DNA, 2X Red dye master mix (Ampliqon®, Denmark), 1µl of each primer (10pmol), and nuclease-free water in a thermal cycler. The details of the primers and the PCR cycling conditions are mentioned in Table 1. The PCR products were analysed on agarose gel as mentioned in Section 2.3.

2.6 Nucleotide sequence and Phylogenetic analyses

The bulk PCR products of the two MDV-specific virulent genes were purified using QIAquick® PCR purification kit (Qiagen, Germany). Both the purified PCR products were sequenced using a BDT v3.0 cycle sequencing kit on an ABI 3730xl Genetic Analyzer (Eurofins India Private Ltd., Bangalore) using their respective forward and reverse primers. The sequences of the MDV-positive sample for the two different oncogenes-were assembled, edited and aligned using Lasergene software (DNAStar). The sequence data were subjected to BLASTn analysis in the NCBI database and the confirmed sequences were submitted to the NCBI GenBank database

The reference homologous gene sequences showing high similarity based on *Meq*, *vIL8* genes were retrieved from the GenBank database. The sequences obtained in this study were aligned with the reference sequences using ClustalW for comparison and a phylogenetic tree was constructed using the maximum likelihood algorithm using 500 bootstrap replicates in MEGA 11 software [18] for evolutionary analysis.

3. Results

3.1 Screening of samples by PCR

The affected chicks and adult quails in this study exhibited generalised clinical signs like ruffled feathers, diarrhoea and a gradually increasing mortality in a week, reaching close to an average of 6% in the farm. Necropsy findings of the dead birds revealed typical tumor-like lesions in proventriculus, liver and spleen suggestive of avian neoplastic diseases. Diagnostic PCR for avian oncogenic viruses like MDV, ALV and REV revealed a positive amplification for 132bp tandem repeat region of serotype-1 specific MDV, with a 434 bp product size and a negative result for detection of ALV and REV (Figure 1, 2).

Fig 1: MDV-132bp PCR M-DNA ladder, 1-Japanese quail sample 2-Negative control, 3-Positive control

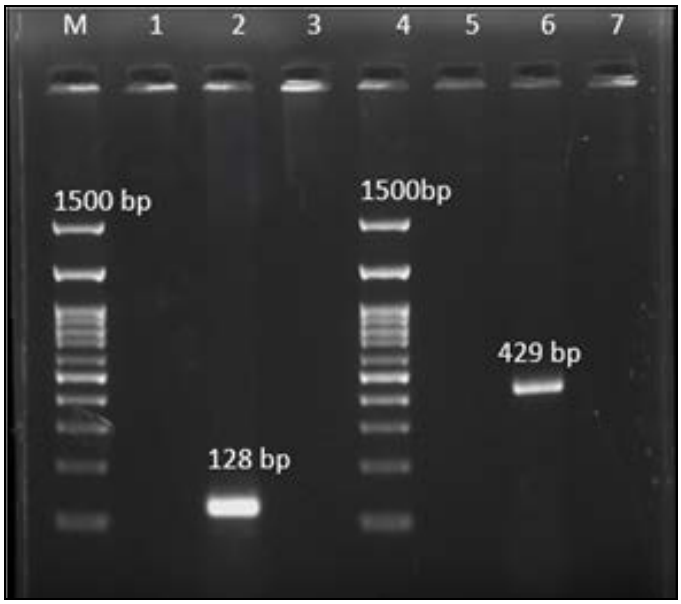

Fig 2: REV and ALV PCR M,4-DNA ladder, 1,5-Japanese quail sample (REV, ALV) 2,6 - Positive controls (REV, ALV) 3,7 - Negative controls (REV, ALV)

3.2 Virus Isolation

The 132bp-specific MDV-positive quail sample (M13) was primarily isolated in successfully passaged in QEF. After five serial blind passages in CEF cells thereafter, the cytopathic effect (CPE) comprising of a slow development of syncytia formation followed by detachment of cells from the culture wells was observed (Figure 3a, 3b). The virus-infected cells were harvested, subjected to repeated freeze-thaw cycles and used for DNA extraction. The genomic DNA was tested positive for MDV with the amplicon size of 434 bp, confirming the presence of serotype-1 MDV in the cell culture supernatants (Figure 4).

Fig 3. Virus isolation in CEF cells

Fig 3a - Control CEF cells

Fig 3b - MDV-infected CEF cells showing syncytia formation

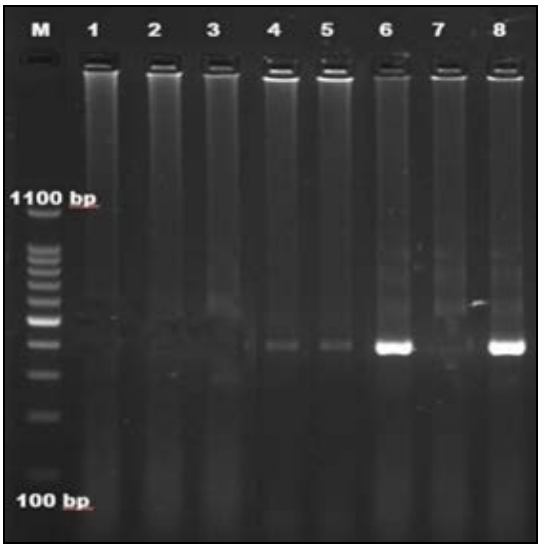

Fig 4: Screening of MDV13-infected CEF culture supernatants by 132bp PCR M-DNA ladder, 1-6: Positive MDV isolate (Passage 1-6), 7-Neg control, 8-Pos control

3.3 PCR amplification of virulence-associated genes

The MDV isolate obtained in this study was subjected to full length amplification of virulence-associated genes of Marek's disease namely, '*Meq*' and '*vIL8*'. Positive amplification of both the target genes were confirmed on agarose gel electrophoresis with specific product sizes of 1080 bp and 887 bp respectively (Figure 5, 6).

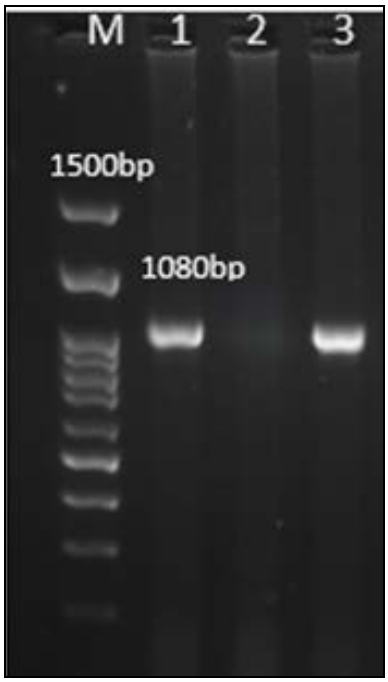

Fig 5: MDV-*Meq* gene PCR
M-DNA ladder, 1-Positive MDV isolate, 2-Negative control, 3-Positive control

Fig 6: MDV-*vIL8* gene PCR
M-DNA ladder, 1-Positive MDV isolate, 2-Positive control, 3-Negative control

3.4 Nucleotide sequencing

The PCR products of the two target genes-*Meq* and *vIL8* were submitted for sequencing. The raw sequence data of the forward and reverse reads were checked manually with the chromatogram. The FASTA sequences for both the genes were trimmed and assembled for the complete 'cds' region. A BLASTn analysis with reference sequences in the GenBank resulted in a percent identity matrix of 98.6% for *Meq* gene and 99.87% for *vIL8* gene. Both the gene sequences of this field isolate of MDV from quails were submitted in GneBank with the following accession numbers; PP943015 for *Meq* gene and PP932047 for *vIL8* gene respectively.

Point mutations at six different nucleotide positions resulting in amino acid substitutions along the *Meq* oncoprotein were observed-71(S→A), 80(D→Y), 88(A→Y), 93(Q→R), 139(T→ A), 180(T→A). Polymorphisms in the tetrad Proline repeats of the Meq oncoprotein also revealed changes in the second Proline residue at different positions (PPPP→^{145}PEPP, ^{175}PAPP, ^{348}PSPP) and changes in the third residue at two other positions (PPPP→^{305}PPQP, ^{327}PPSP).

3.5 Phylogenetic analysis: The *Meq* gene and *vIL8* gene sequences of the MDV isolate in this study was compared with the available sequences in the GenBank from different countries for their evolutionary relationship (Figure 7a, 7b). The quail MDV-Meq sequence of this study is the third sequence in GenBank after the first Indian and second German submissions. The full-length sequences of *vIL8* gene of the same MDV isolate is the only quail viral gene sequence of Indian origin available in GenBank. The phylogenetic tree constructed based on the quail *Meq* oncogenic gene revealed its clustering with the chicken MDV sequences from Nigeria, forming a separate cluster. Based on the *vIL8* gene sequence, the quail isolate clustered with those of the earlier chicken-derived field strains from the Ludhiana (Punjab) strain of India and the Chinese strain.

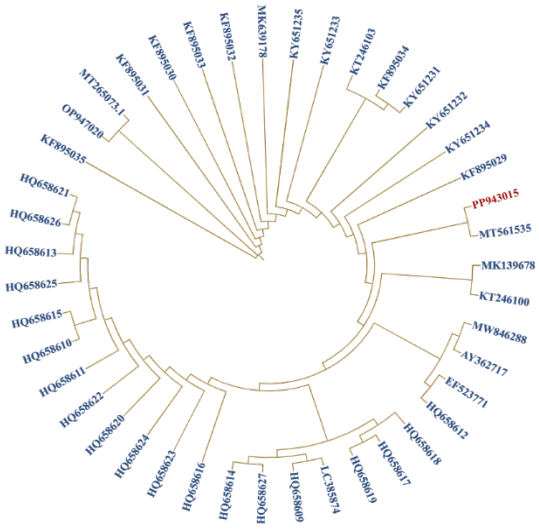

Fig 7a: Phylogenetic tree based on Quail MDV-*Meq* gene sequence in this study

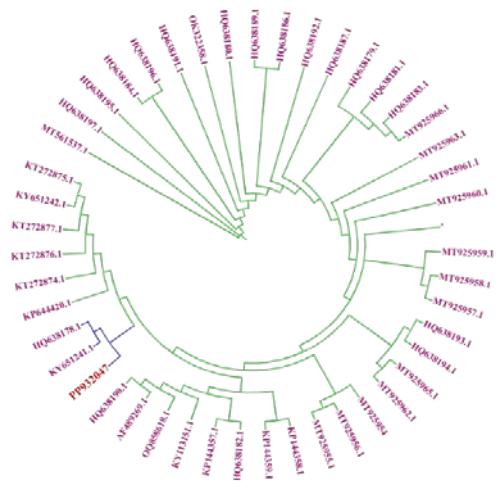

Fig 7b: Phylogenetic tree based on Quail MDV-*vIL8* gene sequence in this study

The evolutionary history was inferred by using the Neighbour-Joining method and the Tamura-Nei model. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 59 nucleotide sequences, using MEGA11 software.

M-DNA ladder, 1-Positive MDV isolate, 2-Negative control, 3-Positive control

The evolutionary history was inferred by using the Neighbour-Joining method and the Tamura-Nei model. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 45 nucleotide sequences, using MEGA11 software.

M-DNA ladder, 1-Positive MDV isolate, 2-Negative control, 3-Positive control

4. Discussion

Marek's disease (MD) has re-emerged in the recent past as an important avian disease, affecting both vaccinated and non-vaccinated poultry flocks in India [13, 19,20] and other countries [21]. Despite the continuous practice of day-old vaccination of chicks against MD, there has been a notable increase in the virulence of MDV strains in chicken, which are able to break vaccine immunity and also induce immunosuppression The disease more commonly reported as a disease of chicks, has witnessed a recent emergence among adult birds too, with different clinical manifestations in various species of poultry [1]. The recent past has reports of the disease in poultry species other than chicken like peafowls, quails and turkeys, caused by more virulent forms of the virus with polymorphisms in the virulence-associated genes. This re-emergence of disease in native and other domesticated species of poultry in many parts of India and other countries has warranted a detailed molecular characterization of MD in the various avian species.

In the present study, both six week-old chicks and adult Japanese quails, with generalized symptoms, diarrhoea and mortality were taken for study. An average mortality of 6% was recorded among the unvaccinated Japanese quails. History of the neighbourhood revealed a recent report of MD in the layers a few weeks back, but could not be subjected to further laboratory investigations. The affected quails in this study neither exhibited any neurological or respiratory symptoms. Necropsy findings included visceral lymphomas especially in proventriculus, liver and spleen, suggestive of any viral neoplastic diseases.

Diagnostic PCR for avian oncogenic viruses (namely MDV, ALV and REV) revealed specific amplification pertaining to

two repeats of the 132-bp tandem repeat region of MDV, pertaining to the virulent serotype-1. Similar findings with single repeats of the 132 bp region have been reported earlier, confirming very virulent MDV from affected chicken [11]. The disease in quails had been reported as early as 1985 in India, later in Japan and Scotland [5] followed by the most recent re-emergences in India [22], Nigeria [23] and Germany [5].

The quail MDV of this study could be isolated primarily in QEF and serially passaged in CEF cells with single to multiple syncytia over a period of time. The MDV isolate was further screened positive for two important virulence-associated genes-namely *Meq* and *vIL8* genes, although a set of other genes are also involved in the pathogenesis [2, 12]. The sequences associated with high virulence from MDV isolates of India and other countries were used in the study according to the BLAST search in GenBank. Point mutations along the Meq gene revealed amino acid substitutions along the 'basic region' and interrupted proline repeat regions of the *Meq* oncoprotein, as reported by earlier researchers in very virulent chicken MDV. The viruses having low number of PPPP repeats and interruptions correlated with high virulence [21, 25]. Therefore, the substitutions at the second and third positions of the proline tetrads in the Meq gene of the quail isolate under study, were suggestive of the very virulent nature. On the other hand, no significant mutations were observed in the vIL8 sequences of the unvaccinated quail MDV, unlike the genetic changes observed in the chicken MDV isolated earlier from vaccinated commercial breeder farms [17]. However, the exact pathotype of the quail MDV can be confirmed only by an *in vivo* pathotyping assay.

Phylogenetic analysis revealed the sequence sharing to an extent of more than 98-99% identity matrix with the reference MDV isolates of chicken and turkeys, despite being a different avian species. The quail MDV-Meq sequences formed a cluster with the chicken Nigerian isolate [25]. The vIL8 sequence clustered with the earlier Chinese isolate [16] and the North Indian [26] MDV isolate in the recent past from chicken. The spread of disease within India or its close borders might be mediated by the migratory birds or by any indirect transmission methods. The virus owing to its tendency to mature and get released along the epithelium of feather follicles, stands a good chance for survival in the poultry house litter, dust and dander. This is readily disseminated in and around the infected premises and inhaled by susceptible avians including migratory birds [27]. However, based on the location of the quail farm in close proximity to the chicken farm, transmission of the virus cannot be excluded. Since quails are not subjected to any vaccination schedule including MD, they tend to be more susceptible to such diseases. The analysis of the virulence-associated genes of such viruses from avian species other than chicken, will aid in devising improved immunization protocols, better control strategies of newer MDV outbreaks and implementation of strict biosecurity measures.

5. Conclusion

The conventional vaccination protocol in chicken has drastically reduced the incidence of Marek's disease. Nevertheless, the evolutionary changes in the recent past leading to emergences of more virulent forms of virus remains a challenge to the growing poultry industry in the other avian species in India, where no such vaccinations are carried out. In this context, this study was carried out for the molecular epidemiology of the virus affecting other avian species like Japanese quails.

In conclusion, typical manifestations of MDV-1 are being reported similarly in chickens and quails but for tissue tropisms between hosts. The immunosuppression caused by MDV can increase the susceptibility of the quails to other infectious diseases too like chicken. Since there are no licensed vaccines in India against MD in quails, understanding the disease pathogenesis, *in vivo* pathotyping, development of effective vaccines for quails, improved biosecurity measures will enable a better control of disease in chicken and other domesticated species of poultry.

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Conflict of Interest

Not available

Financial Support

Not available

7. References

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