Molecular characterization of Marek's disease virus in a poultry layer farm from Colombia

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ABSTRACT Marek's disease (**MD**) is a lymphoproliferative disease caused by an Alphaherpesvirus, genus *Mardivirus*, serotype 1 (*Gallid Herpesvirus 2*, GaHV-2) that includes all known pathogenic strains. In addition to Marek's disease virus (**MDV**) serotype 1, the genus includes 2 distinct nonpathogenic serotypes: serotype 2 (GaHV-3) and serotype 3 (*Meleagridis Herpesvirus 1*, MeHV-1) which are used in commercially available vaccines against MD. As a result of vaccination, clinical signs are not commonly observed, and new cases are usually associated with emerging variant strains against which the vaccines are less effective. In this study, a commercial layer farm showing clinical signs compatible with MDV infection was evaluated. Histological lesions and positive immunohistochemistry in the sciatic nerve and thymus were compatible with cytolytic phase of MD. GaHV-2, GaHV-3 and MeHV-1 were identified by PCR and qPCR in blood samples from 17 birds with suspected MD. Analysis of the *Meq* gene of the Colombian GaHV-2 isolate revealed a 99% sequence identity with Asian strains, and in the phylogenetic analysis clustered with vv+ MDV. The analysis of amino acid alignments demonstrated an interruption of the proline rich region in P176A, P217A and P233L positions, which are generally associated with vv+ strains. Some of these changes, such as P233L and L258S positions have not been reported previously. In addition, primary cell cultures inoculated with lymphocytes isolated from the spleen showed typical cytopathic effect of GaHV-2 at 5 d post infection. Based on the molecular analysis, the results from this study indicate the presence of vv+ MDV infection in commercial birds for the first time in Colombia. It is recommended to perform further assays in order to demonstrate the pathotype characteristics *in vivo*.

Key words: Marek's Disease, Colombia, phylogenetic analysis, field strain

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INTRODUCTION

Marek's disease virus serotype 1 (**MDV-1**) is the causative agent of Marek's disease (**MD**), a neoplastic disease in poultry that results in the formation of lymphomatous lesions in nerves and visceral organs (Baigent and Davison, 2004). MDV-1 (also called *Gallid herpesvirus 2*) is a member of the *Herpesviridae* family, subfamily *Alphaherpesvirinae*, and genus *Mardivirus* (Davison and Nair, 2004; Biarnés et al., 2013). Primary infection in naïve birds occurs via inhalation of virus particles into the respiratory tract. Viral replication in the lungs stimulates immune cell infiltration whereby MDV preferentially infects adaptive immune system cells. Secondary infection and semi-productive viral replication results in an initial acute cytolytic phase in lymphocytes resulting in immunosuppression (Calnek, 2001; Nair, 2005). The virus becomes latent at 6 to 7 d post infection, allowing for immune evasion; the MDV genome integrates into the genome of CD4+ T lymphocytes without detectable expression levels of the potential antigenic proteins, allowing for systemic dissemination to organs, peripheral nerves and feather follicles (Baigent and Davison, 2004). Fully productive viral replication occurs only in feather follicle epithelium which, when sloughed off and disseminated with air currents, becomes the primary source of infectious viral particles to susceptible birds.

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Currently there are 3 MDV vaccine strains commercially available worldwide: serotype 1 (**GaHV-2**) attenuated live vaccine (strain CVI988/Rispens), serotype 2 (**GaHV-3**) strain SB-1, and serotype 3 (**MeHV-1**) *Herpesvirus of turkey* (**HVT**); both serotypes 2 and 3 are considered non-virulent and generate an immune response which is protective against some serotype 1 field strains (Baigent et al., 2011). Vaccination provides a life-long protective immunity against MD; specifically it generates an anti-tumor immune response following infection with field strains which reduces mortality rates, but does not protect against infection, replication, and/or virus dissemination (Baigent and Davison, 2004; Gimeno et al., 2013; Gimeno, 2008). Although vaccine use decreases the presentation of clinical signs associated with MD, the presence of field strains and subsequent immunosuppression observed predisposes chickens to secondary infections such as Chicken Infectious Anemia virus (Schat, 2009; Biarnés et al., 2013). It is considered that clinical signs of MD are associated with the emergence of new variants of MDV strains against which the vaccines are not fully protective (Gimeno, 2008; Davison and Nair, 2004). Based on the lesions, mortality rate and protection offered by the vaccine strains, GaHV-2 strains have been classified into 4 pathotypes: mild (**m**), virulent (**v**), very virulent (**vv**) and very virulent plus (**vv+**) (Davison and Nair, 2004; Witter et al., 2005). In the last 15 years, the vv+ MDV strains have been the predominant pathotype isolated (Zhang et al., 2011) worldwide from vaccinated chickens, for which vaccines do not appear to generate a very robust protection (Gimeno, 2008).

Serological techniques are not useful to evaluate the efficacy of vaccination or to establish the presence of different strains in poultry houses; therefore, it is necessary to use other methods that allow for viral detection and quantification. Molecular tests allow detection of Mardiviruses in feather and blood samples, making it possible to distinguish between serotypes (Biarnés et al., 2013; Baigent et al., 2011) and even to indicate the pathotype of serotype 1 strains (Davidson and Borenshtain, 2003; Becker et al., 1992). Among these techniques the quantitative polymerase chain reaction (**qPCR**) is a highly sensitive test for absolute quantification of GaHV-2 (Baigent et al., 2005), GaHV-3 (Renz et al., 2006) and MeHV-1 (Islam et al., 2006). Through this technique it is possible to quantify the mean number of the viral genomes per cell according to serotype (Baigent et al., 2011). Other techniques like conventional PCR allow the detection and differentiation of the 3 serotypes (Becker et al., 1992).

MD is an economically important disease (Davison and Nair, 2004) and has a great impact on the poultry industry due to its economic losses. In Colombia, the official diagnostic method for MD is based on evaluating clinical signs and assessing histological findings. The Instituto Colombiano Agropecuario (**ICA**) reports the number of MD cases on an annual basis. In 2012, the ICA reported a MDV infection in one farm resulting in 14.3% mortality rate. In spite of being a reportable disease, MD is not considered in the category of official control diseases for chickens in Colombia. As a consequence, although clinical cases of unconfirmed MD are reported in Colombia every year, the virus has not been studied or isolated from clinical cases presented in the country until now, and there is no molecular evidence of viral genome presence in poultry farms in Colombia. The main objective of this study was to investigate and characterize a virus detected in a clinical case compatible with MD in a commercial layer farm from Colombia. The results presented here show the first molecular evidence of MDV and the first viral isolation of a MDV field strain in Colombia.

MATERIALS AND METHODS

Sample Collection

Samples from organs (liver, thymus, spleen, sciatic nerves and blood) and feathers were collected from a suspected case of MD in an 18-week-old flock of approximately 1,000 Hy-Line Brown layers, in a farm from Colombia. The farm had a previous history of impaired condition and clinical signs compatible with Marek's Disease, along with an increase in the mortality rate reaching 1.7% per wk, with a final cumulative mortality rate of 37.7%. The day-old birds are routinely vaccinated with MeHV-1 strain FC126 and GaHV-2 Rispens strain. Economic losses due to poor performance and a decrease in the production parameters were evident for over a year. Previous analysis of macroscopic and microscopic lesions found in tissue samples suggested the presence of MDV. Birds were euthanized by cervical dislocation (Bagust, 2008) and macroscopic changes including splenomegaly, hepatomegaly, renomegaly, and sciatic nerve hemorrhages were observed during necropsy examination. Samples were collected from 8 healthy birds, and 9 birds showing clinical signs (neurological signs: paralysis of legs, depression, lethargy). Tissue samples from liver, spleen, sciatic nerve and thymus were taken in 5×5 mm pieces and fixed in 10% neutral buffered formalin. Spleens were taken for virus isolation, and blood and feather samples were taken from 17 birds for DNA extraction (with clinical signs). The samples were preserved at -20°C until processing and analysis.

Histopathology and Immunohistochemistry

Samples were routinely processed and fixed in 10% neutral buffered formalin, embedded in paraffin wax, sectioned (4 μm), and stained with hematoxylin and eosin. The slides were read and evaluated at PDRC, UGA by light microscopy (Gimeno et al., 2013). For the immunohistochemistry analysis, paraffin tissue sections were deparaffinized in xylene, hydrated

Table 1. Primers used for the PCR and qPCR for detection of the 3 serotypes of MDV.

Target[1]	Sequence	Product size (base pairs, bp)
GaHV-2 - *Meq* gene (PCR and sequencing)	For: CCG CAC ACT GAT TCC TAG GC Rev: AGA AAC ATG GGG CAT AGA CG	1148 bp (RB1B) 1325 bp (Rispens)
BamHI-H and BamHI-D region (Becker et al. 1992) (PCR).	For: TACTTCCTATATAGATTGAGACGT Rev: GAGATCCTCGTAAGGTGTAATATA	434-bp and 566-bp.
GaHV-3: *gD* gene (PCR).	For: TTCTTCGGACACCTTTCGCCT Rev: TTCCTGGACGGGCGTTGAGGT	1040 bp
MeHV-1*: sORF 1* gene (Islam et al. 2006) (PCR).	For: AAGCGCTTGTATGTGTAGG Rev: TATGGACGTCATGCAGTTGG-3	350 bp
GaHV-2 *Meq* gene (qPCR) (Sellers et al. 1998; Jones et al. 1992).	For: GGT CTG GTG GTT TCC AGG TGA Rev: GCA TAG ACG ATG TGC TGC TGA Probe: AGA CCC TGA TGA TCC GCA TTG CGA CT (5′FAM, 3′BHQ1)	73 bp
GaHV-3 *DNA pol* gene (qPCR).	For: AGC ATG CGG GAA GAA AAG AG Rev: GAA AGG TTT TCC GCT CCC ATA Probe: CGC CCG TAA TGC ACC CGT GAC T (5′FAM, 3′BHQ1)	100 bp
MeHV-3 *sORF1* gene (qPCR).	For: GGC AGA CAC CGC GTT GTA T Rev: TGT CCA CGC TCG AGA CTA TCC Probe: AAC CCG GGC TTG TGG ACG TCT TTC (5′FAM, 3′BHQ1)	77 bp
Chicken ovo transferrin Gene (Jeltsch et al. 1987). qPCR	For: CAC TGC CAC TGG GCT CTG T Rev: GCA ATG GCA ATA AAC CTC CAA Probe: AGT CTG GAG AAG TCT GTG CAG CCT CCA (5′Yakima Yellow, 3′TAMRA)	71 bp

For: Forward primer. Rev: Reverse primer.
[1]Name of the target gene.

through graded alcohol, and then steamed for 1 hour in 1.0 mM EDTA solution (pH 8.4) at 60°C. The sections were treated with 0.03% solution of H_2O_2 in PBS (phosphate buffered saline, pH 7.6) at room temperature for 5 min to block the endogenous peroxidase activity. An antibody against the MDV serotype-1 pp38 protein (Mouse monoclonal antibody H19.47, provided by Lucy Lee, USDA-ADOL, East Lansing, MI) (Schat and Nair, 2013) was used to identify the acute cytolytic phase proteins of MDV. Sections were covered with anti-pp38 antibody diluted at 1:250 and incubated in a humid chamber at room temperature for 30 min. The slides were incubated with secondary antibody Mouse/Rabbit UnoVue™ HRP/DAB (Diagnostic Biosystems®, Pleasanton, CA) for 15 min. The peroxidase activity was developed with 3,3-diaminobenzidine tetrahydrochloride, Liquid DAB + substrate Chromogen System (DakoCarpinteria, CA). The sections were counterstained with hematoxylin and then coverslipped.

DNA Extraction

Total DNA was extracted from blood samples using QIAamp® DNA Blood and tissue kit (QIAGEN®), according to the manufacturer's instructions with a final elution volume of 120 μL. Concentration of the extracted total DNA was determined by using a Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington).

PCR amplification of MDV genes

Sets of specifically designed primers were used to amplify sequence specific to each of the MDV serotypes (Table 1). PCR conditions were optimized in a 50 μL reaction volume using 2.5 units of Taq polymerase (Invitrogen®, Waltham, MA), 4 mM of $MgCl_2$, 400 mM of dNTPs, 1 mM of each primer and 10 ng of DNA. Amplification of MeHV-1 was performed using 30 cycles of 94°C for 1.5 min, 60°C for 1 min, and 72°C for 2 min. Amplification of GaHV-3 was performed using 35 cycles of 94°C for 1.5 min, 55°C for 1 min, and 72°C for 2 min, and for GaHV-2 the amplification was performed using 35 cycles of 94°C for 1.5 min, 57°C for 1 min, and 72°C for 2 min.

Analysis by PCR

The PCR products of amplification of the BamHI-H and BamHI-D regions of the virus genome were used to assist in differentiating possible pathogenic strains from non-pathogenic isolates in GaHV-2. This segment flanks the 132 bp tandem repeat region in the viral genome (Becker et al., 1992). The primers used are listed in Table 1. The Forward primer is located in the region 65 bp upstream of the 132 bp repeats, and the reverse primer is located 105 bp downstream of the region. When there are only 2 132-bp repeats these primers amplify a segment of 434 bp. Some authors suggest that the most pathogenic strains have only one or 2 repeats of the 132 bp band, while the mild strains

have between 6 and 7 repetitions. The monomer band is more intense than the other bands, so it is more easily seen (Doosti and Golshan, 2011). PCR conditions were similar to those used in the above, using 35 cycles of 94°C for 1.5 min, 55°C for 1 min, and 72°C for 2 min.

The PCR products were separated on a 1% agarose gel prepared with TAE 1X (40 mM Tris, acetic acid, 2 mM EDTA) buffer and stained with ethidium bromide (1 mg/mL). The gel was run at 70 V for 1 h and the bands were visualized in a UV trans-illuminator and analyzed using the Gel Capture Image Acquisition Software (Bio Imaging Systems).

Sequencing and Phylogenetic Analysis

The *Meq* gene PCR products were purified directly from the gel using the QIAquick Gel Extraction Kit following the manufacturer's recommendations (Qiagen®, MD). In samples with 2 PCR products (2 different bands in the electrophoresis), the bands were cut and processed separately. The purified products were stored at 4°C until required for sequencing. Sequencing oligonucleotides were designed to amplify the full length *Meq* gene, using PyroMark Assay Design Software 2.0 (Qiagen®, Frederick, MD) (Table 1).

Sequence data were assembled and edited over a total length of 1020 bp using the SeqMan program (DNA Star Laser gene software package, WI). DNA sequence accession numbers for *Meq*-encoding genes are summarized in Table 3. Nucleotide BLAST (Basic Local Alignment Search Tool) was used to explore sequence similarity of Marek's disease virus strain UDEACO-2013 strain (name given to the clinical isolate. GenBank: KU058696, KU0558697, KU058701) to some of the available sequences of *Meq* in the NCBI nucleotide databases. Nucleotide and deduced amino acid alignments of the 25 full-length *Meq* gene sequences, along with strain UDEACO-2013 isolated from the present clinical case and several vaccine strains, were performed with MEGA 6 using the Muscle algorithm, and uncorrected (p) distances for nucleotide and amino acid sequences were calculated.

A phylogenetic tree was generated using the neighbor-joining (**N–J**) method, and the liability of internal branches was assessed by 1,000 replicates. The MDV reference sequences were retrieved from the GenBank database, and their accession numbers are listed in Table 3 and Figure 4.

Real-Time PCR

Real-time qPCR was used for absolute quantification of viral genomes (Baigent et al., 2005). Oligonucleotides and probes designed specifically for *Meq* gene (GaHV-2) (Baigent et al., 2005), DNA pol gene (GaHV-3) and *sORF*1gene (MeHV-1) (Islam et al., 2006), were used in the reaction, Table 1 (Baigent et al., 2011). qPCR was performed in 96-well plates (FAST Optical 96-well PCR plates. Applied Biosystems®, CA) in a final volume of 25 μL per well, containing 1X Master Mix (Absolute Blue low ROX qPCR mix, Fisher, # AB4318), Primers 0.4 μM each, 0.2 μM virus probe (5-carboxyfluorescein (FAM) -fluorescent-tagged probe from Sigma-Genosys Ltd., UK), 0.2 μM probe *Chicken Ovotransferrin* Gene (OT) (Yakima Yellow–fluorescent-tagged probe from Eurogentec, CA), 10 μg BSA (Bovine serum albumin, Sigma, 1.6 mg/mL) and 4 μL of DNA (10 ng/μL). An ABI PRISM® 7500 instrument (Applied Biosystems,CA) was used to amplify and detect the reaction products, using the following conditions: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 94°C for 15 sec and 60°C for 1 min (Baigent et al., 2005).

The DNA standards for the qPCR were provided by the Avian Oncogenic Group, Pirbright Institute, UK. Standard curves were prepared using 10-fold serial dilutions of DNA from chicken embryo fibroblast cells (**CEF**) infected with GaHV-2, GaHV-3 or MeHV-1, and non-infected CEF (for ovo reaction), all of which had been accurately calibrated against plasmid constructs of known target gene copy number. Data were analyzed using Microsoft Excel® according to Baigent et al. (2005).

Virus Isolation

Spleens of the affected birds were processed as follows. A half portion of the spleen was stored at −20°C for DNA extraction. The remainder was finely minced with a blade and suspended in 5 mL of DMEM (Powder, high glucose, GIBCO®) supplemented with 2% antibiotic-antimycotic solution (Antibiotic-Antimycotic 100X, GIBCO®). The cell suspension in DMEM + 2% antibiotic-antimycotic solution was macerated and passed through a 40 μm cell sieve using a 10 mL syringe and collected into a 50 mL Falcon tube. The cell suspension was layered onto a Ficoll gradient (Ficoll Paque Premium, GE Health Care,UK) and centrifuged at 450 × *g* for 15 min at 4°C. The white buffy coat was aspirated and washed at 1500 rpm for 5 min. The isolated lymphocytes were then added to primary CEF cultures that were 80% confluent, one of the cultures was a negative control sham infected (0.5 mL DMEM). Three infection doses were used: 6×10^6 spleen lymphocytes per well, 3×10^6 spleen lymphocytes per well and 1×10^6 spleen lymphocytes per well (6-well plate). This allowed the evaluation of different dilutions of the virus. The inoculated cells were incubated at 37°C in an atmosphere with 5% CO_2 and observed daily using an inverted light microscope (Olympus CKX31) for evidence of the cytopathic effect (**CPE**). Once the CPE was observed, DNA was prepared from the cells, and PCR was performed for GaHV-2 to confirm the presence of the virus.

Table 2. Histopathology findings in birds with clinical signs of Marek's Disease.

Bird[1]	Organ	Findings[2]
001	Sciatic nerve	Diffuse lymphocytic neuritis, vasculitis, associated with paralytic changes. Abundant heterophils.
002	Sciatic nerve	Multifocal mononuclear infiltration and edema.
003	Sciatic nerve	Diffuse and multifocal mononuclear infiltration
004	Sciatic nerve	Diffuse lymphocytic neuritis
	Thymus	Severe cortical depletion, lymphoid atrophy.
	Sciatic nerve	Multifocal lymphocytosis
005	Liver	Dilated bile duct.
006	Sciatic nerve	Multifocal lymphoid aggregates
007	Sciatic nerve	Diffuse lymphocytic infiltrates
008	Sciatic nerve	Diffuse and severe lymphocytic neuritis
009	Sciatic nerve	Mild lymphocytic neuritis.

[1]Identification number of the samples.
[2]Description of the lesions found in the histopathology.

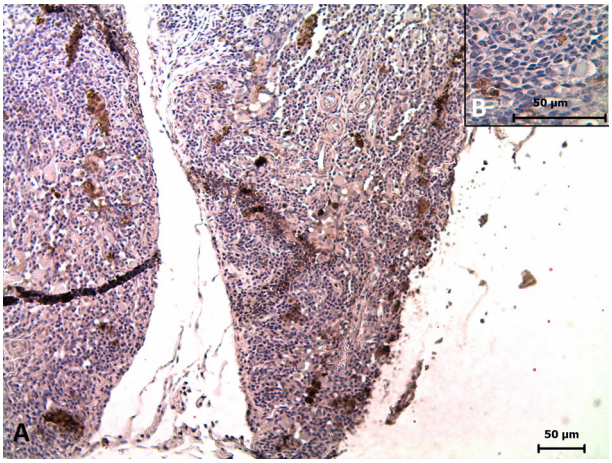

Figure 2. A. Section of thymus from a laying hen with clinical signs of MD from Colombia. Immunohistochemistry with anti-pp38 antibody in formalin fixed tissue. Antibody binding in macrophages and lymphocytes with some background staining observed in fibrous tissue. 100×. B. 400×.

RESULTS

The Histological Lesions were Compatible with Marek's Disease

A diffuse mononuclear infiltrate in the sciatic nerve was observed in birds with clinical signs of MD (Table 2). The infiltrate was interspersed with areas of edema and degeneration of fibers that is compatible with a highly virulent strain of MDV. Severe cortical thymus depletion reflected a state of immunosuppression (Figure 1). The apparently normal birds had mild lymphoid infiltration in the sciatic nerve, which is considered a sign of MD but, because of its low grade, it does not generate neurological signs or paralysis of the legs (Gimeno et al., 2013). In the spleen and thymus no significant changes were evident.

Immunohistochemistry with the anti-pp38 antibody showed positive staining in the cytoplasm of mononuclear cells (lymphocytes and macrophages) in thymus and spleen, as would be expected during the cytolytic phase due to the expression of the phosphoprotein in these cells. This was indicative of active infection with Marek's disease virus (Figure 2) (Gimeno et al., 2013).

Genomic Amplification of the 3 Serotypes of MDV in Blood

The standard PCR for the *Meq* gene (serotype 1, Figure 3A) resulted in 6 positive samples (6/17, 35.29%), which were purified for subsequent sequencing. Only 2 samples were positive for *glycoprotein D (gD)* gene PCR (GaHV-3). All samples were positive for MeHV-1 (data not shown).

A total of 94.11% (16/17) samples tested positive for GaHV-2 by qPCR, with an average of 567 copies of viral genome per 10,000 cells (minimum 0.33 copies, maximum 45,953 copies). The GaHV-3 qPCR detected an average of 2.3 copies per 10,000 cells in 94.7% of the samples. All samples were positive for MeHV-1 qPCR, with an average of 47.79 copies (data not shown).

The GaHV-2 Strain is Pathogenic According to its 132 bp Repeat Pattern

A total of 10 blood samples (59%) from birds with clinical signs of MD were positive for the BamH1-H – BamH1-D 132-bp PCR, 8 of these showed only

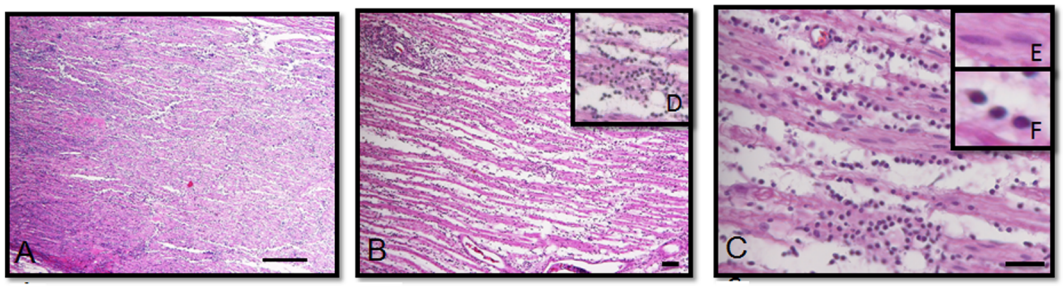

Figure 1. Lymphoid infiltrates in sciatic nerve of a bird with clinical signs of MD in different magnification. H&E stain. A. Sciatic nerve. Severe and diffuse mononuclear infiltrates were observed. bar = 100 μm B. Sciatic nerve. Severe diffuse mononuclear infiltrate was observed. Edema areas between nerve fibers (D). C. Sciatic nerve. Severe and diffuse mononuclear infiltrates. Basophile nucleus characteristic of macrophages (F) was observed. Those were located between nerve fibers. There were signs indicative of edema. Schwann cell nucleus (E). 400×.

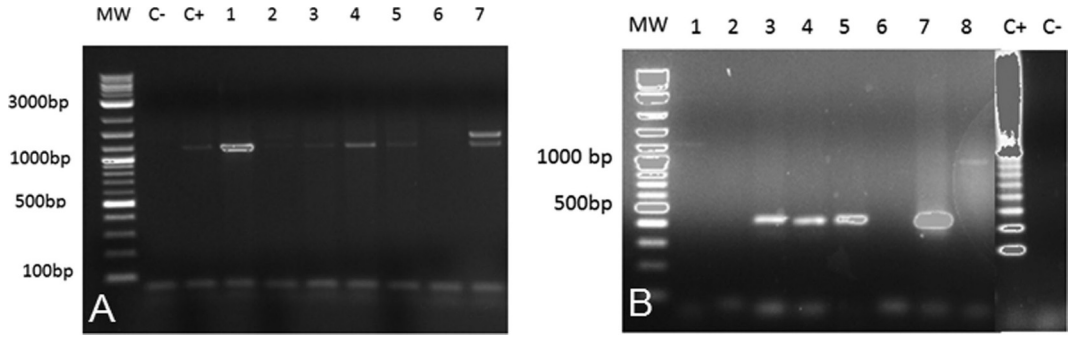

Figure 3. PCR amplification of the *Meq* gene from MD clinical case of this study. A. Expected PCR product band size: 1148 bp (RB1B strain) or 1325 bp (CVI988/Rispens strain). MW: Molecular weight marker 100 bp. C –: negative control (PCR reaction mix with water). C +: Positive control (CEF infected with RB1B strain). DNA blood samples in lanes 1 to 7. Sample with 2 bands corresponding to strains with long *Meq* gene copy was observed (An insertion of 59 amino acids in the proline-rich region) in lands 2 and 7, which is consistent with low virulent strains. Samples that have only one band lack this insertion and are associated with increased virulence; 4 samples exhibit a single band. B. PCR products of the 132 bp repeat segment. Positive Control: CVI988/Rispens strain (8 bands). DNA blood samples in lanes 1 to 8. Image was taken using an UV transilluminator with GelCapture Acquisition Software.

Table 3. Amino acid substitution in the Meq protein of GaHV-2 strains.

Identification			Amino acid position in the Meq protein of MDV														
Strain	Pathotype	Code[2]	71	77	80	93	115	119	153	176	180	217/276[1]	233	258	277/336	283/342	320/379
UDEACO04/2013	vv+MDV	KU058701	A[3]	E	Y	Q	A	C	P	A	A	A	L	S	L	A	I
UDEACO06/2013	vv+MDV	KU058696	A	E	Y	Q	A	C	P	A	A	A	L	S	L	A	I
UDEACO07/2013	vv+MDV	KU058697	A	E	Y	Q	A	C	P	A	A	A	L	S	L	A	I
cu-2	mMDV	AY362708	S	E	D	Q	V	C	P	P	T	P	P	L	L	A	I
567	vMDV	AY362709	A	E	Y	Q	V	R	P	P	T	A	P	L	L	A	I
571	vMDV	AY362710	A	E	Y	Q	A	C	P	H	T	P	P	L	L	A	I
573	vMDV	AY362711	A	E	Y	Q	A	C	P	H	T	P	P	L	L	A	I
617A	vMDV	AY362712	A	E	Y	Q	V	R	P	P	T	A	P	L	L	A	I
JM	vMDV	AY243331	S	A	D	R	A	C	P	P	T	P	P	L	L	A	I
660A	vv+MDV	AY362726	A	K	D	Q	V	R	Q	A	A	A	P	L	P	A	I
686	vv+MDV	AY362727	A	K	D	Q	V	R	Q	A	A	A	P	L	P	A	I
NEW	vv+MDV	AY362719	A	K	D	Q	V	R	Q	A	T	A	P	L	L	V	T
RL	vv+MDV	AY362720	A	K	D	Q	V	R	Q	A	A	A	P	L	L	A	I
TK	vv+MDV	AY362721	A	K	D	Q	V	R	Q	A	A	A	P	L	L	A	I
U	vv+MDV	AY362722	A	K	D	Q	V	R	Q	A	A	A	P	L	P	A	I
For Avian W	vv+MDV	AY362723	A	K	D	Q	V	C	P	P	T	A	P	L	L	V	T
X	vv+MDV	AY362724	A	K	D	Q	V	R	Q	A	A	A	P	L	L	A	I
643p	vvMDV	AY362716	A	K	D	Q	V	R	Q	A	A	A	P	L	F	A	I
RB1B	vvMDV	AY243332	A	K	D	Q	V	C	P	P	T	P	P	L	L	A	I

Position of the amino acid in the Meq protein. Unique changes of the UdeA-2013CO Strain are underlined.
[1]Position according to the isoform: long or short *Meq* gene.
[2]Accession number in the NCBI.
[3]Amino acid's code.

1 band, which is associated with high pathogenicity (Figure 3B). Two samples showed a 1,000 bp band that might be associated with less pathogenic strains. This result suggests that there is more than one strain of GaHV-2 affecting the flock (Becker et al., 1992; Davidson et al., 2002).

Meq Gene Mutations Have Similarities to Those in Highly Virulent (Vv+MDV) Strains

The deduced amino acid sequence of the *Meq* gene for UDEACO-2013 strain (339 aa) was aligned with high- and low-virulence strains isolated in different geographical regions and several vaccine strains (Table 3). The UDEACO-2013 strain, isolated from the clinical case reported in the farm, has point mutations in the proline-rich region: P176A, P217A, and P233L. These exchanges in the second position of proline (PXPP) are associated directly with highly virulent strains. Unique substitutions in P233L and L258S were detected in UDEACO-2013 strain; these specific mutations have not been reported so far in any strain isolated in other geographical regions. It was found, by BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi), that the UDEACO-2013 strain had 99% identity with strains isolated in China during 2006–2008 (Zhang et al., 2015a; Yu et al., 2013; Gong et al., 2013; Zhang et al., 2011) (LDH: HQ658614.1; G2: AF493556.1; LHC4: HQ658618.1). The UDEACO-2013 strain also has a substitution E77K, which has been associated with strains of low virulence (Zhang et al., 2011).

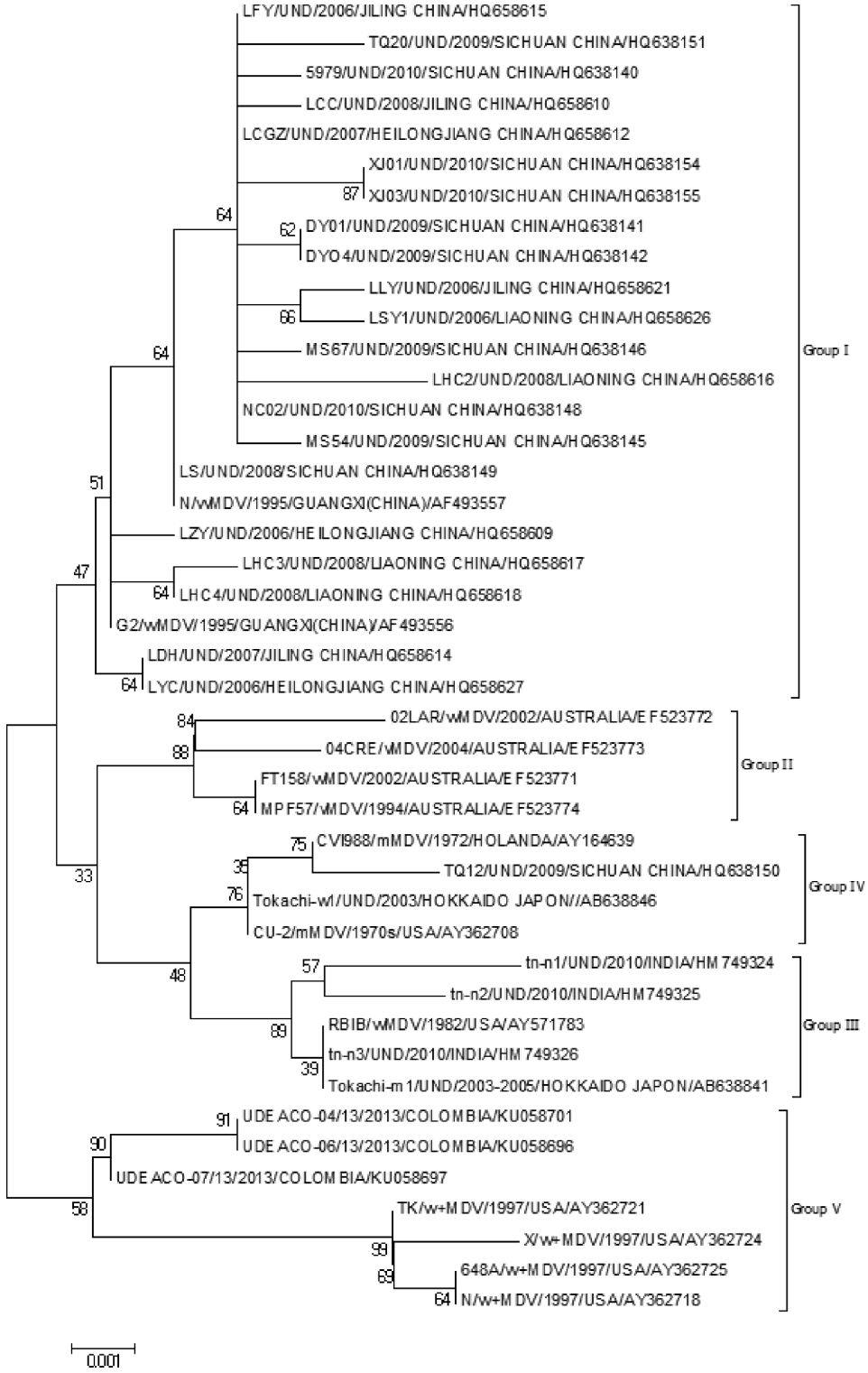

Figure 4. Phylogenetic relationships between 43 GaHV-2 strains based on *Meq* gene sequences. The phylogenetic tree was inferred by the Neighbor Joining Method. Strain name, pathotype, year of isolation, country of origin and GenBank accession numbers are indicated in the tip labels. Numbers at the nodes are bootstrap values for the clade. UND: undetermined pathotype. The analysis was performed in MEGA 6.

The UDEACO-2013 Strain Clustered with vv+ MDV Strains

The phylogenetic relationships, based on the alignment of *Meq* gene sequences inferred by the neighbor joining method, identified 5 monophyletic clusters: Group I including Chinese isolates, Group II comprising Australian isolates, Group IV including attenuated strains and Group III encompassing very virulent strains and Group V including hypervirulent strains. The UDEACO-2013 strain, clustered with hypervirulent strains within group V with a bootstrap value of 58% (Figure 4).

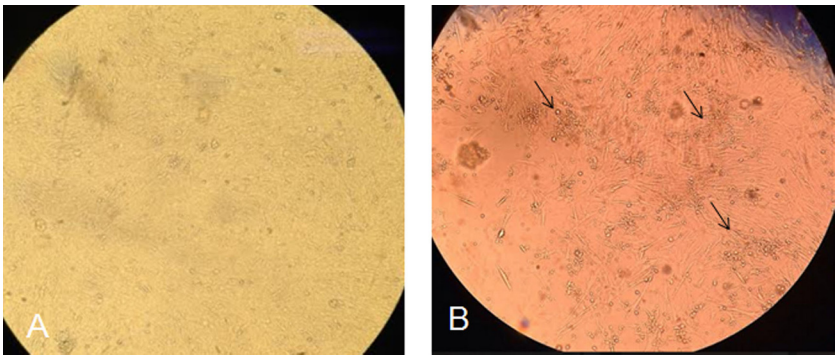

Figure 5. Virus isolation in chicken embryo fibroblast. A. Mock. Intact monolayer. Confluent cell. B. Cells inoculated with 6×10^6 spleen lymphocytes viewed 5 d after inoculation. There are some plaques characteristic of serotype 1 MDV (arrows). 100×.

The Cytopathic Effect in Cell Culture is Characteristic of MDV GaHV-2

Formation of plaques in the monolayer with the presence of large round cells was observed 5 d post inoculation in all 9 wells inoculated with 6×10^6 or 3×10^6 lymphocytes, those plaques are characteristic of *Herpesvirus* (Figure 5). PCR was performed for the *Meq* gene to confirm the presence of the genome of the GaHV-2 in the harvested cells, with a positive PCR result for all samples showing CPE (data not shown).

DISCUSSION

MD is an oncogenic disease that infects predominantly chickens and is estimated to cost $1 to $2 billion annually to the poultry industry worldwide (Atkins, 2010). Although there is no information about economic losses due to MD, it may be one of the most severe viral diseases that is currently affecting the poultry production systems in Colombia. Despite that in the Colombian hatcheries implementing an intensive vaccination program against MDV with the use of CVI988/Rispens + HVT in day-old chickens, poultry flocks still succumb to infection, with sporadic reported cases of Marek's disease like symptoms (Okonkwo, 2015; Okwor and Eze, 2011; Witter et al., 1980; Zhang et al., 2011). Most of the time these cases are undiagnosed causing major problems. For this reason it is necessary to know and to understand how the molecular characteristics of the virus can guide us to understand different clinical outcomes of MD in the field allowing to improve control measures against this important disease. The results presented here contribute to the knowledge of MD in Colombia. Molecular analysis indicates that the characteristics of the virus correspond to very virulent+ strain.

In the classical form of the disease, the nerves are mainly affected, and mortality rates within a flock rarely exceeds 10–15% (OIE, 2016). In this case, the mortality reached over 30% in 50 wk, with no visible lesions or gross visceral lymphomas and the macroscopic and microscopic lesions found in the tissue samples (thymus and sciatic nerves) suggested the presence of MD (Davison and Nair, 2004; Haridy et al., 2009). These findings suggest that the birds presented the classical form of MD probably with secondary infections (not evaluated in this study), which could explain the relatively high mortality (Schat and Skinner, 2014).

The immunohistochemical staining for the MDV pp38 protein in the thymus showed abundant pp38 antigen, confirming the presence of MDV within the lesions (Carvallo et al., 2011). According to Singh et al. (2012) this protein is expressed in all tumor stages of the disease, but Gimeno et al. (2005) observed this antigen widely during the cytolytic phase, where the processes of atrophy and lymphoid depletion occur and can be explained by the reactivation of some of the lymphoid cells in latent phase, which could be the case. In spite of the fact that virus antigens were not detected by IHC, a cellular infiltration in nerves was detected in all the samples (Singh et al., 2012; Gimeno et al., 2005). This is in accordance with Lawn and Payne (1979), who mentioned that inflammatory changes in nerves are observed as early as 5 d after infection, and can be present until the latent phase of the disease (Lawn and Payne, 1979). However, no virus particles can be found in nerves at any time, which supports our findings in the IHC. The infiltration in nerves also correspond to lesions compatible with MD as described in other studies (Gimeno et al., 2013; Haridy et al., 2009; Calnek et al., 1998).

The presence of MDV DNA in the whole blood samples was also demonstrated. The positive results that demonstrate the presence of MeHV-1 and GaHV-2 by PCR can be explained due to the use of recombinant vaccine (HVT + IBD and Rispens) administered at the hatchery by the subcutaneous route in 1 day old chicks. There were some positive samples for GaHV-3, which corresponds to a natural infection with a non-virulent field strain. However, the qPCR revealed high amounts of GaHV-2 in some samples of affected birds. The GaHV-2 vaccine strain genome is quantifiable by qPCR in immune organs as early as 7 d post infection (**dpi**), peaks by 28 dpi, but then gradually decreases by 40 dpi, while the vvMDV strain can still be detected in peripheral blood and tissues by 90 dpi and can cause tissue destruction, resulting in immunosuppres-

sion (Zhang et al., 2015b). We found as many as 45,953 copies of viral GaHV-2 genome per 10,000 cells, which is higher than expected in that age (<1,000 copies) if the detection is due to vaccine strain alone (Baigent et al., 2011; Baigent et al., 2005). It has been found that the number of copies of the genome is low during the first d after infection, but rapidly increases at 14 d and reaches its peak at 28 d (Islam et al., 2006), which explains why there were some birds with low levels of GaHV-2, since the infection could be in the cytolytic phase (approximately 7 dpi). Additionally the Rispens vaccine strain significantly reduces the viral load of vv+ MDV in blood (Haq et al., 2012). Co-infection with other MDV strains has been demonstrated and suggests that short-interval challenge exposure and weak initial exposures may be important factors leading to infection (Dunn et al., 2010; Zhang et al., 2015b). There are also reports of an interaction between pathogenic and vaccine viruses (Haq et al., 2012). Additionally, the BamHI-H PCR amplified a 434 bp fragment (2 132 bp repeats), in 8 of 10 clinical samples suggesting the presence of virulent oncogenic MDV. The Rispens vaccine strain and non-pathogenic strains have many copies of the 132 bp repeats and shows 6 to 8 bands. Based on the results found in this study it could be suggested that an increase in the virulence of the field virus is involved in the present outbreak (Kalyani et al., 2011; Becker et al., 1992).

Furthermore, it is thought that the increase in clinical MD cases from vaccinated birds is attributed to an increased virulence of MDV strains (Gimeno, 2008; Davison and Nair, 2004). It is not clear why virulent pathotypes are able to break vaccine immunity (Gimeno, 2008). GaHV-2 can cause disease in vaccinated chickens due to lowered protection or reduced vaccine virus viability before the administration (inappropriate storage, handling and administration) (Baigent et al., 2006); even in well vaccinated chicks, the very virulent strains of MDV could break through the protection provided by MeHV-1 and GaHV-2 vaccine immunization due to an immune failure caused by co-infections with other pathogens like chicken infectious anemia virus, reovirus and infectious bursal disease virus (Miles et al., 2001; Xiu-guo et al., 2008; Dong et al., 2014; Gong et al., 2013). Furthermore, viral evolution or vaccine escape has been associated with the use of a multitude of vaccines especially in situations whereby sterile immunity is not achieved (Witter, 1997). In Colombia, the presence of MD in poultry flocks can be explained with the emergence of new virulent MDV strains (Witter, 1997), compounded with vaccine mishandling and imperfect vaccination (Read et al., 2015), or co-infection with immunosuppressive agents (Otaki et al., 1987). Nevertheless, we believe that is the first report to demonstrate, with the use of molecular characterization techniques, the presence of MDV in Colombian layer flocks and furthermore, viral evolution that could lead to vaccine escape.

In order to characterize the virus isolate, blood samples were also screened by PCR amplification of the *Meq* full-length gene. It has been described that oncogenic GaHV-2 strains possess a unique basic protein called *Meq* (MDV EcoRI Q) encoded by the internal repeat long and terminal repeat long regions (named IRL and TRL respectively) (Jones et al., 1992); also it has been shown that point mutations and polymorphisms in this protein are directly associated with virulence (Chang et al., 2002; Shamblin et al., 2004). During the GaHV-2 infection, Meq protein is expressed in the transformed cells, and is also associated with the latent phase. This leucine zipper protein (b-ZIP) is located in the nucleus and nucleolus of infected cells; it blocks apoptosis, induces proliferation and activates suppression type genes, these functions being located in the Meq protein carboxy terminal region in a long block of repeats of proline among the amino acid residues 146 and 252 (Jones et al., 1992; Jones et al., 1996). Exchanges in this region are associated with pathotype: thus low virulence strains have proline repeat regions (PPPP), whereas virulent strains present a single exchange in the second amino acid from proline rich region (PA/Q PPP) (Shamblin et al., 2004). The novel mutations that were found in 3 sites (176, 217: A and 253: L) of *Meq* gene of UDEACO-2013, might suggest that this is a highly virulent strain, although we did not find any changes at the position 153 that were reported by other authors in the very virulent strains (Shamblin et al., 2004). On the other hand, the finding of a unique amino acid exchange in 2 positions of the *Meq* gene (P233L and L258S) from the field virus circulating in this farm should be investigated in future analysis of other Colombian isolates of the virus to explore if these changes are regular, a situation that has been demonstrated in countries like China (Yu et al., 2013), where they have observed that amino acid mutations in *Meq* genes of vaccinated birds displayed regularity at certain positions (Zhang et al., 2011). It is important to assess whether these amino acid exchanges correspond to a continuing evolutionary shift of MDVs to greater virulence (Witter et al., 1980).

The causal agent of this outbreak was also confirmed by the virus isolation in CEF. The cell cultures infected with splenic cells of sick birds showed CPE in the monolayer compatible with MDV plaques (Schat, 2005; Awatif et al., 2001; Davison and Nair, 2004) and was further confirmed by PCR of the harvested cells.

The phylogenetic analysis of UDEACO-2013 strain, isolated from the clinical case reported in a commercial farm in Colombia, clustered in the phylogenetic tree within Group V with a bootstrap support value of 58%. This group encompasses highly virulent (vv+) strains of the virus isolated in the United States. Although supported by a lower bootstrap value, the Colombian strain appeared related to these hypervirulent strains and could behave as such based on the phy-

logenetic analysis of *Meq* gene sequences. Future sampling of more clinical cases in Colombia would provide sufficient sequence data to assess whether Colombian strains form a distinct clade closely related to these hypervirulent strains of the virus. The analysis of the DNA sequence of the sample and the amino acid exchanges detected in the proline-rich region allowed the authors to suggest that the outbreak was due to vv+ MDV (Kalyani et al., 2011). However, *in vivo* pathotyping assays in SPF chicken (Walkden-Brown et al., 2013; Dudnikova et al., 2007) to determine the mortality and severity of the lesions are required for confirmation of the pathotype status of these isolates. As we do not currently have the infrastructure to carry out these *in vivo* studies, we are unable to confirm the identification of the MDV pathotype prevalent in Colombia.

In spite of that, the changes in the Meq protein found in this virus are very characteristic of pathogenic MDV and, together with the type of lesions observed in sciatic nerves, presence of the genome of MDV, the quantity of the DNA virus in the samples and the previous history of mortality and vaccination are enough to confirm the highly virulent nature of the MDV strains prevalent in this farm.

This study shows the results of the first molecular analysis of MDV in Colombia suggesting that highly virulent strains of the virus are circulating in the country and could be easily spreading by environmental conditions and biosecurity failures. We demonstrated the presence and quantified the 3 serotypes of MDV in the samples from birds with clinical signs in the Andean region from Colombia. Furthermore, the detection of viral protein pp38 demonstrated in tissues and the histological lesions were consistent with Marek's disease. Additionally, we suggest that the clinical case was the result of an infection with a highly virulent serotype 1 strain (vv+ MDV) because of the predicted amino acid sequence analysis of Meq protein and phylogenetic analysis of the strain.

The results highlight the importance of determining the risk factors for virulence evolution of MDV and consequently to develop control strategies which will prevent the emergence of virulent strains (Witter, 1997). Application of the qPCR for MDV diagnostics in Colombia could significantly increase our understanding of the epidemiology, spread, diagnosis and vaccine control of MD in our country. The identification of the pathotypes is also necessary to establish the standards in the biosecurity of the farms, and to design control programs, like re-vaccination and monitoring the levels of the virus spread during the critical age. Future research should focus on identifying the pathotype present in the farms and verifying the vaccines effectivity against this vv+ MDV strain.

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