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Detection of MDV Very Virulent Strain by Polymerase Chain Reaction and Analysis of its Meq Gene

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Abstract

In this study, virus isolation, polymerase chain reaction (PCR) detection, sequencing and animal test were used to detect the clinical case of chickens infected with Marek's disease (MD). Pathologic changes with tumor lesions could be found on the liver, kidney and spleen of the infected chickens. Results of the virus isolation, PCR detection and Meq gene sequencing showed that the clinical case was caused by Marek's disease virus (MDV). The MDV isolate JZ2014 had Meq amino acid homology at 96.8-99.1% with 11 MDV reference strains. None of the vaccination with seven types of vaccines could completely protect the immunized chickens against JZ2014. The highest mortality was 70.3% in sham vaccine group. The lowest mortality was 23.0% in CVI988+SB1+HVT immunized group. The results indicated that the detected MDV JZ2014 could be a vvMDV strain, and the emergence of vv MDV was a significant problem for the poultry industry in China.

Keywords: Marek's disease virus; Challenge; Virulence; Vaccine

Introduction

Marek's disease virus (MDV) also known as Gallid herpes virus 2 (GaHV-2), was an oncogenic poultry herpes virus, causing lymph proliferative and demyelinating disorder in infected chickens. In the 1970s, herpes virus of turkey (HVT) vaccine, which belonged to serotype 3 vaccines, was mainly used to control the disease. In the mid-1980s, a serotype 2 vaccine, such as SB-1 strain was used in combination with HVT against the enhanced virulence strains. With further increase in virulence of field viruses, CVI988 vaccine, which belonged to serotype 1 vaccine, was introduced for widespread use in the 1990s [1,2]. Recently, the failures of CVI988 vaccination have been reported, when CVI988 vaccine is used either alone or in combination with serotype 2 and/or serotype 3 vaccines, suggesting the emergence of high virulent strains [3,4]. At present, the widespread use of vaccines against Marek's Disease (MD) was suggested to have led to the evolution of field viruses with greater virulence, and a number of patho types classified as vMDV, vvMDV, and vv+MDV have been isolated [5,6], and more virulent strains could overwhelm the protection conferred by currently available vaccines [7]. In recent years, MDV isolates in China have been reported in breeder or layer flocks which have been vaccinated with HVT or CVI988 [8-11]. The virulent Marek's Disease virus strains (MDVs) circulating in China seemed to constitute a separate genotype different from exotic MDV reference strains [10,11], and these isolates were high pathogenic for chickens. In this study, detection of MDV very virulent strain and analysis of its Meq gene were carried out for a clinical case in a chicken farm infected with Marek's disease.

Methods

The following experimental researches on animals were performed with the approval of Experimental Animal Administrative Center of Shandong Province.

Samples collection

A severe MD broke out at a chicken farm, where the infected chickens were found to have MD pathologic changes, including enlargement of spleens, livers and kidneys, with tumors on the surface of the organs. Heparinized blood samples were collected from chickens with tumor lesions for diagnosis.

Virus isolation

The collected samples were kept under refrigeration and transported, then lymphocytes separated from the blood samples by lymphocyte separation medium were inoculated into primary duck embryo fibroblast (DEF) cells prepared from 11-day-old embryonated eggs, and the inoculated DEF cells were incubated at 37°C with 5% CO_2 for five days.

PCR detection

The deoxyribonucleic acid (DNA) for polymerase chain reaction (PCR) detection was purified from DEF cultures of virus isolation and blood samples of the infected chickens using mini-spin column chromatography method, and PCR detections specific for MDV, avian leukosis virus (ALV) and reticuloendotheliosis virus (REV) were carried out individually for the purified DNA. Primers [12,13] for MDV, ALV and REV were shown on Table 1. The MDV target gene of PCR was Meq gene, which was reported to have the greatest possibility which was associated with viral oncogenicity and pathogenicity.

PCR amplification was carried out using 2 μl DNA as template in a total volume of 50 μl containing 25 μl 2×Taq PCR Mixture, 2 μl of 10 μM of each of the two primers, and 19 μl ddH_2O.

The optimum conditions for PCR were as follows: 94°C for 4 min, 35 cycles at 94°C for 1 min, 56°C for 1 min, 72°C for 1.5 min, and final elongation at 72°C for 10 min. The PCR product was analyzed in 0.9% agarose in Tris-borate-EDTA (TBE) buffer gel containing 0.5 mg/ml ethidium bromide.

Virus	Primer sequence		DNA sizes
MDV	F: 5'-GGCACGGTACAGGTGTAAAGAG-3 '	R: 5'-GCATAGACGATGTGCTGCTGAG-3'	1081bp
ALV	F: 5'-AATTCTGCTTGAAATATG-3 '	R: 5'-AGTTGTCAGGGAATCGA-3 '	436bp
REV	F: 5'-CATACTGGAGCCAATGGTGTAAAGGGCAGA-3 '	R: 5'-AATGTTGTAGCGAAGTACT-3 '	291bp

Table 1: Primers for MDV, ALV and REV

Sequencing and analysis of Meq gene

The PCR products from the detected samples, corresponding to the predicted size, were cloned into pMD19-T, and then were sequenced by Sanger dideoxy sequencing method. After sequencing, the deduced amino sequence of Meq gene of the MDV isolate was compared with 11 reference MDVs for homology analysis with the use of Meg Align program. Among these 11 reference strains, two strains were vaccine strains, three strains were isolated from China, and six strains were isolated from USA. The MDV reference strains were retrieved from the Gen Bank database, and the backgrounds of the reference strains used in this study were listed in Table 2.

MDV strains	Virulence	Geographic origin	Year of isolation	Accession number
GX070060	High virulence	China	2008	EU427303
LS	High virulence	China	2008	HQ638149
JZ2014	Very virulence plus	China	2014	KP144355
SD2012-1	Very virulence plus	China	2012	KC511815
814	Vaccine strain	China	N/A	GU354326
CVI988	Vaccine strain	Netherland	1972	DQ534538
CU-2	Mild virulence	USA	N/A	EU499381
GA	virulence	USA	1964	AF147806
Md5	Very virulence	USA	1979	AF243438
RB1B	Very virulence	USA	1982	EF523390
648A	Very virulence plus	USA	1997	AY362725
584A	Very virulence plus	USA	Before 2000	DQ534532

Table 2: MDV reference strains published in Gen Bank

Animal test

270 specific pathogen free (SPF) chickens were divided into nine groupswith 30 chickens in each group. Among the nine groups, seven groups were used as immunization groups, and two groups were used as control groups with sham vaccine. In the seven immunization groups, the one-day-old chickens were vaccinated with seven different types of commodity MDV vaccines, and the immunization dosage for each group was the commodity suggested dosage of the vaccine. In the other two control groups, the one-day-old chickens were vaccinated with sham vaccine. On the 11th day post immunization, the seven immunization groups and one of the sham vaccine groups were intra dermally infected with MDV isolate JZ2014, which was purified by plaque purification and was passaged on DEF, at 2000 PFU. All the tested groups were fed with each group in an isolator and were observed daily for 120 days until experimental termination. The dead chickens were removed from the isolators daily. Carcasses were checked for nodular lesions on the skin. Breast and thigh muscles were inspected for lymphoid tumors or diffuse infiltration. After the opening of the carcasses, the liver, spleen, kidney, gastro-intestinal tract, heart and lungs were examined for gross MD lesions. Chickens with small focal lesions on all organs were considered to be MD positive after histopathological confirmation.

Results

Pathologic changes

No clinical signs were observed on the dead chickens' skin and muscle. After the opening of the carcasses, the main gross lesions of the dead chickens could be found on their organs. Their livers had steatosis and were swollen with white tumor nodules scattered into the surface; their spleens were swollen and brittle with fibrinous exudates on the surface; their kidneys were swollen; their proventriculi were swollen and thick, and the nipples bled with mucus secretion on the surface; the cutin membranes of their muscular stomach were thick and festered; their duodena had hyperemia (Figure 1).

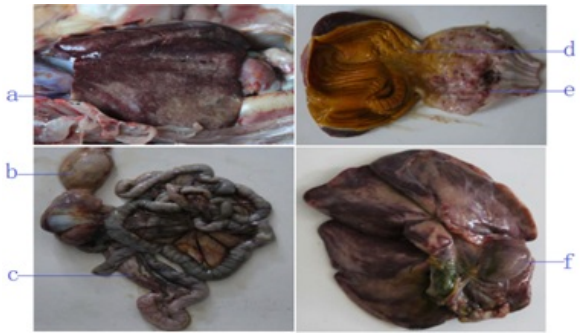

Figure 1: The pathologic changes of the dead chickens a. the white nodule in the liver; b. the enlargement of the proventriculus ; c. the duodenal congestion; d. the swollen muscular stomach with thicken corneum layer which was easy to be peeled; e. mucus secretion of the proventriculus was increased with nipple bleeding; f. the swelling of the spleen.

Results of virus isolation

With inoculation of the lymphocytes from the blood samples of the infected chickens into DEF cells, cytopathic effect (CPE), including the accumulation and detachment of the DEF cells, was developed on the cultured DEF cell layers with incubation at 37ºC. The time of the CPE at the second passage was 90 hours after inoculation, and consistent CPE was developed after 3 passages of the isolate, with the time of the CPE at 86 hours (Figure 2). The MDV strain named JZ2014 was obtained by isolation.

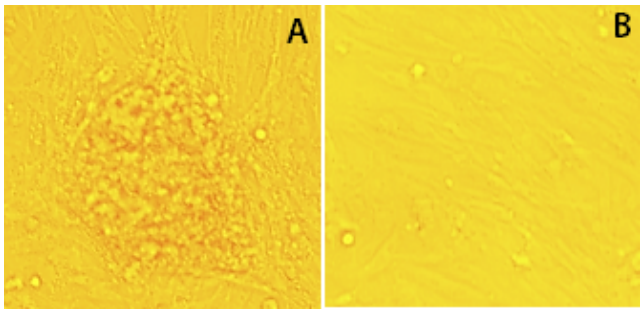

Figure 2: CPE in DEF A. the infected cells 10×40 B. the normal cells10×40.

Results of PCR detection

The PCR detection verified the existence of infection with MDV in chickens and inoculated DEF cells. By PCR, a 1081 bp long DNA product specific to MDV Meq gene was found in the blood samples of the infected chickens and the DEF cell cultures infected with the MDV isolate (Figure 3A). The PCR detection also verified the absence of infection with ALV and REV in chickens and inoculated DEF cells, because the results, which were negative to REV (Figure 3B) and negative to ALV (Figure 3C) by PCR, were found in these samples.

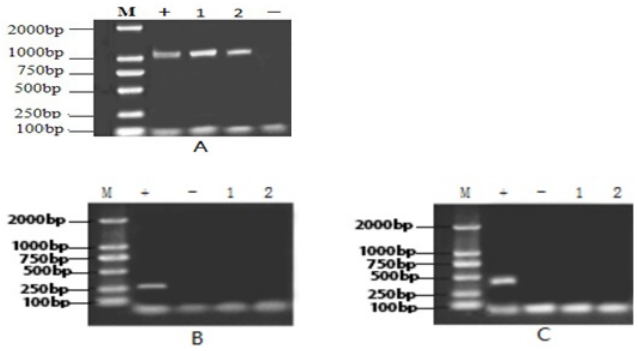

Figure 3: Detection of MDV, ALV and REV by PCR A. PCR detection of MDV; B. PCR detection of REV; C. PCR detection of ALV; M. DL2000 DNA marker; +. Positive control of MDV SD2012-1 in (A), REV in (B) or ALV in (C); -. Negative control (ddH2O); 1. Spleen of infected chickens; 2. DEF culture infected with the MDV isolate.

Sequence of Meq gene of JZ2014

By sequencing, the nucleotide sequence of Meq gene of the MDV isolate JZ2014 and its deduced amino acid sequence was obtained, and the Meq gene sequence of JZ2014 was submitted to the Gen Bank database with the accession number KP144355 for Meq gene.

Homology of JZ2014 with reference MDV strains

The Meq amino acid of JZ2014 was 339aa long, compared with the 11 reference strains in Table 2. The sequence of Meq amino acid of JZ2014 had amino acid homology at 96.8-99.1%. JZ2014 had the highest amino acid homology with SD2012-1 at 99.1%, and had the lowest homology with 584A at 96.8%. Like most strains isolated in China, JZ2014 had the same amino acid mutation of Meq amino acid at position 77(K to E), 80(D to Y), 115(V to A), 119(R to C), 176(P to R) and 233(P to S) (Figure 4).

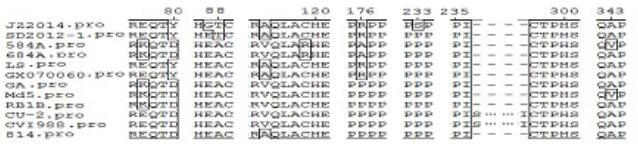

Figure 4: Alignment for amino acid mutation site on Meq gene of 12 MDVs.

Phylogenetic tree

A phylogenetic tree based on the Meq gene sequences of JZ2014 and the 11 reference strains was obtained (Figure 5). In general, the upper end of the tree contained mainly the high-virulence USA isolates while the lower end contained JZ2014 and other 3 Chinese isolates. Two vaccines strains and CU-2 were grouped together in the middle of the tree. JZ2014 was closely aligned with SD2012-1 in the lower end.

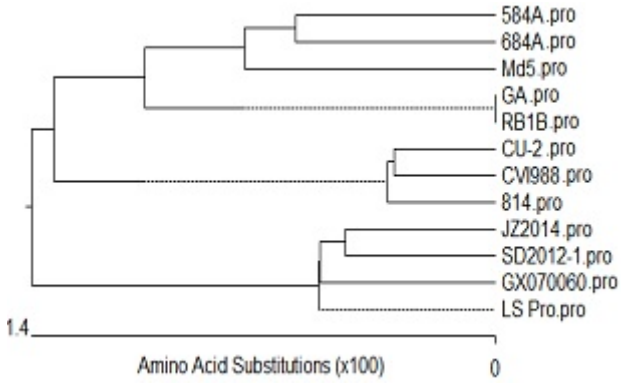

Figure 5: The evolutionary tree of MDV strains by Meq gene.

Results of animal test with JZ2014

Control chickens of sham vaccine without virus challenge had not any MD symptoms or pathologic changes; challenged chickens of sham vaccine had MD symptoms and pathologic changes. None of the vaccination could completely protect the immunized chickens against JZ2014. MD appeared in the seven types of vaccine. The earliest death appeared on 41-44 days post challenge on chickens with sham vaccine, HVT and HVT+SB1 vaccine. The peak of death came on 61-84 days post challenge.

The highest mortality was 70.3% in sham vaccine group. The lowest mortality was 23.0% in CVI988+SB1+HVT immunized group. The mortality was 66.7% in HVT group and SB1 group (Table 3).

Histopathologic lesions

Histopathologic lesions of the chickens challenged with JZ2014 could be observed on histological section (Figure 6). Tumor cells were accumulated in the heart; lots of tumor cells were accumulated around the vessel in the liver; normal cells were necrotic while tumor cells scattered in the spleen; lots of tumor cells were accumulated in the kidney. The Histopathologic lesions verified the infection of MDV in challenged chickens.

Discussion

Marek's disease (MD) is a disease of chickens that occurs worldwide and has serious economic consequences, and in recent years there has been numerous reports of the isolation of vvMDV from vaccinated chickens with the most commonly occurring forms being the lymph proliferative diseases and high mortality syndrome which was confused with AL and RE [14,15].

Group	Bird number	Death with tumor	Tumor lesion	Total (%)
Sham vaccine (no challenge)	30	0	0	0(0.0%)
Sham vaccine (challenge)	30	9	13	22 (70.3%)
HVT	30	8	12	20 (66.7%)
SB1	30	9	11	20 (66.7%)
CVI988	30	4	6	10(33.3%)
HVT+SB1	30	8	11	18 (60.0%)
CVI988+HVT	30	4	4	8(26.7%)
CVI988+SB1	30	5	3	8(26.7%)
CVI988+SB1+HVT	30	2	5	7(23.0%)

Table 3: Statistics of chickens challenged with MDV isolate

In this study, gross tumor lesions were found in the liver, kidney, spleen, proventriculus, muscular stomach of the dead chickens. MDV (JZ2014) was isolated from infected chickens, and was detected by PCR. JZ2014 had the highest Meq amino acid homology with SD2012-1 at 99.1%, and had the lowest amino acid homology with 584A at 96.8%. Neither ALV nor REV was found in the infected chickens. All these tests confirmed the presence of MDV in the infected chickens, and it was MDV (JZ2014) which caused the infection in the field chickens.

It was reported that a piece of 59aa insertion in Meq amino acid in CVI988 could result in suppression of Meq amino acid expression [16,17]. Like most virulent strains, the Meq amino acid of JZ2014 was 339aa long, which was 59aa shorter than Meq amino acid of CU-2 and CVI988. The deletion of the 59aa in Meq amino acid of JZ2014 differentiated this virus from MDV vaccine strains apparently.

It was reported that amino acid mutation of Meq gene at position 77(K to E), 80(D to Y), 115(V to A), 119(R to C), 176(P to R) and 233(P to S), displayed regularity of strains isolated from China. In this study, compared with the reference strains in China in Table 2, JZ2014 had the same amino acid mutation of Meq gene at position 77(K to E), 80(D to Y), 115(V to A), 119(R to C), 176(P to R) and 233(P to S), which further confirmed the regularity of strains isolated from China.

It was reported that amino acid change at position 77 (E to K) was the feature of high virulent MDV strains, and a glutamic acid (E) at position 77 was associated with lower virulence [18]. However, JZ2014 and SD2012-1, which had glutamic acid (E) at position 77, were the

high virulence strains. This could demonstrate that a glutamic acid (E) at position 77 was not necessarily a feature of MDV strains of lower virulence.

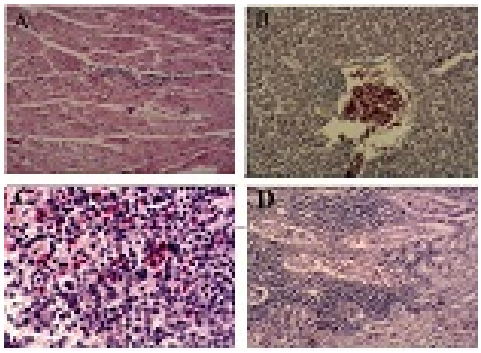

Figure 6: Histopathologic lesions on chickens challenged with JZ2014 A. tumor cells accumulated in the heart of the SPF chickens infected with virus (H.E, ×100) B. lots of tumor cells around the vessel in the liver of the SPF chickens infected with virus (H.E, ×100) C. normal cells necrotic and some tumor cells scattered in the spleen of the SPF chickens infected with virus (H.E, ×100) D. lots of tumor cells accumulated in the kidney of the SPF chickens infected with virus (H.E, ×100).

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CVI988 vaccine was widely used now in China, but immune failure still occurred in some chickens. A number of vvMDVs were recently isolated from different areas [19], and it was poorly understood whether the currently available vaccines were able to protect against these very virulent MDVs. In this study, the immunized chickens in the seven groups were challenged with JZ2014. The result showed that CVI988 vaccine or CVI988 combined vaccine could not completely protect the immunized chickens against JZ2014, with the lowest mortality at 23.0% in CVI988+SB1+HVT immunized group. HVT and HVT+SB1 could hardly protect the immunized chickens against JZ2014, with the mortality at 66.7% in HVT group and SB1 group (mortality at 70.3% in sham vaccine group). It was the poor protective effect of the MD commodity vaccines against vv MDV that resulted in outbreaks of MD in China. The emergence of MDVs with increasing virulence is a significant problem for the poultry industry in China.

Acknowledgments

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