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Detection of the Marek Virus in Cockfight Roosters by the Polymerase Chain Reaction (PCR) Technique

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Abstract: Marek Disease (MD) is a lymphoproliferative disorder with polyneuritis in birds of the *Gallus domesticus* sp. The direct presence of the virus has remained unknown in Mexico. In order to determine its presence, a Polymerase Chain Reaction (PCR) was established, from the ICP4 conserved gene of the Gallid Herpesvirus-2, CVI988 attenuated strain (serotype 1) and the turkey Gallid Herpesvirus-2, FC126 strain (serotype 3). The PCR assay was done on the brain, nerve, lung, spleen, liver and feather calamus tissues from 24 cockfight roosters that showed signs suggestive to MD, such as: paralysis, ataxia, torticollis, fallen wings, neck and wings laxity. Tissues were also collected from 19 roosters without clinical neurological signs. Results showed that 6/25 roosters (25%) with clinical signs were positive by PCR to the ICP4 gene, from which 6/19 (31%) were positive by histopathology to MD and 4/24 (16%) were negative to the ICP4 gene, but positive by histopathology. A group of 5/24 roosters (21%), with signs suggestive to MD, were negative by PCR and histopathology to MD. One hundred percent of the roosters positive by PCR corresponded to the serotype 1, while one rooster was positive to serotype 3. Regarding roosters without MD signs, only 1/19 (5.2%) was positive by PCR corresponding to serotype 3. This study points out the presence of the Marek virus genome in cockfight roosters for the first time, detecting the highest frequency for the serotype 1.

Key words: Marek disease, serotype 1, cvi988, fc126, icp4 gene, pcr, cockfight rooster

INTRODUCTION

Marek Disease (MD) is caused by a Gallid herpesviurs-2 of the Alphaherpesviridae family, characterized by a 120-200 nm virion with a glycoproteic envelope that contains a DNA genome of 174 kb, organized in several sections called Long Unique (LU) and Short Unique (SU), a Long Internal Repetition (LIR) and Short Internal Repetition (SIR), along with the Short (STR) and Long Terminal Repetition (LTR) sections. The genome has 77 open reading frames from which 55 are genes characteristic of the Simplex Herpesvirus (SHV) and 12 are unique for the MD virus (Brunovskis and Velicer, 1995; Lee *et al*., 2000).

This virus is classified into 3 serotypes: Serotype 1, pathogenic for chicken, Serotype 2, non-pathogenic and Serotype 3, non-pathogenic and isolated from turkey (Witter and Schat, 2003).

The MD virus (MDV) causes a lymphoproliferative and nervous disease characterized by the formation of lymphomas in internal organs, muscle, skin and nerves of birds of the *Gallus gallus domesticus* sp. (Witter and Schat, 2003). The severity of the disease has been classified as virulent strains, very virulent strains and very virulent plus strains, based on the pathogenicity on commercial poultry vaccinated with the MDV (Witter, 1997). Very virulent plus strains have been subdivided into neurologic strains characterized by temporary paralysis, ataxia, torticollis and spasms (Gimeno *et al*., 1999, 2002).

The virus is localized in the epithelial cells of the feather follicle (Eidson *et al*., 1971), renal tubule, lymphocytes inside the bursa of Fabricius (Fujimoto *et al*., 1974) and in those lymphocytes around nerve periphery (Ubertini and Calnek, 1970).

MD in Mexico is localized in the commercial poultry and cockfight roosters, which are a Mexican tradition characterized by its wide moving along the country and the absence of vaccination. Morphological diagnosis is based on the presence of neoplastic lymphocytic infiltrate

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in nerves and bursa of Fabricius among other organs (Witter and Scaht, 2003) however, there is no direct evidence of the virus.

The present study aimed to detect the presence of the Gallid herpsvirus-2 in roosters with nervous sings or lymphoproliferative changes through out the Polymerase Chain Reaction (PCR) technique, as evidence of its presence in Mexico. For this, the ICP4 gene that is inside a group of 5 early genes called ICP0, ICP4, ICP22, ICP27 and ICP47, was used (Pereira *et al*., 1977; Smith and Schaffer, 1986). The ICP4 gene is a conserved sequence among the different strains of the MDV localized in the short internal region. This region codifies for a molecule that joins the ATCGTC consensus sequence of the DNA, needed to activate the expression of early and late genes in the nucleus of the infected cells during the latency phase (Muller, 1987; Kattar-Cooley and Wilcox, 1989). The ICP4 gene has been experimentally used for MDV detection (Endoh *et al*., 1996; Handberg *et al*., 2001; Davidson *et al*., 2002).

MATERIALS AND METHODS

Organ and tissue samples: A total of 24 adult cockfight roosters (eight clinical cases) that showed nervous clinical signs and 19 roosters (five clinical cases) without nervous signs were studied through out the diagnostic service for avian pathology at the Avian Department at the FMVZ, UNAM, during the 2003-2005 period. The samples collected from carcasses included: brain, nerve, spleen, liver and feather calamus. Organ samples were frozen at -70°C until use.

Vaccines: The CVI988 attenuated strain was used as positive control of the ICP4 gene of the MDV of chicken (serotype 1); while the FC126 strain was used for the ICP4 gene of the Gallid Herpesvirus of turkey (serotype 3).

Primers: The PCR was done using the oligonucleotides designed by Handberg *et al*. (2001), that amplify a 247 bp fragment of the ICP4 gene of the MDV serotype 1 (GenBank U17701). Amplification of the serotype 1 was carried out with the 5′-GGA TCG CCC ACC ACG ATT ACT ACC-3′ primers (sense) and 5′-ACT GCC TCA CAC AAC CTC ATC TCC-3′ (antisense). The primers for the amplification of the serotype 3 were 5′-ATG GAA GTA GAT GTT GAG TCT TCG-3’ (sense) and 5’-CGA TAT ACA CGC ATT GCC ATA CAC-3’ (antisense).

DNA extraction: Viral DNA was extracted form lung, liver and spleen tissues by the phenol-chloroform method. Viscera were macerated at a 2:10 ratio with phosphate saline solution (pH 7.4) and centrifuged at 325×g for 10 min. Then 250 μL of the supernatant were transferred into a tube plus 250 μL of the Tris-EDTA buffer (0.001M EDTA, 0.01M Tris-HCl, pH 8.0), sodium dodecyl sulfate (1%) and K proteinase (1 mg mL^{-1}). Samples were homogenized and incubated for 18 h at 37 °C, followed by extraction with the phenol-chloroform-isoamyl method (25:24:1). DNA was precipitated with cold ethanol and resuspended in 100 μL of distilled water treated with diethylpyrocarbonate (0.2%). DNA was kept at -20°C until use.

Alkaline extraction of the cellular DNA used on feather, brain and nerve: Viral DNA extraction from brain, nerve and feather calamus was done by the alkaline extraction method described by Malagro *et al*., (2002). The procedure consists on depositing 5 mm^2 of the tissue in a 1.5 mL tube with 20 μL of sodium hydroxide (0.2 M), followed by incubation at 65 °C for 10-15 min. Then 180 μL of Tris-HCL (pH 7.5) were added, homogenized and 5 μL were transferred into a PCR reaction tube.

PCR: The reaction volume for PCR was 50 μL that contained 50 μL of the purified DNA sample, 100 pmol of each primer, 0.5 U of Taq (Fermentas, USA), 1X of polymerase buffer (Fermentas, USA), 1 mL of $MgCl_2$, 2 mM of triphosphate deoxynucleotide (Fermentas, USA) and distilled water up to 50μL. Amplification conditions were done on a thermocycler (Termo Hybaid PCR Express) for 28 cycles at 94°C for 30 sec, followed by 55°C for 90 and 72°C for 60 sec. The last cycle included one extension 72°C at for 5 min. Observation of the amplified fragment was done in a 2 % agarose gel dyed with ethidium bromide (1 μg mL^{-1}) through out a transilluminator of UV light.

DNA sequenciation of the MDV (CVI988 strain): The PCR fragment was purified from the incision of the band in the 2% agarose gel and purified by a commercial purification system (QIAQUICK, QIAGen, USA) as follows: DNA was purified through out a column, washed several times with buffer, eluded with distilled water and kept at -20°C until sequenciation.

The nucleotide sequence was obtained with an automatic sequencer (PERKIN ELMER model 310) using the primers previously described and at 57 °C as annealing temperature. Nucleotide sequence was compared to that of the ICP4 gene (GenBank U17701) through out WorkBench 3.0 software.

RESULTS AND DISCUSSION

The expected fragments of the ICP4 gene were amplified from the MD vaccines CVI988 and FC126 strains. However, the fragment of the CVI988 strain had an

approximated size of 288 bp, in contrast with the 247 bp size reported by Handberg *et al*. (2001). The 505 bp fragment of the FC126 strain was identical to the reported by Handberg *et al*. (2001) (Fig. 1). Regarding the size difference of the CVI988 strain, it would suggest a variation between the CVI988 strain used by Handberg *et al*. (2001) and the one used for the vaccine in Mexico. Nonetheless, the nucleotide sequence of the 288 bp fragment had a 100% similarity with the sequence of the ICP4 gene published by McKie *et al*. (1995) (Fig. 2).

ICP4 gene detection in roosters with nervous signs: The ICP4 gene serotype 1 was detected in 4 out of 8 different clinical cases in cockfight roosters (50%) showing nervous signs, from which the gene was detected in 25% of the birds with nervous signs (Table 1). Based on the histopathology records of these roosters, only one clinical case (2/4 birds) positive to the ICP4 gene did not presented the characteristic anatomical-pathological changes of MD. Meanwhile, 4 clinical cases positive to nervous signs and to the characteristic anatomical-pathological changes of MD resulted negative to the ICP4 gene.

Localization of the ICP4 gene in tissues and organs form roosters with clinical signs: Only three out of 8 cases (37.5%) of roosters with nervous clinical signs, positive to the ICP4 gene, showed the gene in the feather calamus, representing 16% of all roosters with nervous signs. In contrast, only one clinical case presented the gene exclusively in the nerve in 2/3 roosters. It is important to point out that there was only one case of a rooster positive to the ICP4 gene serotype 1 (288 bp) with nervous signs and characteristic anatomical-pathological changes of MD, showing also the ICP4 gene serotype 3 (FC126).

Detection of the ICP4 gene in rosters without nervous signs: Detection of the ICP4 gene by PCR was observed in 5% (1/19) of the roosters without nervous signs and represented in five different clinical cases. The only rooster positive to the ICP4 gene corresponded to serotype-3 (FC126) (Table 2). It is important to remark that this rooster was clinically healthy, while the rest of the birds had several diagnoses different form MD.

ICP4 gene localization in tissues and organs without nervous signs: The only case positive to the ICP4 gene in roosters without nervous signs was detected in the feather calamus.

Experimental studies have detected more precisely the kinetics of the Gallid herpesvirus-2 along its pathogenesis, using the ICP4 gene amplification by PCR. Infection in one-day-old chicken with the very virulent RB-1B strain (serotype 1) has been detected until 84 days after infection in feather and spleen. Meanwhile, the CVI988 strain (serotype 1) is found in feathers until 56 days, in contrast to the HVT strain (serotype 3) which has been seen until day 42 in feathers (Handberg *et al*., 2001). A similar behavior has been observed in one-day-old chicken infected with the CVI988 strain (serotype 1), detecting the virus in thymus, bursa of Fabricius and

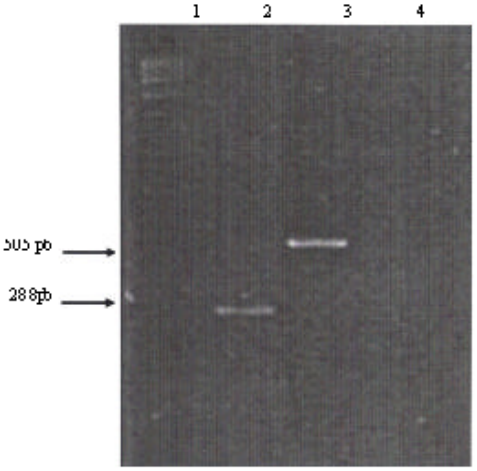

Fig. 1: Amplification of two fragments of the ICP4 gene after standardization of the PCR test. Lane 1 molecular weight marker; lane 2 CVI988 vaccine serotype 1; lane 3 vaccine with FC126 strain, turkey Herpesvirus serotype 3; lane 4 negative control without DNA

Table 1: Tissues positive to the ICP4 gene of the MDV by PCR in cockfight roosters with nervous signs

Case number	Nerve	Brain	Lung	Liver	Spleen	Feather	Diagnosis
1	0/3	0/3	0/3	0/3	0/3	0/3	MD
2	0/1	0/1	0/1	0/1	0/1	1/1*	MD
3	0/1	0/1	0/1	0/1	0/1	0/1	MD
4	0/4	0/4	0/4	0/4	0/4	2/4*	ND
5	2/3*	0/3	0/3	0/3	0/3	0/3	MD
6	0/2	0/2	0/2	0/2	0/2	1/2*≠	MD
7	0/5	0/5	0/5	0/5	0/5	0/5	ND
8	0/5	0/5	0/5	0/5	0/5	0/5	ND

*CV 1988, ≠FC 126; ND. Not determinated

Table 2: Tissues positive to the ICP4 gene of the MDV by PCR in cockfight roosters without nervous signs

Case number	Nerve	Brain	Lung	Liver	Spleen	Feather	Diagnosis
1	0/2	0/2	0/2	0/2	0/2	0/2	Pneumonia
2	0/5	0/5	0/5	0/5	0/5	0/5	Enteritis
3	0/6	0/6	0/6	0/6	0/6	0/6	Stomatitis and air sacculitis
4	0/5	0/5	0/5	0/5	0/5	0/5	Enteritis
5	0/1	0/1	0/1	0/1	0/1	0/1≠	Healthy

≠ FC 126

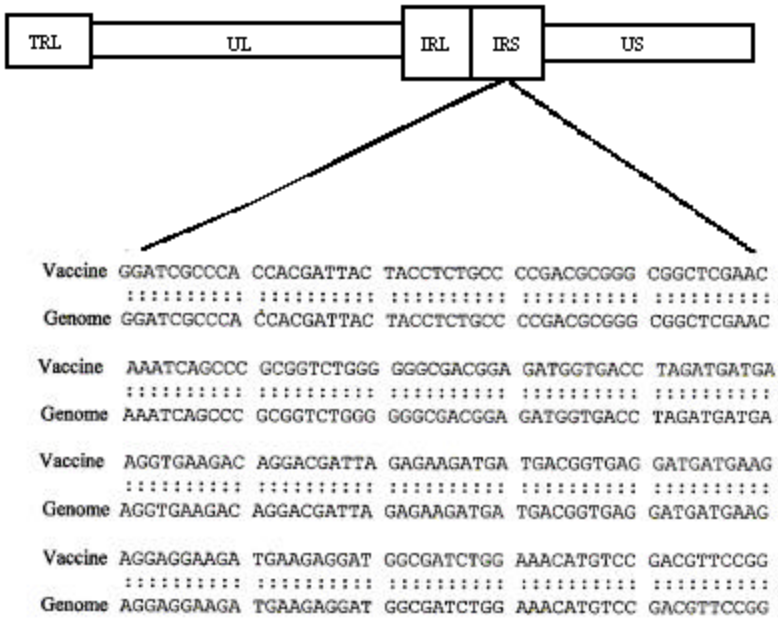

Fig. 2: Results from the sequenciation of the CVI988 vaccinial virus. The first line correspond to the sequenciation of the CVI988 vaccine and the second to the genome reported for the Marek disease herpesvirus serotype 1, localized in the GeneBank with the accession number U17701

spleen from day 3 until day 28 after infection. At the same time, detection in feather was from day 14, persisting until day 28 after the experiment was finished (Baigent *et al*., 2005). The CVI988 strain has been detected in other studies by PCR in spleen until day 30 after infection in one-day-old chicken, using other genes such as meq and gB (Davidson *et al*., 2002). This study detected serotypes 1 and 3 of the Gallid herpesvirus through out the ICP4 gene in adult cockfight roosters with nervous signs suggestive to MD. Results showed that most of the cockfight roosters with nervous signs presented the virus regarding those without nervous signs, pointing out that the test may support differential diagnosis. Regarding MD, isolation and molecular detection are necessary to differentiate the circulating strains in cockfight rosters for 2 reasons: tumors were not observed macroscopically in none of the cases as it has been described in non-vaccinated chicken, which might suggests the presence of neurotrophic strains that cause different nervous signs and death in chicken (Gimeno *et al*., 1999). These are classified into 3 groups called A, B and C (Gimeno *et al*., 2002) corresponding to very virulent plus, very virulent and virulent strains (Witter, 1997) and circulation of the CVI988 strain that is widely used to vaccinate broiler or laying chicken that is characterized by avoiding tumor developing (Baigent *et al*., 2005).

Viral detection in feather and not in viscera, along with the presence of nervous signs, can be explained by 3 reasons the scarce viral presence in visceral tissue, presence of the CVI988 vaccinial strain and presence of another strain of the MDV serotype 1.

The presence of the vaccinial virus in feathers and not in viscera concurs with the reported by Handberg *et al*. (2001) who detected the ICP4 gene by PCR in feathers of one-day-old chicken between days 3-56 after infection. However, the presence of the ICP4 gene fragment exclusively amplified from nerve was an unexpected result. Research on MDV kinetics and pathogenia have been done by experimental studies on commercial chicken from one to seven days old, in contrast with this study on adult roosters. The relevance of the aforesaid relies on the fact that it is known that there are chicken genetically susceptible to most of the virulent and very virulent strains of the MDV, while others are susceptible to the very virulent plus strains.

The 505 bp fragment of the ICP4 gene (serotype 3) was detected in feather of one of the roosters without nervous signs. Serotype 3 is used as vaccine in one-day-old chicken and, based on Handberg *et al*. (2001) kinetic studies, can only be detected in feather.

The presence of the disease in cockfight-rooster aviculture, fulfills the characteristics that favor the maintenance of MD: Cockfight roosters do not have an active vaccination program as poultry do; they have a longer life expectance, they are widely moved across the country in a discrete way and viral disease diagnosis is

limited. The present study verified that PCR supports indirect localization of the virus in birds with nervous signs or suspected to MD, which can be used for identification, characterization and isolation of the field virus and its impact on production, cockfight and wild Galliforms.

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