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The genome evolution of Marek's disease viruses in chickens and turkeys in China

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Abstract

The virus that causes Marek's disease (MD) is globally ubiquitous in chickens, continuously evolving, and poses a significant threat to the poultry industry. Although vaccines are extensively used, MD still occurs frequently and the virus has evolved increased virulence in China. Here, we report an outbreak of MD in vaccinated chickens and unvaccinated turkeys in a backyard farm in Guangdong province, China, in 2018. Phylogenetic analysis revealed two lineages of MDVs at this farm, with one lineage, containing isolates from two turkeys and five chickens, clustering with virulent Chinese strains and displays a relatively high genetic divergence from the vaccine strains. These new isolates appear to have broken through vaccine immunity, yielding this outbreak of MD in chickens and turkeys. The second lineage included four chicken isolates that clustered with the CVI988 and 814 vaccine strains. The large diversity of MDVs in this single outbreak reveals a complex circulation of MDVs in China. Poor breeding conditions and the weak application of disease prevention and control measures make backyard farms a hotbed for the evolution of viruses that cause infectious diseases. This is especially important in MDV as the MD vaccines do not provide sterilizing immunity, which allows the replication and shedding of virulent field viruses by vaccinated individuals and supporting the continuous evolution of MDVs. Hence, constant monitoring of the evolution of MDVs is necessary to understand the evolution of these field viruses and potential expansions of their host range.

Abbreviations:

MD   Marek's disease
MDV   Marek's disease virus
HVT   Turkey herpesvirus

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Introduction

Marek's disease (MD) is highly contagious in chickens and is characterized by immunosuppression and neurological disorders, with eventual tumor formation involving the peripheral nerves, visceral organs, eye, muscle or skin [1]. The causative agent of MD is the Marek's disease virus (MDV). MDV is a member of the genus *Mardivirus* and belongs to the *Alphaherpesvirinae* subfamily of the family *Herpesviridae*.

MD is widespread in chickens [6]. In China, MD vaccines are used extensively, however, they have limited effect on viral infection and transmission [7]. The vaccines protect against tumors but do not provide sterilizing immunity, thus, vaccinated chickens still support the replication and shedding of virulent field viruses. The MD vaccine program frequently fails, and occasionally virulent MDVs are isolated from vaccinated chickens in China [8], which has two major consequences. First, virulent virus shed by the vaccinated chickens is still pathogenic to the non-vaccinated [9]. Second, with the widespread use of MD vaccines, MDV strains

are continuously evolving leading to the generation of strains with greater virulence [10].

MDV encodes more than 100 genes, including MDV EcoRIQ (*meq*), phosphorylated protein 38 kDa (*pp38*), virus-encoded interleukin 8(*vIL8*), glycoprotein B (*gB*), *gE*, and *gI*, with *meq* and *pp38* being of utmost importance for the pathogenicity and tumorigenicity of MDV [11]. *pp38* may be involved in immune modulation against MD and the absence of the *pp38* gene can reduce lymphoproliferative lesions [12]. The *meq* gene is involved in the transformation of lymphocytes, and the absence of the *meq* gene prevents the formation of lymphomas in chickens after MDV infection [13].

Here, we report an outbreak of MD in chickens and turkeys in a backyard farm in Guangdong province, China, in 2018. Whole genome sequences of 11 MDVs were generated and analyzed to examine the evolution of MDVs in China. The aim of this study was to understand the transmission and evolution of Marek's disease virus (MDV) in poultry. Our results emphasize the importance of continuous monitoring of MDV evolution and provide a basis for the control of MD in poultry.

Materials and methods

Samples and virus detection

Vaccinated chickens and unvaccinated turkeys with severe MD-like symptoms, including paralysis of the feet and emaciation, were collected from a backyard farm in Guangdong Province, China, in April 2018. The clinical symptoms in this flock began at about day 90 after hatching. Livers and feathers from the sick birds were collected and kept at -80℃. DNA from the livers and feathers was extracted using the Universal Genomic DNA Extraction Kit Ver.3.0 (TaKaRa Biotechnology Dalian Co., Ltd.) according to the manufacturer's instructions. PCR was performed with primer sets for MDV viral *meq* (F 5′-ATGTCTCAGGAGCCAGAGCC-3′; R 5′-TCAGGGTCTCCCGTCACCTG-3′) [14] and *pp38* (F 5′-TTAATTTGATTCAGATTTTG-3′; R 5′-ATGGAATTCGAAGCAGAACA-3′) genes. PCR products for *meq* and *pp38* of the anticipated sizes were isolated and purified from agarose gels using the E.Z.N.A.® Gel Extraction Kit (Omega Bio-tek, Inc., Guangzhou, China).

PCR products were cloned into the pJET1.2 vector using the conditions defined by the manufacturer (Wuhan Miaoling Bioscience & Technology Co., Ltd, Wuhan, China). All ligation products were transformed into *E. coli* DH5a cells and were selected on LB agar plates containing 100 mg/ml of ampicillin. Colonies were screened and those with inserts of the appropriate size for the *meq* or *pp38* PCR products were used for DNA sequencing.

Genome sequencing

MDVs from nine chickens (designed MDV-C1, MDV-C2, MDV-C3, MDV-C4, MDV-C5, MDV-C6, MDV-C7, MDV-C8, and MDV-C9) and from two turkeys (designed MDV-T6 and MDV-T7) were selected for genome sequencing. Sequencing libraries were prepared with the NEBNext Ultra DNA Library Prep Kit for Illumina (New England Biolabs), and sequencing was performed on the Illumina NovaSeq platform with 6000 and 150 bp paired-end reads generated.

Raw reads were cleaned to exclude adaptor and low-quality sequences using fastp (v.0.19.7) [15]. Clean reads that mapped to the host genome sequence (NCBI reference genomes: chicken, GCA_000002315.5; turkey, GCA_000146605.4) with BWA-MEM (v.0.7.17) were filtered out [16]. For each sample, the viral genome was de novo assembled using Spades (v.3.13.1) with the parameter "-k 21,33,55,77 –careful"[17]. The assembled contigs were aligned against the reference MDV genome (strain name: Md5 NCBI. Accession no. NC_002229.3) using the nucmer program from the MUMmer package [18] to generate contiguous viral genomes. Newly assembled viral genomes were deposited into GenBank (Accession numbers: OP887017- OP887027).

Phylogenetic analysis

In addition to the genomes sequenced in this study, genomes of other MDV strains were obtained from GenBank (Table S1). MAFFT (v 7.0) was used to obtain a multiple sequence alignment with the iterative refinement method (FFT-NS-i) [19]. For phylogenetic analysis, alignment gaps associated with incomplete genomic data were removed using Trimal (v1.4) [20]. IQ-TREE (v1.6.12) [21] was used to reconstruct maximum likelihood (ML) trees for the MDV genome sequences, *meq* gene sequences, and *pp38* gene sequences. The parameters for IQ-TREE were iqtree-s input. fasta– m MFP-bb 1000.

Recombination analysis

RDP4 was used to detect potential recombination events between the sequences using seven different methods: RDP, GENCONV, BOOTSCAN, MAXCHI, CHIMERA, SISCAN, and 3SEQ [22]. Only recombination events that had significant signals from at least three different methods were considered to be potential recombinations. To further analyze the possibility of recombination, the genomes were analyzed with Simplot software (version 3.5.1) [23]. Parameters for the similarity plots are: window, 200 bp; step, 50 bp.

Results and discussion

Upon dissection, sick chickens and turkeys with symptoms of MD were found to possess multifocal nodules on their livers, with many more multifocal nodules observed in the chickens compared to the turkeys (Fig. 1). PCR amplification and sequencing of the target genes *meq* and *pp38* confirmed that the sick turkeys and chickens were infected with MDVs. The occurrence of MDV-induced tumors in turkeys is unusual. In this case, the turkeys had been raised with chickens in a backyard farm, thus, they were likely in contact with a high density of MDV from the chickens, leading to infection and a more serious disease in these turkeys. In our study, the turkeys infected by MDV possessed tumors with fewer multifocal nodules on their livers compared to their companion chickens (Fig. 1B). Turkey herpesvirus (HVT) is reported to be ubiquitous in domestic turkeys, and is used as a vaccine against MD in chickens, thus it might also protect turkeys against natural infection by MDV [24, 25]. However, due to recent evolutionary changes in MDV, HVT no longer fully protects chickens against tumors and mortality from MDV. These evolutionary changes may also be a factor in the infection of turkeys by MDV at this farm. In addition to chickens and turkeys, MDV has been reported in other species of wild birds [26–30]. The expansion of the host range of MDVs requires additional attention.

To further explore the evolution of these MDVs, MDV isolates from nine chickens and two turkeys were selected for genome sequencing. Homology analysis showed the nucleotide identity of the genome, *meq* and *pp38* genes were 97.14–99.94%, 99.02–100%, and 98.67–100%, respectively, among the nine chicken isolates, 99.90%, 100%, and 100%, respectively, between the two turkey isolates, and 97.14–99.94%, 99.71–100%, and 98.67–100%, respectively, between the chicken and turkey isolates. *meq* and *pp38* genes have very important functions for the pathogenicity and tumorigenicity of MDV [12, 13]. They may under more strict selection pressure than other parts of the genome. Therefore, these two genes and the full-genome were selected for further phylogenetic analyses. Phylogenetic relationships based on whole genome sequence and the *meq* gene showed similar topologies, where the MDVs were separated into two clusters (Fig. 2A, B). Five chicken isolates and two turkey isolates belonged to clade I and the four remaining chicken isolates belonged to clade II. The clade 2 sequences included MDV-C1 and MDV-C6, which clustered with the CVI988 vaccine sequence, and MDV-C4 and MDV-C5, which clustered with the 814 vaccine sequence. The *meq* gene is associated with the evolution of MDV virulence and considered to be an MDV oncogene that plays a role not only in tumor formation but also in its immunosuppressive effects [31, 32]. In this study, the nucleotide sequences for the *meq* gene identified in the clade 1 genome sequences (MDV-T6, MDV-T7, MDV-C2, MDV-C3, MDV-C7, and MDV-C8) had relative low nucleotide identities (99.02–99.12%, Table 1) when compared with the sequences from the genomes of the two commercial vaccine strains (CVI988 and 814). In addition, the *meq* gene sequences from these isolates encode amino acid substitutions K77E, D80Y, V115A, T139A, P176R, and P217A that are characteristic of MDV strains isolated from China, and isolates with these substitutions have been found to yield higher morbidity [33]. It is also worth noting that these amino acid substitutions, with the exception of K77E and V115A, were exclusive to these isolates and are not present in the vaccine sequences. Point mutations can influence the transcriptional activity of the *meq* protein, thereby affecting pathogenicity [34]. Notably, substitutions P176R and P217A occur at the second-position in the four proline repeat (PPPP) sequences found in
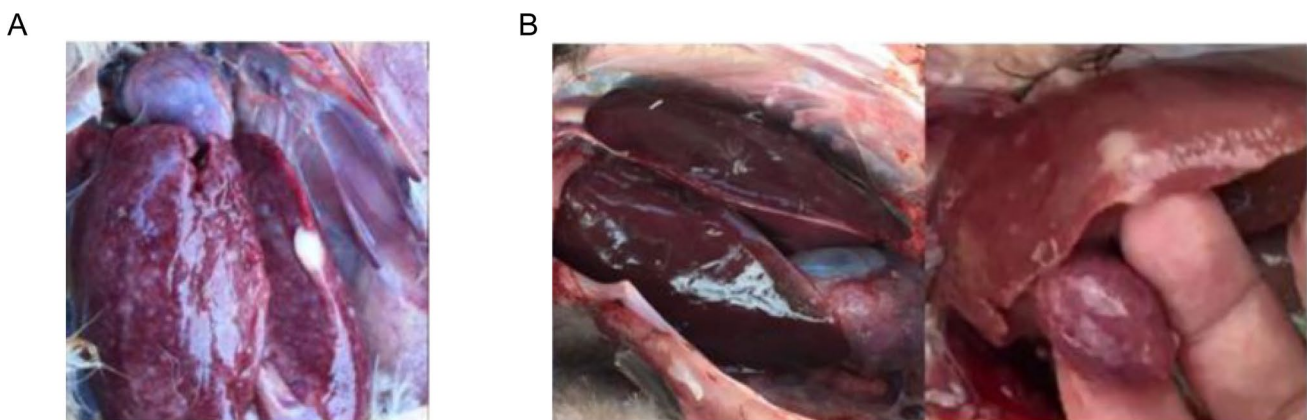
Fig. 1 Pathological lesions of MD seen in the livers of sick birds. **A** chickens. **B** turkeys. Representative livers of sick birds display multifocal nodules, with many more multifocal nodules of varying size observed in the chicken compared to the turkeys
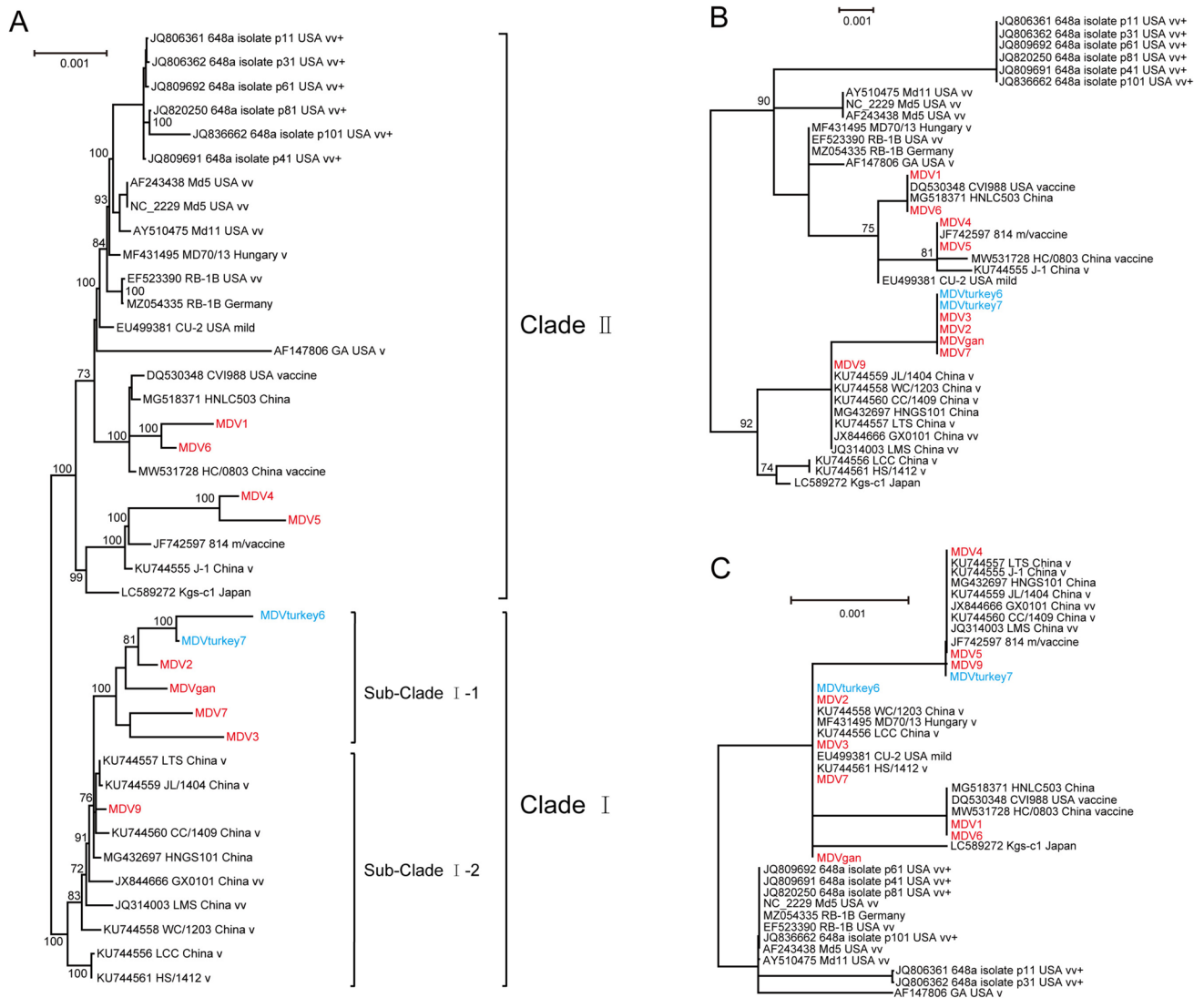
Fig. 2 Phylogenetic analysis of MDVs estimated using maximum likelihood (ML). **A** whole genome, **B** *meq* gene, **C** *pp38* gene. Phylogenetic trees were estimated with IQ-TREE (Minh et al. 2020) using the best fit substitution model and 1000 bootstrap replicates. Numbers (> 70) above branches are percentage bootstrap values for the major nodes. Scale bars depict the number of amino acid substitutions per site. Strains isolated from different hosts are in different colors, Red = chicken, Blue = turkey. Vaccine strains are marked in green

Table 1 Percent nucleotide and amino acid identity (% identity) between MDV strains sequenced in this study and two vaccine strains (CVI988 and 814)

Strains	Nucleotide			Amino acid	
	Genome	*Meq*	*pp38*	*Meq*	*pp38*
MDV-C1	99.82–99.94	99.75–100	99.86–100	99.25–100	99.59–100
MDV-C2	99.78–99.79	99.02–99.12	99.89	97.34–97.63	99.66–100
MDV-C3	99.69	99.02–99.12	99.89	97.34–97.63	99.66–100
MDV-C4	99.83–99.93	99.75–100	99.77–100	99.25–100	99.66–100
MDV-C5	99.71–99.81	99.75–100	99.77–100	99.25–100	99.66–100
MDV-C6	99.82–99.94	99.75–100	99.87–100	99.25–100	96.85–97.24
MDV-C7	99.73–99.74	99.02–99.12	99.89	97.34–97.63	99.66–100
MDV-C8	99.79–99.80	99.02–99.12	99.85	97.34–97.63	99.56–100
MDV-C9	99.83–99.84	99.31–99.41	99.87–100	97.93–98.22	99.60–100
MDV-T6	99.64–99.66	99.02–99.12	99.89	97.34–97.63	99.66–100
MDV-T7	99.76–99.78	99.02–99.12	99.86–100	97.34–97.63	99.59–100
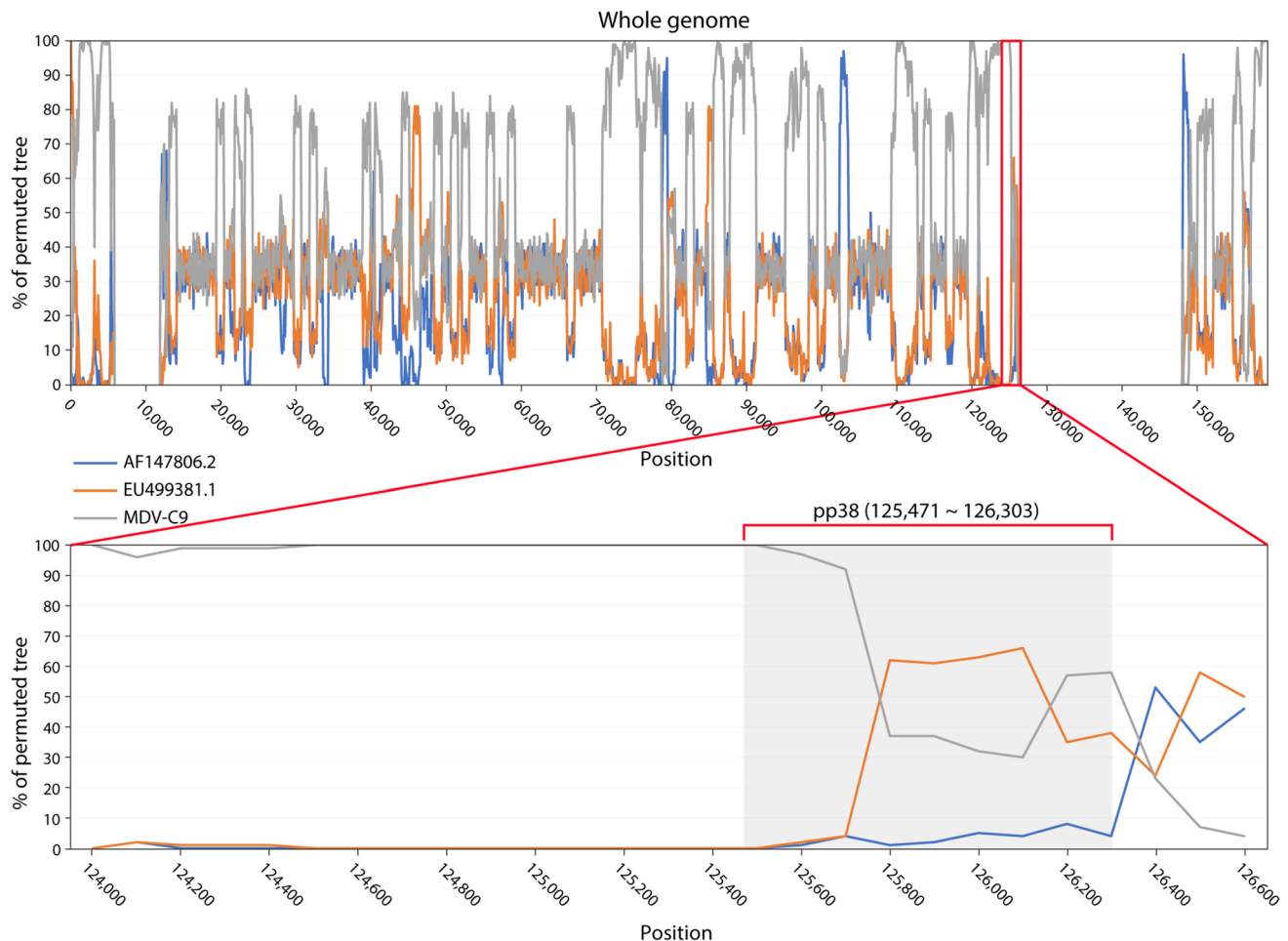
Fig. 3 Identification of potential recombination events in the MDV genomes. Similarity plot of the *pp38* gene of MDV-C8 against sequences from MDV-C9, AF147806, and EU499381. Parameters for the similarity plots are: window, 200 bp; step, 50 bp

the proline-rich central region of the protein, and may be associated with the increase in virulence of these strains [4, 35]. The functional consequences of other substitutions need further study. The virulence and genetic characteristics of MDV strains have been changing with the introduction of vaccines [36, 37]. In the past few years several highly virulent MDV strains have been isolated from vaccinated chickens in China [38]. Like them, the virulent strains described here appear to have broken through vaccine immunity, leading to an outbreak of MD in chickens and turkeys in this farm.

The phylogenetic tree for the *pp38* gene showed a different topology compared to those for the genome sequence or the *meq* gene (Fig. 2C). Sequences for the MDV-C4, C5, C9, and MDV-T7 *pp38* gene clustered with the 814 vaccine sequence and some Chinese virulent strain sequences such as CC/1409, JL/1404, LTS, J-1, GX0101, and LMS, but did not cluster with MDV-T6, MDV-C2, MDV-C3, MDV-C7, MDV-C1, MDV-C6, and MDV-C8. Since *pp38* and *meq*

sequences display different phylogenetic patterns, this suggests that recombination occurred within the MDV genomes. RDP4 software [22] detected evidence for 21 recombination events (Table S2) in these genomes, with one of the potential recombination events located in the region surrounding the *pp38* gene. According to these results, the strain MDV-C8 was further used as the query to against strains MDV-C9, AF147806, and EU499381 in the SimPlot program [23]. The results indicated potential multiple recombination events in the region surrounding the *pp38* gene (Fig. 3). These recombination events explained the different topologies between the *pp38* and *meq* genes. Although MDVs are DNA viruses, and have relatively lower mutation rates compared with RNA viruses, recombination can facilitate their evolution and allowing them to adapt to new hosts [39]. Research has demonstrated that recombination increases the complexity of disease diagnosis, prevention, and control [40].

Marek's disease is globally ubiquitous in chickens, necessitating a strategy of comprehensive vaccination. In China,

chickens are inoculated after hatching with commercial vaccines, such as CVI988, HVT, and 814 [10, 41, 42]. Although MD vaccines have proven to be very successful in protecting chickens from tumor development and mortality, they do not provide sterilizing immunity. Some reports have shown that MDV field viruses have continuously evolved toward greater virulence and resistance to immune responses [43], leading to vaccinated chickens retaining the ability to support the replication and shedding of virulent field viruses [10]. In this study, our 11 new MDV genome sequences from chickens and turkeys show that these isolates had a deep divergence from the vaccine strains, which suggests rapid evolution of MDVs in China. Rapid evolution and large divergence may explain the outbreak of MD in these birds. Generally, HVT is ubiquitous in domestic turkeys, and protects turkeys from infection by MDVs [44]. The turkeys and chickens examined here were raised in the same backyard farm and it appears that HVT failed to provide protection to the turkeys from these newly evolved MDV strains, hence, constant monitoring of the evolution of MDV is necessary to control future outbreaks of MD in both chickens and turkeys.

Author contributions YS conceptualized, designed and and wrote the manuscript. WL, HM, JP and XS performed the experiments. XL analyzed the data. DMI participated in revising the manuscript. All authors read and approved the final manuscript.

Data availability The sequences generated in the current study were deposited to the GenBank under the accession numbers OP887017-OP887027.

Declarations

Conflict of interest The authors declare no conflict of interest.

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