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Original

Detection and characterization of *chicken anemia virus* in Colombia

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ABSTRACT

Objective. identify the presence of chicken anemia virus (CAV) in poultry farms and backyard chickens from Antioquia, Colombia. **Materials and Methods.** Blood and feather samples were taken from laying chickens; in each farm, three birds of six different ages (1, 15, 30, 60, 90, and 120 days old) were chosen randomly. Backyard chicken samples were also obtained near the research farms. We used serology and molecular techniques to analyze the samples. **Results.** By PCR, the 84% of the birds were positive in whole blood and 66% were positive in feather samples. The 60% of backyard chickens tested were positive in blood and 40% in feather follicle. By serology, the 22% of the poultry farm birds presented high antibody titers and 19% moderate antibody titers. In the backyard chickens, 43% of them presented high antibody titers and 29% moderate antibody titers. In addition, results from the RFLP test and sequencing showed that the circulating virus found in this study was different from the Cux-1 vaccine strain used in Colombia. **Conclusions.** CAV is present in Colombia in both commercial and backyard chickens. According to the findings, a high percentage of the birds tested positive for viral detection, whereas the number of birds that tested positive for antibodies was low. Thus, the characteristics of the circulating virus need to be determined to explain the antibody response observed in this study.

Keywords: Chicken anemia virus; molecular detection; phylogenetic analysis; sequencing (*Source: NLM*).

RESUMEN

Objetivo. identificar el virus de la anemia infecciosa aviar (*chicken anemia virus*, CAV) en granjas avícolas y aves de traspatio en Antioquia, Colombia. **Materiales y métodos.** Se tomaron muestras de sangre y plumas de gallinas ponedoras; en cada granja se eligieron tres aves de seis edades diferentes (1, 15, 30, 60, 90 y 120 días de edad). También se obtuvieron muestras de aves de traspatio ubicadas cerca de las granjas estudiadas. Se realizó ELISA y PCR para el análisis de las muestras.

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Resultados. Mediante PCR, el 84% de las aves resultaron positivas al CAV en sangre total y el 66% en muestras de plumas. El 60% de las aves de traspatio dieron positivo en sangre y el 40% en folículo de pluma. Mediante ELISA, el 22% de las aves de las granjas avícolas presentó títulos de anticuerpos altos y el 19% moderados. En las aves de traspatio, el 43% presentó títulos de anticuerpos altos y 29% moderados. Además, los resultados de la prueba de RFLP y la secuenciación mostraron que el virus circulante encontrado en este estudio era diferente del de la cepa vacunal Cux-1 utilizada en el país. **Conclusiones.** El CAV está presente en Colombia en aves comerciales como de traspatio. Según los hallazgos, un alto porcentaje de las aves dieron positivo para la detección viral, aunque el número de aves positivas por anticuerpos fue bajo. Se requiere determinar las características del virus circulante para explicar la respuesta de anticuerpos obtenida.

Palabras clave: Análisis filogenético; estudio molecular; secuenciación; virus de la anemia infecciosa aviar (*Fuente: NLM*).

INTRODUCTION

Avian infectious anemia (AIA) is caused by chicken anemia virus (CAV), which occurs in poultry under 2 weeks old and without maternal antibodies (1). CAV is transmitted both vertically and horizontally (2), being excreted through feces (3) and probably through feathers (4). The virus infects T cell lymphoblasts and hemocytoblasts (5,6) causing immunosuppression (7) that may allow other infectious agents to enter (8).

CAV is one of the ten members of the genus *Gyrovirus* within the family *Anelloviridae* (9). Its 2.3 kb genome is circular, with negative single-strand DNA (1). It encodes a polycistronic RNA (5) with three open reading frames for three viral proteins: VP1, VP2 and VP3 (10). The fragment encoding VP1 is characterized by its high variability (2,11), making it useful for CAV characterization and phylogenetic analysis (12). Although the virus has been detected and characterized in various parts of the world (1), no studies on it have been carried out in Colombia, even though breeders are vaccinated before laying.

Due to the lack of information in the country, this study aimed to identify the presence of CAV, perform a phylogenetic characterization of the circulating strains and determine antibody levels in commercial farms of laying birds of different ages and in backyard chickens in the east and north of Antioquia, Colombia.

MATERIALS AND METHODS

Sampling area. Blood and feathers samples were taken between 2014 and 2015 from Lohman Brown and Isa Brown laying birds clinically healthy with no history of having been vaccinated against CAV which was the object study virus. The birds belonged to four poultry farms located in the department of Antioquia, Colombia. In each farm, three birds at six different ages (1, 15, 30, 60, 90 and 120 days old) were randomly selected. Samples of backyard chickens located near the study farms were also included. The sampling method was for convenience, and it was no possible to sample all birds at 90 days old neither backyard. The animal procedures and methods used in the study were approved by the ethics committee for animal experimentation at the Universidad de Antioquia (Act 99 of 2015).

ELISA detection of antibodies against CAV. To identify animals with antibodies against CAV, a commercial kit (IDEXX, United States) was used, following the manufacturer's instructions. Blood samples were centrifugated at 2000 x g for 5 minutes to obtain the serum fractions, which were diluted 1:100 for the test. The results were read on the spectrophotometer (BioTek. Vermont, United States) at 650 nm wavelength.

The analyses were performed according to the instructions of the kit manufacturer (IDEXX, United States). The ratio of the optical density readings of the samples to that of the negative

control (S/N) was used to calculate the results. Samples with values less than 0.2 were defined as positive and those with values greater than 0.8 as negative. A relation of the result value, the corresponding titer and protection was established. Very low titers below 1000 were assumed to confer no protection; moderate titers between 1000 and 8600 conferred low protection, and high titers for results greater than 8600 were related to high protection titers (13).

PCR detection of CAV in blood and feather samples. DNA from blood samples was extracted using a commercial kit (Qiagen, United States) following the manufacturer's instructions. For the extraction of DNA from the feather, the same kit was used with the following amended protocol. Briefly, the feather follicles were cut into fragments of approximately 2 mm mixed with 180 µL of the ATL buffer and 20 µL of proteinase K. The mixture was incubated at 56°C for one hour with the product being mixed every 15 minutes. DNA was stored at -20ºC.

A total of 81 whole blood DNA samples (3 farms with 18 samples each, 1 farm with 17 samples, and 10 backyard chicken samples), 29 feather DNA samples (6 pools per farm, and 5 backyard poultry pools), and 78 blood serum samples (3 farms with 18 samples each, 1 farm with 17 samples, and 7 backyard chicken samples) were analyzed.

For detection, a PCR was carried out to amplify a 713-bp fragment of the gene that encodes the CAV VP2 protein, with the primers 5-GCG CAC TAC CGG TCG GCA GT-3 and 5-GGG GTT CGG CAG CCT CAC ACT AT-3 (14). Amplification was carried out in PCR buffer containing 2 mM $MgCl_2$, 0.4 mM of deoxynucleotide 5'-triphosphate, 0.4 pM of each primer and 5 U of *Taq* DNA Polymerase (ThermoFisher Scientific, United States) in a final volume of 25 µL. The reaction was carried out in an automated thermal cycler (MJ Research, Massachusetts, United States) with the following profile: initial denaturation of 94°C for 5 minutes, followed by 34 denaturation cycles, annealing and extension of 94°C for 1 minute, 63°C for 1 minute and 72°C for 1 minute, respectively.

PCR targeting the VP1 gene. In the CAV-positive samples, PCR assays were performed to independently amplify two fragments of the gene encoding the VP1, one of them used the primer pair forward 5-GAC TGT AAG ATG GCA AGA CGA GCT C-3 and reverse 5-GGC TGA AGG ATC CCT CAT TC-3 to amplify a 675-bp fragment (15), and the other used the primer pair forward 5-AGC CGA CCC CGA ACC GCA AGA A-3 and reverse 5-TCA GGG CTG CGT CCC CCA GTA CA-3 to amplify a 1390-bp fragment (14). The reactions were carried out in PCR buffer containing 2 mM $MgCl_2$, 0.4 mM of deoxynucleotide 5'-triphosphate, 0.4 pM of each primer and 5 U of Taq DNA Polymerase (ThermoFisher Scientific, United States) in a final volume of 25 µL.

The PCR conditions for the amplification of the 675-bp fragment consisted of: initial denaturation of 95°C for 5 minutes, followed by 30 cycles of denaturation, annealing and extension of 95°C for 30 seconds, 50°C for 30 seconds and 72°C for 1 minute, respectively. On the other hand, for the amplification of the 1390-bp fragment the conditions were as follows: initial denaturation of 94°C for 5 minutes, followed by 34 cycles of denaturation, annealing and extension of 94°C for 1 minute, 56°C for 1 minute and 72°C for 2 minutes, respectively.

RFLP for strain characterization. The 675-bp fragment PCR products of the VP1 gene from the study samples and the Cux-1 vaccine strain were independently digested with endonucleases: *Dde* I (HpyF3I), *Hae* III (BsuRI), *Hha* I, *Mbo* I, *Hpa* II y *Hinf* I (ThermoFisher Scientific, United States) (15,16). The reaction in a final volume of 32 mL contained 10 mL of the PCR product, 1X of the recommended buffer for each enzyme and 20 U of the enzyme. The reaction was incubated at 37°C for 6 hours and denaturated at 80°C for 30 minutes. The digested products were analyzed and separated by electrophoresis with 1X TBE buffer in 15% polyacrylamide gel and stained using the Silver Kit (ThermoFisher Scientific, United States) according to the manufacturer's recommendations. The number of bands and their weight was determined from the 50-bp and 100-bp molecular weight markers (GeneRuler DNA Ladder, ThermoFisher Scientific, United States)

Phylogenetic analysis. The sequences M5S3_N_CO_2015, D5S2_N_CO_2015 and C+_vaccine_Cux_1_RegICA8649BV derived from the VP2 gene were edited and assembled using the Geneious® 9.1.8 (Biomatters Ltd., New Zealand). After that, they were aligned with each other and with respect to homologous sequences available in GenBank, including the sequence of the CAV strain Cux-1 (NC_001427.1), as the reference (only the fragment corresponding to the length of the sequences gotten in this study was included), using the ClustalW algorithm

integrated into the MEGA 7.0 software (17). The nucleotide and amino acid positions were identified according to the reference sequences NC 001427.1 and NP 056774.1.

With the purpose of establishing the relationship between the sequences obtained in the present study and other sequences from strains circulating worldwide, a phylogenetic tree was inferred through the MEGA 7.0 software, by using the Maximum Likelihood (ML) method with the nucleotide substitution model of Kimura-2-parameters (17,18). The *Bootstrap* support method was used with 1000 replicates to establish the confidence for the tree topology. The nucleotide sequences generated in the present study were deposited in GenBank database.

RESULTS

PCR amplification of the CAV VP2 from blood samples. According to the PCR results of amplification of the VP2 gene, 84% (68/81) of the total samples analyzed were positive for CAV detection. Positive CAV birds were found at all ages: 92% (11/12) of the 1-day-old birds; 83% (10/12) of the 15-, 30- and 60-day-old birds; 82% (9/11) of the 90-day-old birds and 100% (12/12) of the 120-day-old birds were all positive. In backyard chickens, 60% (6/10) tested positive for CAV (Table 1).

PCR amplification of CAV VP2 from feather samples. Of the total feather samples studied, 66% (19/29) were positive for CAV. In this study, positive feather samples were found from commercial birds at all ages. The results were as follows: 100% (4/4) of 1- and 60-day-old birds; 50% (2/4) of 15-day-old; 75% (3/4) of 30- and 90-day-old; and 25% (1/4) of 120-day-old birds tested positive. In addition, 40% (2/5) of backyard chickens tested positive for the virus (Table 1).

Serology for CAV. Of the total serum samples tested 41% (32/78) were positive for CAV antibodies. There were 22 percent (17/78) of positive birds with high antibody titers and 19 percent (15/78) with moderate titers. Percentages of animals who presented high antibody titers against CAV were: 50% (6/12) of 1- and 120-day-old birds, 8% (1/12) of 15-day-old birds and 9% (1/11) of 90-day-old birds. Percentages of birds who only presented moderate antibody titers were: 17% (2/12) 30-day-old birds and 8% (1/12) of 60-day-old birds. Of the total backyard chickens tested 43% (3/7) presented high antibody titers and 29% (2/7) moderate titers (Figure 1).

Table 1. PCR results of the VP2 fragment and Serology.

Age	PCR of the VP2 (713 bp)				Serology for CAV			
	PCR in blood		PCR in feather		S/N <0.2[a]		S/N 0.8-0.2[b]	
1-day-old	92%	(11/12)	100%	(4/4)	50%	(6/12)	17%	(2/12)
15-day-old	83%	(10/12)	50%	(2/4)	8%	(1/12)	25%	(3/12)
30-day-old	83%	(10/12)	75%	(3/4)	0%	0	17%	(2/12)
60-day-old	83%	(10/12)	100%	(4/4)	0%	0	8%	(1/12)
90-day-old	82%	(9/11)*	75%	(3/4)	9%	(1/11)	27%	(3/11)
120-day-old	100%	(12/12)	25%	(1/4)	50%	(6/12)	17%	(2/12)
Backyard chicken	60%	(6/10)*	40%	(2/5)	43%	(3/7)	29%	(2/7)
Total positives	84%	(68/81)	66%	(19/29)	22%	(17/78)	19%	(15/78)

[a]Positives with high antibody titers; [b]Positives with moderate titers; *The sampling was carried out for convenience, but in these farms it was no possible to sample 12 birds as in the other farms.

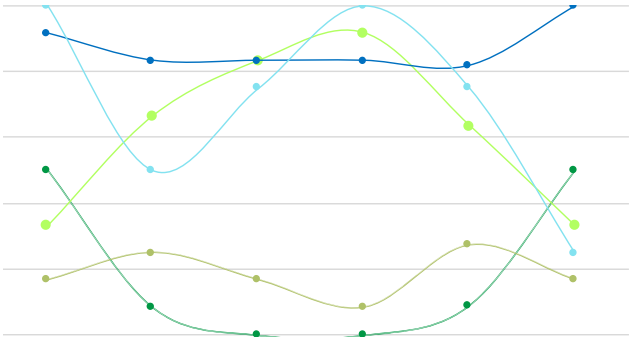

Figure 1. Percentage of CAV-positive animals *versus* animals with antibodies against the virus (birds from 1 to 120 days of age).

The percentage of CAV-positive animals in blood by PCR is indicated by blue line. The percentage of CAV-positive animals in feather follicle by PCR is indicated by light blue line. The percentage of antibodies by ELISA is indicated by green lines (high antibody titers: S/N <0.2; moderate antibody titers: S/N 0.8-0.2; low titers or absence of antibodies: S/N> 0.8).

Molecular characterization of the CAV strain circulating in Colombia. The RFLP results for Colombian samples showed a pattern that differed from that of the control strain (Cux-1 vaccine strain)(Figure 2).

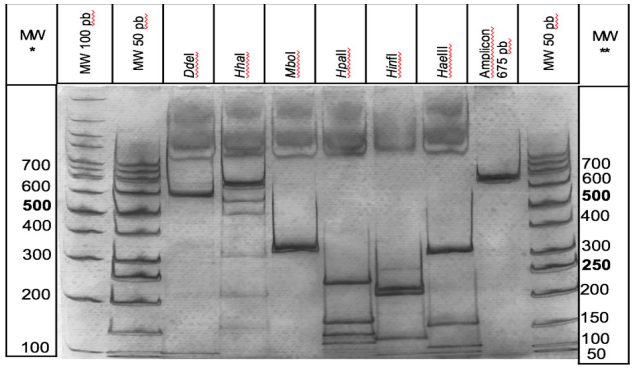

Figure 2. RFLP characterization of the positive control (Cux-1 strain) by Polyacrylamide gel (15%) through *Hha* I, *Mbo* I, *Dde* I, *Hae* III, *Hinf* I and *Hpa* II enzymes. Column 1* and 12**: molecular weight markers of 100 pb and 50 pb, respectively. Column 2, 3 and 11: bands positions of molecular weight markers. Columns 4 to 9: bands positions of Cux-1 digestion with endonucleases. Column 10: 675 pb PCR fragment of the VP1 gene from the Cux-1 vaccine strain.

A restriction pattern like the Cux-1 strain was only obtained for the *Hha* I and *Mbo* I enzymes. A different restriction pattern of Cux-1 was obtained for *Dde* I, *Hae* III, *Hinf* I and *Hpa* II. Interestingly, *Hae* III and *Hpa* II showed more cuts than *Hinf* I and *Dde* I (Table 2).

Table 2. Summary of molecular bands obtained by RFLP of the studied samples (M) in comparison with the positive control (C+).

Endonuclease	Number of bands with respect to molecular weight (pb)													
	=50 –<200		≥200 –<300		≥300 –<400		≥400 –<500		≥500 –<600		≥600		Total bands	
	M	C+	M	C+	M	C+	M	C+	M	C+	M	C+	M	C+
***Dde* I**	3	-	1	-	1	-	-	-	-	1	-	-	5	1
***Hha* I**	-	1	**1**	**1**	**1**	**1**	-	-	-	2	-	1	2	6
***Mbo* I**	-	-	-	-	**1**	**1**	-	-	-	-	-	-	1	1
***Hpa* II**	2	3	1*	1*	-	-	2	-	-	-	-	-	5	4
***Hinf* I**	-	1	-	2	-	-	-	-	1	-	-	-	1	3
***Hae* III**	-	2	3	-	-	1	-	-	-	-	-	-	3	3

*: this result had the same number of bands but their position in the range was different.

The nucleotide sequences of the partial VP2 gene of positive samples obtained from the study were identified as M5S3_N_CO_2015 and D5S2_N_CO_2015 and deposited in GenBank* with access numbers MF357710 and MF357711. These sequences corresponded to CAV circulating in 90-day-old laying birds on farms located in the north of Antioquia, Colombia. The positive control of the reaction was identified as C+_vacuna_Cux_1_RegICA8649BV and deposited in GenBank* with access number MF357712. The length of these sequences, excluding the hybridization regions of the oligonucleotides used during the PCR amplification, was 221 bp, located between nucleotides 640 and 860 of the reference genome. Two large lineages were observed in the constructed tree, which were identified as A and B (Figure 3). The sequences from Colombia belonged to lineage A along with most of the analyzed sequences, except for Mhd8 and Mhd6 from Iran, which were the only sequences located in lineage B.

*ID GenBank of sequences from this study: MF357710, MF357711, MF357712.

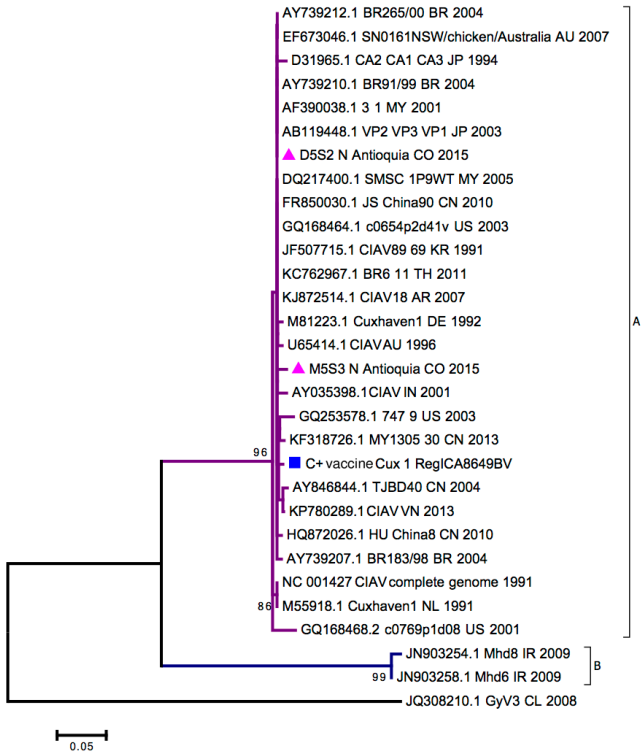

Figure 3. Molecular phylogenetic analysis of 26 nucleotide sequences of CAV.

Phylogenetic tree built by the Maximum Likelihood method with the 2-parameter Kimura nucleotide substitution model (18) of the MEGA 7.0 software (17), with 1000 *bootstrap* replicates; the relevant values of the branches are indicated (≥60). Two clades (clade A, and clade B) were identified; most of the sequences were in group A. The sequences identified in the farms studied are indicated with pink triangles (D5S2-N-Antioquia-CO-2015 and M5S3-N-Antioquia-CO-2015), the control sequence with a blue square (vaccine strain, C+_vaccine_Cux_1_RegICA8649BV), and the 26 sequences available in GenBank were identified with the access number followed by the name, country, and year.

Three nucleotide positions were variable when the M5S3_N_Antioquia_CO_2015, D5S2_N_Antioquia_CO_2015 and C+_vacuna_Cux_1_RegICA8649BV were pairwise compared. Interestingly, two of the three substitutions were shared by the D5S2_N_Antioquia_CO_2015 and C+_vacuna_Cux_1_RegICA8649BV sequences and the other substitution was shared by the sequences M5S3_N_Antioquia_CO_2015 and D5S2_N_Antioquia_CO_2015 (Table 3).

At the amino acid level, changes occurred at positions 93, 118, 138, 153 of VP2 (K/L, A/K, K/S and A/R, respectively) and 83 of VP3 (P/K) from the reference sequence (NC 001427). In particular, the M5S3 N Antioquia CO 2015 sequence presented a variation compared to the sequences identified in this study and the one used as a reference. Minor variations were found compared to the other sequences analyzed, except for the sequences from Iran and the sequence of the external group. The nucleotide and amino acid changes are presented in table 3.

According to the estimate of evolutionary divergence, the Colombian sequences D5S2_N_Antioquia_CO_2015 and M5S3_N_Antioquia_CO_2015 showed a distance of 0.005 and 0.014 against the positive control (C+_vaccine_Cux_1_RegICA8649BV), respectively, being less than or equal to the distance found between this control and strains from other parts of the world, such as Japan (VP2 VP3 VP1 JP 2003), Malaysia (3 1 MY 2001; SMSC 1P9WT MY 2005), Brazil (BR265/00 BR 2004; BR91/99 BR 2004), China (TJBD40 CN 2004; JS China90 CN 2010), United States (c0654p2d41v US 2003), Korea (CIAV89 69 KR 1991), Thailand (BR6 11 TH 2011), Argentina (CAV 18 AR 2007) and Australia (SN0161NSW/chicken/Australia AU 2007). The greatest distance was found in comparison to the sequences from Iran (Mhd8 IR 2009; Mhd6 IR 2009) (Matrix to estimate evolutionary divergence between sequences, supplementary material).

Table 3. Amino acid changes of the selected study sequences.

N[a]	NC 001427 CAV complete genome 1991	M5S3 N Antioquia CO 2015		D5S2 N Antioquia CO 2015		C+ vaccine Cux 1 RegICA8649BV	
656	Leucine	**K**	ORF2: 93	-		-	
		L	ORF3: 57				
732	Lysine	**A**	ORF2: 118	-		-	
		P	ORF3: 83				
794	Serine	**K**	ORF2: 138	-		-	
		S	ORF3: 103				
838	Arginine	**A**	ORF2: 153	**A**	ORF2: 153	**A**	ORF2: 153
		R	ORF3: 118	R	ORF3: 118	R	ORF3: 118

[a] N: nucleotide. Position of the nucleotide change according to the length of the CAV reference sequence (NC 001427) The changes are shown according to the length of the CAV reference and its position in each ORF (Open Reading frame).

DISCUSSION

A high number of animals was found positive for CAV PCR (84%), despite there being no history of vaccination or signs of disease. The presence of the virus in blood and tissue samples has been associated with migration of infected lymphoid cells to the bloodstream, and then to other lymphoid tissues or organs (19). Despite the evidence of active infection, the percentage of birds that tested positive for antibodies was low (41%), possibly due to previously reported lymphoid tissue involvement (20) that hinders seroconversion, making these birds highly susceptible to presenting with AIA.

The results in 1-day-old birds that maintained the infection for little over 15 days could be explained assuming that they were able to acquire it from the mother vertically (21), since the virus invades and remains in the gonads (22) and reproductive tract for up to 12 months post-infection without signs of disease and with transmission of the virus to the progeny (23).

However, although 100% of the 1-day-old birds were expected to present antibodies against CAV due to passive immunity (transfer of antibodies from the mother), this percentage was 50%, which was probably associated with a variation in the mother's antibody titers that affects transmission to the progeny and makes them susceptible to AIA (24). Seroconversion in birds can be altered by factors such as strain type, nutrition, incubation conditions, stress, mycotoxins, infectious agents, and contaminated environments (25,26,27), these reasons could promote the poor transmission of antibodies from mothers to chickens (21,28).

Considering the period of the infection from the mother, birds from ages after 15 days could be horizontally reinfected, where the probability of acquiring the virus increases as age increases (29) and passive immunity decays at 21 or 42 days (28). Along with the involvement of the immune tissue (20), this last condition would explain why only a few chicks in the intermediate ages of 30 and 60 days presented antibodies despite testing positive for the virus in their blood, making the birds susceptible to the effects of the virus and facilitating the entry of other agents such as Infectious Bronchitis virus (30) or Marek's Disease virus (MDV) (31). Particularly, the presence of MDV might increase the susceptibility to CAV or its persistent infection.

Reinfections with CAV or virus reactivation could stimulate the production of antibodies that occurs 2 weeks post-infection (32,33), which would result in more animals testing positive for ELISA at advanced ages (34), like in the case of this study where it was at 120 days of age. At this stage, the onset of sexual maturity could promote the reactivation of the virus because of hormonal changes, inducing its replication (22,35) and subsequent seroconversion. However, more studies are necessary to confirm this.

Regarding backyard chickens, positive animals were not expected to be found as they are part of small productions, reared for the owner's consumption (36) and without vaccination programs. Nevertheless, a large number had

antibodies that could theoretically neutralize the circulating strain, resulting in only a low percentage being positive by PCR. Also, the circulating virus in these birds could be the same or different from the vaccine strain found in this study, due to the fact that commercial birds that finish their productive stage are sold and become backyard chickens. Additionally, if these birds did not have antibodies, they could promote the spread of the virus in the countryside (37). Consequently, it is essential to characterize the CAV circulating in backyard chickens and identify its effects on commercial poultry production as they can maintain the virus's transmission cycle (38).

Sampling feathers has been useful for identifying CAV at the population level since the virus is eliminated by feces up to 5 dpi (4,39); additionally, it is a non-invasive technique that allows the easy evaluation of CAV horizontal infection in poultry productions (33,40). So, a possible explanation for the 1-day-old birds that resulted 100% positive by feather might be an environment contaminated with the virus between hatching and arrival at the farm. In the case of 90-day-old and 120-day-old birds, the PCR results were low but the ELISA percentages were high so it could be proposed that the antibodies were able to stop CAV in such animals. However, there were positive PCR results at all ages and these results may suggest a fault on the biosecurity measures applied in the studied farms (41) and the failure to monitor health status, since feathers work as a vehicle for the virus and maintain it for up to 32 days at 4ºC (4,40). Thus, the obtained results in this study indicated that the virus was found in farm birds at all ages and in backyard chickens. Therefore, more work is needed to assess the role of feathers in transmitting the identified strain under natural conditions and its relationship with antibodies.

In addition, biosecurity measures in these farms should be assessed, and vaccination should be studied as a useful measure against CAV, especially in breeders as it is the first passive immunity against the virus that the progeny will have (42). On the other hand, a timely diagnosis with the appropriate techniques would favor health surveillance and avoid economic losses due to immunosuppression (24).

Characteristics of the Colombian CAV strain. Considering the infection characteristics, it is expected that the virus transmitted from the mother to the progeny arises from the vaccine. In this work, the restriction pattern presented by the samples in the RFLP results was different from the Cux-1 vaccine strain, in other words this difference could affect the animal's immune response (43). This is consistent with the results that have been reported by other authors (44,45,46,47). Likewise, it was determined that useful enzymes for differentiating the Colombian from the Cux-1 strain through RFLP are *Dde* I, *Hha* I, *Hae* III, *Hinf* I and *Hpa* II; some of which have been reported by other authors (14,48). The usefulness of *Hae* III and *Hpa* II is highlighted due to the number of cuts that can be made, which increases the possibility of classifying the strains. Similarly, the little information that *Hinf* I could provide due to the low number of fragments it generates. Even though the pattern obtained with *Mbo* I for the study samples was the same as for the vaccine strain Cux-1, the circulating strain was not considered to correspond to Cux-1 due to the results obtained with other endonucleases used in the study.

Despite the limitation in the length of the analyzed fragment, the RFLP results are supported by DNA sequencing, confirming that the circulating strains from the study farms are not 100% identical to the Cux-1 (vaccine) strain. This is further supported by the phylogenetic distance of the identified strains that was shorter as compared with strains that have caused AIA outbreaks, for example in Argentina (44,46). The evolutionary distance presented by the Colombian CAV sequences could be related to the effects they generate in animals, the ability to spread in cell culture (49) or the subclinical course of the disease (44,46). The nucleotide changes of the identified strains should therefore be evaluated further, along with their pathogenicity and ability to stimulate seroconversion, even more so when they are associated with other immunosuppressive agents (50).

In this sense, it can be concluded that the CAV circulated in the farms studied over the growth stages with low seroconversion in the animals and that according to the sequences obtained, the virus was not identical to the Cux-1 vaccine strain used for preventing the virus in Colombia.

It is worth mentioning that this constitutes the first report of CAV in Colombia using molecular techniques, where the molecular differences between the Colombian and vaccine strains might be related to immunosuppression patterns that could promote a subclinical course of the

infection. It might be more investigated, because as we mentioned, CAV could favor the entry and permanency of secondary agents such as MDV, a poor response to vaccination and economic losses yet to be determined. Finally, more studies are needed to deepen the information known about the strain, its pathogenicity, and the economic consequences of its presence on affected farms.

Conflict interest

All authors declare no conflicts of interest.

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