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Research article

Prevalence of Marek's disease virus in unvaccinated healthy backyard chickens in Cantho city, Vietnam and genetic characterization of *meq* gene

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Abstract

An investigation was carried out to survey the prevalence of Marek's disease virus serotype 1 (MDV-1) on unvaccinated healthy backyard chicken flocks from four districts in Cantho city and to analyze the genetic characterization of the *meq* gene. A total of 353 feather follicle samples were collected from 50 unvaccinated chicken flocks for Marek's disease to investigate prevalent MDV-1 by polymerase chain reaction (PCR). The four represent strains were chosen for *meq* gene sequencing. The results revealed that 26 out of 353 samples were positive for MDV-1 accounting for 7.37%. There were significant differences in the prevalence of MDV-1 on chickens among districts ($P<0.05$). The Meq protein of the four strains (VT, CD, PD, and TL) contained 20.14% proline and three regions of proline repeats. Besides, all four strains occurred with amino acid point mutations. These strains had only a substitution of proline at position 217 as well as interruption of consecutive proline at site 2. Phylogenetic analysis indicated that these strains were within a group relating to virulent Italian isolates.

Keywords: Backyard chickens, Cantho, Marek's disease, *meq* gene, Unvaccinated

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INTRODUCTION

Marek's disease (MD) is caused by the infection of Marek's disease virus serotype 1 (MDV-1). MD causes economic losses due to lower feed conversion, weight loss, and egg production decrease (Rozins et al., 2019). Besides, the virus has impacted an indirect economy by increasing the requirement for hygiene. It has also caused immunosuppression, which leads to animals being more susceptible to secondary infections (Gimeno and Schat, 2018; Rozins et al., 2019). Overall, economic losses have been caused by MD of about $1–2 billion in industrial poultry annually (Morrow and Fehler, 2004).

Chickens are infected by inhalation of contaminated dust from the poultry houses, and the infected chickens will shed the virus from the feather follicles (Baigent and Davison, 2004). MDV is also considered to be a major cause of mortality in backyard chickens, and the virus has persisted in the environment (Pohjola et al., 2015; Mete et al., 2016). Backyard chickens are not generally vaccinated, and the applied biosecurity is not constantly concerned. This permits the virus to be present in the environment, and it causes a threat to industrial chicken farms nearby (Cecchinato et al., 2011). The *meq* gene is one of the genes related to an increase of virulent MDV-1 (Trimpert et al., 2017). Circulation and genetic characterization of MDV-1 in healthy backyard chickens without vaccination against MD in Cantho city have been not reported. Therefore, the study was conducted to determine the status of MDV-1 infection in unvaccinated healthy backyard chickens for MD and genetic characterization of the *meq* gene of MDV-1.

MATERIALS AND METHODS

Sample collection

Chicken feather follicles were obtained from 50 flocks of apparently healthy backyard chickens in 4 districts (Phong Dien, Co Do, Thoi Lai, Vinh Thanh) in Cantho city, Vietnam, and all the flocks were unvaccinated for Marek's disease. Seven to eight chickens were collected from each flock, from every chicken, 5 wing feather follicles were collected (López-Osorio et al., 2019). The chickens were treated in accordance with Vietnam's Animal Husbandry Law (32/2018/QH14).

DNA extraction

Five feather follicles from each bird were pooled into a sample. All pooled samples were then processed to detect the *meq* gene by polymerase chain reaction (PCR). Total DNA was extracted from the pulp of feather follicles using a commercial kit TopPURE® Tissue viral extraction (ABT Biomedical, Solution Company, Vietnam). The process of DNA extraction was performed to the manufacturer's instructions.

PCR amplification of *meq* gene

A total of 353 processed samples were tested by PCR, using serotype 1 specific primer (Table 1). The *meq* gene (1148 bp) of MDV-1 was amplified, according to López-Osorio et al. (2017). PCR reaction was performed in a final

volume of 25 µl, comprising 12 µl of Master Mix 2X Tracking dye (2X), 3 µl of template DNA, 8 µl of H_2O for molecular biology, 1 µl of forwarding primer, and 1 µl of the reverse primer. PCR conditions included initial denaturation at 94°C for 5 mins, followed by 35 cycles of denaturation at 94°C for 1.5 mins, annealing at 57°C for 1 min, and extension at 72°C for 2 mins. A final elongation step at 72°C for 5 mins completed the reaction. The PCR products were separated on a 1.5% agarose gel prepared with TAE 1X (40 mM Tris, acetic acid, 2 mM EDTA) buffer and stained with Safe Dye (Phusa biochem, Vietnam) (1 mg/ml). The gel was visualized under ultraviolet light after electrophoresis at 150V and 400 mA for 40 mins. Samples were considered positive for MDV-1 by amplification of products of 1148 bp.

Table 1 Primer used for the PCR for detection of the serotypes 1 of MDV.

Primer	Target gene	Sequences	Product size (bp)
MDV-1 (GaHV-2)	*meq*	F: 5'-CCGCACACTGATTCCTAGGC-3' R: 5'-AGAAACATGGGGCATAGACG-3'	1148

Sequencing and phylogenetic analysis

From positive samples with PCR, the representatives were chosen for sequencing and phylogenetic analysis. Each of them was presentative for each district which was named VT, CD, PD, and TL. Amplification of the *meq* gene was conducted by Sanger dideoxy sequencing. The obtained nucleotide sequences and the deduced amino acid sequences of the *meq* genes of MDV-1 were edited computationally using the Bioedit Sequence Alignment Editor Version 7.2.5.0 (Tom Hall, Ibis Therapeutics, Carlsbad, CA). Alignment and comparison were conducted by using Clustal W software (Thompson et al., 1994). The phylogenetic tree was generated by the Maximum-Likelihood tree method using MEGA.X (Kumar et al., 2016), and the liability of internal branches was assessed by 1000 bootstrap replications. The reference sequences of MDV-1 were retrieved from the GenBank database, and their accession number was listed in Table 2.

Table 2 MDV-1 (GaHV-2) strains retrieved from GenBank which were included in the molecular analysis.

GenBank accession number	Strains/isolate	Country of origin	Year	Pathotype
AF493556	G2	China	1995	*vv*
AY362708	CU-2	USA	2004	*m*
AY362717	L	USA	1997	*vv+*
AY362718	N	USA	1999	*vv+*
AY362723	W	USA	2004	*vv+*
AY362724	X	USA	2002	*vv+*
AY362725	648A	USA	2004	*vv+*
DQ534538	CVI988	USA	1969	*att*
EF523771	FT158	Australia	2002	*v*
EF523772	02LAR	Australia	2004	*v*
EF523773	04CRE	Australia	2007	*v*
EF523774	MPF57	Australia	2007	*v*
EU032468	3004 MEQ	Russia	2007	*m*
HF546084	HNGS101	China	2013	*vv*
HG328238	Anhui001	China	2013	*vv*
HQ638141	DY01	China	2011	*vv*
KJ949618	MDV/2/SA/2013	India	2013	*vv*
MK139661	GaHV-2/Italy/CK/507/15	Italy	2015	*v*
MK139662	GaHV-2/Italy/CK/509/15	Italy	2015	*v*
MK139663	GaHV-2/Italy/CK/510/15	Italy	2015	*v*
MK139672	GaHV-2/Italy/CK/847/17	Italy	2017	*v*
MK139678	GaHV-2/Italy/CK/855/15	Italy	2017	*v*
MN943294	GX18LZM1	China	2020	*vv*
MN943299	GX18NNM5	China	2018	*vv*
MT799507	GX20GG2	China	2021	*vv*

Statistical analysis

The comparison of prevalence of MDV-1 on chicken flocks among districts was statistically analyzed by Chi-square test using Minitab 16.0 software.

RESULTS

Prevalence of MDV-1 in unvaccinated healthy chickens

A total of 353 feather samples from unvaccinated healthy chicken flocks were examined, and the results showed that 26 out of 353 samples were positive for MDV-1 comprising 7.37%. The prevalence of MDV-1 in chicken in Phong Dien district was highest with 16.07%. followed by Co Do (8.60%), Thoi Lai (5.43%) and Vinh Thanh (3.57%). There were significant differences in the prevalence of MDV-1 in the surveyed chickens among the districts.

Molecular characteristics of the *meq* gene

Meq gene sequencing was completely successful, and four strains were from each district, namely VT, CD, PD, and TL. Among representative strains, the highest similarity of amino acid (aa) sequences was observed between PD and TL (99.68%), followed by VT and CD strains with 99.22% identity of aa sequences. Aligning the sequence of all these nucleotides showed that the four strains had higher similarity to vaccine CVI 988 (88.41%-99.48%) mild CU-2 (88.41%-99.48%) and virulent Italian strains (88.98%-99.48%) (data not showed). Besides, amino acid substitutions were also recorded in Meq protein of the four investigation strains at positions 80 (D →H), 93 (Q →R), 98 (H→D), 101 (K→N), and 217 (P→A). Moreover, the substitution of proline was recognized in Meq protein of all four surveyed strains at position 217 (P217A) (Table 3), and interruption of the PPPP sequence at position 2 (PPPP → PAPP) compared to vaccine strain CVI 998 (Intervet).

The CVI 998 has encoded 318 aa, whereas the four strains VT, CD, PD, and TL have encoded 278 aa. Proline was encoded by the *meq* gene of the four strains accounting for 20.14%. The number of proline repetitions, as well as proline content of these strains and reference isolates were shown in Table 4. The most virulent strains had some proline repetitions from 2 to 5 whereas attenuated and mild strains had a higher number of these repeats (7 to 8).

Table 3 Amino acid substitutions in the *Meq* protein of MDV-1 (GaHV-2) strains.

Identification		**Amino acid position in the *Meq* protein of MDV-1**												
Strain	Pathotype	71	77	80	93	98	101	115	119	139	153	176	180	217/ 276
CVI988	*att*	S	E	D	Q	H	K	V	C	T	P	P	T	P
CU-2	*m*	S	E	D	Q	H	K	V	C	T	P	P	T	P
FT158	*v*	S	A	A	Q	H	K	A	C	T	P	P	T	A
02LAR	*v*	S	A	D	Q	H	K	A	C	T	P	P	T	A
Italy/507	*v*	S	E	E	R	D	N	V	C	A	P	P	T	-
Italy/510	*v*	S	E	E	R	D	N	V	C	A	P	P	T	-
GX18LZM1	*vv*	A	E	Y	Q	H	K	A	C	A	P	R	T	A
G2	*vv*	A	E	Y	Q	H	K	A	C	T	P	P	T	A
HNGS101	*vv*	A	E	Y	Q	H	K	A	C	A	P	R	T	A
DY01	*vv*	A	E	Y	Q	H	K	A	C	A	P	R	T	A
L	*vv+*	A	K	D	Q	H	K	V	R	T	Q	A	A	A
X	*vv+*	A	K	D	Q	H	K	V	R	T	Q	A	A	A
648A	*vv+*	A	K	D	Q	H	K	V	R	T	Q	A	A	A
VT		S	E	H	R	D	N	V	C	T	P	P	T	A
CD		S	E	H	R	D	N	V	C	T	P	P	T	A
TL		S	E	H	R	D	N	V	C	T	P	P	T	A
PD		S	E	H	R	D	N	V	C	T	P	P	T	A

Table 4 Meq protein features of MDV-1 (GaHV-2) strains.

Strain/isolate	Pathotype	Size of Meq (aa)	Number of proline	Number of proline repeat	Proline content (%)
G2	*vv*	339	72	4	21.18
CU-2	*m*	398	92	7	23.06
L	*vv+*	339	70	2	20.59
N	*vv+*	339	71	2	20.88
W	*vv+*	339	72	3	21.18
X	*vv+*	339	70	2	20.59
648A	*vv+*	339	71	2	20.88
CVI988	*att*	318	76	8	23.90
FT158	*v*	398	90	5	22.56
02LAR	*v*	398	90	5	22.56
04CRE	*v*	398	90	5	22.56
MPF57	*v*	398	90	5	22.56
3004 MEQ	*m*	479	50	0	10.44
HNGS101	*vv*	339	71	3	20.88
Anhui001	*vv*	339	71	3	20.88
DY01	*vv*	339	71	3	20.88
MDV/2/SA/2013	*vv*	273	55	3	20.15
GaHV-2/Italy/CK/507/15	*v*	190	34	3	17.89
GaHV-2/Italy/CK/509/15	*v*	190	34	3	17.89
GaHV-2/Italy/CK/510/15	*v*	190	34	3	17.89
GaHV-2/Italy/CK/847/17	*v*	190	34	3	17.89
GaHV-2/Italy/CK/855/15	*v*	273	53	2	19.41
GX18LZM1	*vv*	339	71	3	20.88
GX18NNM5	*vv*	339	71	3	20.88
GX20GG2	*vv*	339	71	3	20.88
VT		278	56	3	20.14
CD		278	56	3	20.14
TL		278	56	3	20.14
PD		278	56	3	20.14

Phylogenetic tree analysis (Figure 1) was based on the nucleotide sequences of the *meq* gene of the four strains VT, CD, PD, TL, and the reference MDV-1 strains in the world were given in Figure 1. The strains from the USA have been classified into two groups including mild (m) and very virulent plus (vv+) whereas the isolates from Italy and Australia were virulent (v). In contrast, all Chinese isolates fall into the very virulent (vv) group. Alignment of *meq* gene sequences revealed that the four strains VT, CD, PD, and TL clustered with virulent Italian isolates with a bootstrap value of 90.0%.

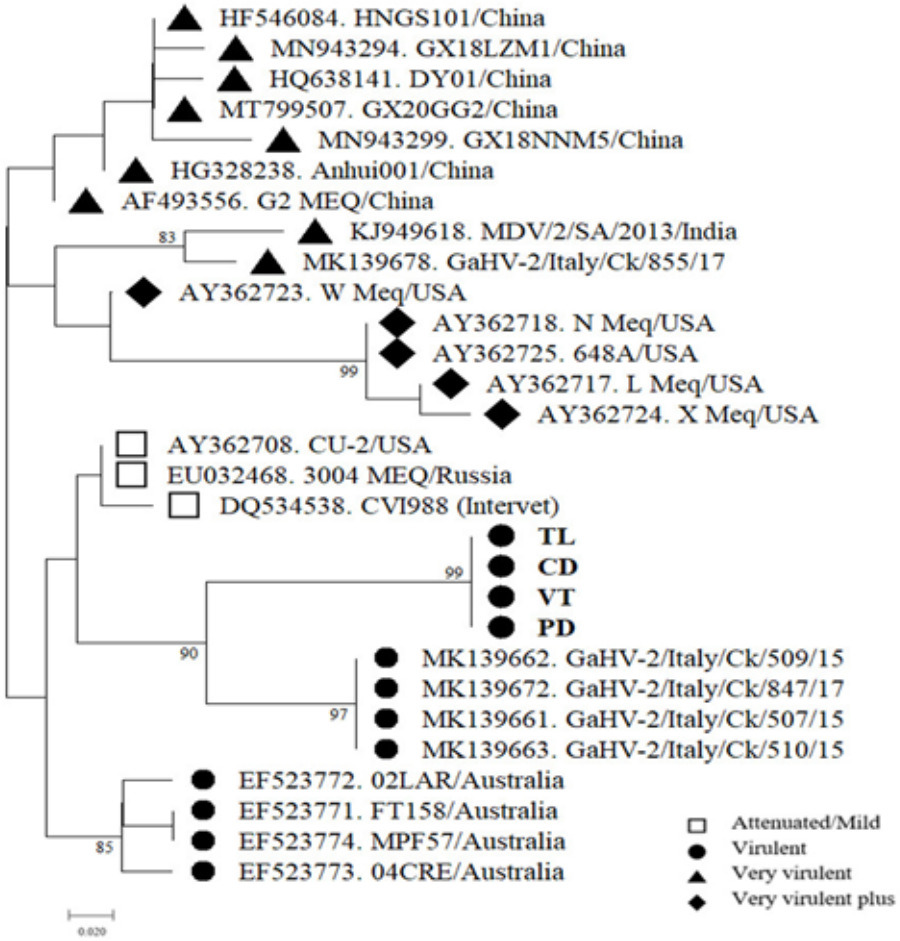

Figure 1 Phylogenetic tree of MDV-1 (GaHV-2) based on the *meq* gene. The tree was constructed using the maximum-likelihood algorithm implemented in MEGA.X with 1000 bootstrap values ≥ 65 replicates.

DISCUSSION

Prevalence of MDV-1

All surveyed chickens were not vaccinated against MD so the positive chickens could be infected by the field virus strains. The virus has been shed into the environment from the feather follicle of infected chickens that have been considered a major source for transmission to other chickens in nature (Couteaudier and Denesvre, 2014). Chickens have been infected by inhalation of dust contamination (Baigent and Davison, 2004). Calnek et al. (1970) have demonstrated that cells of feather follicles can keep virus and virus transmission for a long time. Ho et al. (2021) determined that prevalence of MDV-1 in unvaccinated chickens against MD in Dong Thap province was 27.27%. In addition, the study also indicated that the prevalence of MDV-1 in the chickens from the four locations was significantly different. This could be different genetics of chicken breeds. Bumstead and Kaufman (2004) reported that chicken genetics have been associated with resistance to MDV infection.

Meq gene sequencing

The *meq* gene of MDV-1 from 4 strains VT, CD, PD, and TL had a high degree of identity although they were from distinct areas of Cantho city. They appeared on point mutations at some positions of *Meq* protein that was confirmed in some previous research (López-Osorio et al., 2017; Mescolini et al., 2019; Shi et al., 2021).

Mutant point of *meq* gene associated with virulence of MDV (Shamblin et al., 2004). Moreover, interruption of four consecutive prolines at position 2 (PPPP → P(Q/A)PPP) in Meq protein is related to virulent strains (Shamblin et al., 2004). All four strains had only the interruption of four consecutive prolines at position 217. Proline interruption at position 217 was reported by Lachheb et al. (2020) when they analyzed Meq protein from Tunisian isolates (TN1013/16, TN1014/16). The interruption of four consecutive prolines was observed in all virulent strains except RB1B (Lachheb et al., 2020). In addition, Renz et al. (2012) reported that v, vv, vv+ strains have been characterized by the presence of two to five repeats, and a low proline content has been shown to correlate with high virulence. The four strains in this study contained 20.14% proline and three PPPP, which were relatively similar to the v, vv reference strains. Besides, a phylogenetic tree was based on the *meq* gene of the four strains VT, CD, PD, and TL within a group that related to virulent Italian isolates (Fig. 1).

CONCLUSIONS

The study has determined the circulation of MDV-1 in the unvaccinated healthy backyard chicken flocks. All these four strains were within a group that related to virulent isolates from Italy. Point mutations of amino acid occurred in all these strains and second proline molecular substitution at site 217.

AUTHOR CONTRIBUTIONS

This work was carried out with the contribution of all authors. HNT and NTPC collected and analyzed the samples. HNT and HTVT interpreted the data and prepared the manuscript. All authors read and approved the final manuscript.

CONFLICT OF INTEREST

We have no conflict of interest.

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