

Molecular characteristics and evolutionary analysis of field Marek's disease virus prevalent in vaccinated chicken flocks in recent years in China

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Abstract Marek's disease is a highly contagious, oncogenic, and immunosuppressive avian viral disease. Surveillance of newly registered Marek's disease virus (MDV) isolates is meaningful for revealing the potential factors involved in increased virulence. Presently, we have focused on the molecular characteristics of all available MDVs from China, including 17 new Henan isolates. Based on Meq, gE, and gI genes, we found that most Chinese isolates contain conserved amino acid point mutations in Meq, such as E⁷⁷, A¹¹⁵, A¹³⁹, R¹⁷⁶, and A²¹⁷, compared to USA virulent MDVs. However, the 59-aa or 60-aa insertions are only found in a few mild MDVs rather than virulent MDVs in China. Further phylogenetic analysis has demonstrated that a different genotype of MDV

has been prevalent in China, and for virulent MDVs, their recent evolution has possibly been geographically restricted. Our study has provided more detailed information regarding the field MDVs circulating in China.

Keywords China · MDV · Isolate · Meq · gE · gI · Phylogenetic analysis

Introduction

Marek's disease virus (MDV) is phylogenetically classified as a herpesvirus of the subfamily *Alphaherpesvirinae* [1], and is one of the few oncogenic herpesviruses that can induce tumors in its natural host [2]. The disease caused by MDV was first reported by the Hungarian veterinarian József Marek in 1907 [3]. It is a highly contagious, lymphoproliferative disorder, and neoplastic immunosuppressive disease of poultry known as the Marek's disease

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(MD). Since MDV was identified as the pathogen, three distinct serotypes, 1, 2, and 3, have been characterized [2]; while in the newest taxonomy of herpesviruses, MDV has been reclassified as species of *Gallid herpesvirus 2* (GaHV2), *Gallid herpesvirus 3* (GaHV3), and *Meleagrid herpesvirus 1* (MeHV1), respectively [1]. Serotype 1 (GaHV2) includes the pathogenic and oncogenic isolates and their attenuated progeny, while strains of serotype 2 (GaHV3) are nonpathogenic. The strains of herpesvirus of turkeys (HVT) are classified into the serotype 3 (MeHV1), which are nonpathogenic and can be used as vaccines to control MD.

Similar to other oncogenic herpesviruses, such as *Human herpesvirus 4* (HHV4), MDV infection is first established and then maintained in the host before finally inducing transformation and the production of lymphoma [4]. Worldwide, MD has caused huge losses in the poultry industry and is one of the most important avian tumorigenic and immunosuppressive diseases. During the past several decades, anti-viral vaccination has contributed effectively to its control. However, due to high-density poultry production and possible selection pressure from vaccination, the virulence of MDV field strains has been seen to have increased [5]. Based on mortality rates in flocks, lesion frequency, and protection by existing vaccines, GaHV2 isolates have been grouped into four further pathotypes designated as classic or mild (mMDV), virulent (vMDV), very virulent (vvMDV), and very virulent plus MDVs (vv+MDV) [5]. The evolution of GaHV2 strains with higher virulence, which can possibly break the immunoprotection of presently available vaccines, have introduced fresh challenges. Thus, research on the characteristics of MDV strains circulating in chicken flocks will be extremely important for understanding the epidemiology of MDV and help in looking for possible measures to further control of MD.

As one of the largest bases of the poultry industry in the world, China covers a large and diverse geographic area and historically has been a serious epidemic region of MD. Although at hatching, chickens are inoculated with commercial vaccines, such as CVI988/Rispens, HVT, and/or 814, a mMDV isolate from the northeast China and has been used as a vaccine strain, MD outbreaks happen sporadically every year in China. However, except for the recent few reports in Guangxi (GX) [6], Sichuan (SC) [7], and the northeast provinces [8], the overall prevalence of MDV among chickens in most regions of China is still not known. During the winter from 2011 to 2012, MD outbreaks happen in many small poultry farms in China, especially throughout the central province of Henan, which has historically been the hub of both road and rail transport routes in China, and is one of the biggest bases of the poultry industry in China. Herein, we have recently

focused on the molecular epidemiology of MDV in vaccinated chicken flocks from poultry farms, which outbreak MD in Henan. In addition, we have analyzed the molecular characteristics and phylogenetic evolution of all previously reported MDVs from China, with particular emphasis on those isolated over the years 1995–2012. This has provided more detailed information regarding the nature of endemic MDVs circulating in China.

Materials and methods

Cells and virus

Primary chicken embryo fibroblast (CEF) cells were prepared from 9-day-old embryonated eggs and then seeded into cell plates or flasks. CEFs were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 5 % new-born calf serum (NCS) (Hangzhou Sijiqing Biological Engineering Materials Co., Ltd., China), and incubated at 37 °C with 5 % CO₂ for 24 h to prepare confluent CEF monolayers for MDV isolation or propagation. MDV GX0101, a field strain of GaHV2 isolated from an egg production unit in Guangxi province of South China [9], is used as a positive control, which is propagated in CEFs, harvested and stored in liquid nitrogen for further use. For virus isolation, blood samples from diseased layers showing suspected MD clinical symptoms were collected in anticoagulant from each bird and 1 ml of blood was mixed with 9 ml of DMEM medium supplemented with 1 % NCS and centrifuged at 500×g for 5 min. White blood cells were collected and seeded onto three duplicate 12-well plates containing confluent CEF monolayers. After 5 to 6-day interval, CEFs were digested and transferred to new monolayers in 6-well plates, and then, for two passages in 25-cm² flasks. The final culture was incubated for further 3–5 days until the plaque formation. Cell cultures presenting typical viral plaques were collected and stored in liquid nitrogen for further characterization.

DNA extraction

Total cellular DNA was extracted from CEF cultures using the Universal Genomic DNA Extraction Kit Ver.3.0 (TaKaRa Biotechnology Dalian Co., Ltd.) according to the manufacturer's instructions. Concentrations of the extracted total DNA were determined using a NanoDrop ND-1000 spectrometer (NanoDrop Technologies, Wilmington, DE) and the integrity of the DNA was assessed using 0.8 % agarose gel electrophoresis. Finally, the concentration of

total DNA was adjusted to 0.1 mg/ml in d_3H_2O and stored at $-20\text{ }^\circ\text{C}$ for further use.

PCR amplification

Specific primers for amplifying MDV Meq (Marek's EcoQ-encoded protein), gE (glycoprotein E), and gI (glycoprotein I) genes, as listed in Table 1, were designed and synthesized on the basis of the viral genome sequence of vvMDV strain Md5 (GenBank Acc. No. AF243438). PCR amplification was carried out using 1 μl DNA as template in a total volume of 20 μl containing 10 μl Premix Ex *Taq* (TaKaRa Biotechnology Dalian Co., Ltd.), 1 μl of 10 μM of each of the two primers, and 7 μl d_3H_2O . For amplifying Meq gene, the optimum conditions were as follows: 94 $^\circ\text{C}$ for 4 min, 30 cycles at 94 $^\circ\text{C}$ for 1 min, 60 $^\circ\text{C}$ for 30 s, 72 $^\circ\text{C}$ for 2 min, and final elongation at 72 $^\circ\text{C}$ for 10 min. The optimum conditions for amplification of gE and gI genes were as follows: 94 $^\circ\text{C}$ for 4 min, 30 cycles at 94 $^\circ\text{C}$ for 1 min, 58 $^\circ\text{C}$ for 30 s, 72 $^\circ\text{C}$ for 3 min, and final elongation at 72 $^\circ\text{C}$ for 10 min. The PCR products were analyzed using 1 % agarose gel electrophoresis.

DNA cloning and sequence analysis

PCR products of Meq, gE, and gI genes with anticipated size were isolated and purified from agarose gels using the Agarose Gel DNA Fragment Recovery Kit Ver.2.0 (TaKaRa Biotechnology Dalian Co., Ltd.) and then were cloned using pMD18-T vector (TaKaRa Biotechnology Dalian Co., Ltd.). Three clones of each sample were sent to Sangon Biotechnology (Shanghai, China) for DNA sequencing. The obtained nucleotide sequences were edited using the software EditSeq (DNASTar, USA), blasted against the NCBI databases (<http://www.ncbi.nlm.nih.gov/BLAST>) and compared with other reference MDVs for homology analysis using the software MegAlign (DNASTar, USA). Finally, all the validated new nucleotide gene sequences of MDV isolates were deposited in the GenBank/EMBL/DDBJ databases and the corresponding Acc. Nos. are listed in Table 2.

Phylogenetic analysis

Phylogenetic analysis was performed with the neighbor-joining method using MEGA version 4.0 software packages [10]. The bootstrap values were determined from 1,000 replicates of the original data. The MDV gene sequences were obtained from PubMed publications or from direct submissions to the GenBank database. For the analysis of Meq gene, a total of 84 sequences of MDV isolates or strains which includes the 17 new isolates presently reported, 46 reference isolates previously reported in China from 1995 to 2010 [6–9], eight representative strains reported in the USA [11], four virulent strains reported in Australia [12], five isolates from Japan [13], as well as three isolates from India along with that of the vaccine strain CVI988. While for the phylogenetic analysis of gE and gI genes, only a total of 33 sequences of MDV isolates were used due to the unavailable information of most Chinese isolates and reference isolates from other countries except USA. The detailed information and backgrounds of the reference strains used in this study are listed in Table S1.

Results

Virus isolation from vaccinated chickens in poultry farms in China

To investigate the correlation between MD outbreaks and the current prevalence of MDV in vaccinated chickens in central regions of China, blood samples from 40 live clinically diseased chickens with suspected MD, which were pre-vaccinated with CVI988/Rispens vaccines on 1-day-old, were collected from 18 chicken farms in Henan (HN) province during 2011–2012 (Fig. 1a). Using culture on CEFs, a total of 17 MDV isolates were obtained from layer chickens of 18 poultry farms, distributed in the local regions of Guangshan (GS), Shangcai (SC), Luohe (LH), Luanchuan (LC), Xinzheng (XZ), and Linzhou (LZ) as

Table 1 Primers for amplifying the viral genes of MDV used in this study

Gene	Primer	Type	Sequence (5'–3')	Length (nt)	Genomic location ^a	Amplicon (bp)
meq	meq-Forward	5'	ATGTCTCAGGAGCCAGAG	18	134867–134884	1,020
	meq-Reverse	3'	TCAGGGTCTCCCGTCACC	18	135869–135886	
gE	gE-Forward	5'	ATTGTTGGAAAGAGAAGAATGTGTA	25	162223–162247	1,813
	gE-Reverse	3'	TAGCAAGAGTGGATGTGTTGAATGA	25	164011–164035	
gI	gI-Forward	5'	GGTATGGCGTGTGCTATAGTGCA	25	161086–161110	1,262
	gI-Reverse	3'	AACTTACTGCCTTGTGTTCTGTCT	25	162323–162347	

^a Nucleotide numbering corresponds to the viral genome sequence of very virulent strain Md5 (GenBank Acc. No. AF243438)

Table 2 Detailed information of the field MDV isolates newly obtained from the province of Henan (HN), China

No.	Isolate	Year & month	Geographical location	Host specie ^a	Ages for virus isolation (days)	Passage on CEF	GenBank Acc. Nos.		
							Meq	gE	gI
(1)	HNGS101	2011, Oct	GS	Hyline	150	4	HF546084	HF546101	HF546118
(2)	HNGS201	2011, Oct	GS	Hyline	150	4	HF546085	HF546102	HF546119
(3)	HNGS206	2011, Oct	GS	Hyline	150	4	HF546086	HF546103	HF546120
(4)	HNLH302	2011, Oct	LH	Hyline	120	4	HF546087	HF546104	HF546121
(5)	HNLH303	2011, Oct	LH	Hyline	120	4	HF546088	HF546105	HF546122
(6)	HNLH304	2011, Oct	LH	Hyline	120	4	HF546089	HF546106	HF546123
(7)	HNLH305	2011, Oct	LH	Hyline	120	4	HF546090	HF546107	HF546124
(8)	HNLC107	2011, Nov	LC	Huangshan	145	4	HF546091	HF546108	HF546125
(9)	HNLC202	2011, Nov	LC	Huangshan	145	3	HF546092	HF546109	HF546126
(10)	HNLC203	2011, Nov	LC	Huangshan	145	3	HF546093	HF546110	HF546127
(11)	HNLC401	2011, Nov	LC	Huangshan	145	3	HF546094	HF546111	HF546128
(12)	HNLC502	2011, Nov	LC	Huangshan	145	3	HF546095	HF546112	HF546129
(13)	HNLC503	2011, Nov	LC	Huangshan	145	3	HF546096	HF546113	HF546130
(14)	HNXZ101	2012, Jan	XZ	Kondarl	130	3	HF546097	HF546114	HF546131
(15)	HNXZ103	2012, Jan	XZ	Kondarl	130	3	HF546098	HF546115	HF546132
(16)	HNXZ106	2012, Jan	XZ	Kondarl	130	3	HF546099	HF546116	HF546133
(17)	HNSC105	2012, Feb	SC	Hyline	120	8	HF546100	HF546117	HF546134

GS Guangshan, LH Luohe, LC Luanchuan, XZ Xinzheng, SC Shangcai

^a Hyline, Hyline-brown chicken; Huangshan & Kondarl, Huangshan yellow chicken and Kondarl yellow chicken, local varieties in China, respectively

shown in Fig. 1b, in where represented approximate average mortalities of 20, 30, 15, 70, 15, and 35 %, respectively. The total viral isolation rate from clinical diseased chickens was 42.5 %. The isolates adapted to CEFs very well and most produced typical plaque formation after 2–3 blind passages at 3–5 days post-infection (dpi). Details of the source, year, distribution, and case history of the new isolates are listed in Table 2.

PCR amplification of Meq, gE, and gI genes from MDV Henan isolates

For further characterization, the total DNA of CEFs infected by 17 new isolates was extracted and subjected to the PCR amplification of the complete sequences of MDV Meq, gE, and gI genes. Most of the 17 isolates gave corresponding PCR amplification products of 1,020 bp for Meq, 1,813 bp for gE, and 1,262 bp for gI genes (Fig. 2a, b, c), except for the isolates of HNGS201 and HNLC503 that gave a slightly longer Meq gene products than anticipated (Fig. 2a). After gene cloning and DNA sequencing, all the validated Meq, gE, and gI gene sequences of the new MDV isolates were submitted to the GenBank database and the Acc. Nos. are listed in Table 2.

Analysis of the nucleotide sequence of Meq, gE, and gI genes between isolates and reference MDVs

A high level of nucleotide sequence identity between newly isolated MDVs and the reference MDVs for Meq, gE, and gI genes was observed. Analysis of Meq gene showed that the similarity among the 17 new Henan isolates was 99–100 %. The similarity between the new isolates and MDVs from other areas in China, such as Guangxi, Sichuan, and northeast Chinese provinces, was 97–100 %. However, compared to the MDVs from the USA, the Meq gene similarity of Henan isolates were 97.3–98.8 %. For the gE gene, the similarity of the nucleotide sequences of 17 new isolates was 98.8–100 %, and between MDVs from Henan and Guangxi provinces or the USA were 99.1–100 % and 98.9–99.9 %, respectively. Similarly for the gI gene, the sequence similarity for the 17 isolates was 99.1–100 %, and 99.1–100 % and 98.8–100 % between MDVs from Guangxi province and those from the USA, respectively.

Comparison of deduced amino acid mutation of Meq, gE, and gI genes between isolates and reference MDVs

Alignment analysis of deduced Meq complete amino acid sequences of the 17 isolates and other reference MDVs, as

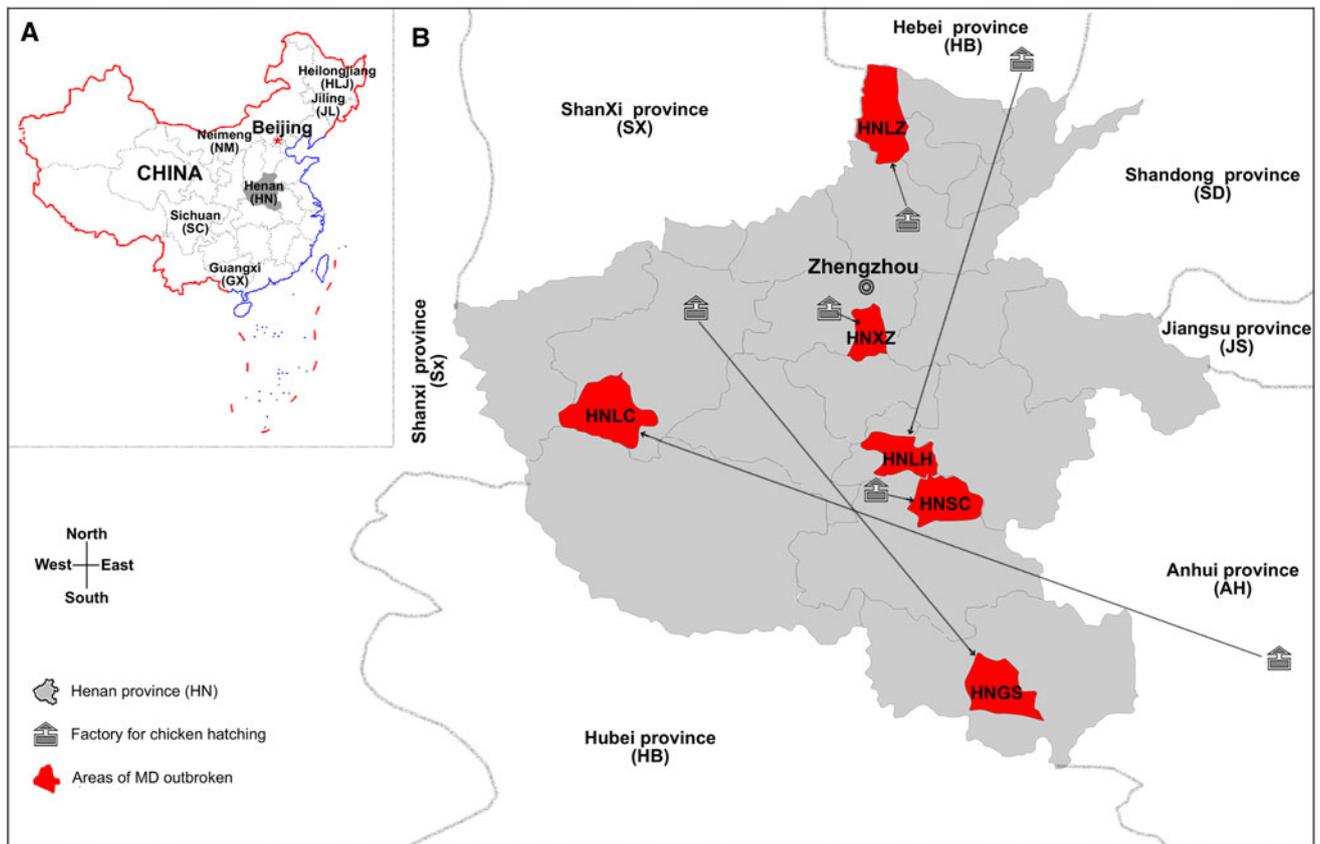
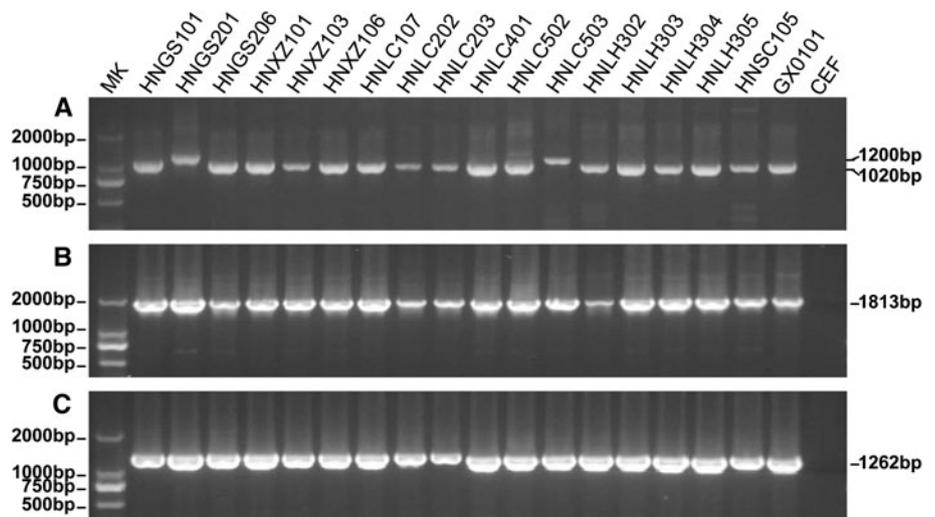


Fig. 1 Distribution of MDV isolates in China. **A.** Location of Henan province and other representative provinces; **B.** Sources and distributions of the new MDV isolates within Henan province, central China. MD outbreaks in the regions of Guangshan (GS), Shangcai

(SC), Luohe (LH), Luanchuan (LC), Xinzheng (XZ), and Linzhou (LZ) in Henan (HN) province are shown by red plaques, respectively. Migrations of the chickens from hatching factory to poultry farm are shown by dashed lines with arrows (Color figure online)

Fig. 2 PCR products of the Meq, gE, and gI genes of 17 MDV isolates from China. **a** amplification of Meq gene; **b** amplification of gE gene; **c** amplification of gI gene. Lane MK DNA molecular weight marker; GX0101 total DNA from CEFs infected by MDV strain GX0101 used as positive controls; CEF total DNA from MDV-uninfected CEFs used as negative controls



shown in Table S1 from China or USA together with the vaccine strains, was performed (Table S2). As demonstrated in Table 3, amino acid mutations in Meq genes were observed to display regularity at ten positions, including 71, 77, 80, 115, 119, 139, 153, 176, 180, and 217,

and mutations at position 77 (K → E), 80 (D → Y), 115 (V → A), 139 (T → A), 176 (P → R), and 217 (P → A) occurred in most field MDVs from China except the isolates HNGS201, HNLH304, HNLG503, J-1, LCGZ, and TQ12, which represented similar mutations to mMDVs

Table 3 Amino acid substitutions in the Meq oncoproteins of the new isolates and reference MDVs

Pathotype ^a	Strains/isolates	71(A)	77(E)	80(Y)	115(A)	119(C)	139(T)	153 PPPP(P)	176(R)	180(T)	217/276 ^b PPPP(A)
vv+MDV	648A, N, TK	–	K	D	V	R	–	Q	A	A	–
vvMDV	Md5	–	K	D	V	–	–	–	P	–	–
	RB1B	–	K	D	V	–	–	–	P	–	P
vMDV	GA	–	K	D	V	–	–	–	P	–	P
mMDV	CVI988 ^c , CU-2 ^c	S	–	D	V	–	–	–	P	–	P
	814	S	–	D	–	–	–	–	P	–	P
New isolate	HNGS201 ^c , HNLC503 ^c , HNLH304	S	–	D	V	–	–	–	P	–	P
	HNLH302, HNLH303, HNLH305	–	–	–	–	–	–	–	P	–	–
	HNLC202, HNLC203, HNLC401	–	–	–	–	–	–	–	–	–	–
	HNGS101, HNGS206, HNLC107	–	–	–	–	–	A	–	–	–	–
	HNLC502, HNXX101, HNXX103	–	–	–	–	–	A	–	–	–	–
	HNXX106, HNLC105	–	–	–	–	–	A	–	–	–	–
CHN ref. MDV	GX0101, GXY2, GX070060	–	–	–	–	–	A	–	–	–	–
	YL040920, LSY1, LQQHR, LHC2	–	–	–	–	–	A	–	–	–	–
	N, LYC	–	–	–	–	–	–	–	–	–	–
	G2, LZ Y	–	–	–	–	–	–	–	P	–	–
	J-1	S	K	D	–	–	–	–	P	–	P
	LCGZ	S	–	D	V	–	–	–	P	–	P
	TQ12	S	–	D	V	–	–	–	P	–	P

“–” Mean same to the consensus amino acids shown in brackets below corresponding sites

^a Pathotypes of vv+MDV, very virulent plus MDV; vvMDV, very virulent MDV; vMDV, virulent MDV; mMDV, mild MDV

^b Denote strains containing 59-aa proline-rich repeat amplification: mutations predominantly in lower virulence trains

^c Strains or isolates of MDV containing longer Meq genes

CVI988, CU-2, and 814. The amino acid mutations at positions 119 (R), 153 (Q), 176 (A), and 180 (A) were unique in the vv+MDV strains 684A, N, and TK from the USA. Notably, the substitution at position 139 (T → A) was found in only field isolates in China. All the Henan isolates, except HNGS201, HNLH304, and HNLC503, had 176 (R) and/or 217 (A), which interrupted stretches of the proline-rich repeat (PRR). Besides, similar to mMDV CU-2, a 60-aa and a 59-aa insertion were also observed in the Meq gene of isolates HNGS201 and HNLC503 between positions 194-253 and 194-252 of virulent strains, respectively (Table S2).

As for gE and gI genes, high identities of amino acid sequences were observed between most Henan isolates and Chinese reference MDVs from Guangxi except the isolates HNGS206, HNLC502, and strain N (Table 4). Compared to MDVs from USA, mutations in gE genes of 13 Henan isolates displayed regularity at six positions, including 23

(H → R), 28 (A → V), 392 (I → L), 453(D → G), 455 (T → A), and 469 (A → D). However, no mutations were found at these positions in gE gene in isolates HNGS201, HNLH304, and HNLC503. Similar phenomenon was also observed in gI gene and the amino acid mutations displayed regularity at six positions, including 3 (L → V), 112 (D → G), 142 (A → S), 155(A → V), 223 (I → V), and 240 (F → S), but mutations at these positions were also not found in isolate HNLC502 as well as HNGS201, HNLH304, and HNLC503.

Phylogenetic and evolutionary analysis of MDVs from China based on Meq, gE, and gI genes

A phylogenetic tree based on the Meq gene sequences of 84 MDVs (Table S1), including the 17 new isolates, has been prepared to analyze their relationships, particularly

Table 4 Amino acid substitutions in the gE and gI proteins of the new isolates and reference MDVs

Pathotype ^a	Strain/isolate	gE						gI						
		23(R)	28(V)	392(L)	453(G)	455(A)	469(D)	3(V)	112(G)	142(S)	155(V)	223(V)	240(S)	
vvMDV	Md5, RB1B	H	A	I	D	T	A	L	D	A	A	I	F	
vMDV	GA	H	A	I	D	T	A	–	–	–	–	–	F	
mMDV	CVI988, CU-2	H	A	I	D	T	A	L	D	A	A	I	F	
	814	H	A	–	D	–	–	–	–	–	–	–	F	
New isolate	HNGS201, HNLH304, HNLC503	H	A	I	D	T	A	L	D	A	A	I	F	
	HNLC502	–	–	–	–	–	–	L	D	A	A	I	F	
	HNGS101	–	–	–	–	–	–	–	–	–	–	–	F	
	HNLH302, HNLH303, HNLH305	–	–	–	–	–	–	–	–	–	–	–	–	
	HNLC107, HNLC202, HNLC203	–	–	–	–	–	–	–	–	–	–	–	–	
	HNLC401, HNXZ101, HNXZ103	–	–	–	–	–	–	–	–	–	–	–	–	
	HNXZ106, HNSC105	–	–	–	–	–	–	–	–	–	–	–	–	
	HNGS206	H	A	–	D	–	–	–	–	–	–	–	F	
	CHN ref. MDV	N	H	A	–	D	–	–	L	–	–	–	–	F
		GX070060, GX070079	–	–	–	–	–	–	L	–	–	–	–	–
GXY2, G2, YL040920		–	–	–	–	–	–	L	–	–	–	–	F	

“–” Mean same to the consensus amino acids shown in brackets nearby corresponding sites

^a Pathotypes of vvMDV, very virulent MDV; vMDV, virulent MDV; mMDV, mild MDV

those from China. It was confirmed that a total of four groups occurs and that all 63 isolates from China fall into two separate groups (Fig. 3). All the 17 new isolates, except HNGS201, HNLH304, and HNLC503, occur in the first group (group I), which contains all of the other 43 Chinese isolates, including seven from Guangxi (GX), 22 from Sichuan (SC), one from Neimeng (NM), six from Liaoning (LN), and three from Heilongjiang (HLJ), along with four isolates from Jiling (JL). No clear geographic associations feature among these isolates, although, they are obtained from widely distributed locations of different geographic natures, within China as shown in Fig. 1a. The virulent strains from Australia are clustered into an adjacent separate group II. All the v, vv, and vv+MDVs from USA, including strains GA, RB1B, Md5, N, TK, and 648A, together with six isolates from India or Japan, are clustered into group III. The other 11 MDVs fall into the closely related fourth group (group IV), which contains three vaccines or mMDV strains (CVI988, 814 and CU-2), six

isolates HNGS201, HNLH304, HNLC503, J-1, LCGZ, and TQ12 from China, and two isolates from Japan.

Phylogenetic analysis of the gE gene sequences of 33 MDVs showed that all the analyzed strains and isolates could be separated into two groups, groups I and II (Fig. 4). In this tree, 14 new isolates from Henan and six vvMDVs from Guangxi, together with vaccine strain 814, were clustered into group I while all the other MDVs, including isolates HNGS201, HNLH304, and HNLC503 from China, all the m, v, vv, and vv+MDVs from USA, and the vaccine strain CVI988, were included in group II.

Further analysis based on the gI genes showed that except for the GA strain from USA and isolate HNLC502 from China, all the other same MDVs were also clustered into two groups, similar to that of gE phylogenetic tree (Fig. 4). However, differently from the phylogenetic tree of gE gene, the Chinese MDVs recently isolated from either Henan (except HNGS206) or Guangxi provinces formed a separate cluster within group I, implying a geographically

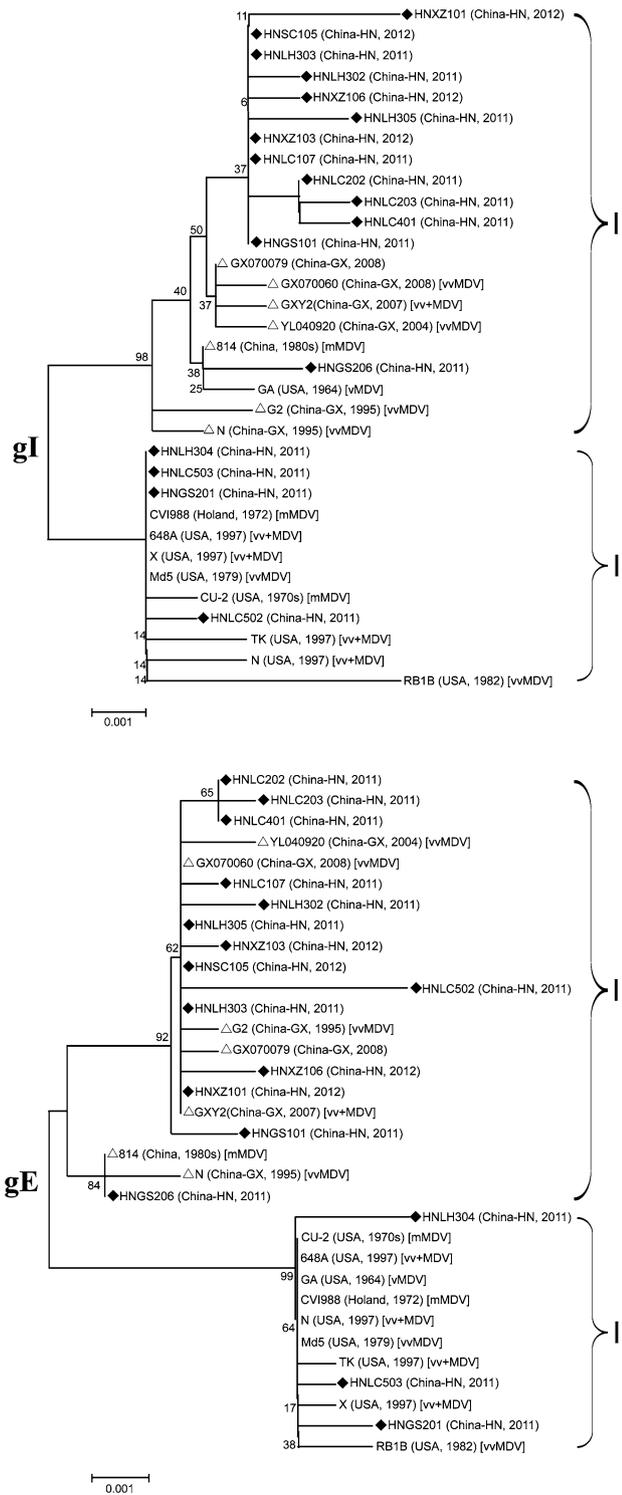


Fig. 4 Phylogenetic analysis of the MDV isolates based on the gE and gI gene sequences. Phylogenetic analysis was performed by the neighbor-joining method using MEGA version 4.0 software packages. Bootstrap confidence limits for 1,000 replicates are indicated above each branch. Viruses were identified using the nomenclature of strain or isolate name/country–province abbreviation/year of isolation. The new Henan (HN) isolates are marked by filled rhombuses while the Chinese reference isolates from Guangxi (GX) and mMDV strain 814 are marked by open triangles. Pathotypes of the reference strains, which had been previously characterized in vivo, are indicated using brackets on the right side

attachment, cell-to-cell spread, and other aspects of the virus lifecycle [18]. In addition to the basic functions, some of the surface glycoproteins present as potent immunogens (gB) and others have evolved immune evasive functions, such as gE and gI [19]. However, when compared the representative MDV strains of all the distinct pathotypes, researchers found that no consistent mutations in surface glycoprotein genes were correlated with virulence, unlike the Meq gene [11]. A recent report has however shown that these genes might be used as good genetic markers for phylogenetic evolutionary analysis [6]. Similar to previous reports, our research has showed that all the genes of both gE and gI are highly conserved in all available Chinese isolates from both Henan and Guangxi provinces, and showed similar point mutations compared to USA MDVs. Phylogenetic analysis also revealed significant genetic diversity. Notably, two main lineages were formed in both the gE and gI phylogenetic trees, and most of the Chinese MDVs fall into a separate group (apart from one USA strain in the gI tree) more distant from the MDV reference strains from USA. Unfortunately, there is no more available information on both of the gE and gI genes from other regions in China or other countries, such as Australia, India, and Japan.

The same distribution of strains is seen in the analysis of the Meq phylogenetic tree, which has shown that most of the Henan isolates fall into a single group along with all the other Chinese reference isolates (except J-1, LCGZ, and TQ12). However, the other three Henan isolates, HNGS201, HNLH304, and HNLC503, were clustered into another distinct group together with J-1, LCGZ, TQ12, mMDV CU-2 and the vaccine strains CVI988 and 814, showing features of mild or attenuated MDV different from virulent MDVs. Except for Meq gene, we also found that three isolates, HNGS201, HNL304, and HNLC503, consistently lie outside of this pattern and fall into the same group for both of the gE and gI genes as the “CVI988/Rispens” vaccine strain. The reasons resulting in such a phenomenon remain unknown. Considering the wide application of CVI988 as a vaccine for a long time in China, it’s possible that they are variants from this strain, but more verification needs to be performed. It is undoubtedly that based on the phylogenetic analysis of all

180-bp insertions alone is an indicator for distinguishing virulent and attenuated MDVs and their possible role involved in the pathogenicity of MDV deserves to be revealed in future studies.

The viral glycoproteins of herpesviruses play critical and fundamental roles in virus infection, maturation,

the three viral genes, it appears that a different, independently evolved, genotype of MDV has become endemic in China.

Another interesting phenomenon is that either in the gE or gI phylogenetic trees, the predominant Chinese field MDVs were evolutionarily clearly more adjacent to the Chinese vaccine strain 814, but far away from the vaccine strain CVI988/Rispens. These differences had been suggested to be partly responsible for the most recent outbreaks in Guangxi [6], but in another recent report of field MDVs in Sichuan, the researchers stated that CVI988/Rispens vaccine could provide enough protection against the challenge of prevalent MDVs and should be widely used commercially [7]. Thus, the effectiveness of commercial vaccines on the prevalent Chinese MDVs, such as “CVI988/Rispens” and 814 strains used either alone or in combination with other GaHV3 and/or MeHV1 vaccines, needs to be further studied. It is undoubtedly true that for the future control of MD, constant surveillance of newly isolated field MDVs and characterization of their pathotypes are necessary to reveal the characters of endemic Chinese MDVs and hence, to develop better vaccines and control programs.

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