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SEQUENCING AND ANALYSIS OF THE *MEQ* ONCOGENE OF MDV CAUSING MAREK'S DISEASE IN BAC NINH PROVINCE FROM 2019 TO 2022

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SUMMARY

Marek's disease is a contagious avian viral disease that is caused by a Marek's disease virus serotype 1 (MDV-1) or gallid herpesvirus 2 (GaHV-2), a member of the genus *Alphaherpesvirus*, family *Herpesviridae*. Up to now, the disease has caused significant losses to the chicken farming industry in many provinces and cities across the country. In this study, four MDV-1 (GaHV-2) strains causing the disease in Bac Ninh province during the 2019–2022 period were identified by multiplex Polymerase Chain Reaction (PCR). Subsequently, the segments of 1020 bp encoding 339 amino acids of *Meq* gene (Marek's disease virus EcoRI fragment Q) from four samples were collected and sequenced. The rate of identity and homology between the four Vietnamese strains and other global strains ranged from 89.4% to 100% and 87.6% to 100%, respectively. These rates were higher between the four Vietnamese strains and MDV-1 Chinese strains, ranging from 99.2% to 100% and 98.2% to 100%, respectively. The molecular characterization of the *Meq* gene revealed that all four of the GaHV-2 strains infecting chickens in the Bac Ninh province belonged to the highly virulent group, with a low proline ratio in *Meq* protein (ranging from 20.59–21.18%), consisting of three PPPP motifs and three interrupted motifs that contain mutations at the second position of the proline rich region (PRR): PPPP>P (Q/A/R)PP. Overall, this study provides valuable information on molecular characteristics of MDV-1 strains in Bac Ninh province during the 2019–2022 period.

Keywords: *Bac Ninh province, GaHV-2, MDV, Meq, Vietnam*

INTRODUCTION

Marek' disease (MD) is a dangerously infectious disease that has caused significant economic losses to the global and Vietnamese poultry industries. The disease was firstly described in Hungary in 1907, and it later spread throughout Europe and the United States (Kennedy *et al*., 2017). In Asia, the disease was discovered in Japan in 1930

and quickly spread to other Asian countries, including Vietnam (Le Van Nam, 2003). The virus is a member of the genus *Alphaherpesvirus*, family *Herpesviridae*, that has the ability to multiply and cause tumors in poultry internal organs (Witter *et al*., 2005). The virus has three serotypes based on their serological characteristics: serotype 1 (MDV-1), serotype 2 (MDV-2) and serotype 3 (MDV-3). Serotype 1 is caused by Gallid herpesvirus 2 (GaHV-2), which is a virus with high virulence, immunosuppressive ability, and the ability to form tumors, resulting in significantly economic losses in many countries (Lee *et al*., 2008; Kennedy *et al*., 2017). Serotype 3 is caused by meleagrid herpesvirus 1 (MeHV-1), also known as HVT, which is less virulent and has no tumorigenesis. In particular, serotype 2 caused by Gallid herpesvirus 3 (GaHV-3) (also known as turkey herpesvirus) is not capable of causing disease in chickens and is thus of little concern.

MDV is a large DNA virus with a size of approximately 174 kb. Among MDV serotypes, GaHV-2 has the largest genome (174,076 bp), followed by GaHV-3 (164,270 bp) and MeHV-1 (160,673 bp) (Kingham *et al*., 2001). All three MDV serotypes' genomes are similarly organized, with long unique (UL) and short unique (US) regions, connected by long terminal repeats (TRLs) and short terminal repeats (TRSs), long internal repeats (IRLs) and short internal repeats (IRSs); and at least 90 open reading frames. Some genes, such as those encoding gB (glycoprotein B), gC, gD, and gH, have significant similarities across serotypes (Lee *et al*., 2000).

MDV serotype 1 (GaHV-2) encodes over 100 genes, the majority of which are present in the UL and US regions. Many genes involved in virus virulence have been identified within the genome, including the *Meq*, icp4 and vil-8 genes. Among them, the *Meq* gene has been identified as the virulence gene, which is primarily responsible for the virus's virulence (Davison and Nair, 2004). The *Meq* gene is 1020 bp in length, located in the genome's long repeat region, and has been linked to virus carcinogenicity and pathogenicity (Renz *et al*., 2012). Previous research showed that different polymorphisms and point mutations in the *Meq* gene region were linked to MDV virulence, in particular, the number of proline repeat (PPPP) motifs. Virulent viruses (v, vv and vv+ strains) are characterized by the presence of two to five repeats (Renz *et al*., 2012). The attenuated strains and vaccines have from 7-8 motifs motif (Renz *et al*., 2012; Shamblin *et al*., 2004; Mescolini *et al*., 2019). In addition, mutations in the second position of the PPPP motif (P(Q/A/R) PP) have also been found in virulent MDV strains (Shamblin *et al*., 2004).

Based on mortality and morbidity in infected animals, MDV-1 has five disease patterns as described in the following: attenuated (a), mild virulence (m), virulent (v), very virulent (vv) and extremely virulent (vv+) (Witter *et al*., 2005). Recently, molecular epidemiological studies around the world have discovered evidence of recombination between MDV strains.

In order to understand the molecular epidemiology of the virus and contribute significantly to effective disease prevention and control, it is crucial to identify the virus strains that are spreading and causing disease in Vietnam.

In this work, we have identified the MDV virus strains circulating in Bac Ninh

province in 2019–2022 period as the very virulence strains (vv) by using the molecular biology approach of decoding and analysis the *Meq* gene sequence.

MATERIALS AND METHODS

Sample collection and DNA extraction

Samples of hair follicles and liver obtained from 25 chickens suspected of being infected with MDV during the 2019-2022 period, from different farms in Bac Ninh province. The samples were refrigerated and transported to the laboratory within 24 hours, and they were preserved at -80°C until be used. These birds had been vaccinated with CVI-988 vaccine when they were one day old.

Purification, isolation, and identification of virus

Organs were homogenized, and the mixture was clarified by centrifugation at 1500 rpm for 15 min and passed through a 0.22-µm filter. The viral suspensions were stored at -80°C. Primary cultures of chicken embryo fibroblasts (CEFs) were prepared according to standard protocols (Hernandez, Brown, 2010).

A volume of 100 µL of purifed 10% virus suspension was used to inoculate the cells, which were then incubated at 37°C and 5% CO_2 for 7 days. The cells were harvested after the appearance of a cytopathic effect (CPE) and lysed by two cycles of freezing and thawing.

DNA extraction and detection of the viral genome by PCR

Total genomic DNA was extracted using the DNeasy Blood & Tissue kit (Catalog 69504, Qiagen, Germany) following the manufacturer's instructions. The DNA content was quantified by a NanoDrop® ND-1000 UV-Vis Spectrophotometer to determine the concentration of DNA template for PCR reactions. For each PCR reaction with a volume of 50 µL, 2 µL (approximately 100 ng) of DNA template was used.

Design primers for PCR amplification

Two primer pairs were designed for multiplex-PCR to diagnose MDV. These primer pairs were designed to capture different PCR products. A Marek-F and Marek-R primer pair with a size of 773 bp with field samples and about 950 bp with CVI988 vaccine samples were designed based on the *Meq* gene sequences available in the Genbank (http://www.ncbi.nlm.nih.gov). The specific primer pair for MDV serotype 3 is HVT1-HVT2 with a size of 505 bp according to Handberg *et al*. (2001) (Table 1). The whole *Meq* gene acquisition primer pair was designed to amplify the PCR product with a size of about 1.6 kb in order to obtain the entire *Meq* gene (1020 bp) (Table 1). The Taq PCR master mix (Catalog 201443, Qiagen, Germany) was used to perform PCR reaction. The multiplex-PCR reaction mixture had a total volume of 25 µL, consisting of 12.5 µL PCR master mix (Qiagen, Germany), 4 µL primer mix, 2 µL DNA template, 1 µL DMSO (dimethyl sulfoxide), and DEPC deionized water up to 25 µL. The PCR reaction mixture had a total volume of 50 µL, consisting of 25 µL PCR master mix (Qiagen, Germany), 2 µL each primer (10 pmol/µL), 3 µL DNA template, 2 µL DMSO (dimethyl sulfoxide), and 16 µL DEPC deionized water. PCR amplification reaction was performed on MJ PTC-100 (USA), and the thermal cycles included 1 cycle at 95^{o}C/5 min, 35 cycles at (95^{o}C/1 min,

50°C/1 min; 72°C/1 min), last cycle at 72°C/10 min. The PCR products (10 µL of each) were examined on a 1% agarose gel, stained with ethidium bromide and visualized under UV light (Wealtec, Sparks, NV, USA). Single-band PCR products were purified using the QIAquick PCR purification kit (Catalog 28104, Qiagen, Germany) or isolated the PCR product DNA bands of the intended size (if multiple bands present) using the QIAquick Spin Columns (Catalog 28115, Qiagen, Germany).

Table 1. List of primers used in this study for serotyping and sequencing.

Primers	Sequence (5' → 3')	Target gene	Length
Marek-F	YGACGGCCTATCTGAGGAGG	*Meq* gene	773 bp/ serotype 1, virulent strain
Marek-R	GGATCCTCGGTAAGACGAGC		950 bp/ serotype 1, vaccine CVI988 strain
HVT1	ATGGAAGTAGATGTTGAGTCTTCG	US3 gene	505 bp/ serotype 3
HVT2	CGATATACACGCATTGCCATACAC		
GaHV2 - MeqF	AATTGTGACCGTTCGCGAACG	*Meq* gene	1.6 kb/ PCR and sequencing
GaHV2 - MeqR	TTCCGAGTCTAAGCTACACGG		

Nucleotide sequencing and computational analysis

The PCR products were cloned into the cloning pCRTMII vector (Thermo Fisher Scientific). The recombinant plasmid DNA was extracted and sequenced using primers M13F-M13R. The sequences were identifed using Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST). Multiple nucleotide and amino acid sequence alignments of the MDV-1 strains and previously reported global MDV-1 sequences in the GenBank database were performed using GENEDOC 2.7 (http://iubio.bio.indiana.edu/soft/molbio/ibmpc/genedoc/readme.html) (Table 2).

RESULT AND DISCUSSION

Diagnosis of MDV by multiplex-PCR

Of the total 25 field samples, by using multiplex-PCR with a mixture of two pairs of diagnostic primers to determine MDV serotype 1 and MDV serotype 3 (Table 1), we identified four samples containing virulent MDV-1 (GaHV-2), including samples BN1-VN, BN3-VN, BN5-VN and BN22-VN (Table 1, Figure 1).

The PCR product of the studied MDV samples gave a unique PCR product of 773 bp in size, indicating the presence of MDV serotype 1, a virulent field strain, not a vaccine strain.

Table 2. List of MDV-1 strains used in this study for analysis of *Meq* gene.

	Strain	Genbank	MDV virulence	Country	Year
1	CU-2	AY362708	-	USA	-
2	CVI-988	DQ534538	att	Netherlands	-
3	584a	DQ534532	vv+	USA	-
4	648A	AY362725	vv+	USA	1994
5	660-A	AY362726	vv+	USA	-
6	L	AY362717	vv+	USA	-
7	New	AY362719	vv+	USA	-
8	N	AY362718	vv+	USA	-
9	RL	AY362720	vv+	USA	-
10	TK	AY362721	vv+	USA	-
11	X	AY362724	vv+	USA	-
12	GX0101	JX844666	vv	China	2001
13	YA	HQ638156	vv	China	-
14	LMS	JQ314003	vv	China	2007
15	WS03	HQ638152	vv	China	-
16	GX070060	EU427303	vv	China	2008
17	YLO40920	DQ174459	vv	China	2005
18	GXY2	EF546430	vv	China	2007
19	JL-1404	KU744559	vv	China	2014
20	WC-1203	KU744558	vv	China	2012
21	GX070079	EU427304	vv	China	2008
22	LTS	KP888838	vv	China	2012
23	MDJ3-1301	KP888849	vv	China	2013
24	1409	KU744560	vv	China	2014
25	BN1-VN	This study		Bac Ninh, Vietnam	2019
26	BN3-VN	This study		Bac Ninh, Vietnam	2019
27	BN5-VN	This study		Bac Ninh, Vietnam	2020
28	BN22-VN	This study		Bac Ninh, Vietnam	2022

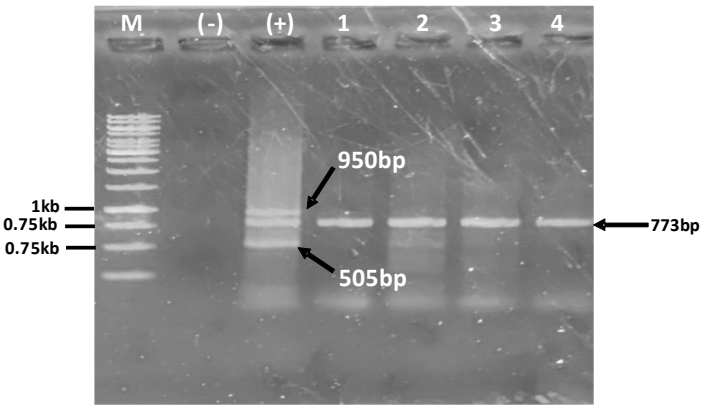

Figure 1. Detection of the *Meq* gene of MDV-1 strains using *Meq*-specific PCR. PCR was performed using 2 pairs of primers as described in Table 1. PCR was also performed with positive control (950 bp band is from the MDV serotype 1 vaccine sample; 773 bp band is from the virulent strain MDV sample; 505 bp band is from the MDV serotype 3 sample) and negative control (DNA template is pure water).

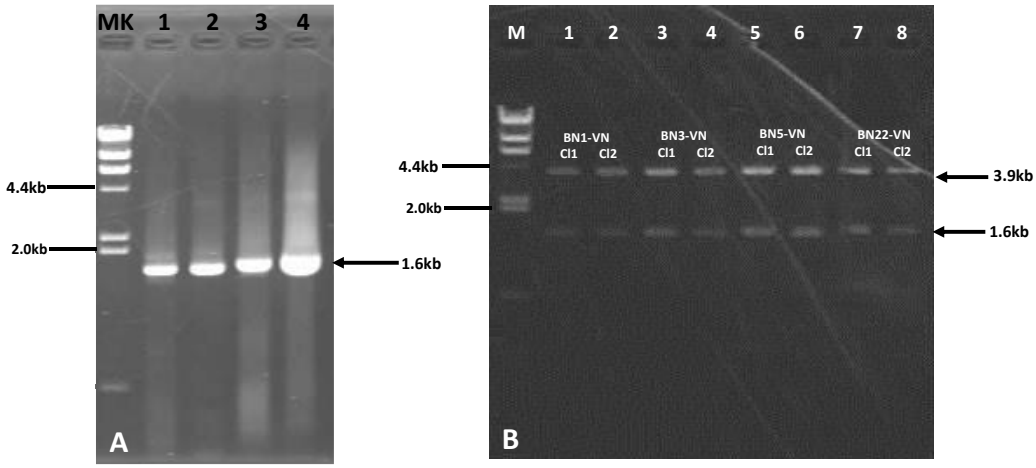

Figure 2. PCR products using primer pairs GaHV2-MeqF and GaHV2-MeqR **(A)** and restriction enzyme plasmid DNA cutting products of four MDV-1 strains studied **(B)**

Sequence analysis of the *Meq* gene of four Vietnamese MDV-1 strains

Using primer pairs GaHV2-MeqF and GaHV2-MeqR, PCR products of about 1.6 kb in size were obtained from the four samples. Electrophoresis results showed that the PCR product was a bright, clear, good quality single band (Figure 2A). The PCR products were then purified and cloned into the pCRTMII vector. Plasmid DNA was extracted using the QIAprep Spin Miniprep Kit, and the cloning product was checked using the restriction enzyme *Eco*RI (Figure 2B) before being sequenced with the M13F-M13R primer pair.

Based on the sequencing results, the length of the *Meq* gene for all MDV-1 strains was identified as 1020 nt coding for 339 aa. The complete *Meq* gene sequences of 4 Vietnamese isolates were aligned with 40

other MDV sequences for MDV-1 and MDV-3 vaccine available in Genbank. The results of the sequence comparison showed that for nucleotide and amino acid, there was 91%–100% identity between the 4 Vietnamese samples and 35 strains of MDV-1, and 84.2%–84.7% identity between these 4 Vietnamese samples and 5 strains of MDV-3. The intergenotypic variation between MDV-1 and MDV-3 reached over 15%. Four Vietnamese strains have 3 mutations in nucleotide at positions 79 (T><C); 417 (A><G), 884 (A><C), and all three nucleotide mutations lead to amino acid changes at positions 27(S><P), 139(A><T) and 295 (Q><P). The Vietnamese strains in this study have a high identity of nucleotide and homogeneity with Chinese strains (99.2%–100% and 98.2%–100%, respectively) and has the low identity with attenuated vaccine strains CVI-988 and CU-2 (84.2%–82.6% and 83.2%–83.7%, respectively). The CVI-988 vaccine is widely used around the world as well as in Vietnam. The amino acid differences between MDV serotypes 1 and 3 are shown in Figure 3.

The dot (.) indicates a nucleotide that is identical to the corresponding sequence in the att-CVI988 strain, which is the reference strain. The nucleotide differences of the following species are indicated by their symbolic letters. The samples from Vietnam directly used in this study are bolded. The consistent differences from all other MDV strains are shown in a box.

The field MDV-1 strains have a standard *Meq* gene size of 1020 bp encoding 339 aa, while the attenuated strains have a large *Meq* (L-*Meq*) size (399 aa) due to the insertion of at least 177 nucleotides from position 562 to 739 (Lee *et al*., 2000; Mutara *et al*., 2007; Renz *et al*., 2013). The L-*Meq* were also found in the strains of China (strain 814) and Russia (strain 3004), but not in the highly virulent strains of US origin or the field MDV-1 strains of Vietnam.

The pathotype and virulence of MDV-1 strains are typically determined by specific features of the Meq gene, including its length, proline percentage, number of PPPP motifs, and number of PR/S/APPP motifs (Lachheb *et al*., 2020; Wajid *et al*, 2013). The highly virulent strains had a lower proline ratio than the lowly virulent strains. The highly virulent strains have fewer PPPP motifs, while attenuated strains have more PPPP motifs. According to published data, highly virulent US strains have two PPPP motifs, while vaccine attenuated strain CVI 988 have eight PPPP motifs and moderately virulent strains including the CU-2 vaccine strain have seven PPPP motifs. In addition, the highly virulent strains have additional interrupted motifs P(Q/A/R)PP (Lachheb *et al*., 2020).

The results of *Meq* gene analysis showed that all four strains of GaHV-1 circulating in Bac Ninh from 2019 to 2022, which had low proline rates, from 20.59 to 21.18%; three PPPP motifs (at positions 152-155; 191-194 and 232-235) and three interrupted PPPP motifs in the second position (PRPP at positions 176-179, PAPP at positions 217-220 and PQPP at positions 246-249) (Figure 4), which are similar to the highly virulent Chinese strains: GX070079 (EU427304), YA (EU427304) and LTS (KP888838). The data demonstrated that the GaHV-1 strains circulating in Bac Ninh, Vietnam are highly virulent (vvMDV). So far, there are not many publications on molecular characterization of MDV in Vietnam.

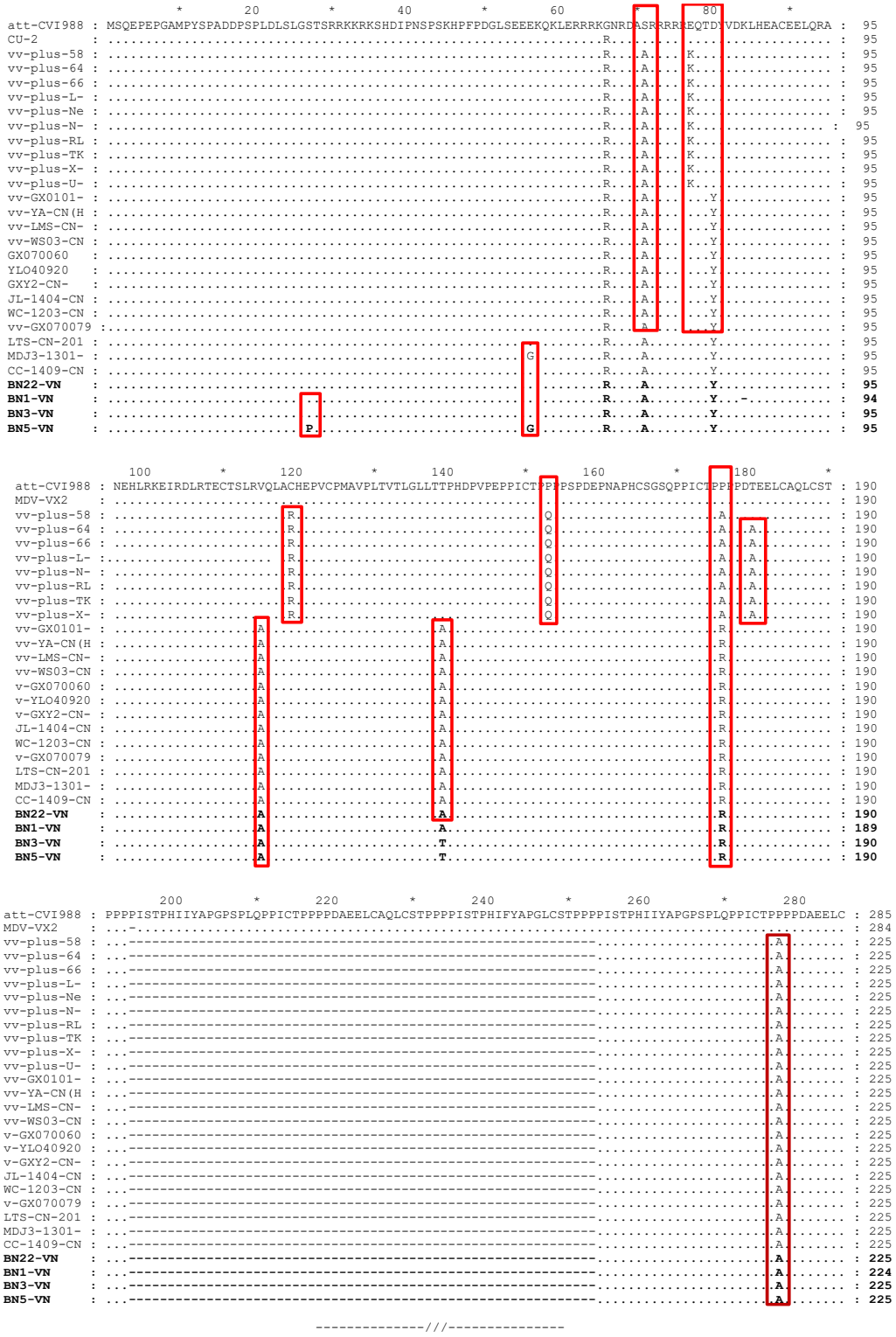

Figure 3. The variable positions in the *Meq* protein of the MDV-1 strains with different geographical origins

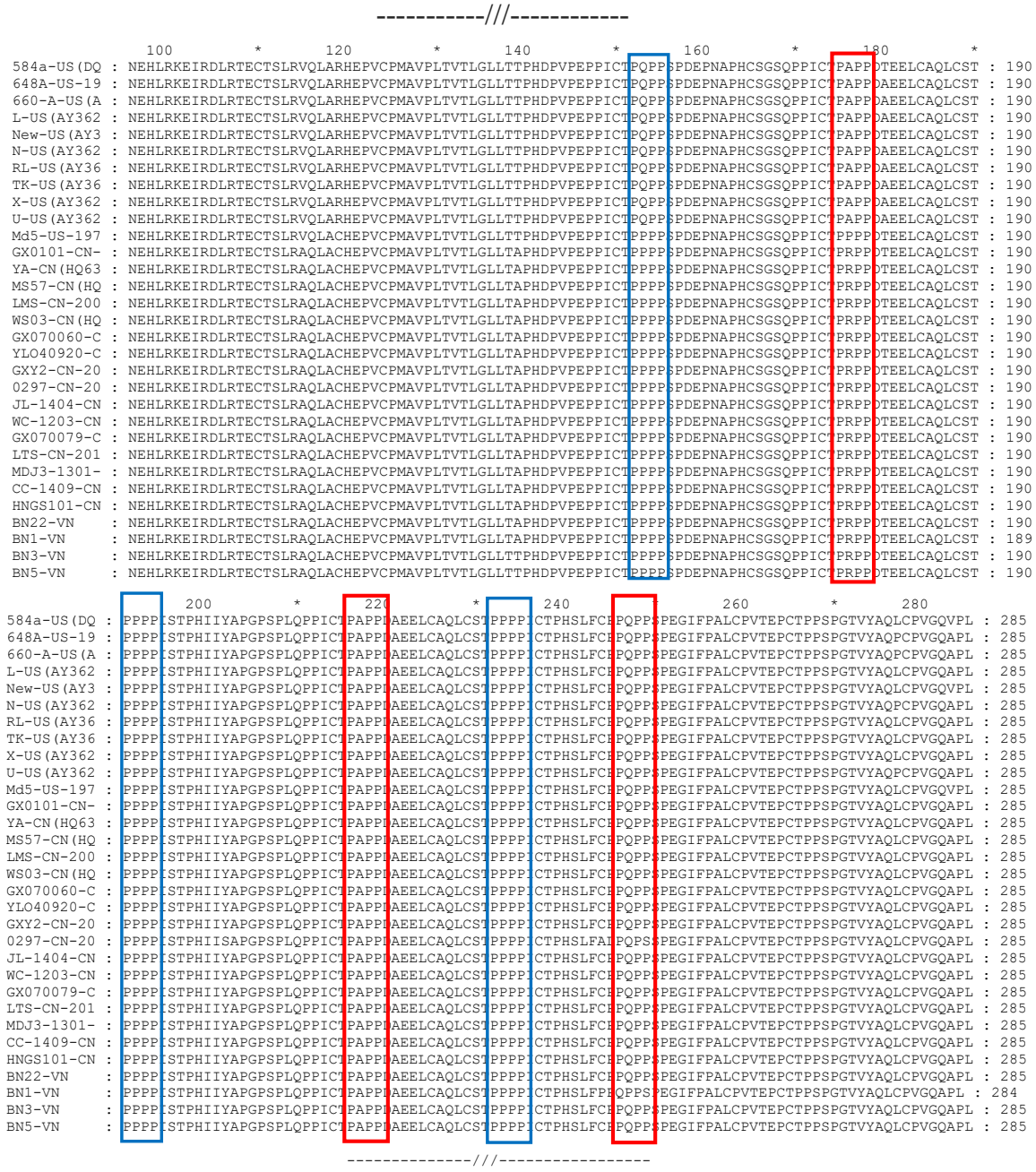

Figure 4. PPPP motifs and interrupted motifs in the *Meq* gene of MDV-1 strains. The PPPP motifs are shown in the blue box. The interrupted motifs are shown in the red box.

A previous publication revealed the MDV-1 strains circulating in chickens in Can Tho province also belong to the highly virulent group (Huynh *et al.*, 2022). However, compared to three interrupted PPPP motifs in the Bac Ninh's strains, the Can Tho's strains had only one this motif, indicating that MDV-1 strains circulating

in Bac Ninh province have higher virulence.

The extremely virulent group of GaHV-2 (vv+MDV) has only been announced in the US. These highly virulent strains were not detected in Asia.

Worldwide, Marek's disease is largely controlled through a series of immunization programs with live attenuated GaHV-2 vaccines. Despite great efforts by countries to control the spread of Marek's disease through mass vaccination strategies, there is ample evidence that GaHV-2 has been evolving to become more virulent (Feng *et al*., 2012; Atkins *et al*., 2013, Katherine *et al*., 2013). The biggest concern of the poultry industry is that current vaccines do not protect chickens against many new strains of field viruses (Hao *et al*., 2021). The application of PCR for the diagnosis and sequencing analysis of MDV can significantly improve our understanding of the epidemiology, transmission, diagnosis, and control vaccines of MD. In addition, the identification of disease patterns is also necessary to monitor and design effective vaccination programs in Vietnam.

CONCLUSION

The study has determined the circulation of high virulent MDV-1 (vvMDV) in Bac Ninh province from 2019 to 2022. Four of the strains in the study have high similarity with vvMDV strains from China but low similarity with vaccine strain CVI988. Our results will be helpful for understanding the molecular characterization of MDV in Vietnam.

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