Survey of coccidia on commercial broiler farms in Colombia: frequency of *Eimeria* species, anticoccidial sensitivity, and histopathology

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ABSTRACT Avian coccidiosis continues to be one of the costliest diseases of commercial poultry. Understanding the epidemiology of *Eimeria* species in poultry flocks and the resistance profile to common anticoccidials is important to design effective disease prevention and control strategies. This study examined litter samples to estimate the prevalence and distribution of *Eimeria* species among broiler farms in 4 geographic regions of Colombia. A total of 245 litter samples were collected from 194 broiler farms across representative regions of poultry production between March and August 2019. The litter samples were processed for oocysts enumeration and speciation after sporulation. End-point polymerase chain reaction **(PCR)** analysis was conducted to confirm the presence of *Eimeria* species. Anticoccidial sensitivity was determined with 160 Ross AP males in 5 treatment groups: noninfected, nonmedicated control **(NNC)**, infected, nonmedicated control **(INC),** infected salinomycin treated **(SAL, dose: 66 ppm)**, infected diclazuril treated **(DIC, dose: 1 ppm),** and infected methylbenzocuate-Clopidol treated **(MET.CLO, dose: 100 ppm),** All birds were orally inoculated with 1×10^6 sporulated oocysts using a 1 mL syringe, except for the NNC- group who received 1ml of water**.** *Eimeria* spp. were found in 236 (96.3%) out of 245 individual houses, representing 180 (92.8%) out of 194 farms. *Eimeria acervulina* was the most prevalent species (35.0%) followed by *Eimeria tenella* (30.9%), *Eimeria maxima* (20.4%), and other *Eimeria spp.* (13.6%). However, mixed species infections were common, with the most prevalent combination being mixtures of *E. acervulina, E. maxima, E. tenella,* and other species in 31.4% of the *Eimeria*-positive samples. PCR analysis identified *E. acervulina, E. maxima, E. tenella, Eimeria necatrix, Eimeria mitis,* and *Eimeria praecox* with variable prevalence across farms and regions. Anticoccidial sensitivity testing of strains of *Eimeria* isolated from 1 region, no treatment difference ($P > 0.05$) was observed in final weight (BW), weight gain (BWG) or feed conversion (FCR). For the global resistance index (**GI**) classified SAL and MET.CLO as good efficacy (85.79 and 85.49, respectively) and DIC as limited efficacy (74.52%). These results demonstrate the ubiquitous nature of *Eimeria* spp. and identifies the current state of sensitivity to commonly used anticoccidials in a region of poultry importance for Colombia.

Key words: broiler chicken, *Eimeria* monitoring, oocyst, litter, anticoccidial sensitivity

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INTRODUCTION

Coccidiosis is the costliest parasitic disease in commercial poultry. Global estimates of the economic loss caused by coccidiosis in chickens range from USD 3 billion in 1995 (Williams, 1999) to over USD 13 billion annually in 2016 (Blake et al., 2020). Such losses include decreased feed consumption and growth rate, increased feed conversion (Williams, 1999; Dalloul and Lillehoj, 2006), and the cost of prophylactic and therapeutic control of the disease (Blake and Tomley 2014; Blake et al., 2020). Currently, intensive chicken farming relies heavily on anticoccidial drugs and live vaccines to control coccidiosis (Jenkins et al., 2017a). Anticoccidial control programs can be optimized by knowing the severity and timing of challenge as well as the species present and their anticoccidial drug sensitivity profile

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(Jenkins et al., 2017a). The number of *Eimeria* oocysts per g of litter follows a general pattern throughout the life of a flock and these data can be used to evaluate coccidiosis control programs (Chapman et al., 2002, 2016; Williams, 2002). In addition, *Eimeria* can be identified and speciated by visual evaluation of species-specific intestinal lesions (Johnson and Reid, 1970) and through oocysts morphology as assessed by the microscopic evaluation of intestinal scrapings (Hadipour et al., 2011; Györke et al., 2013). However, under field conditions, the common occurrence of mixed infections makes it difficult to reach a specific diagnosis (Long and Joyner, 1984; Györke et al., 2013) that is essential for optimal prevention and control of coccidiosis. Molecular techniques such as the polymerase chain reaction (**PCR**) have proven to be effective tools for *Eimeria* diagnosis in broilers (Györke et al., 2013; Moraes et al., 2015). Molecular techniques reduce possible misdiagnosis between species with similar morphometric characteristics and can identify infections that might be missed through gross evaluation (Kučera, 1990). Nevertheless, PCR is not commonly used in field conditions.

Apart from accurately assessing the presence and severity of coccidia in the field, it can be helpful to understand the sensitivity of field isolates against commonly used anticoccidials (Abbas et al., 2011). Sensitivity testing usually consists of infecting groups of medicated and nonmedicated birds with *Eimeria* isolated from the litter of commercial farms (Chapman, 1998). Sensitivity tests can provide valuable information to help producers design effective *Eimeria* control strategies (Abbas et al., 2008).

In Colombia the broiler and layer sectors have an annual growth rate of approximately 7 to 8%, as reported by the Colombian Agriculture Institute (ICA: Instituto Colombiano Agropecuario in spanish) (ICA, 2019), with a current population of 187.5 million birds, of which 104.8 million are broilers (ICA, 2019). The production of chicken meat in 2019 was 1.69 million tons (Fenavi, 2019). Poultry production is concentrated in 4 States: Santander (24.4%), Cundinamarca (17.06%), Valle del Cauca (15.98%) and Antioquia (6.25%) -. Each state has different climates, general management conditions and production environments. The ubiquitous nature of coccidia and concentrated poultry production can make disease control a challenge (Carvalho et al., 2011).

In Colombia there is no information on the incidence or prevalence of coccidiosis and only production companies know which anticoccidial drugs are used. In addition, vaccines are not commonly used to control coccidia (personal communications, March to October 2019). Having more information on *Eimeria* prevalence and the sensitivity of field isolates to commonly used anticoccidials can help producers make better treatment and control decisions. Therefore, the objective of this work is to survey the prevalence of *Eimeria* spp. on broiler farms in 4 important broiler-producing regions of Colombia and perform an anticoccidial sensitivity test with strains isolated from one of the surveyed areas.

MATERIALS AND METHODS

Ethics Committee Approval

The work was approved by the animal ethics committee of Universidad de Antioquia (Act No. 122 of February 5, 2019).

Sample Area and Farms

The study was conducted between March and August 2019. Using data from the MAPS-Fenavi - (2018) national database on poultry farms, the sample size was calculated (Grisales, 2011) for studies with known populations assuming an error of 10%. A total of 219 farms were sampled: 62 in Cundinamarca, 61 in Santander, 58 in Valle del Cauca and 38 in Antioquia. In each state, commercial farms that were in the finishing phase of the productive cycle (beyond d 21) were selected. A survey was used to collect additional information from each farm (e.g., location, size, number of birds, number of sheds, management practices and biosecurity).

Litter Sampling and Analysis of Eimeria spp.

Samples were collected from 194 of the 219 proposed farms, corresponding to 88.6% of the initial objective.

It was not possible to comply with the entire sampling because confidentiality of the farms. Litter samples were randomly collected from each shed while walking in a zigzag pattern (Goan, 2009). Depending on the size of the shed, between 6 and 12 grab samples of approximately 100g were taken, pooled, and homogenized. A 500g subsample was taken from each composite sample, placed in a hermetically sealed plastic bag, and transported to the laboratory where it were kept refrigerated at 4°C for 1 to 3 days. Oocysts per g of litter (**OPG**) was calculated by microscopic (Olympus CH30, Olympus Optical Co., Ltd., Tokyo, Japan) enumeration using a McMaster chamber (Conway and McKenzie, 2007). To identify the *Eimeria* species, positive litter samples were washed in tap water, kept at room temperature overnight and filtered through sieves (Endecotts, London, England) of different diameters in a descending manner: 500, 212, 180, 75 and 45 μm. The resulting liquid was diluted in a proportion 1:1 with 5% potassium dichromate ($K_2Cr_2O_7$) [200 mL liquid in 200 mL potassium dichromate in an Erlenmeyer flask], and aerated with an aquarium pump (DG-302A, Aquarium air pump CE, China) at room temperature (approximately 27°C) for 1 wk. Oocysts were isolated in saturated saline using a flotation technique (López et al., 2018) and 100 sporulated oocysts were microscopically (100X objective. Olympus Optical Co., Ltd., Tokyo, Japan) speciated using morphometric measurements of length, width and shape index (Castañon et al., 2007; Haug et al., 2008).

Determination of Eimeria species by Polymerase Chain Reaction (PCR)

Sample Collection Litter samples from each of the 4 geographic regions were processed as described previously except a saturated sugar solution (density 1.18−1.20 g/mL- Sheather) was used for oocyst flotation prior to the addition of 2.5% potassium dichromate (K2Cr2O7) for storage at 4°C until analysis. Prior to the DNA extraction, 500 μL of the final solution was washed 5 times with PBS and centrifuged at 847 RCF (3000 rpm) for 5 minutes (Hettich Mikro 120 centrifuge [Tuttlingen, Germany]), to remove the potassium dichromate.

DNA Extraction DNA extraction was carried out for Antioquia, Santander, Cundinamarca and Valle del Cauca, taking 200 μL of the suspension containing 100 oocysts from each zone and using the commercial NucleoSpin Soil KIT (Macherey -Nagel, Düren, Germany). Following the methodology of López et al. (2018), the lysis time of the sample was modified by adjusting the agitation time in A-NucleoSpin Bead tubes (50 preps., Cat. No. 74078050, Macherey-Nagel GmbH & Co.KG, Düren, Germany) to 20 minutes. The quality of the DNA was verified and quantified in an Epoch-spectrophotometer using a nucleic acid quantification Take 3 plate (BioTek Instruments, Inc., Winooski, VT), with Gen5 2.04 software (BioTek Instruments, Inc., Winooski, VT).

PCR Analysis PCR analysis was performed using a modified method of Moraes et al. (2015). The reaction was performed in a T3-Thermoblock thermocycler (Biometra GmbH, Gottingen, Germany), with initial denaturation at 96°C for 5 min, followed by 35 cycles of 1 minute at 94°C and 2 minutes at 55°C (50°C for *Eimeria necatrix, Eimeria tenella,* and *Eimeria acervulina*), an extension at 72°C for 30 seconds and a final extension at 72°C for 10 minutes. The PCR products were mixed with DNA-6X loading buffer (Thermo scientific, Waltham, MA) and separated on a 1% agarose gel with the electrophoresis technique, marking them with GelRed (Bioutium, SF). The specific size fragments were identified, using a molecular weight pattern with a 100 bp ladder under ultraviolet light (UV Transilluminator Model M-20, Upland, CA).

The PCR was performed for each *Eimeria* species, preparing a solution with 200 μM of dNTps, 2.5 mM of $MgCl_2$; 5 U of Taq DNA polymerase (Thermo Scientific-Waltham, Massachusetts, USA), 10 pM Primer R, 10 pM Primer F and 10 ng of DNA. The solution for the species *E. necatrix, Eimeria maxima, Eimeria praecox,* and *Eimeria mitis* was brought to a final volume of 33 μL and for *E. acervulina* and *E. tenella* to a final volume of 20 μL. The primers used are shown in Table 1. As positive controls, Evant-Hipramunet vaccine (HIPRA-The Reference in Prevention for Animal Health) was used for *E. acervulina, E. maxima, E. tenella, E. praecox,* and *E. mitis* and Livacox Q vaccine (BIOPHARM, Research Institute of Biopharmacy and Veterinary Drugs) was used for *E. necatrix. Eimeria brunetti* is not reported since there was no positive control available.

Table 1. Forward (F) and reverse (R) primers in end-point PCR for detection of 6 *Eimeria* species and size of generated amplicon (TA).[1]

Name of primer	Primer sequence	Size (pb)
ac-A03-F	AGT CAG CCA CAC AAT AAT GGC AAA CAT G	811
ac-A03-R	AGT CAG CCA CAG CGA AAG ACG TAT GTG	
tn-K04-F	CCG CCC AAA CCA GGT GTC ACG	539
tn-K04-R	CCG CCC AAA CAT GCA AGA TGG C	
mt-A03-F	AGT CAG CCA CCA GTA GAG CCA ATA TTT	460
mt-A03-R	AGT CAG CCA CAA ACA AAT TCA AAC TCT AC	
pr-A03-F	AGT CAG CCA CCA CCA AAT AGA ACC TTG G	354
pr-A03-R	GCC TGC TTA CTA CAA ACT TGC AAG CCC T	
mx-A09-F	GGG TAA CGC CAA CTG CCG GGT ATG	272
mx-A09-R	AGC AAA CCG TAA AGG CCG AAG TCC TAG A	
nc-A18-F	TTC ATT TCG CTT AAC AAT ATT TGG CCT CA	200
nc-ENec-R	ACA ACG CCT CAT AAC CCC AAG AAA TTT TG	

[1]Eimeria species: E. acervulina (ac), E. tenella (tn), E. mitis (mt), E. praecox (pr), E. maxima (mx), E. necatrix (nc). (Pb): base pair. Adapted from Moraes et al., 2015.

In vivo Anticoccidial Sensitivity Test With Field Strains Isolated from Cundinamarca (Colombia)

Eimeria Strain Isolation Cundinamarca isolate was chosen for being one of the most important states between the higher poultry production states in Colombian. The 64 samples of litter containing *Eimeria* from Cundinamarca state was used to isolate oocysts for sensitivity testing. Litter were processed as described previously and oocysts were isolated by flotation in a saturated sugar solution (density 1.20 g/mL-Sheather). The saturated solution was removed by 5 serial washes with buffered water and centrifugation at 1211 RCF (2,500 rpm) for 10 minutes (Thermo Scientific IEC Centra GP8R, Needham Heights, MA). The oocysts of the 64 samples were mixed and deposited in 2.5% potassium dichromate, at room temperature and with constant stirring for 4 days to achieve at least 70% sporulation. Oocyst concentration, speciation, and sporulation rate was determined using a hemocytometer (Neubauer Chamber). Subsequently, the potassium dichromate was removed replacing it with buffered water, performing 5 centrifugations at 1211 RCF (2,500 rpm) X 10 minutes (Thermo Scientific IEC Centra GP8R, Needham Heights, MA), and the challenge inoculum was adjusted to 1×10^6 sporulated oocysts/mL (Gerhold, et al., 2011). The mixed inoculum had a similar composition of oocysts of *E. acervulina, E. maxima* and *E. tenella*, with approximately 33% of each of these species (333.000 oocyst for each *Eimeria*), differentiated on the basis of oocyst morphology (Stephan, et al., 1997).

The poultry farms in this study belong to different owners. They report to use anticoccidial drugs via feed,

but do not know the type of anticoccidial. In direct communication with technical personnel in the area, they report that the basic anticoccidial plan for the area is the supply of ionophore for the starter period (D. 1−21) and a combination of ionophore with chemical for the growth period (D. 21−42).

Anticoccidial Sensitivity Test The test was carried out following the guidelines for evaluation of anticoccidial efficacy by the World Association for the Advancement of Veterinary Parasitology (Holdsworth et al., 2004) and the methodology used by Stephan et al. (1997) and Thabet et al. (2017). The anticoccidial sensitivity test lasted 21 days and was carried out during October and November 2019, in an experimental farm located in the town of Rionegro (Antioquia, Colombia) at an altitude of 2,125 m, with an average temperature of 17°C.

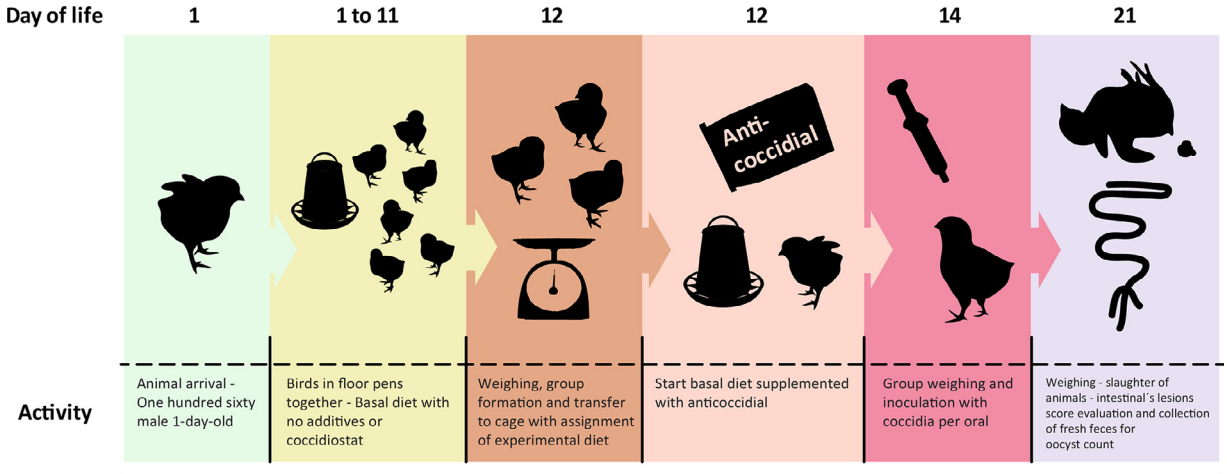
A total of 160 one-day-old Ross AP males were used to test the sensitivity of the *Eimeria* spp. strain to 3 commonly used anticoccidial medications. Broiler chickens were sourced from a commercial hatchery, received a standard vaccination program, and were housed together in floor pens previously flamed and sanitized with Farm Fluid S (NEOGEN, Lansing, MI) with *ad libitum* access to water and starter feed without anticoccidials or antibiotic growth promoters. Feed was formulated to complying with Ross 308 AP nutrient recommendations (Aviagen, 2019). At 12 d of age, animals were weighed, randomized into 4 replicates of 8 birds each (Table 2) and placed in wire-floor cages previously randomized to treatment, without blocking by cage. The wire-floor cages had been thoroughly cleaned and fumigated with concentrated ammonia solution prior to use. Birds received experimental diets from 12 d of age to trial termination at 21 d of age. The following experimental groups were evaluated: Noninfected nonmedicated control (**NNC**), Infected nonmedicated control (**INC**) and 3 infected medicated treatments including Salinomycin (**SAL, dose: 66 ppm**), Diclazuril (**DIC, dose: 1 ppm**), and Methylbenzocuate Clopidol (**MET.CLO, dose: 100 ppm**).

Table 2. Description of treatments for in vivo anticoccidial sensitivity test in Broilers from Colombia.

Treatment[1]	Number of replicates	Dose (ppm)
NNC	4	
INC	4	
SAL	4	66
DIC	4	1
MET.CLO	4	100

[1]Experimental group of 4 replicates with 8 individuals each. Each challenged bird was gavaged with 1×10^6 sporulated oocysts containing equal portions of *E. acervulina, E. maxima,* and *E. tenella,*333.000 oocysts each specie). Non-infected non-medicated control (NNC), Infected non-medicated control (INC) and 3 infected medicated treatments including Salinomycin (SAL-66 ppm), Diclazuril (DIC-1 ppm), and Methylbenzocuate Clopidol (MET.CLO -100 ppm).

On day 14, each bird was weighed and orally inoculated with 1×10^6 sporulated oocysts using a 1 mL syringe. On day 21 birds were group weighed and 2 individuals per replicate were randomly selected, for a total of 8 individuals per experimental group, to evaluate intestinal lesion scores (Johnson and Reid 1970) and fresh feces were collected directly from the bird at death to perform the count of oocysts per gram of feces (OPG) (Figure 1; experimental test scheme).



Day of life	Activity
1	Animal arrival - One hundred sixty male 1-day-old
1 to 11	Birds in floor pens together - Basal diet with no additives or coccidiostat
12	Weighing, group formation and transfer to cage with assignment of experimental diet
12	Start basal diet supplemented with anticoccidial
14	Group weighing and inoculation with coccidia per oral
21	Weighing, slaughter of animals for evaluation intestinal´s lesions score evaluation and collection of fresh feces for oocyst count

Figure 1. Experimental test scheme.

Calculation of the Global Resistance Index The global resistance index (**GI**) for each experimental group was calculated using a modified version of the formula developed by Stephan et al. (1997) where oocyst index (**OI**) was obtained from the OPG count in fresh feces not from mucosa scrapings and used the following 0 to 5 categorical ranking: 0 = no, 1 = 1−10,000, 2 = 10,001−49,999, 3 = 50,000−79,999, 4 = 80,000−99,999, and 5 >99,999 OPG of feces.

$$\mathbf{GI}(\%) = \mathbf{BWG_{NNC}} - [(\mathbf{FCR_M} - \mathbf{FCR_{NNC}}) \times \mathbf{10}] - (\mathbf{OI_M} - \mathbf{OI_{INC}}) - [(\mathbf{LS_M} - \mathbf{LS_{INC}}) \times \mathbf{2}] - (\%\mathbf{MR}/\mathbf{2}).$$

Where, OI = Oocyst index (Score 0-5), BWG_{NNC} = relative weight gain calculated as the percentage (%) gain of the NNC group, LS = Lesion score (score 0-4), FCR = feed conversion, MR = mortality (%), M = medicated, NNC = noninfected, nonmedicated control, and INC = infected, nonmedicated control.

In addition, the GI for each test group was calculated as a percentage of the GI for the NNC (Table 3).

Histopathological Analysis On the final day of the test, 3 birds per experimental group were randomly chosen for intestinal histopathology at approximately 4 cm from the duodenal loop, 5 cm after Meckel's diverticulum (ileum), and from the cecum. Tissue samples were fixed in 10% buffered formalin, routinely processed, embedded in paraffin, sectioned, and stained with hematoxilyn and eosin (H&E). Microscopic evaluation was conducted at 100X and 400X magnification after staining with hematoxylin and eosin and was performed in duplicate by an expert avian pathologist who was blinded to treatments. Histopathological lesions and the presence or absence of *Eimeria* spp were categorized (Table 4) and summed across birds and intestinal locations for each experimental group. *Finally, the total score is presented, a higher score indicates a greater degree of damage and a greater amount of intestinal Eimeria.*

Table 3. In vivo sensitivity rating parameters.

Efficacy	GI_{NNC} (%)
Very good	≥90
Good	80−89
Limited	70−79
Partial resistance	50−69
Resistance	<50

The global resistance index (GI). Non-infected non-medicated control (NNC).

Adapted from Thabet et al. (2017).

Table 4. Categorization histopathological of intestinal lesions and presence of *Eimeria* spp.

Score	Intestinal microscopic-lesions	Presence of *Eimeria*
0	Without lesions	No presence
1	Mild lesion	Sparse - scattered
2	Moderate lesion	Moderate - medium
3	Severe lesion	Numerous

Statistical Analysis

Survey data (OPG and *Eimeria* species present) were entered manually into Microsoft Excel (Microsoft Office 2016, Microsoft Corporation, Redmond, WA) to determine frequencies and prevalence of species by state. R version 4.0.2 was used to calculate a Pearson correlation coefficient between flock age and logarithmically transformed OPG. Results of the sensitivity test were analyzed using JMP 15 (SAS Institute Inc., Cary, NC). Growth performance data were analyzed as a randomized block (for high and low weights) design with cage as the experimental unit. For intestinal lesions and oocyst count the nonparametric Wilcoxon test was used, with bird as the experimental unit. The OPG data were transformed by (Ln + 1) (Oviedo et al., 2006) and evaluated by a mixed model with cage as the random factor. Histopathology results are presented descriptively.

RESULTS

Field Survey Study

The density of birds in most farms was between 12 to 14 birds/m2, with an average number of 5.5 (± 3.0) sheds per farm, an average flock size of approximately 80,000 birds (range 10,000−330,000), and an average slaughter age of 37.5 days (Table 5). The most common building design was traditional open-sided sheds with tarpaulin or curtains with concrete floors using rice hull or wood shaving litter. In regions with warmer temperatures, mechanical fans are used to regulate the climate inside the sheds. Very few farms had controlled environment systems in the sampling areas that coincide with the warmest areas in the departments of Santander and Valle del Cauca. The commercial broiler lines used in the 194 farms studied were: Ross AP 51.2% (83/160), Ross AP x Cobb 500 33.8% (54/160), Cobb 500 13.7% (21/160) and others 1.3 % (2/160) (Table 6). Except for farms in Santander, which milled their own feed, most of the farms used contract feed suppliers that manufacture diets for prestarter (hatching until day 10, starter from 11−21 days, and grower/finisher from 22 to 35−42 days). In Colombia, the use of anticoccidial vaccines is not very frequent and none of the farms included in the study were using coccidial vaccines at the time of sample collection. The use of recycled litter between flocks was only reported as a frequent practice in the region of Cundinamarca, where litter was typically reused for 3-5 cycles; 80% of the farms in the other sampling areas used new litter between each flock. At the end of each flock farms are chemically cleaned and disinfected, with an average down time of 10 to 15 days.

The overall prevalence of *Eimeria* spp. was 92.8% (180/194) and OPG of litter varied widely between

Table 5. Management characteristics of broiler farms in 4 regions of Colombia during 2019.

Department	Cundinamarca	Santander	Valle del Cauca	Antioquia
Sampled/registered farms[1]	37/803	72/648	57/379	28/86
Annual average temperature (°C)	24.2	26.5	24.5	23.2
Altitude (masl)	1,781	1,098	1,150	1,679
Genetic line in number of sampled farms[2]				
Ross AP	14	45	11	13
Ross 308	0	1	0	1
Cobb 500	2	5	5	9
Mix of multiple lines	2	21	26	5
Density, birds per m^2 (min. to max.)	14.0 (13.0−15.0)	12.2 (8.8−17.0)	12.6 (11.0−17.9)	14.1 (13.5−14.5)
Litter composition	44.4% WS 55.6% RS	100% RS	56.1% WS 43.9% RS	92.9% WS 7.1% M
Birds placed per farm (min.to max.)	36.591 (10.200−162.000)	56.346 (5.700−195.000)	126.715 (9.180−330.000)	83.672 (20.400−147.600)
Sheds per farm (min.to max.)	2.7 (1−7)	4.4 (1−14)	6.4 (1 to 18)	7.6 (3−14)
Average age of birds at sampling (min.to max.)	29 (21−38)	30 (21−39)	31 (25−43)	32 (22−41)

Abbreviations: M, mix of wood shavings with rice hulls; RS, rice hulls; WS, wood shavings.
[1]Register of farms by department - MAPS-Fenavi.
[2]160 total surveys.

Table 6. Frequency of *Eimeria* species and average (± SD) oocysts per gram (OPG) of farm litter in 4 regions of Colombia.

Department	Cundinamarca	Santander	Valle del Cauca	Antioquia	Total
Number of litter samples analyzed[a]	67	72	62	44	245
Number of farms positive for *Eimeria* spp/total analyzed (%)	29/37 (78.4%)	69/72 (95.8%)	55/57 (96.5%)	27/28 (96.4%)	180/194 (92.8%)
Average OPG values of positive samples (min. to max.)	1,592 ± 4,735 (0−32,800)	1,386 ± 3,185 (0−24,300)	1,018 ± 3,711 (0−28,044)	4,728 ± 9,827 (0−48,960)	1,931 ± 5,543 (0−48,960)
Eimeria spp. (%)					
E. acervulina	39.3	38.9	37.3	20.2	35.0
E. maxima	13.6	21.4	8.5	35.5	20.4
E. tenella	35.3	22.6	36.1	36.0	31.0
Other spp.	11.8	17.2	18.2	8.3	13.6

Percentage of diversity and distribution of species.

farms and departments (Figure 2), with an average of 1,931 (±5,543) (Table 6). *Eimeria* species identified in descending order of frequency were: *E. acervulina* (35.0%), *E. tenella* (30.9%), *E. maxima* (20.4%), and other *Eimeria* spp. (13.6%). Infections of mixed species with 2, 3, and more species were common and the most frequently found combination was the mix of at least 4 *Eimeria* spp., of which 3 are *E. acervulina, E. tenella, E. maxima,* and others, in 74 of 236 positive samples (Table 7).

No association was found between the age of the flock of the litter samples collected and the number of OPG found (R2 = 0.00 and $P = 0.2227$) (Data not shown).

Confirmation of the Presence of Eimeria *Species by the PCR Technique in 4 Regions of Poultry Importance in Colombia*

The end-point PCR results showed that Antioquia and Santander were positive for the 6 *Eimeria* species analyzed *E. necatrix, E. maxima, E. praecox, E. mitis, E. acervulina,* and *E. tenella*; Cundinamarca was positive for *E. necatrix, E. maxima, E. praecox, E. acervulina,* and *E. tenella* and negative for *E. mitis* while, Valle del Cauca was positive for *E. maxima, E. praecox, E. mitis, E. acervulina,* and *E. tenella* and negative for *E. necatrix* (Figures 3A−3F).

In vivo Anticoccidial Resistance Test With Field Strains Isolated From Department of Cundinamarca (Colombia)

No treatment difference ($P > 0.05$) were observed in final weight (BW), weight gain (BWG) or feed conversion (FCR) (Table 8).

No intestinal lesions, oocyst excretion, or mortality was observed in the NNC group (Table 9). The oocyst index presented a statistical difference between the SAL and MET.CLO groups. The only death recorded was in the DIC group but there were no treatment differences ($P > 0.05$) in mortality. SAL and MET.CLO treatments received a good efficacy with GI scores of 85.79 and 85.49, respectively. With a GI score of 74.52, the DIC treatment received a limited efficacy rating (Table 10).

Histopathology Analysis

The NNC group showed a degree of inflammation categorized as enteritis and minimal eosinophilic typhlitis, but no presence of *Eimeria* (Score 18) (Table 11). The INC group showed evidence of intestinal damage, with tissue erosion and severe focal cecal hemorrhage and *E. acervulina, E. maxima* and *E. tenella* organisms were observed in each segment (Score 18); coccidial typhlitis was diagnosed. The groups medicated with SAL (Score 25) and with DIC (Score 23) showed mild to moderate

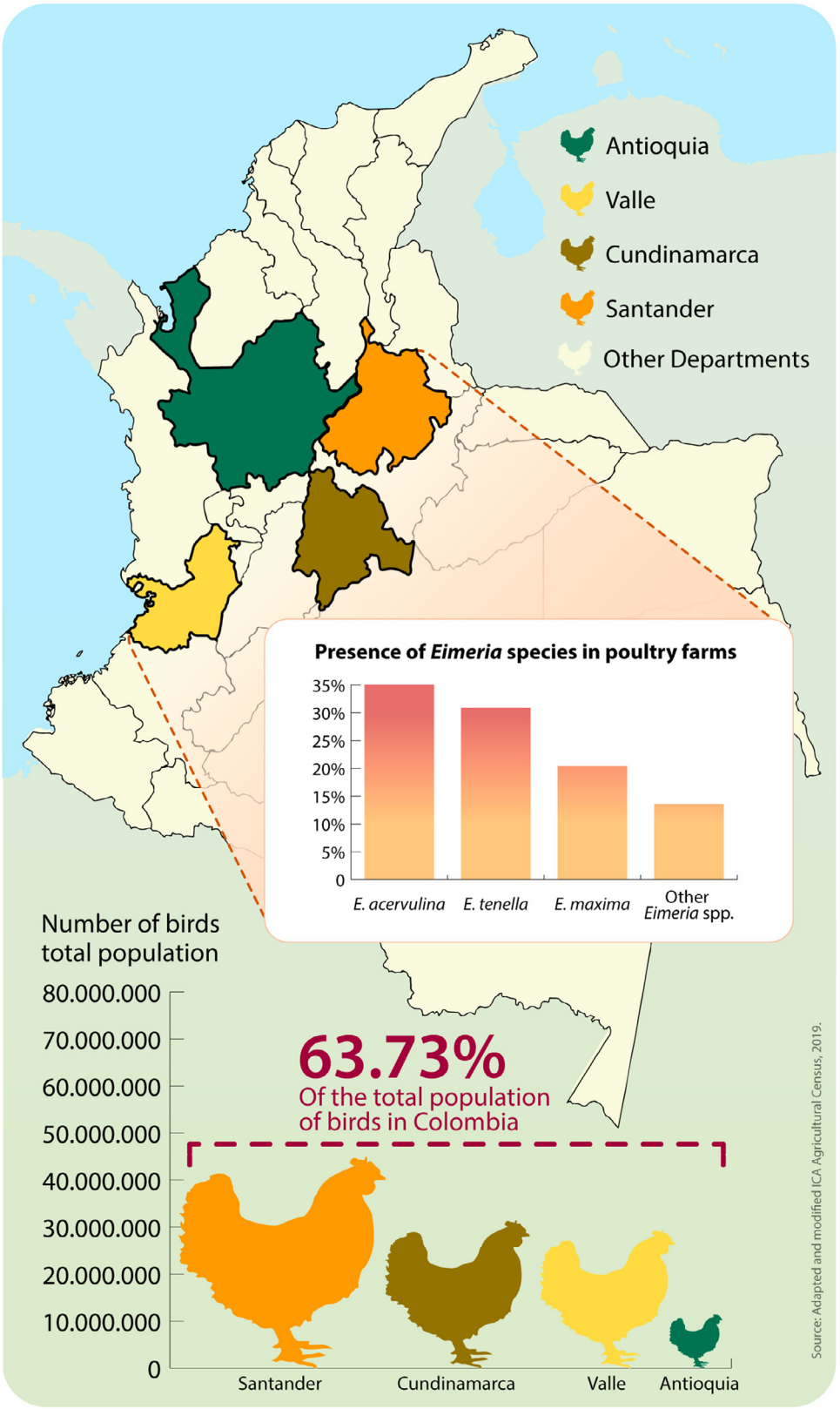


Figure 2. Eimeria species distribution in 4 departments of Colombia.

hyperplasia of gut-associated lymphoid tissue (**GALT**) in the lamina propria, slight necrosis in some segments (ceca) and *E. acervulina, E. maxima,* and *E. tenella* organisms were identified. *Eimeria necatrix* was only found in 1 bird receiving DIC. In the DIC group, there were large oocysts that coincide with *E. necatrix*, where severe coccidial typhlitis was diagnosed. The group treated with MET.CLO (Score 33) showed moderate hyperplasia of the GALT in the lamina propria, moderate necrosis of the cecal tissue, and all the sections evaluated had coccidia: *E. acervulina, E. maxima,* and *E. tenella.* Unidentified *Eimeria* species were found in the

Table 7. Number of litter samples with different combinations of *Eimeria* species for each department sampled.

Region	No. of farms	No. of litter	No. of Posit.	No. of Negat.	a+m+t+o	a+m+t	a+t+o	m+t+o	a+m	a+t	a+o	m+t	m+o	t+o	a	m	t	Not identified[1]
Ant	28	46	44	2	9	6	1	1	1	2	1	8	0	1	0	1	2	13
San	72	72	69	3	35	7	1	0	0	0	0	0	0	0	0	0	0	26
Vall	57	62	59	3	15	0	6	0	0	0	0	0	0	0	0	0	0	36
Cun	37	75	64	11	15	3	3	0	0	0	0	0	0	0	0	0	0	43

Abbreviations: a, *E. acervulina*; Ant, Antioquia; Cun, Cundinamarca; m, *E. maxima*; o, other spp.; San, Santander, t, *E. tenella*; Vall, Valle del Cauca.
[1]Not identified: samples with an OPG ≤170 species identification was not possible.

ileum of all 3 anticoccidial treated groups but not in the infected control group. In the matters of histological score, the group with MET.CLO shows the highest score indicating greater intestinal damage and greater amount of coccidia.

DISCUSSION

This is the first study we are aware of that reports the prevalence and anticoccidial sensitivity of *Eimeria* on commercial broiler farms in Colombia. In this study, litter samples were taken since it can be difficult to get fresh feces from commercial farms (Chapman et al., 2016). Chapman et al. (2016) reported that sampling built-up litter allows the evaluation of accumulated oocysts across several grow-outs and indirectly verifies the conditions of the litter to know whether the parasite sporulation process is taking place (Venkateswara et al., 2015). In this work at the time of sampling, it was not possible to sacrifice birds to identify scores of intestinal lesions by *Eimerias* spp, as in other reported studies (Gharekhanietal, 2014; Belal, 2017; Gazoni et al., 2020) to be able to complement the OPG found in litter with the intestinal health of the birds. Of the total litter sampled, 92.8% were positive for *Eimeria* spp. This high frequency of *Eimeria* spp. has also been reported in other studies using litter or fecal samples: 90% in Argentina (Mattiello et al., 2000); 92% in Romania (Györke et al., 2013), 79.4% in North India (Prakashbabu et al., 2017), 65.8% in East China (Sun et al., 2009) and 78.7% in South Korea (Lee et al., 2010); but different from the reports of Gharekhani et al., 2014 in Hamedan (Western Iran) and Kaboudi, et al., 2016 in Sidi Thabet, (Tunisia), where the general prevalence of coccidia was 31.8%. Additionally, 41.1% (74/180) had mixed *Eimeria* (*E. acervulina, E. maxima, E. tenella* and others) infections, although 1 species was always predominant in each farm. Results agreed with other studies in various regions of the world, such as Iran, India, Alegeria, Brazil (Gharekhanietal., 2014; Prakashbabu, et al., 2017; Debbou-Iouknane et al., 2018; Gazoni et al., 2021). This determination of the distribution of coccidial species is important because it depends not only on the level of oocysts ingested, but also on the species involved and the host's immune response (Yun et al., 2000). It is known that *E. acervulina* can develop resistance to anticoccidials at a faster rate, due to its high reproductive potential and short life cycle (Williams, 2001; Chapman, et al., 2010). This behavior could explain the higher frequency of *E. acervulina* (35.0%) in all sampling areas of this study, as well as in the study by Haug et al. (2008) in Norwey with 100%, Györke et al. (2013) in Romania with 91%, and in the work by Gazoni et al. (2015) in Brazil with 13.5%.

Others have reported that there is an increase in the number of oocysts in litter between 3 and 4 weeks after bird placement and OPG can be a good way to monitor the course of infection throughout the life of a flock (Chapman et al., 2016; Jenkins et al., 2017a). In this work, there was no evidence of a relationship between the age of the flock at the time of sampling and the oocyst count, possibly because only 1 sample was taken per farm. In contrast, Chapman et al. (2016) evaluated the course of infection at weekly intervals in 6 successive flock showing a bell-shaped curve with a oocyst peak around 21 to 33 d of age. The average OPG in the current study (1.9×10^3), similar to that reported in Hamedan (Western Iran) by Gharekhani et al. (2014) (1.8×10^3), was lower than the 52×10^3 (range 35.8×10^3 to 73.6×10^3) reported by Chapman et al. (2016), but relatively similar considering production difference between the US and Colombia and the fact we only sampled farms once and potentially missed the peak of oocyst production. Of the regions sampled, Antioquia presented the highest oocyst count (average 4.73×10^3, range $0-48.96 \times 10^3$), this result could be speculatively due to particular conditions of the area, such as handling a higher density of birds in the sheds (average 14.1 birds /m^2), where 92.9% wood shavings are used as litter material and the climatic conditions of the area at the time when the sampling was carried out, coinciding with the rainy months of the year between March and May 2019. These results agree with the work of Balel (2017) in Bangladesh, showing a higher occurrence of coccidiosis in the rainy season, with higher relative humidity and temperatures. These conditions favor humidity, facilitating the spread of the parasite, reflecting in a higher oocyst count (Bachaya, et al., 2012, Lawal et al., 2016) from this sampling area.

As reported by other authors, coccidia is ubiquitous in poultry farms (Bachaya, et al., 2012; Debbou-Iouknane et al., 2018). The variation in terms of hygiene conditions, management and geographic location of the farms can be the main causes of the differences in the presence of the parasite in the field (Bachaya et al., 2012; Gharekhani et al., 2014), as reflected in the results of the OPG counts in the 4 sampling areas in this work. In addition, as a differential management practice, the use of recycled litters was reported more frequently in

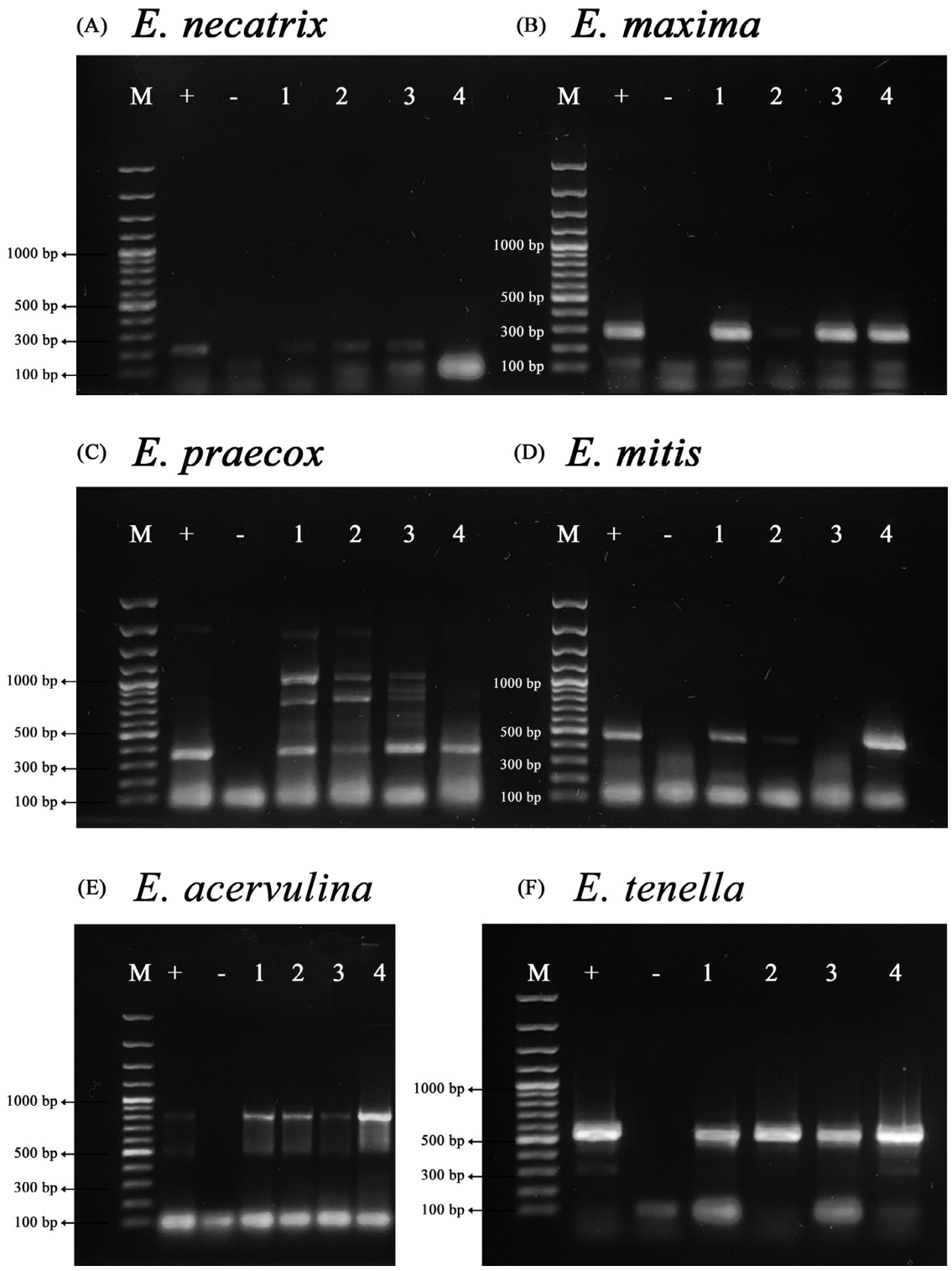


Figure 3. Gel electrophoresis. PCR result: molecular weight (marker 100 base pairs (bp)) (A) *Eimeria necatrix* (amplicon size 200bp) + (positive control) ¥/*; - (negative control); 1 (Antioquia); 2 (Santander); 3 (Cundinamarca); 4 (Valle del Cauca). (B) *Eimeria maxima* (amplicon size 272bp). (C) *Eimeria praecox* (amplicon size 354bp). (D) *Eimeria mitis* (amplicon size 460bp). (E) *Eimeria acervulina* (amplicon size 811bp). (F) *Eimeria tenella* (amplicon size 539bp). ¥Positive control LIVACOX Q vaccine with *E. necatrix.* *Positive control EVANT-HIPRAMUNET with *E. maximum, E. praecox, E. mitis, E. tenella,* and *E. acervulina.*

the Cundinamarca zone, a condition that can directly affect the amount of oocysts in litter (Chapman, 2016). Garcés et al (2018) in Ecuador, showed that the use of recycled litter more than twice, improves productive parameters on day 35 of the chickens' life, compared to the use of new litters between each batch. This results possibly due to the immunity generated by early exposure to oocysts in the litter, in addition to the effect of earlier colonization with microbiota that can act as a probiotic or direct feed microbial supplement, leading to better bird performance. Another condition that can alter the results of the oocyst counts is the anticoccidial treatment program implemented in each farm at the time of sampling, which was not possible to obtain due

Table 8. Summary of the zootechnical results for the In vivo Anticoccidial sensibility test.

Group	BW	BWG	FCR
NNC	760.48 ± 36.78	403.62 ± 35.24	1.33 ± 0.110
INC	746.28 ± 36.78	395.59 ± 35.24	1.38 ± 0.110
SAL	750.12 ± 36.78	398.25 ± 35.24	1.39 ± 0.110
DIC	731.60 ± 36.78	356.81 ± 35.24	1.59 ± 0.110
MET.CLO	732.53 ± 37.17	379.49 ± 35.90	1.44 ± 0.121

Measurements taken 7 days after the challenge with 1×10^6 sporulated oocysts/mL of field *Eimeria* spp.

Final weight (BW), weight gain (BWG) represented in grams, and feed conversion (g/g) (FCR) (Least squares mean ± standard error). Non-infected non-medicated control (NNC), Infected non-medicated control (INC) and 3 infected medicated treatments including Salinomycin (SAL-66 ppm), Diclazuril (DIC-1 ppm), and Methylbenzocuate Clopidol (MET.CLO-100 ppm).

to the confidentiality claimed for the producer (Chapman and Jeffers, 2015; Garcés et al., 2018).

Epidemiological studies on the frequency of *Eimeria* spp. complemented with molecular analyzis are valuable tools for the prevention and control of coccidiosis (Haug et al., 2008; Ogedengbe et al., 2011; Györke et al., 2013; Jenkins, et al., 2019). They can identify in a short time and with precision, for example the viability of oocysts capable of causing infection (Jenkins, et al., 2019) and which species of *Eimeria* are compromising the productive parameters in poultry farms, so an effective control strategy can be developed. This study, like the study by Hamidinejat et al. (2010) and Moraes et al. (2015), used the PCR technique to identify the species of *E. acervulina, E. maxima, E. praecox, E. mitis, E. necatrix* and *E. tenella*. Despite the differences in the sampling locations and methods, both studies showed that it is common to find mixed infections which can be difficult to characterize through clinical signs alone which require more specialized training (Carvalho et al., 2011). Finally, in this work, due to morphological characteristics of the oocysts in order of prevalence, the species of *E. acervulina* (35.0%), *E. tenella* (31.1%) and *E. maxima* (20.4%) were identified, data similar to those presented by Debbou-Iouknane et al (2018) in Algeria, with prevalence of 32.05%, 26.92% and 11.53% and to the work of Gharekhani et al (2014) in Western Iran, with higher but consistent prevalence in the order of frequency 75.7%, 54.3 % and 20% for *E. acervulina, E. tenella* and *E. maxima*, respectively; species classified between moderate and high pathogenicity (Kaboudi et al., 2016). Additionally with the PCR *E. praecox, E. mitis* and *E. necatrix* were identified, the latter being highly pathogenic (Kaboudi et al., 2016). These findings are of great importance, since the species with moderate and high pathogenicity are possibly interfering with the productive response of the birds in the sampled farms (Debbou-Iouknane et al., 2018), in addition to this subclinical form (mild infections that do not show symptoms) of coccidiosis is responsible for causing a reduction in the feed conversion of birds (Gazoni et al., 2021) and taking into account that the feed account for approximately 70% of the cost of broiler chicken production, the economic impact is very high (Jordan et al., 2002; Molla & Ali 2015; Gazoni et al., 2021) only due to the reduction in this productive parameter.

Table 10. Relative weight gain (% BWG_{NNC}), global resistance index (GI) and Global index of NNC (%), estimated average values (± standard deviation) for field *Eimeria* spp.

Group	BWG_{NNC} (%)	GI	Global index of NNC (%)	Status
INC	98.01 ± 3.75			
SAL	98.67 ± 5.09	97.27	85.79	Good efficacy
DIC	88.40 ± 25.79	84.49	74.52	Limited efficacy
MET.CLO	96.93 ± 9.02	96.93	85.49	Good efficacy

In this work, the PCR technique complements the diagnosis of *Eimeria* species, which due to morphological characteristics of the oocysts was not easy to identify, for this reason the PCR technique is a methodology that recently has gained relevance for its rapid and accurate diagnosis (Brown et al., 2018).

In countries such as Norwey (Haug et al., 2008), Romania (Györke et al., 2013), Brazil (Moraes et al., 2015), and India (Brown et al., 2018), the PCR technique is frequently used for the identification and diagnosis of *Eimeria.* Additionally, data from PCR can complement control programs against coccidia, which, due to the reduced use of antibiotic feed additives in the European Union and other regions of the world (Regulation EC N° 1831/2003), have been focused on reinforcing biosecurity and hygiene practices in poultry farms, combined with the prophylactic use of live vaccines (Thabet et al., 2017) and natural feed additives (Peek and Landman, 2010).

Although there is a trend to reduced chemotherapy in poultry production, anticoccidial products are still used frequently (Thabet et al., 2017) and are considered critical for broiler production in many regions of the world

Table 9. Gross lesion score (LS) for *E. acervulina, E. tenella* and total mean lesion score (TMLS), oocyst index (OI) and mortality rate (MR).

Group	LS - *E. acervulina*	LS - *E. tenella*	TMLS[1]	OI	MR (%)
NNC	0.00[b]	0.00[b]	0.00[b]	0.0[b]	0
INC	0.50 ± 0.53[a]	1.37 ± 1.19[a]	1.88 ± 1.46[a]	2.2 ± 0.64[a]	0
SAL	0.62 ± 0.52[a]	1.37 ± 0.74[a]	2.00 ± 1.07[a]	2.8 ± 1.19[c]	0
DIC	0.50 ± 0.53[a]	0.62 ± 0.74[a]	1.13 ± 1.55[a]	3.4 ± 1.69[a]	3.21
MET.CLO	0.37 ± 0.52[a]	1.50 ± 1.31[a]	1.88 ± 2.07[d]	1.2 ± 1.13[d]	0

Measurements taken 7 days after the challenge with 1×10^6 sporulated oocysts of field *Eimeria* spp.

[a-d]Different letter indicates statistical significance ($P < 0.05$).

[1]TMLS: Sumatory of the average score of *Eimeria* species intestinal lesions, 8 chickens per treated group. Non-infected non-medicated control (NNC), Infected non-medicated control (INC) and 3 infected medicated treatments including Salinomycin (SAL-66 ppm), Diclazuril (DIC- 1 ppm), and Methylbenzocuate Clopidol (MET.CLO- 100 ppm).

Table 11. Histological description of lesions and presence of *Eimeria* in 3 intestinal segments of individuals challenged with coccidia.

	Experimental group				
Histopathological findings[1]	NNC	INC	SAL[66 ppm]	DIC[1 ppm]	MET.CLO[100 ppm]
Hyperplasia GALT[2]	4	0	2	1	2
Hyperplasia lamina propria	3	0	2	1	2
Eosinophils	9	0	1	0	0
Heterophiles	0	0	0	0	1
Lymphocytes	1	0	0	0	0
Fibrin	0	0	1	1	1
Necrosis	1	0	1	0	2
Erosion	0	1	0	0	0
Hemorrhage	0	1	0	0	4
E. acervuline	0	2	4	4	4
E. maxima	0	7	6	6	7
E. tenella	0	7	6	6	8
E. Necatrix	0	0	0	1	0
Other Eimerias	0	0	2	3	2
Total score	18	18	25	23	33

[1]Sum of 3 individuals and 3 intestinal segments (duodenum, ileum and cecum) per treatment. The table shows the number of individuals that presented different cell types in the different intestinal layers and segments; different type of lesions: necrosis, erosion, hemorrasge; and the presence of different *Eimeria* spp. stages.

[2]GALT: gut-associated lymphoid tissue, NNC: non-infected non-medicated, INC: infected non-medicated, SAL: infected treated with Salinomycin, DIC: infected treated with Diclazuril, MET.CLO: infected treated with Methylbenzocuate Clopidol.

(Chapman et al., 2010; Peek, 2010). Widespread use of anticoccidial products has led to the appearance of resistant strains, which is a serious challenge for the poultry industry in general (Quiroz-Castañeda and Dantán-González, 2015). In this study, representatives of 3 classes of anticoccidials were evaluated in a sensitivity test. For synthetic compounds popularly known as "chemicals" (Chapman and Jeffers, 2015), DIC was used. In the category of ionophores that are produced by fermentation (Noack et al., 2019), SAL was evaluated (Chapman and Jeffers, 2015). And MET.CLO was used as an example of a combined product (Peek, 2010; Noack et al., 2019). Strain sensitivity was measured using the global resistance index methodology, which considers several relevant parameters related to the pathogenic effects of coccidia and zootechnical performance (Thabet et al., 2017). This methodology considers oocyst excretion, lesion score, growth performance, and mortality, with weight gain and feed conversion weighted heaviest because of their economic impact (Stephan et al., 1997; Arabkhazaeli et al., 2013).

Oocyst excretion is influenced by various factors such as the reproductive potential of the various species, overcrowding, and the host's immune response (Fayer, 1980), therefore, as the only variable to measure anticoccidial efficacy, it can be misleading (Reid, 1975). Several publications have shown that oocyst production has little correlation with weight gain, lesion score and, in highly pathogenic species (*E. tenella* and *E. necatrix*), even with mortality (Fayer, 1980; Stephan et al., 1997). The intestinal lesion score is one of the most common methods for evaluating intestinal damage caused by coccidia, but it involves the evaluator's subjectivity and experience (Conway et al., 1990; Stephan et al., 1997; Li et al., 2004). Under field conditions for highly pathogenic species, the mortality variable is the strongest sign of resistance (Bedrnik, 1983; Stephan et al., 1997).

In this work, a modification was made in the IO calculation of the formula by Stephan et al. (1997), since the oocyst count was done from fresh feces and not from intestinal scrapings. However, they were categorized on a scale of 0 to 5 to be able to include them in the same way in the formula for sensitivity analysis. The DIC group presented the highest IO values (3.4 ± 1.69). However, it was not found to be statistically significant with the other treated groups, and although the DIC group had higher IO, poorest weight gain and feed conversion, it did not present the highest intestinal lesion scores. This result is similar to Chapman's (1998) report that high count oocyst is not always related to greater intestinal damage.

Similarly, in this study, it was not possible to find a statistical difference in the intestinal lesion score for any groups evaluated, which could possibly reflect a low pathogenicity of the field strains used (Thabet et al., 2017).

E. maxima is classified as a species with a moderate to high pathogenicity (Chapman, 2014, Quiroz-Castañeda and Dantán-González, 2015) and the infective dose used in this work was high compared to other reports such as Oviedo et al. (2006), (3.33×10^5 vs. 100×10^3 oocysts, respectively), but during the experimental period, lesions caused by *E. maxima* could not be observed. Although, in the evaluation of coprological analysis on the final day of the test, it was possible to oocysts count of this species, which, due to their large size, shape, and color, are difficult to confuse. To corroborate these findings, the histology analysis performed on different intestinal sections was of great help, as it showed the presence of *Eimeria maxima* and other species such as *E. mitis* and *E. necatrix*. Possibly, on the day of the final evaluation, this variety of *Eimeria maxima*, was just reaching its oocyst excretion peak, which, as reported by Jenkins et al. (2017b), can occur between 130 and 162 hours post inoculation. Another possibility is that this field strain of *E. maxima* was not pathogenic and had little replication capacity (Dalloul and Lillehoj, 2006) to show intestinal lesions. This is according

to results reported by Schwarz et al. (2009), that indicated there are genetic variants with different pathogenic capacities among the same species of *Eimeria maxima.*

The values of the global index of resistance with respect to the NNC group were in the range of good efficacy for the groups medicated with SAL and MET.CLO (85.79% and 85.49% respectively) and the group treated with DIC reported a score of 74.52%, classified as limited efficacy. These data is consistent with the field results evaluated, since this last group was the only one that presented mortality caused by coccidia. According to Stephan et al. (1997), this is the greatest evidence of resistance problems, what generated a decrease in the value of the index of this group, down to the category of limited efficacy. The results in this work are alike with other results under similar evaluation conditions: strains from the field in a mixture of diverse species, where drugs such as DIC, for coccidia control reported limited efficacy to total resistance but differs for SAL and MET. CLO who report resistance (Stephan et al., 1997; Abbas et al., 2008; Arabkhazaeli et al., 2013; Lan et al; 2017; Thabet et al., 2017).

For future work, performing a dose titration to confirm the estimated level of challenge, include others anticoccidial treatments and make a comparison with different methodologies for evaluating anticoccidial sensitivity. For example: Anticoccidial index (**ACI**), percentage of optimum anticoccidial activity (**POAA**), Reduction of lesion scores (**RLS**) and Relative oocyst production (**ROP**) (Arabkhazaeli et al., 2013; Lan et al; 2017), that allow broadening the criteria to determine anticoccidial sensitivity.

This work is the first epidemiological report of coccidia in 4 regions responsible for 63.7% (ICA, 2019) of broiler production in Colombia. In addition, this study provides information on the identification of the most frequently identified coccidial species and their distribution, along with information on the sensitivity of field strains to some of the most commonly used anticoccidials in the region. More research is needed, but this is a great first step to provide more knowledge about the current state of coccidial challenge in Colombia.

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DISCLOSURES

The authors declare no conflict of interest

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