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Marek's Disease in Chicken Flocks in JuRong, Jiangsu Province, China: Pathology, Virus Isolation, and Molecular Characteristics Analysis

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Authors' Contribution
XL, HM and CQZ designed the research. CHH and YZ performed laboratory examinations. XL, HM, YZ, YQP, JBY, LZH, and WD analysed the data. XZX and SFW initiated the study and organized the samples. XL and CQZ wrote the paper. All the authors have read and approved the final manuscript.

Key words
Marek's disease virus, Clinical necropsy, Virus isolation, Meq sequence

ABSTRACT

Marek's disease (MD), one of the most important avian immunosuppressive and neoplastic diseases, causes enormous economic losses in China. In 2023, seven 3-month-old chickens were determined to be infected with Marek's disease virus (MDV) by histopathological examination, polymerase chain reaction (PCR), and gene sequencing. All of them originated from chicken flocks in JuRong (Jiangsu, China) and were immunized with the CVI988/Rispens (CVI988) vaccine. The novel MDV strain identified in these chickens was named JuRong 2023 and was isolated from feather follicle samples. Sequence analysis revealed that the Meq gene was most closely related to the SD-2012 strain, which was detected in Shandong Province. Furthermore, compared with the reference strain CVI988, the amino acid sequence of JuRong 2023 had mutations at positions 71, 80, 115, 176, 217 and 326, which may affect the function of the virus. Constant surveillance of newly isolated field strains in China is essential to evaluate the immune protection efficacy of different MD vaccines.

INTRODUCTION

Marek's disease (MD) is an avian-specific disease that causes immunosuppression, neurological disorders, CD4+ T-cell transformation and, eventually, tumour formation in peripheral nerves and visceral organs (Calnek, 2001). Marek's disease virus (MDV), which causes MD, belongs to the genus *Mardivirus* in the subfamily Alphaherpesvirinae (Gatherer *et al*., 2021). Previously, MD been well controlled by live vaccines. Although MDV is has a relatively stable dsDNA virus for which commercially available vaccines are available, poultry farming in JuRong, Jiangsu Province, has led to pathogen evolution as a result of cramped living conditions and shorter rearing periods.

MDV was first detected by Josef Marek in 1907. There are three serotypes of MDV, MDV-1 (GaHV-2), MDV-2 (GaHV-3) and MDV-3 (MeHV-1), of which only MDV-1 induces tumours in birds (Bulow and Biggs, 1975). There are four pathotypes of MDV-1 as evaluated by monovalent HVT or bivalent HVT+SB-1 vaccinations: mild (m) MDV, virulent (v) MDV, very virulent (vv) MDV and very virulent plus (vv+) MDV (Witter, 1997, Witter *et al*., 2005). In addition, chickens, similar to turkeys (Davidson and Borenstein, 1999), quail (Kobayashi *et al*., 1986) and pheasant (Lesnik *et al*., 1981), have been found to

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Abbreviations
MDV, Marek's disease virus; MD, Marek's disease; PCR, polymerase chain reaction.

be infected with MDV. All chickens, including gamefowl (Kenzy *et al*., 1964), native breeds (Grewal *et al*., 1977) and jungle fowl (Weiss and Biggs, 1972), are susceptible to tumour development induced by MDV infection. The transmission of MDV is horizontal rather than vertical, and MDV cannot be transmitted from chicken to egg (Boodhoo *et al*., 2016). Skin dander contains viruses that can persist for at least several months at room temperature (Calnek, 1986).

However, due to the continued evolution of MDV, MD remains a serious threat to poultry and causes substantial economic losses annually worldwide (Yu *et al*., 2023). The current live vaccines allow MDV to exist in vaccinated hosts and be shed into the environment (Davison and Nair, 2005). Thus, the present study analysed the pathogenicity of newly isolated MDV strains from tumour-bearing chickens in China and revealed that the protection indices (PIs) of four commercial MD vaccines (CVI988, HVT, CVI988+HVT, and 814) for the new isolates were less than 50% (Liu *et al*., 2023). Although MD vaccines can effectively prevent disease occurrence, including the occurrence of tumours, they cannot completely prevent the transmission of the virus, which may lead to the evolution and enhancement of virulence (Read *et al*., 2015). In recent years, several countries have reported that emerging strains of vvMDV, vv+MDV, and other variants in many chicken flocks are thought to be the major reasons for MD vaccine failures (Song *et al*., 2022).

In recent years, especially between 2019 and 2023, many chicken flocks, particularly in the southern to northeastern regions of China, have suffered from neoplastic avian diseases, which are caused mainly by MDV infection (Zheng *et al*., 2022). In this study, we report a recent case of MD in yellow feathered chickens from a small poultry farm (containing 300 chickens) in JuRong, Jiangsu Province. Necropsy of seven chickens from this flock was performed, and the pathologic organs were collected. Detection of MDV was performed using histopathological assays and PCR. Meq and 132-bp tandem repeat region genes were selected for the detection of MDV. These genes, which are associated with oncogenicity and virus replication, are usually mutated in virulent MDVs. In this study, conducted in JuRong, Jiangsu Province, MDV1 was isolated from immunized chickens and molecularly characterized. The results show that MD outbreaks occur frequently in MD-vaccinated chicken flocks, but the key pathogenic determinants and influencing factors remain unclear.

MATERIALS AND METHODS

Clinical samples

Seven 3-months old chickens were obtained from a chicken flock in JuRong, Jiangsu Province, in April 2023. The chickens belonged to a small family-type flock (the capacity was 300 chickens) that also included ducks. All chickens were necropsied for gross pathological examination and PCR. The chickens were previously immunized with the MDV vaccine CVI988. Cages were not isolated from the outside, so the chickens were able to mingle with wild birds. These chickens were free-range chickens that moved outside during the day and lived in cages at night.

Histopathological assay

The spleen and liver from the sick chickens were fixed in 10% neutral formalin, dehydrated in alcohol, and embedded in paraffin. The tissues were sliced, stained with HE and visualized via light microscopy.

Virus isolation from feather follicles

The feather follicles from the dead chickens were used to isolate the virus. Accordingly, feather tips approximately 5 mm in length were suspended in phosphate buffer (pH 7.2) and freeze–thawed three times. The suspension was filtered through a 0.45 µm membrane filter and cultured in CEFs. Then, the medium was added, and the cultures were incubated for 7 days. Cells infected with the field strain were cultured for three blind passages. PCR was used to confirm infection in cultured cells with CPE.

Genomic DNA extraction and PCR amplification of MDV genes

Genomic DNA extracted from feather follicle samples was used for the diagnosis of MDV. The samples were digested in lysis buffer (10 mM Tris, 100 mM EDTA, 0.5% SDS, 0.2 mg/ml proteinase K), extracted three times with phenol/chloroform, precipitated in 100% ethanol and washed twice with 70% ethanol. Finally, the DNA was dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0). In the PCR system, a 50 µl reaction mixture, consisting of 25 µl of 2 × Phanta Max Buffer, 2 µl of each of the two primers (10 µmol), 1 µl of Phanta Max Super-Fidelity DNA Polymerase (Vazyme, China), 19 µl of ddH_2O, and 1 µl of DNA template, was used. The PCR conditions for the Meq and 132-bp tandem repeat region genes were as follows: 95°C for 3 min; 33 cycles of 95°C for 15 s, 54°C for 15 s, and 72°C for 40 s; and 72°C for 5 min. The PCR conditions for the full-length Meq gene were as follows: 95°C for 3 min; 33 cycles of 95°C for 15 s, 54°C for 15 s, and 72°C for 60 s; and 72°C for 5 min. The primers used for amplification are listed in Table I.

Table I. Primers used for amplification of MDV viral genes.

Name	Sequence (5' → 3')	Length
132-bp tandem repeat regions	F: TGCGATGAAAGTGCTATGGAGG R: GAGAATCCCTATGAGAAAGCGC	317bp
MDV-Meq- partial	F: TTCCCTGACGGCCTATCTGA R: TTCGGGATCCTCGGTAAGAC	786bp
MDV-Meq	F: AGAGATGTCTCAGGAGCCAGAGCC R: ATCATCAGGGTCTCCCGTCACCTG	1020 bp

Table II. GenBank accession numbers of the Meq gene sequences used in this study.

Isolate	Country of origin	Accession number	Pathotype
CVI988/ Rispens	Netherlands	AY243334.1	attMDV
GA	USA	AF147806.2	vMDV
Md5	USA	AF243438.1	vvMDV
RB/1B	USA	HM488349.1	vvMDV
648A	USA	AY362725.1	vv+MDV
SD-2012	China	KC511815.1	vvMDV
LMS	China	AEM63531.1	vvMDV
HNXZ105	China	HF546100.1	N.A.
HNGS101	China	HF546084.1	N.A.
HNGS206	China	HF546086.1	N.A.
HNLC107	China	HF546091.1	N.A.
HNLC202	China	HF546092.1	N.A.
HNLC203	China	HF546093.1	N.A.
HNLC401	China	HF546094.1	N.A.
HNLC502	China	HF546095.1	N.A.
CC/1409	China	KU744560.1	N.A.
WC/1203	China	KU744558.1	N.A.
JL/1404	China	KU744559.1	N.A.
GX0101	China	JX844666.1	vvMDV
LTS	China	KP888838.1	N.A.
HS/1412	China	KU744561.1	vMDV

Att, attenuated; N.A., not available; v, virulent; vv, very virulent; vv+, very virulent plus.

Sequencing and phylogenetic analysis

The PCR products were gel purified and sequenced by a commercial sequencing service (Sangon Biotech, China). The nucleotide sequence data of the JuRong 2023 strain were obtained from the company, and those of other strains were collected from GenBank. The data were subsequently analysed with MegAlign software. Additionally, phylogenetic trees for the Meq genes were constructed with MEGA 5.0 software. At this stage, the neighbour–joining method with 1000 bootstrap replicates was used. The MDV-1 strains in Table II were used to construct the phylogenetic tree and calculate pairwise sequence identity.

RESULTS

Clinical necropsy and histopathological assays

Sick chickens approximately 3 months of age that had already been immunized with the CVI988 vaccine were found in JuRong, Jiangsu Province. Clinical symptoms of MD, such as anorexia, inability to stand up, and loss of weight, were recorded by the owners in approximately 30 chickens. Seven of the chickens were subsequently sent to our laboratory, and the samples were numbered 1 to 7. One of them was dead on arrival and was labelled sample 1. All the chickens were necropsied for gross pathological examination in the Veterinary Pathology Laboratory. Feather samples were collected from these chickens. The nodules in the spleen and liver of four out of the seven chickens were revealed by clinical necropsy. Figure 1A and B shows the nodular lesions, which were white or grey in colour, in the spleen and liver of chicken sample 1. No obvious lesions were found in other organs or leg nerves. The histopathological features included pleomorphic lymphoid cells and other mitotic features in the spleen (Fig. 1C). Infiltrating lymphoid cells were present in the liver, which led to destruction of the tissue structure, corresponding to the classic lesions observed for MDV (Fig. 1D).

Detection of the MDV genome by PCR

Genomic DNA extracted from feather follicle samples of sick chickens was used to detect the MDV genes. The 132-bp tandem repeat regions (Fig. 2A) and *Meq* (Fig. 2B) genes were detected in all the samples via PCR. CEFs infected with Md5 and CVI988, which are MDV serotype 1 strains, were used as MDV-positive controls. The negative control was CEF cells. Following PCR using primers specific for 132-bp tandem repeat regions, positive amplification products were obtained from all the samples.

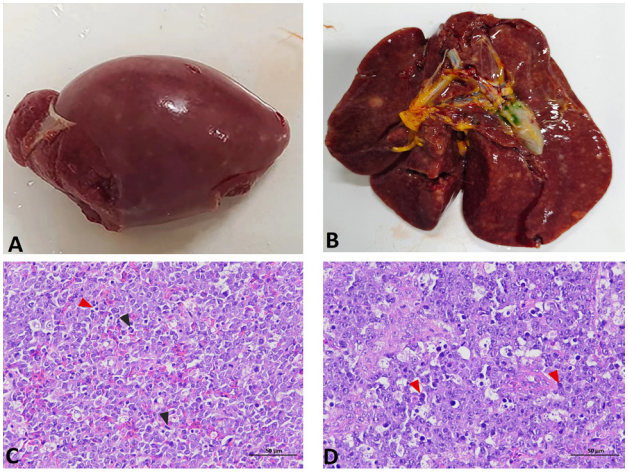

Fig. 1. Pathological lesions and histopathological sections (HandE staining) of the spleen and liver of chicken sample 1. A-D, Spleen (A), liver (B), lymphomatous infiltration in the spleen (C), and lymphomatous infiltration in the liver (D). The red arrow shows pleomorphic leukocytes, and the black arrow shows malignant cells with mitotic figures.

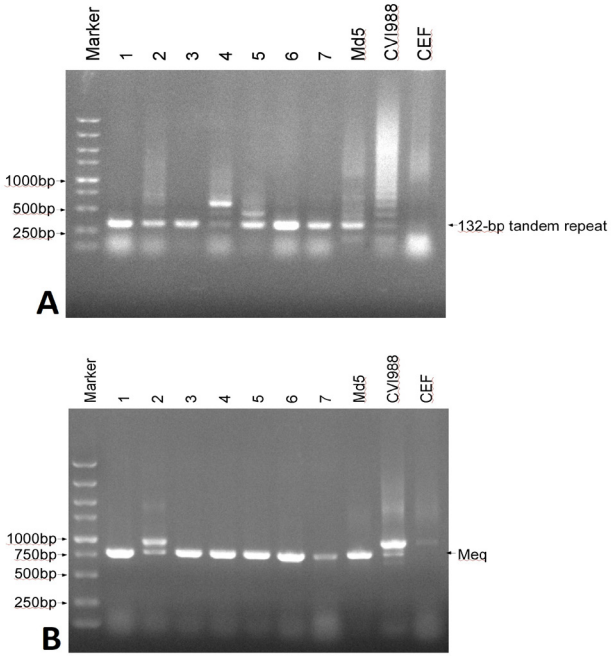

Fig. 2. Agarose gel electrophoresis of MDV from PCR of 132-bp tandem repeat regions and Meq. A, 132-bp tandem repeat regions (317 bp or multiple bands) were amplified from the genomes of feather pulp. B, Meq (786 bp, partial) was amplified from the genome of feather pulp. Samples 1-7 were obtained from sick chickens. The whole-cell genomes of Md5- and CVI988-infected CEF cells were used as positive controls. cDNA from the CEF cells was used as a negative control.

Except for samples 4 and 5, in which multiple bands were amplified, the amplified bands of the other samples were similar to those observed in the Md5-positive control (Fig. 2A). The designed sequence of the Meq primers was used to distinguish between Meq and L-Meq to identify field strains and the vaccine strain CVI988. The amplification product of Meq, approximately 786 bp in length, was detected in six samples. This product was still detected in sample 2, along with the additional larger L-Meq product, as in CVI988. This indicated that the viruses from the seven samples all belonged to MDV serotype 1, which included the virulent strain and the vaccine strain.

Isolation of the MDV field strain

In this study, a total of 60 pooled feather follicle samples were collected from six chickens, except for sample 2, and were cultured on CEFs. MDV was isolated from sample 1. There were very small plaques on the CEFs after 7 days of culture in the first passage (Fig. 3A). We also found small plaques after 5 days of culture in the second passage (Fig. 3B). The infected cells were plaque-cloned, and the isolates were propagated in 6-well plates. The formation of bright and enlarged plaques, which is a typical cytopathogenic effect (CPE), was observed beginning at three days of the third passage (Fig. 3C-F). Four separate wells from the plaque-purified samples were collected for PCR detection of the Meq sequence. As demonstrated in Figure 4, Meq bands could be detected in 3 wells of CEFs infected with MDV, similar to what was observed for the Md5-positive control. There were two bands in well 2, which were similar to the band for CVI988. The resulting isolated virus was called JuRong 2023.

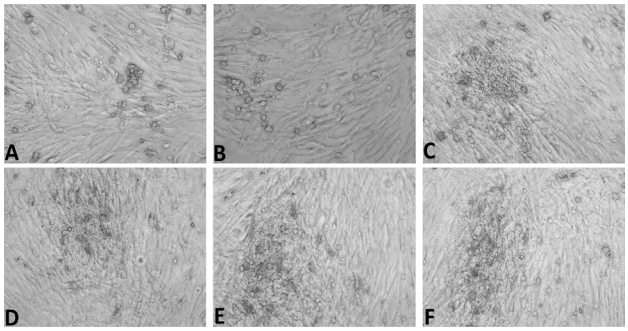

Fig. 3. Typical virus plaques of the MDV isolates (100×). A, JuRong2023 (Passage 1). B, JuRong2023 (Passage 2). C-F, JuRong2023 (Passage 3).

Phylogenetic and evolutionary analysis of MDV based on Meq

Based on the examination of the MDV genome by PCR and postmortem examinations, we amplified the

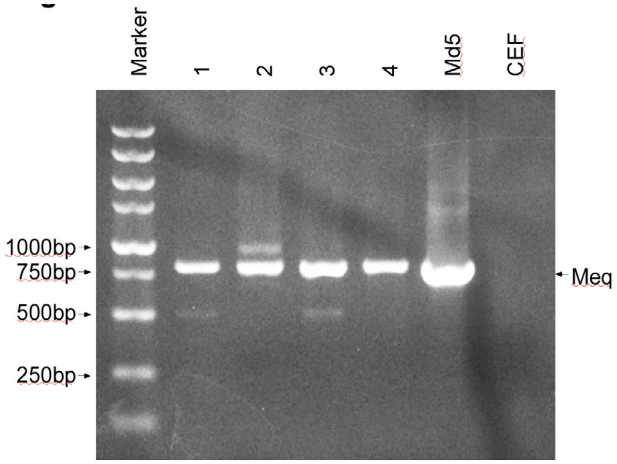

Fig. 4. Agarose gel electrophoresis of MDV from PCR of Meq for detecting MDV isolates. Samples 1-4 were obtained from 4 separate wells containing plaque-purified samples of the JuRong2023 strain (Passage 3). The whole-cell genome of Md5-infected CEF cells were used as a positive control. The cDNA from the CEF cells was used as a negative control.

Meq gene from sample 1. The complete sequences of the Meq genes, approximately 1020 bp in length, were successfully amplified from the genome of feather pulp. A phylogenetic tree based on the Meq gene sequences of 24 MDVs, including 19 isolates from China, was constructed to analyse the evolutionary relationships between strains. The remaining 5 strains, except for CVI988 from the Netherlands, were all isolated from the USA. Among them, HNLC107, HNLC502, HNXZ105, HNGS101, HNGS101, HNGS206, HNLC202, HNLC401 HNLC203 and SD-2012 were able to bypass the protective effects of the CVI988 vaccine (Song *et al*., 2022). Phylogenetic analysis of Meq revealed that our MDV strain (JuRong 2023) was most closely related to SD-2012, which induced more than 50% mortality under vaccine protection (Fig. 5). This may partially explain why JuRong2023 bypassed the protective effects of the CVI988 vaccine.

Amino acid sequence analysis of virulent strains

To identify associated changes, we aligned the Meq amino acid sequences of the 14 strains with the CVI988 sequence. Among them, the American strains GA, RB1B and Md5 were standard virulent strains. The remaining were Chinese isolates that could evade the CVI988 vaccine. As shown in Table III, we detected a total of 9 amino acid site substitutions in JuRong 2023 compared with CVI988. The D to Y substitution at position 80, the V to A substitution at position 115, the P to R substitution at position 176, and the P to A substitution at position 217 were the most common substitutions among the 11 Chinese strains that have been reported to bypass the protective effects of the CVI988 vaccine.

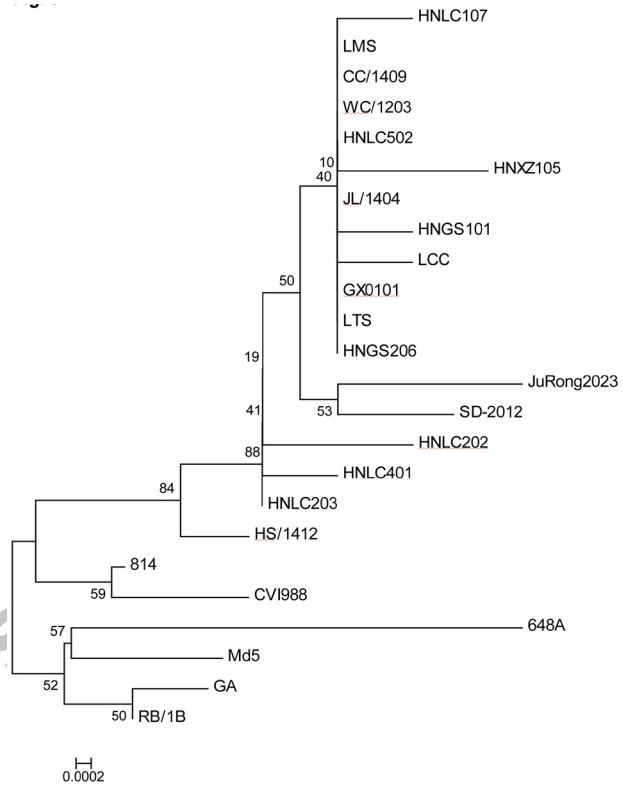

Fig. 5. Phylogenetic profile showing the relationships among MDV isolates based on a comparison of the *Meq* gene sequences. The tree was constructed by using the N-J analysis method in the MEGA5.0 program with bootstrapping (1000).

DISCUSSION

In recent decades, MD has essentially been controlled by vaccines. However, GaHV-2 strains have evolved continuously in terms of virulence and can overcome the immune responses induced by the currently available vaccines (Zhang *et al*., 2016). In recent years, outbreaks of MD have been frequently reported in many regions in China, such as Henan, Shandong, and Jiling (Zhang *et al*., 2016; Deng *et al*., 2021; Song *et al*., 2022). Usually, MD is detected at 4-6 weeks of age in broilers and 10-12 weeks of age in hens (Okwor and Eze, 2011). In general, young chickens infected with virulent MDV strains may have high mortality rates at 8–16 dpi (Stephens *et al*., 1980). In the present study, gross and histopathological examinations revealed lymphomatous lesions in visceral organs, such as the liver and spleen; these findings were

Table III. Amino acid substitutions of virulent MDV.

Isolate	Amino acid site																
	27	**71**	**77**	**80**	**88**	**93**	**115**	**133**	**139**	**176**	**217**	**277**	**283**	**318**	**320**	**326**	**332**
CVI988/Rispens	S	S	E	D	A	Q	V	T	T	P	P	L	A	I	I	I	W
JuRong2023	·	A	·	Y	T	R	A	·	A	R	A	·	·	·	·	T	·
SD-2012	·	A	·	Y	T	·	A	·	·	·	A	·	·	V	·	T	·
GA	·	A	K	·	·	·	·	·	·	·	·	·	·	·	·	T	·
Md5	·	A	K	·	·	·	·	·	·	·	A	·	V	·	T	T	·
RB1B	·	A	K	·	·	·	·	·	·	·	·	·	·	·	·	T	·
LMS	·	A	·	Y	·	·	A	·	A	R	A	·	·	·	·	T	·
HNXZ105	·	A	·	Y	·	·	A	P	A	R	A	·	·	·	·	T	·
HNGS101	·	A	·	Y	·	·	A	·	A	R	A	·	·	·	·	T	·
HNGS206	·	A	·	Y	·	·	A	·	A	R	A	·	·	·	·	T	·
HNLC107	·	A	·	Y	·	·	A	·	A	R	A	F	·	·	·	T	·
HNLC202	·	A	·	Y	·	·	A	·	·	R	A	·	·	·	·	T	G
HNLC203	·	A	·	Y	·	·	A	·	·	R	A	·	·	·	·	T	·
HNLC401	P	A	·	Y	·	·	A	·	·	R	A	·	·	·	·	T	·
HNLC502	·	A	·	Y	·	·	A	·	A	R	A	·	·	·	·	T	·

·, means the amino acid type in this site was the same as the CVI988/ Rispens amino acid sequence. The most commonly found substitutions in China virulent strains are labeled in red.

mostly consistent with previously reported cases of MD (poultry diseases). Furthermore, molecular sequencing is one of the most reliable methods for classifying genotypes.

Latent infection is a prerequisite for lymphocyte transformation, and Meq plays an important role in this process. It has various functions, including functions in DNA binding, chromatin remodelling and transcriptional regulation (Jones *et al*., 1992). Animal experiments have indicated that the Meq-negative mutant MDV BAC does not induce the formation of tumours in chickens (Lupiani *et al*., 2004). Thus, Meq was used as a molecular marker for typing virus strains. The 132-bp tandem repeat region can be used as an important target gene to differentiate field strains from vaccine strains (Tian *et al*., 2011). In this study, we used Meq and 132-bp tandem repeat regions as target genes for detecting field strains in pathological materials collected from chicken flocks in JuRong. MDV was isolated from pooled feather follicles of chickens. Bright and enlarged cells were observed beginning five days after the second passage, and a clear CPE was observed at three passages; these findings are characteristic of MDV and were confirmed by PCR.

A phylogenetic tree based on the Meq amino acid sequences of the JuRong 2023 isolate and 23 MDV reference strains in the GenBank database was generated via the neighbour–joining method. Bootstrap confidence limits for 1,000 replicates are indicated above each branch and are presented in Figure 5. It has been reported that four strains (LTS, HNGS101, 206, and HNXZ105) have mortality rates ranging from 30% to 50%, and six strains (HNLC202, 203, 401,502, and 107; SD-2012) have mortality rates greater than 50% (Yu *et al*., 2013). The same distribution of strains was observed in the analysis of the phylogenetic tree, which showed that the JuRong 2023 isolate and the SD-2012 isolate belong to the same group. Although the infected chickens in the flock from which the JuRong 2023 strain was isolated were euthanized, the mortality rate was unknown. It was speculated that JuRong 2023 is a virulent strain. This indicates that the transmission of virulent strains occurs across different regions in China. This may be partly responsible for the recent outbreaks in immunized chicken flocks in JuRong, Jiangsu Province. Overall, these findings indicate that the efficacy of the most frequently used CVI988 vaccine has started to decrease over the last 10 years in China.

The Meq sequence has been shown to correlate with GaHV-2 virulence and contains numerous repeat sequences of four prolines (PPPP repeats), which have been shown to have a strong negative association with viral oncogenicity and pathogenicity (Renz *et al*., 2012). It has been reported that positions 176 and 217 of the Meq protein harbour the second proline in proline-rich repeat regions, and mutations at these positions (PPPP to PNPP) may lead to increased virulence (Shamblin *et al*., 2004).

As demonstrated in Table III, compared with the reference strain CVI988, the strain JuRong 2023 had mutations at positions 71 (S to A), 80 (D to Y), 115 (V to A), 176 (P to R), 217 (P to A) and 326 (I to T). According to a previous study, the changes at positions 71, 176 and 217 are positively related to increases in virulence (Shamblin *et al*., 2004). Mutations at positions 80 (D to Y) and 115 (V to A) were widely found in Chinese isolates (Tian *et al*., 2011). These mutations have been detected in several Chinese isolates and may be characteristic of GaHV-2 isolates in China (Yu *et al*., 2013). This evaluation showed that the mutations at positions 80, 115, 176, and 217 affect the function of the virus and are worthy of attention in China.

The detection of virulent MDV from the Jurong chicken farm further demonstrated that the Meq genes of GaHV-2 strains isolated from commercial flocks in China exhibit significant genetic diversity and are correlated with increased virulence. It seems that most of the virulent Chinese strains share a similar Meq amino acid sequence, although they differ from the non-Chinese strains and vaccine strains. For the control of MD in chicken flocks, constant surveillance of newly isolated field strains is necessary to reveal the characteristics of endemic Chinese MDVs to develop better vaccines and control programs. The field strain used to challenge MD-vaccinated birds to evaluate the immune protection efficacy of different MD vaccines, CVI988, HVT, CVI988+HVT, and 814, needs to be further studied.

DECLARATIONS

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IRB approval and consent to participate

This study was approved by the author's institution (Nanjing Agricultural University ethics committee), and owner consent was obtained for the animals used for the postmortem examinations.

Availability of data and materials

Our findings are contained within the manuscript.

Consent for publication

Consent was obtained from the owner of the animal for publication of this case report.

Statement of conflict of interest

The authors have declared no conflict of interest.

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