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Full Length Article

Populations of *Eimeria tenella* express resistance to commonly used anticoccidial drugs in southern Nigeria

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ABSTRACT

Coccidiosis is one of the most economically important diseases of poultry. This study determined the preponderance of chicken *Eimeria* in southern Nigeria and assessed the parasite's resistance to three anticoccidial drugs: Amprolium hydrochloride; Amprolium hydrochloride + Sulfaquinoxaline-Sodium; and Toltrazuril. Multiplex PCR amplification of the SCAR region was used to confirm *Eimeria* preponderance. Resistance was assessed following the inoculation of 2.32×10^5 infective oocysts into broilers. Data on weight gain, feed intake, feed conversion and fecal oocyst shed were recorded. At 7 days post inoculation 9 birds per treatment were sacrificed and assessed for macroscopic lesions in four intestinal regions. Percent optimum anticoccidial activity (POAA), Anticoccidial index (ACI) and Anticoccidial sensitivity test (AST) were used to access resistance. The preponderance of *Eimeria* spp. were *E. tenella* (77%), *E. necatrix* (55%), *E. acervulina* (44%) and *E. mitis* (11%), with multi-species infection occurring in 55% of samples assessed. Fecal oocyst shedding was low ($P < 0.05$) in the medicated groups. Lesions in the cecal region were present in all infected groups regardless of treatment and accounted for 27.8% of lesion scores by severity and 37.5% of lesion scores by frequency. Overall, lesion scores were less ($P < 0.05$) in birds of the medicated groups compared with the infected-unmedicated group. The high preponderance of *E. tenella* in the field, and the occurrence of cecal lesions – caused mainly by *E. tenella*- despite drug administration, indicate resistance in populations of this species in our isolate. Based-on the POAA, ACI and AST values, the *Eimeria* isolate showed reduced sensitivity to toltrazuril.

1. Introduction

Poultry coccidiosis caused by the Ampicomplexan parasite *Eimeria*, is one of the world's most economically important diseases of poultry birds. *Eimeria* primarily targets the tissues of the intestinal epithelium which consequently results in a decline in growth and feed utilization in poultry[1,2]. The disease is therefore associated with high production losses, high morbidity and mortality rates of above 50% [3,4]. In broilers, mild or subclinical infections are also important as even minor intestinal lesions can significantly impede feed efficiency and profitability [5].

The control of poultry coccidiosis can often be achieved by a combination of several strategies including; the use of anticoccidial drugs, delivery of live *Eimeria* vaccines, good husbandry practice and optimum biosecurity standards [6,7]. Inclusion of anticoccidial drugs in the diet of poultry birds is unarguably the most widespread means of controlling poultry coccidiosis [8,9]. Usage rates upward of 70% have previously been reported in poultry farms across the USA and Europe [10], and up to 100% in Nigeria [11,12]. This is particularly so because modern poultry systems are characterized by high stocking density that encourages parasite accumulation and transmission [13] and anticoccidial drugs are a convenient, and affordable method for keeping parasite challenge at a minimum [14]. In Nigeria, anticoccidial drug use is very popular, particularly because the majority (over 60%) of our poultry farms are characterized by poor hygiene conditions and low biosecurity levels [15]. These drugs seem to be indispensable for the sustainability of poultry production systems.

Unfortunately, as with the problems encountered with the widespread use of antimicrobials in veterinary and public health, the extensive use of chemotherapeutic drugs unavoidably leads to the development of drug resistance among *Eimeria* species [16]. Heavy drug dependence could have profound impact on the biology of *Eimeria* species in Nigeria with regards to the development of drug resistance, which can limit the effectiveness of such products [17].

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Following personal conversations with some local veterinarians in Rivers state, it was established that the anticoccidials Toltrazuril, Amprolium hydrochloride and Amprolium hydrochloride + Sulphaquinoxaline Sodium, are the drugs of choice for the control of chicken coccidiosis in the state. The presence of resistant *Eimeria* spp. in Rivers state are a threat to local poultry production and could compromise local control efforts. This study was therefore embarked upon to assess drug resistance of *Eimeria* spp. to these three drugs using preponderance studies and drug sensitivity trials.

2. Materials and methods

2.1. Ethical approval

The sampling protocol and the use of animals for experimental studies applied here were approved by the University of Port Harcourt Research Ethics Review Committee with ethics approval number UPH/CEREMAD/REC/04.

2.2. Field study

2.2.1. Sampling location

Port Harcourt is the capital of Rivers State and the third largest city in southern Nigeria (Fig. 1). It is a diverse city located in the Niger Delta region (within latitude $4^0 49'27''$N $7^0 2'1''$E) and is economically significant as the centre of Nigeria's oil industry. Port Harcourt metropolis is home to two local government areas (LGA): Port Harcourt LGA and Obio-akpor LGA. Nine major Live Bird Markets (LBMs) in Port Harcourt city were selected purposively based on the fact that being a densely populated city, live poultry trade traffic would very likely flow into the city from different parts of the state including the rural, urban as well as suburban areas.

2.2.2. Sample collection and processing

From September to November 2017, field studies were conducted and fresh fecal droppings were randomly collected from across cages where live chickens were housed. Samples from each market were pooled into a bulk sample. Each bulk sample was homogenized (in a blender or by stirring with a rod) and filtered using a 106 μm mesh sieve. *Eimeria* oocysts were harvested using the saturated NaCl floatation method (10 min at $1000 \times g$). The harvested oocysts were re-suspended in distilled water and washed by centrifugation three times to remove the flotation solution ($500 \times g$ for 5 min). The sediment containing the oocysts was transferred into beakers, suspended in 2.5% (w/v) $K_2Cr_2O_7$ solution and allowed to sporulate at room temperature for seven days with regular stirring. After sporulation, oocysts from each sample were cleaned with sodium hypochlorite (4% active chlorine) and washed with distilled water three times as described before [18].

2.3. Molecular characterization of chicken Eimeria

2.3.1. Genomic DNA extraction

Total genomic DNA extraction from oocysts of *Eimeria* species was done as follows: 20 mL of each oocyst suspension, were centrifuged at 750*g* for 10 min to pellet the oocysts. Each pellet was re-suspended in the minimum volume residual supernatant and transferred to a 2 mL screw top plastic tube containing glass beads (0.1–0.5 mm; ZR BashingBead™, SA) and covered with sterile phosphate buffered saline (PBS; pH 8.0). The pelleted oocysts were then disrupted using a Mini Beadbeater-8, (Biospec Products Bartlesville, USA) for three minutes. Total genomic DNA (gDNA) was isolated from the smashed oocysts homogenate using a Quick-DNA™ extraction kit (ZYMO RESEARCH) following the manufacturers protocol. Briefly, 1200 μL of Genomic Lysis Buffer were added to the filtrate and 800μL of the filtrate plus Lysis Buffer suspension transferred to a Zymo-Spin™ IIC column in a collection tube and centrifuged at $10{,}000 \times g$ for 60 s. Then 500 μL of genomic DNA Wash Buffer were added to the previous step and centrifuged at $10{,}000 \times g$ for 60 s. The suspension was then transferred to a 1.5 mL centrifuge tube and 100μL of DNA Elution Buffer added directly to the column matrix and centrifuged at $10{,}000 \times g$ for 30 s to elute the DNA. Finally, the eluted DNA was transferred into a prepared Zymo-Spin™ IV-HRC Spin Filter placed in a 1.5 mL micro-centrifuge tube and

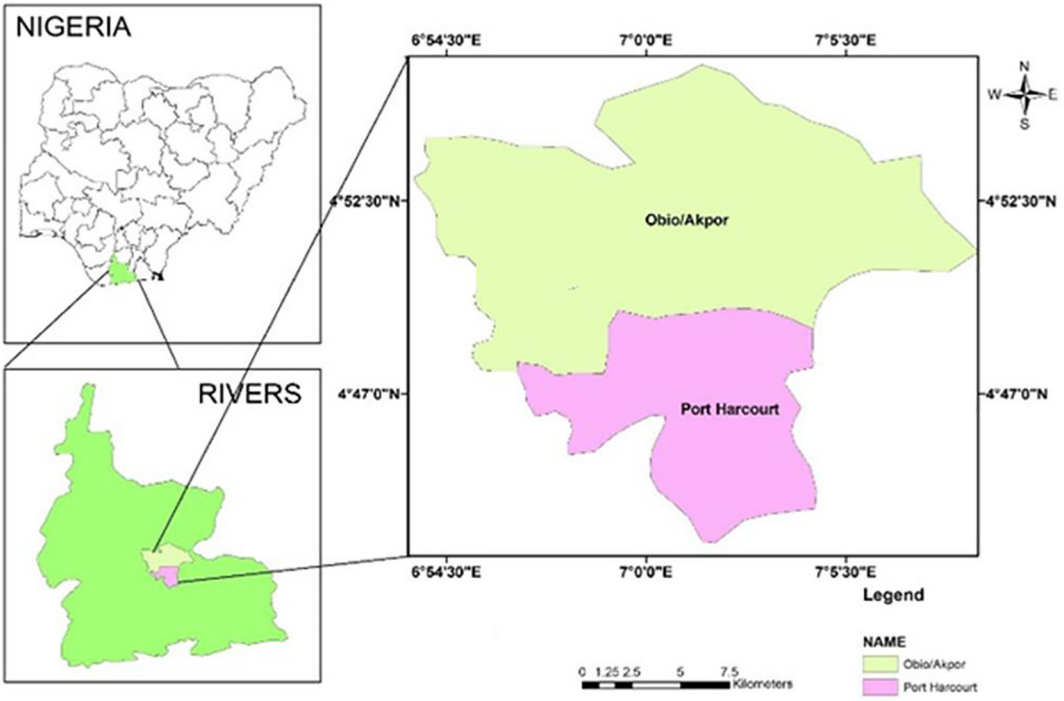

Fig. 1. Map of Nigeria: The study area is in Rivers state (highlighted in green) in the southern region of Nigeria. The sampling locations cover Port Harcourt and Obio/Akpor local government areas (The Cartography Unit, University of Port Harcourt).

Table 1
Nucleic acid content in each sample following DNA extraction.

Sample ID	ng/μL
Oil Mill	28.94
Choba	24.95
Slaughter	39.15
Mile 1	27.85
Rumuokuta	28.53
Fruit garden	66.81
Creek-road	263.33
Rumuokoro	23.83
Rumuomasi	36.58

Nucleic acid was quantified using the Nano drop nucleic acid spectrophotometer as explained in the text. Nucleic acid concentration is quantified here in ng/μL.

centrifuged at 8000 × *g* for 60 s. The amount of DNA extracted from each sample (Table 1) was quantified in Nano grams/μL using a NanoDrop nucleic acid quantification UV spectrophotometer (Thermo fisher Scientific).

2.3.2. Multiplex PCR amplification

The *Eimeria* species was verified by a multiplex PCR assay as earlier described by Fernandez *et al.*, [19]. Six pairs of species-specific primers were used (Table 2). The PCR amplification was based upon a 25 μL volume consisting of 1 μL genomic DNA template, 0.5 μL of each primer, 12.5 μL Taq DNA polymerase (Sigma, USA) and made up to 25 μL with nuclease free water. The standardized cycling conditions consisted of initial denaturation: 96 °C for 5 min followed by 30 cycles of 95 °C denaturation for 1 min, 59 °C annealing for 2 min; 72 °C extension for 1 min, and a final extension: 72 °C for 7 min.

2.3.3. Agarose gel electrophoresis

Agarose gel was prepared as follows: 1.5% (w/v) Agarose was prepared with 100 mL of distilled water and TBE buffer. The mixture was microwaved for 3 min. The Agarose solution was allowed to cool and 1μL of ethidium bromide added per 100 mL of agarose solution. The solution was loaded onto a casting tray with well position casting combs and refrigerated for 20 min to solidify.

The solidified Agarose gel was placed into the gel electrophoresis unit. The unit was filled with TBE until the gel was completely submerged. DNA samples extracted previously were well loaded with loading dye. A molecular ladder of 1000 base pairs was loaded into lane 4 and the other samples loaded into additional wells. Gel was run at 120 V for 25 min. DNA fragments (Bands) were visualized under UV light and images captured using a digital camera.

Table 2
The primers used in multiplex PCR amplification.

S/N	Species		Primer sequences 5′–3′	Expected amplicon size (bp)
1	*E. acervulina*	F	AGTCAGCCACACAATAATGGCAAACATG	811
		R	AGTCAGCCACAGCGAAAGACGTATGTG	
2	*E. tenella*	F	CCGCCCAAACCAGGTGTCACG	539
		R	CCGCCCAAACATGCAAGATGGC	
3	*E. mitis*	F	AGTCAGCCACCAGTAGAGCCAATATTT	460
		R	AGTCAGCCACAAACAAATTCAAACTCTAC	
4	*E. maxima*	F	GGGTAACGCCAACTGCCGGGTATG	272
		R	AGCAAACCGTAAAGGCCGAAGTCCTAGA	
5	*E. necatrix*	F	TTCATTTCGCTTAACAATATTTGGCCTCA	200
		R	ACAACGCCTCATAACCCCAAGAAATTTTG	
6	*E. brunetti*	F	TGGTCGCAGAACCTACAGGGCTGT	626
		R	TGGTCGCAGACGTATATTAGGGGTCTG	

(Adapted from Fernandez *et al.* [19]).
F: forward primer; R: reverse primer; bp: base pairs.

2.4. Drug sensitivity of chicken Eimeria species in Rivers state.

2.4.1. Isolate selection, propagation and enumeration

Eimeria oocysts isolated from Slaughter market were selected from amongst the others for propagation and drug sensitivity trials. Based on the results of our field survey, Slaughter market is the second largest live bird market in Port Harcourt in terms of the number of live poultry traders present and the sources of live birds. It was therefore expected that *Eimeria* oocysts from this market would be more representative of oocysts from across the state.

The isolate selected was inoculated into five 2-3-week - old susceptible chickens to provide sufficient oocysts for drug sensitivity trials. Fecal droppings were collected between days 5 and 7 post inoculation and processed for total *Eimeria* oocysts using standard procedures [20]. The concentration of oocysts and total number of oocysts collected were estimated using a Neubauer counting chamber as described previously [21]. On day eight post inoculation, birds were killed by decapitation. The intestines were removed and oocysts harvested as previously described [21]. The recovered oocysts were washed three times with distilled water, suspended in $K_2Cr_2O_7$ (2.5% w/v) and allowed to sporulate at room temperature with regular stirring. The total number of sporulated oocysts were enumerated using a Neubauer counting chamber as described by Holdsworth *et al.* [21].

2.4.2. Anticoccidial drugs

Three kinds of anticoccidial drugs were studied. Toltrazuril, Amprolium and Sulphaquinoxaline Sodium. Toltrazuril is a broad spectrum anticoccidial used in planned treatment programmes of poultry by inclusion in their drinking water [14]. Amprolium affects cofactor uptake and synthesis and is particularly effective against *E. acervulina, E. necatrix* and *E. tenella* [22]. Sulphaqunoxaline like other sulphonamides is a broad spectrum anticoccidial drug with a broad spectrum activity and the longest history of use in the preventative and curative management of Coccidiosis in poultry [22].

These drugs were administered according to the recommended dosage and usage as follows: 2.5% Toltrazuril (Bio-Pharmachemie, Vietnam) (Tolt): 1 mL per L of water for 2 days; 250 mg Amprolium hydrochloride (DSL Pharma, Nigeria) (Amp): 0.5 g per 2.5 L of water for 7 days; and 165 mg Amprolium hydrochloride + 185 mg Sulfaquinoxaline sodium (Bremer Pharma, GMBH Germany) (Amp + Sul): 1 g per L of water for 5 days. The anticoccidial drugs above were used in the state according to the local veterinarians.

2.4.3. Experimental design

The study performed involved 135 one-day-old MARSHAL broiler chicks purchased from a local breeding center (Obasanjo farms, Otta Nigeria). Chicks were housed under coccidian free condition at a simulated animal biosafety level 2 containment area in the Department of Animal and Environmental Biology facility, University of Port Harcourt. Birds were offered tap water and a basal diet ad libitum throughout the trial. The temperature within each cage was maintained at 30 to 35 °C by electric heating using 100 W bulbs. At 12 days of age the chicks were randomly assigned to five groups of 27 chicks. Each group comprised three replicates of nine birds. The various coccidiostats mentioned above were administered to the medicated groups (Amp, Amp + Sul and Tolt) from day 12 and continued up to day 19 under the recommended usage stated above. At 14 days of age, all birds except those in the non-infected-unmedicated groups (NegCntr) were orally infected with 2.32×10^5 sporulated oocysts of the *Eimeria* spp. isolate. All birds were weighed individually on the day they were inoculated, and 7 days post inoculation. Data regarding weight gain, feed intake, feed conversion and oocysts shed per gram of faeces were recorded. On the seventh day post inoculation, three chickens were randomly

selected from each group, euthanized, necropsied and lesion scores recorded for the four regions of the intestine as described by Johnson and Reid, [23].

2.4.4. Evaluation of resistance

Drug resistance of *Eimeria* was evaluated using three indexes:

1. The Anticoccidial Index (ACI). ACI = (rate of relative body weight gain + survival rate) – (lesion score + oocyst value). An ACI value of ≥ 160 indicated sensitivity; a value < 160 indicated resistance [24]. Oocyst value was calculated as follows: (OPG output of each group/OPG output of PosCntr group) × 100.
2. The Anticoccidial Sensitivity Test (AST) [25]. AST = (average lesion score in infected-unmedicated group – average lesion score in medicated group)/average lesion score in infected-unmedicated group × 100%. AST ≥ 50% was judged to be sensitive and < 50% was resistance; and
3. Percent Optimum Anticoccidial Activity (POAA). POAA = (GSR in medicated group – GSR in infected-unmedicated group)/(GSR in uninfected-unmedicated group – GSR in infected-unmedicated group) × 100%. GSR (Growth and Survival Ratio) was defined as final body weight divided by initial body weight. POAA > 50% was judged to be sensitive and ≤50% was resistance [5].

The overall assessment of drug resistance of our LBM *Eimeria* isolate was adapted from Lan *et al.*, [26]. Briefly, if 3 of 3 indexes showed resistance, our isolate was considered as severely drug resistant (+ + +). Two of 3 meant moderate drug resistance (+ +), 1 of 3 meant slight drug resistance (+) and none meant no drug resistance.

2.5. Statistical analyses

Data were analyzed by the software R 3.1.0 using a Windows XP system. The body weights, feed intake, lesion scores and oocysts shed per gram of feces were analyzed by one-way ANOVA and a post hoc analysis using Tukey's multiple comparison test to identify statistically significant variations. The difference was considered significant if $P < 0.05$. Box plots and bar charts were generated using R 3.1.0.

3. Results

3.1. Preponderance of chicken Eimeria spp. in Rivers state

The results of the molecular identification showed that *E. tenella, E. necatrix, E. acervulina* and *E. mitis* are the predominant *Eimeria* species in Rivers state. *E. tenella* was present at 7 of 9 locations (77%), *E. necatrix* at 5 of 9 (55%), *E. acervulina* at 4 of 9 (44%) and *E mitis* at 1 of 9 (11%). The primers used allowed for the discrimination of all four *Eimeria* species present in the samples. The different sizes of DNA fragments amplified were displayed on Agarose gel as follows: *E. acervulina* (811 bp), *E. tenella* (539 bp) *E. mitis* (310 bp) and *E. necatrix* (200 bp) (Fig. 2). Multi - *Eimeria* species infections were found in 5 of 9 (55%) locations with *Eimeria tenella* being the dominant species.

Table 3
Average body weight gain (g) of experimental birds between 14 days old and 21 days old.

Treatment	Replicate 1	Replicate 2	Replicate 3
Amp	161.08 ± 57.98*	163.06 ± 21.78*	157.23 ± 36.36*
Amp + Sul	176.22 ± 42.31*	165.27 ± 18.78*	173.34 ± 50.24*
Tolt	149.34 ± 35.11 ns	167.94 ± 55.75 ns	142.51 ± 50.51 ns
NegCntr	185.56 ± 9.75*	182.43 ± 10.43*	183.00 ± 17.85*
PosCntr	112.27 ± 39.95	140.41 ± 21.35	131.02 ± 45.10

Tolt, toltrazuril; Amp, amprolium hydrochloride; Amp + Sul: Amprolium hydrochloride + sulfaquinoxaline sodium; NegCntr: uninfected-unmedicated negative control; PosCntr: infected-unmedicated positive control. Data represent mean ± SD (*: $P < 0.05$ vs. *PosCntr; ns:* no significant differences).

3.2. Drug sensitivity of chicken Eimeria spp. isolate in Rivers state.

3.2.1. Performance results: Body weight gain (BWG), feed intake and feed conversion ratio (FCR)

In all treatments, body weight gain (BWG) of the medicated groups and the uninfected-unmedicated groups (NegCntr) were significantly higher than the infected-unmedicated group (PosCntr) (Table 3). In Amprolium hydrochloride and Amprolium hydrochloride + Sulfaquinoxaline sodium treated groups, BWG, was better than PosCntr group. Toltrazuril did not result in a significant increase in BWG. Feed consumption was highest in the NegCntr group and lowest in the PosCntr group (Table 4). The highest weight gain and the lowest feed conversion ratio was recorded in the NegCntr group (Table 5).

3.2.2. Lesion scores

Intestinal macroscopic lesions were completely absent in birds of the NegCntr group, and present in the PosCntr group. Figs. 3 and 4 show the occurrence and specific mean lesion scores for the four regions of the intestine assessed respectively. Lesions in the cecal region were present in all medicated groups as well as the PosCntr group. However, lesion scores of birds in the PosCntr group were markedly higher. The three anticoccidial drugs were effective in reducing the occurrence of lesions to varying degrees in the lower, middle and upper intestinal

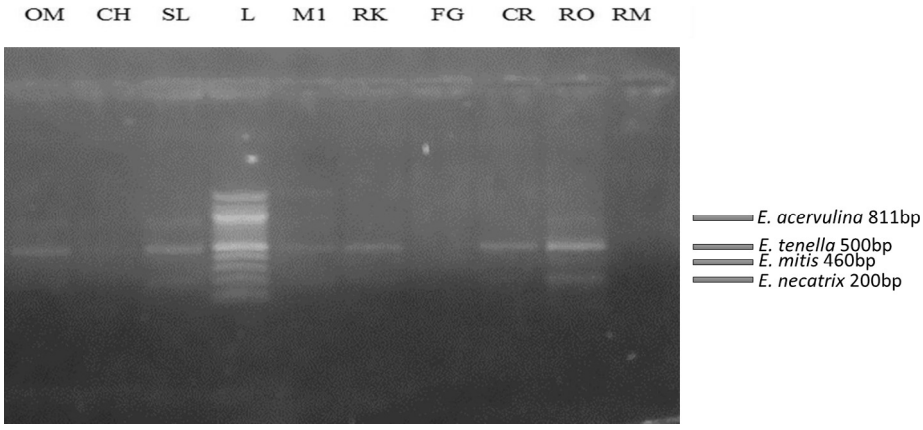

Fig. 2. Agarose gel electrophoresis showing the amplified SCAR gene of the *Eimeria* spp. **Lane OM**: *E. acervulina* (811 bp), *E. tenella* (500 bp); **Lane CH**: *E. acervulina* (811 bp), *E tenella* (500 bp); **Lane SL**: *E. acervulina* (811 bp), *E. tenella* (500 bp), *E. necatrix* (200 bp); **Lane M1**: *E. tenella* (500 bp), *E. necatrix* (200 bp); **Lane RK**: *E. tenella* (500 bp), *E. necatrix* (200 bp); **Lane FG**: No band; **Lane CR**: *E. tenella* (500 bp), *E. necatrix* (200 bp); **Lane RO**: *E acervulina* (811 bp), *E tenella* (500 bp), *E. mitis* (460), *E. necatrix* (200 bp); **Lane RM**: No band; **Lane L**: 1000 bp molecular ladder. Abbreviations: OM: Oil Mill; CH: Choba; SL: Slaughter; M1, Mile 1: RK, Rumuokuta; FG, Fruit garden; CR, Creek road; RO, Rumuokoro; RM, Rumuomasi; bp, base pairs.

Table 4
Total feed consumed within the infection period.

Treatment	Feed consumed (g)
Amp	4090.59 ± 86.20
Amp + Sul	4227.52 ± 135.48
Tolt	4036.94 ± 76.39
NegCntr	4389.27 ± 75.06
PosCntr	3561.93 ± 136.38

Tolt, toltrazuril; Amp, amprolium hydrochloride; Amp + Sul: Amprolium hydrochloride + sulfaquinoxaline sodium; NegCntr: uninfected-unmedicated negative control; PosCntr: infected-unmedicated positive control. Data represent mean ± SD.

Table 5
Feed Conversion ratio of birds between 14 days old and 21 days old.

Treatment	Replicate 1	Replicate 2	Replicate 3
Amp	2.76	2.78	2.95
Amp + Sul	2.75	2.75	2.71
Tolt	3.01	2.72	3.08
NegCntr	2.66	2.69	2.61
PosCntr	3.40	2.80	3.14

Tolt, toltrazuril; Amp, amprolium hydrochloride; Amp + Sul: Amprolium hydrochloride + sulfaquinoxaline sodium; NegCntr: uninfected-unmedicated negative control; PosCntr: infected-unmedicated positive control.

regions but not in the cecal region. A combined assessment of the average intestinal lesion scores of birds in our drug sensitivity trial showed that, Amp + Sul group had the least lesion score i.e. most effective, and Tolt group had the highest scores, i.e. least effective. Overall, intestinal lesion scores were significantly less ($P < 0.05$) in birds treated with the anticoccidial drugs (Table 6.) compared with birds in PosCntr group.

3.2.3. Fecal oocyst shedding

Oocysts shed in the feces of birds from 5 days post infection (dpi), 6 dpi and 7 dpi in all treatment groups is shown in Figs. 5, 6 and 7 respectively. Fecal oocysts shedding increased with the duration of infection with the least oocysts count on day 5 and the highest oocyst count on day 7. Among the medicated groups, Amp group recorded the least number of fecal oocysts excreted with $1.46 \times 10^5 \pm 0.24$ oocysts shed per gram of feces. Tolt group had the highest with $3.17 \times 10^5 \pm 1.98$ oocysts shed per gram of feces. Overall, fecal oocyst counts in all three medicated groups were significantly less ($P < 0.05$) than that in the PosCntr group (Table 7).

3.2.4. Anticoccidial index (ACI), anticoccidial sensitivity test (AST) and percent optimum anticoccidial activity (POAA).

The results of ACI, AST and POAA are summarized in Tables 8 and 9. No bird mortality was recorded throughout the trial. The ACI values of Amp and Amp + Sul groups (> 160) indicated that these drugs were able to treat the chickens infected with our isolate while maintaining bird productivity. Resistance to toltrazuril was expressed as the value of 150.99. Both POAA and AST values (> 50%) showed that our isolate is sensitive to all three coccidiostats tested, however, sensitivity to toltrazuril was much less. In the present study, an overall drug sensitivity assessment based on a combined analysis of the three indexes revealed slight resistance of *Eimeria* isolate to toltrazuril (Table 10).

4. Discussion

Eimeria spp. isolates were identified by the multiplex PCR amplification of the SCAR rDNA region using species-specific primers [19]. The preponderance of chicken *Eimeria* species recorded in Rivers state were *E. tenella*: 77%, *E. necatrix*: 55%, *E. acervulina*: 44% and *E. mitis* 11%. Our results are in agreement with a related study involving the molecular characterization of chicken *Eimeria* in northern Nigeria where preponderance rates of 75% for *E. tenella*, 25% *E. necatrix*, 33% *E acervulina* and 50% *E. mitis* were reported [27]. Our study further revealed that *E. burnetti* and *E. maxima* were absent from our samples. This result is also in agreement with the study by Jatau *et al.*, [27] where these species were also absent. The absence of *E. maxima* and *E burnetti* in these two regions is an indication that these species may not play significant roles in the epidemiology of chicken coccidiosis in Nigeria.

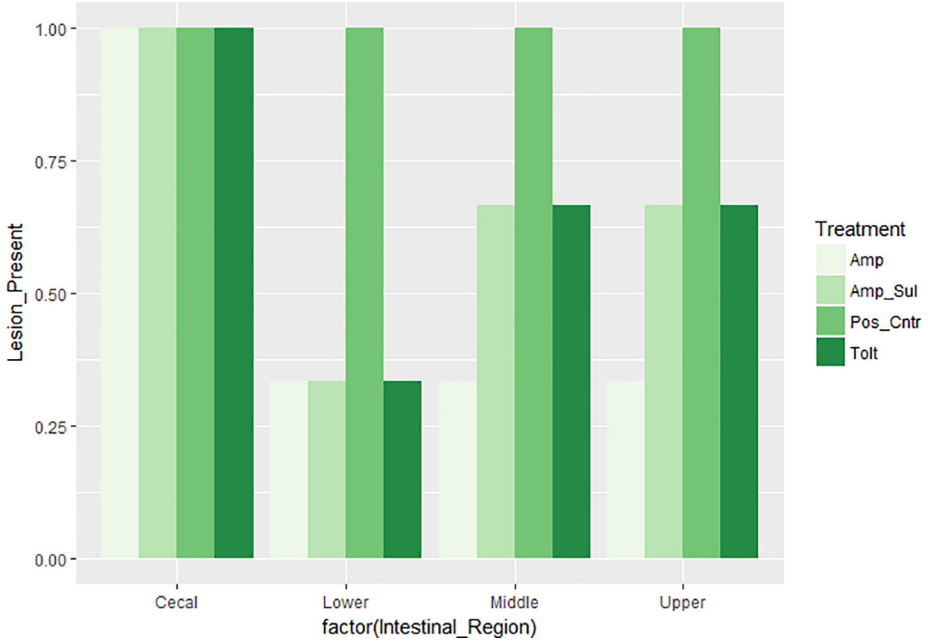

Fig. 3. Occurrence of lesions in the four regions of the intestine. Keys: Tolt, toltrazuril; Amp, amprolium hydrochloride; Amp + Sul: Amprolium hydrochloride + sulfaquinoxaline sodium; PosCntr: infected-unmedicated positive control.

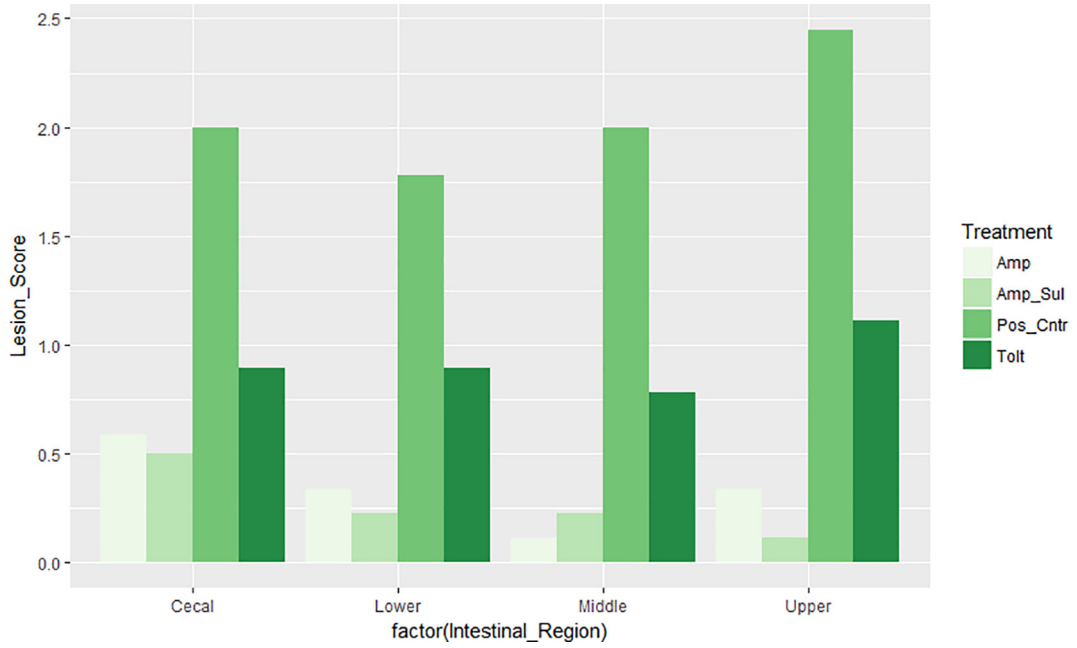

Fig. 4. Mean lesion scores in the four regions of the intestine. Keys: Tolt, toltrazuril; Amp, amprolium hydrochloride; Amp + Sul: Amprolium hydrochloride + sulfaquinoxaline sodium; PosCntr: infected-unmedicated positive control.

Table 6
Overall lesion score of birds infected with a mixed *Eimeria* spp. isolate assessed by taking an average of the individual scores for the four intestinal regions.

Treatment	Lesion score
Amp	0.333 ± 0.36*
Amp + Sul	0.305 ± 0.21*
Tolt	0.944 ± 1.28*
NegCntr	0
PosCntr	2.083 ± 0.25

Tolt, toltrazuril; Amp, amprolium hydrochloride; Amp + Sul: Amprolium hydrochloride + sulfaquinoxaline sodium; NegCntr: uninfected-unmedicated negative control; PosCntr: infected-unmedicated positive control. Data represent mean ± SD (∗: $P < 0.05$ vs. PosCntr).

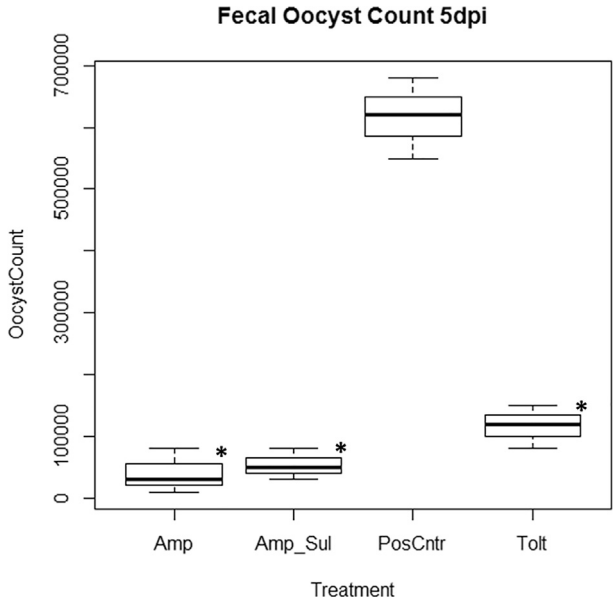

Fig. 5. Oocysts shed per gram of feces 5 days post inoculation. Keys: Tolt, toltrazuril; Amp, amprolium hydrochloride; Amp + Sul: Amprolium hydrochloride + sulfaquinoxaline sodium; PosCntr: infected-unmedicated positive control. ∗: $P < 0.05$ vs. PosCntr.

Analysis of our results showed that *E. tenella* was present at every positive sample location and had the highest prevalence (77.8%). The same observation was reported by Jatau *et al.* [27] in northern Nigeria. High preponderance rates such as these are often an indication of drug resistance in this species [10]. *E. tenella* therefore remains highly invasive amongst others and is possibly the most economically important *Eimeria* species causing chicken coccidiosis in southern Nigeria. Similar results have been obtained in Ethiopia [28], India [29] and China [9,26,30] where *E. tenella* was reported as the most prevalent.

The co-occurrence of multiple *Eimeria* species in a given location was also evident in this study. Our finding of the high prevalence of mixed *Eimeria* species infections (55%) is in agreement with related results of 68% and 67% found in northern Nigeria by Jatau *et al.*, [31] and Jatau *et al.*, [27] respectively.

E. tenella and *E. necatrix* are considered as highly pathogenic species of *Eimeria* [32,33]. While the presence of *Eimeria* oocysts in chicken feces is not a definitive diagnosis of chicken coccidiosis [28], the circulation of such pathogenic species can contribute to high morbidity and mortality in young chickens particularly when conditions of poor nutrition, poor sanitation and hygiene and co-infection with other pathogens persist [34]. Pathogenic species such as these could compromise poultry productivity in the majority of Nigerian chicken farms, particularly among the medium to small scale farms that are often characterized by poor sanitation and minimal-to-no biosecurity [15]. Based on our result, the species with the least preponderance is *E. mitis*. *E. mitis* is not often associated with severe disease in chickens, however, it can result in the reduction of feed efficiency in chickens [32].

Given the high prevalence of poultry coccidiosis reported in Nigeria, Nigerian poultry farms rely heavily on the use of anticoccidials for the control of coccidiosis [11]. It has been established that such practice as

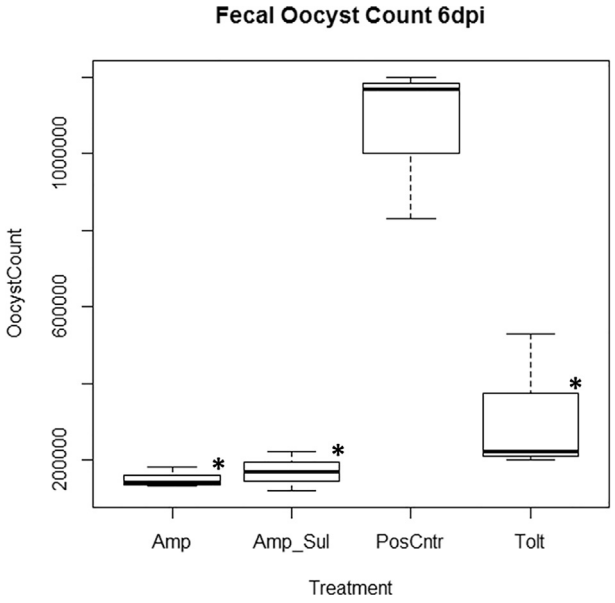

Fig. 6. Oocysts shed per gram of feces 6 days post inoculation. Keys: Tolt, toltrazuril; Amp, amprolium hydrochloride; Amp + Sul: Amprolium hydrochloride + sulfaquinoxaline sodium; PosCntr: infected-unmedicated positive control. ∗: $P < 0.05$ vs. PosCntr.

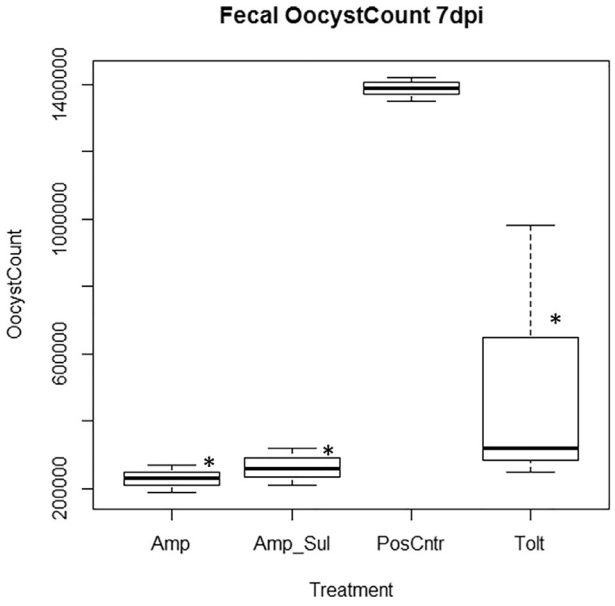

Fig. 7. Oocysts shed per gram of feces 7 days post inoculation. Keys: Tolt, toltrazuril; Amp, amprolium hydrochloride; Amp + Sul: Amprolium hydrochloride + sulfaquinoxaline sodium; PosCntr: infected-unmedicated positive control. ∗: $P < 0.05$ vs. PosCntr.

the extensive use of anticoccidial drugs could greatly influence the development of anticoccidial resistance in poultry *Eimeria* [35,36].

In this study, *Eimeria* oocysts were isolated from chicken fecal samples collected at a live bird market in Port Harcourt. By targeting all *Eimeria* species from a live bid market instead of a single poultry farm, our parasite detection rates should be higher [37] and our anticoccidial profile should be more representative of what is happening in the average chicken population in the field [35]. This is particularly so because live birds for sale at the market are often sourced from multiple farms [38], making such places important pathogen accumulation and distribution points [39].

The *Eimeria* oocysts were isolated, propagated, characterized and assessed for sensitivity to three anticoccidial drugs – Amprolium hydrochloride, Amprolium hydrochloride + Sulfaquinoxaline sodium and Toltrazuril. The sensitivity of these drugs was accessed on a prophylactic basis as experimental birds were inoculated with the *Eimeria* spp. isolate two days after drug administration commenced. The isolates were subsequently confirmed to contain *E. tenella*, *E. necatrix*, and *E. acervulina*, as described above. The common assay of including the respective anticoccidial drugs into the diet of infected birds was employed.

The extent to which an anticoccidial drug can result in a marked reduction or in some cases, the complete elimination of intestinal lesions is often explored as an indication of drug sensitivity and/or parasite resistance [5,9]. Also, presence and characteristic nature of lesions in specific areas of the intestine can be used to identify the species expressing resistant traits in a mixed inoculum of *Eimeria* [23]. Analysis of our results showed that all three medicated groups resulted in marked reduction in the occurrence of lesions in the upper, middle and lower intestinal regions (Fig. 3). The putative *Eimeria* species in our isolate that cause lesions in these parts of the intestine are *E. acervulina* and *E. necatrix* [40]. This indicates that populations of *E. acervulina* and *E. necatrix* in our isolate are sensitive to the three anticoccidial drugs tested. On the contrary, cecal lesions due primarily to infection by *E. tenella* [40] were present in all birds inoculated with *Eimeria* spp. isolate despite medication. Cecal lesions accounted for 27.8% of lesion scores by severity and 37.5% of lesion scores by frequency. These results indicated reduced sensitivity of all three anticoccidial drugs to populations of *E. tenella* in our study. Overall, birds in the medicated groups had significantly less severe lesion scores, which is an indication of a healthier gut and a greater chance of recovery from disease [41].

Fecal oocyst shedding was apparent in all groups except the uninfected-unmedicated group. Our results are in agreement with similar studies conducted in China [26], Egypt [42] and Iran [5] where birds continued to shed large amounts of oocysts regardless of anticoccidial medication. Oocysts shed per gram of feces by birds in all three medicated groups was however, significantly less than those in the infected-unmedicated groups. Our results therefore showed that the transmission of coccidiosis was sustained in the presence of drug pressure though the transmission potential (amount of oocysts shed) is reduced. This is very important in the epidemiology of the disease and should be factored in when considering disease control options.

Birds in all three medicated groups had higher body weight gains than those in the infected-unmedicated group. However the difference was statistically significant in Amprolium hydrochloride and Amprolium hydrochloride + Sulfaquinoxaline treated groups only. Our results further showed that the body weight gains and feed efficiency of birds in the uninfected-unmedicated groups were significantly better than those in the medicated groups. These results are in agreement with similar studies [9,26] and therefore indicates that regardless of the prophylactic treatment of birds with anticoccidial drugs, coccidiosis can still reduce production efficiency in infected birds [5]. This highlights the economic importance of chicken coccidiosis.

Three anticoccidial efficacy indexes were adopted. An overall assessment of the drug sensitivity of our field isolate using the ACI, AST and the POAA showed sensitivity to Amprolium hydrochloride and Amprolium hydrochloride + Sulfaquinoxaline sodium and slight resistance to Toltrazuril. Our results are markedly different from a related study in China where severe resistance to Amprolium hydrochloride, Toltrazuril and Sulfaquinoxaline sodium was reported [26]. The

Table 7
Overall oocyst shed per gram (OPG) of feces assessed by taking an average of the oocyst shed from day 5 to day 7 post inoculation.

Treatment	OPG feces (10^5)
Amp	1.40 ± 0.24*
Amp + Sul	1.62 ± 0.39*
Tolt	3.17 ± 1.98*
NegCntr	–
PosCntr	10.23 ± 0.92

Tolt, toltrazuril; Amp, amprolium hydrochloride; Amp + Sul: Amprolium hydrochloride + sulfaquinoxaline sodium; NegCntr: uninfected-unmedicated negative control; PosCntr: infected-unmedicated positive control. Data represent mean ± SD (∗: $P < 0.05$ vs. PosCntr).

Table 8
Results for Anticoccidial Sensitivity Test and Anticoccidial Index of each drug.

Treatment	Survival rate%	BWG rate %	Oocyst value	AST	ACI
Amp	100	87.36	13.68	84.01	173.35
Amp + Sul	100	93.44	15.85	85.36	177.29
Tolt	100	82.88	30.95	54.68	150.99
NegCntr	100	100	0	–	200
PosCntr	100	69.64	100	–	67.56

AST: Anticoccidial sensitivity test = (average lesion score in infected-unmedicated group – average lesion score in medicated group)/average lesion score in infected-unmedicated group × 100%. AST ≥ 50% was judged to be sensitive and < 50% was resistance; **ACI**: Anticoccidial index = (rate of relative body weight gain + survival rate) – (lesion score + oocyst value). An ACI value of ≥160 indicated sensitivity; a value < 160 indicated resistance, **Oocyst value** = (OPG output of each group/OPG output of PosCntr group) × 100; **Tolt**: toltrazuril; **Amp**: amprolium hydrochloride; **Amp + Sul**: Amprolium hydrochloride + sulfaquinoxaline sodium; **NegCntr**: uninfected-unmedicated negative control**; PosCntr**: infected-unmedicated positive control.

Table 9
Result for Percentage Optimum Anticoccidial Activity (POAA).

Treatment	Initial Body Weight (g)	Final Body Weight (g)	GSR	POAA %
Amp	213.74 ± 2.44	374.20 ± 0.66	1.75	86.58
Amp + Sul	227.25 ± 1.59	398.86 ± 4.10	1.76	88.96
Tolt	217.55 ± 14.52	369.77 ± 21.97	1.70	59.49
NegCntr	236.69 ± 4.53	420.35 ± 3.40	1.78	100.00
PosCntr	217.64 ± 7.15	345.54 ± 7.85	1.59	0.00

POAA: Percent Optimum Anticoccidial Activity = (GSR in medicated group – GSR in infected-unmedicated group)/(GSR in uninfected-unmedicated group – GSR in infected-unmedicated group) × 100%, **GSR** (growth and survival ratio) = final body weight divided by initial body weight. POAA > 50% was judged to be sensitive and ≤50% was resistance; **Tolt**, toltrazuril; **Amp**, amprolium hydrochloride; **Amp + Sul**: Amprolium hydrochloride + sulfaquinoxaline sodium; **NegCntr**: uninfected-unmedicated negative control; **PosCntr**: infected-unmedicated positive control.

differences reported in poultry *Eimeria* drug sensitivity results perhaps express the unique ecological and/ or epidemiological factors that modulate the development of drug resistance between regions.

5. Conclusions

The study aimed to evaluate drug resistance of *Eimeria* spp. in southern Nigeria using preponderance and drug sensitivity assays. Four species, *E. tenella, E. necatrix*, *E. acervulina* and *E. mitis* were identified with *E. tenella* being the most dominant species present in 7 of 7 *Eimeria* positive locations. The higher preponderance of *E. tenella* was an indication of drug resistance in this species and the drug sensitivity results corroborated this finding as populations of *E. tenella* causing cecal lesions resulted in varying degrees of pathology in birds despite treatment with anticoccidial drugs. Overall, slight resistance to Toltrazuril was observed within the experimental period.

Table 10
Overall assessment of the sensitivity of *Eimeria* LBM isolate.

Treatment	ACI	AST	POAA	Resistance
Amp	–	–	–	None
Amp + Sul	–	–	–	None
Tolt	+	–	–	Slight

(+): resistance expressed within experimental period; (–): no resistance expressed within experimental period; Tolt, toltrazuril; Amp, amprolium hydrochloride; Amp + Sul: Amprolium hydrochloride + sulfaquinoxaline sodium; ACI: Anticoccidial Index; AST: Anticoccidial Sensitivity Test; POAA: Percentage Anticoccidial Activity.

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