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Morphometric and molecular identification of *Eimeria* species from commercial chickens in Nigeria

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Abstract

Eimeria species are important coccidian parasites causing significant economic losses from clinical and subclinical coccidiosis in poultry worldwide. The objective of this study was to identify the abundance of *Eimeria* species responsible for coccidial infections in commercial chicken flocks in Nigeria. Morphometric methods by oocyt sedimentation and microscopy were used to identify *Eimeria* species from pooled faecal samples randomly obtained from commercial chicken farms. While Real-Time quantitative Polymerase Chain Reaction (RT-qPCR) targeting the Sequence Characterized Amplified Region (SCAR) from the genomic DNA extracted from the faecal samples was used to determine the specific species of Eimeria.

Morphometry identification resulted in three groups of oocyst category *Eimeria* based on the length were identified (BM group: *E. brunetti* and *E. maxima*; AM group: *E. acervulina* and *E. mitis* and NTP group: *E. necatrix, E. tenella* and *E. praecox*). The relative abundance of 3.0%, 52.0% and 45.0% were obtained for BM group, AM group and NTP group respectively. Molecular method detected Five *Eimeria* species were identified by real-time qPCR from the samples with species-specific relative abundance of 37.8%, 1.1%, 7.1%, 3.1% and 50.9% *E. acervulina*, *E. maxima, E. necatrix*, *E. tenella* and *E. mitis*; while *E. praecox* and *E. brunetti* were not detected. All markers utilized in these qPCR assays were absolutely species-specific and support reproducible quantification across a wide linear range, unaffected by the presence of non-target species or other contaminating DNA. The sensitivity of this assay indicated that DNA equivalent to a single sporulated oocyst can be consistently detected.

Only five out of the conventional pathogenic species of Eimeria were detected in commercial chickens sampled in Nigeria. The epizootiological significance of the identified circulating *Eimeria* species is to enhance coccidiosis vaccination prophylaxis in Nigeria.

Keywords: morphometry, molecular, coccidiosis, *Eimeria,* chicken

Introduction

Coccidiosis is one of the most economically devastating diseases of domestic poultry caused by protozoan parasites of genus *Eimeria*. Domestic fowls are infected by seven distinct *Eimeria* species which are usually both site and host-specific resulting in enteric diseases of a variable degree of severity[1] are recognized namely *Eimeria brunetti*, *E. maxima*, *E. necatrix*, *E. tenella, E. acervulina*, *E. mitis*, and *E. praecox.*[2]

However, the existence of *Eimeria hagani* and *Eimeria mivati* has been questioned by many researchers.[3,4] These two species are also overlooked in papers that deal with species discrimination techniques.[4,5] Vrba et al.,[6] reported that *Eimeria mivati* is a variant of *Eimeria mitis* and not distinct specie. The authenticity of *E. hagani* is still doubtful. For morphometric identification, *Eimeria* species was categorized according to oocysts size, into three groups namely: AM group (small oocysts, <18.8µm tentatively *E. acervulina* and *E. mitis*), an NTP group (medium-sized oocysts 18.9µm to 23.8µm tentatively *E. necatrix*, *E. tenella* and *E. praecox*), or a BM group (large oocysts, >23.9µm, tentatively *E. maxima* and *E. Brunetti*).[7]

Eimeria species cannot be precisely distinguished by simply using oocyst morphology. There are specie specific molecular methods that are used to achieve precise identification of *Eimeria* species. Classical non-quantitative PCR methods for molecular diagnosis of the seven *Eimeria* species that infect chickens have been developed using targets including internal transcribed spacer or ITS-1sequences[8,9] or sequence characterized amplified regions – SCARs.[10] Diagnosis can be complicated in chickens with mixed specie infections and polymorphism within the target region can affect the sensitivity of the PCR.[5] Real-Time Quantitative PCR is capable of identifying, differentiating among and quantifying the *Eimeria* species present in chickens even with mixed-species infections. It is more sensitive than conventional PCR because while Real-Time PCR can detect as little as two-fold amplification, Agarose gel resolution required about 10 folds.[5]

Material and methods

Sampling

The study was conducted on commercial chicken flocks in Nigeria. Fresh faecal samples were collected from minimum of six different spots in the poultry pens. The faeces were homogenized and a representative sample of 100g was transported to the laboratory with ice packs for processing.

Laboratory processing

The homogenized faecal suspension was passed through a sieve (50 microns) and nylon tissue to exclude helminth eggs. The supernatant was decanted and the sediment was centrifuged at 4000 x g for 10

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minutes. The sediment was re-suspended in saturated sucrose solution (containing 685 g sucrose per liter of solution). It was then centrifuged at 1500 x g for 30 minutes to concentrate the oocysts. The collected supernatant was subsequently diluted up to 5 times with distilled water, shaken vigorously and centrifuged at 4000 x g for 10 minutes. The supernatant was decanted and the *Eimeria* oocysts present in the sediment were preserved in 2.5% Potassium dichromate.

Identification of *Eimeria* species

The oocysts enumerated above (both sporulated and unsporulated) were identified by morphometry carried out by measurement of 50 oocysts using calibrated ocular micrometer at 400x magnification according to Long and Reid.[11] They were categorized in accordance with Haug et al.,[7] into three groups: *Acervulina-Mitis* (AM) group (small oocysts, <18.8µm; tentatively *Eimeria acervulina* and/or *Eimeria mitis*); Necatrix, Tenella & Praecox (NTP) group (medium-sized oocysts, 18.9 µm to 23.8 µm; tentatively *Eimeria necatrix, Eimeria tenella* and/or *Eimeria praecox*) or a Brunetti-Maxima (BM) group (large ovoid oocysts, >23.9µm; tentatively *Eimeria brunetti* and/or *Eimeria maxima*).

Extraction of DNA from *Eimeria* Oocysts

One µl of the pooled oocyst sample was washed with an equal volume of Phosphate Buffer Saline (PBS) in a 2 ml Eppendorf tubes three times by centrifuging at 13,000 g for 1 minute in order to remove the potassium dichromate used in preserving the oocysts. The resulting pellet was re-suspended in 100 µl of PBS. It was transferred into a screw cap tube with 0.5µg of glass bead added and vortexed for 3 minutes to break the resilient oocyst walls in order to extract the DNA from the sporozoites. A drop of the sample was examined under the microscope to confirm disruption of the oocyst wall under x40 objective. After confirmation of the disruption of the oocyst wall, 200µl of ATL from Qiagen DNeasy blood and tissue kit was added and vortexed briefly. After this, 20µl proteinase K was added to the mixture and vortexed. It was incubated at 56^{0}C for 30 minutes to enable efficient tissue lysis. The lysate was put in a new tube and 200µl of AL buffer was added and vortexed. Also, 200µl of ethanol was added to the sample and vortexed. The resulting suspension was transferred into a DNeasy column and centrifuged at 13,000 g for 1 minute. The flow-through was discarded and the spin column put inside a new 2ml collection tube. It was then washed with 500µl AW1 and spin at 13,000 g for 1 minute. The flow-through was was also discarded and the spin column put inside another new 2 ml collection tube. This was washed with 500µl AW2 and spin at 13000 g for 3 minutes to dry the DNeasy membrane of residual ethanol so as not to interfere with subsequent reactions. It was then eluted with 100µl AE buffer into clean 2 ml micro centrifuge tubes and centrifuge for 1 minute at 8000 g. The flow-through contained the genomic DNA samples of the oocysts and stored in a refrigerator for preservation.

Quantitative real-time polymerase chain reaction technique

The DNA sample was removed from the fridge and 1µl was taken by pipette into a well in the 96 plate qPCR plate in triplicates. 1 mL of the positive and negative controls was put in the 11th and 12th column respectively. The following were measured, vortexed and mixed together in a 2ml qPCR tube (A): 500µl master mix containing 150mM Tris-Hcl, 40mM (NH4)2 SO4, 0.02% Tween 20, 5mM MgCl2, 400µM d ATP, 400µM d CTP, 400µM d GTP, 400µM d TTP and 50µ/mL Taq DNA polymerase; 297µl qPCR water and 3µl ROX dye. It was briefly vortexed after mixing. Into another 2ml qPCR tube (B) was measured the following after being vortexed initially: 10µl of Primer, 5µl of the probe and 175µl of nuclease free water and vortexed again. Subsequently 8µL from the mixture in A and 1µL of B was added to all the rows and columns of the qPCR plate. Table 1 shows the panel of primers and probe sequences used.

Table 1 qPCR primer panel of and probe sequences specific for the seven chicken *Eimeria* species

Species	Sequence source (SCARdb ID)	Primer and probe sequences	Amplicon size [bp]
E. acervulina	Ac-AD18-953	ACE-F: GCAGTCCGATGAAAGGTATTTG; ACE-R: GAAGCGAAATGTTAGGCCATCT; ACE-P: [6-FAM]ACAGTCCCCGCTGATGGTGTAACG[BHQ1	103
E. brunetti	Br-J18-626	BRU-F: AGCGTGTAATCTGCTTTTGGAA; BRU-R: TGGTCGCAGACGTATATTAGGG ; BRU-P: [6-FAM]CAACCGCAGCAAGCGAAGTTGA[BHQ1]	118
E. maxima	EmMIC1	MAX-F: TCGTTGCATTCGACAGATTC; MAX-R: TAGCGACTGCTCAAGGGTTT; MAX-P: [6-FAM]ATTGTCCAGCCAAGGTTCCCTTCG[BHQ1]	138
E. mitis	Mt-A09-716	MIT-F: CAAGGGGATGCATGGAATATAA; MIT-R: CAAGACGAATGGAATCAATCTG; MIT-P: [6-FAM]CCCGCGAGGGTTTCAGTTGATG[BHQ1]	115
E. necatrix	Nc-AD10-702	NEC-F: AACGCCGGTATGCCTCGTCG; NEC-R: GTACTGGTGCCAACGGAGA; NEC-P: [6-FAM]CCGTAGCATAGCTCAGGCAGCCAC[BHQ1]	134
E. praecox	Pr-A09-1108	PRA-F: CACATCCAATGCGATATAGGG; PRA-R: ACAGAAAAACGCAAAGAGCAA; PRA-P: [6-FAM]AGCAG-CAGCTGCCTCTCATTGACC[BHQ1]	117
E. tenella	Tn-E03-1161	TEN-F: TCGTCTTTGGCTGGCTATTC; TEN-R: CAGAGAGTCGCCGTCACAGT; TEN-P: [6-FAM]CTGGAAAGCGTCTCCTTCAATGCG[BHQ1]	100

The qPCR plate was covered with the optical cover provided and pressed down without allowing finger print impression marks on it. The plate was centrifuged briefly for a few seconds to allow a proper settlement of the constituents in the wells of the qPCR plates. The thermocycler was already put on to warm it up to the required temperature of 95^{0}C. The qPCR plate was put inside the thermo-cycler. The thermal cycling program used was initial denaturation at 95^{0}C for 1 minute followed by 40 cycles of denaturation at 95^{0}C for 15 seconds and combined annealing and extension at 60^{0}C for 30 seconds.

Fluorescence data were collected at the end of each cycle. Each sample was run in triplicate. The result was then analysed on the computer after the end of the cycling period. The quantitative real-time PCR reactions were performed in a Stratagene Mx3005P® real-time qPCR cycler. Both positive and negative template controls were included. The resulting data were processed using Mx ProTM software (Stratagene, USA). Threshold cycles (Ct) were calculated from baseline-corrected normalized fluorescence (dRn) data (Figure 1).

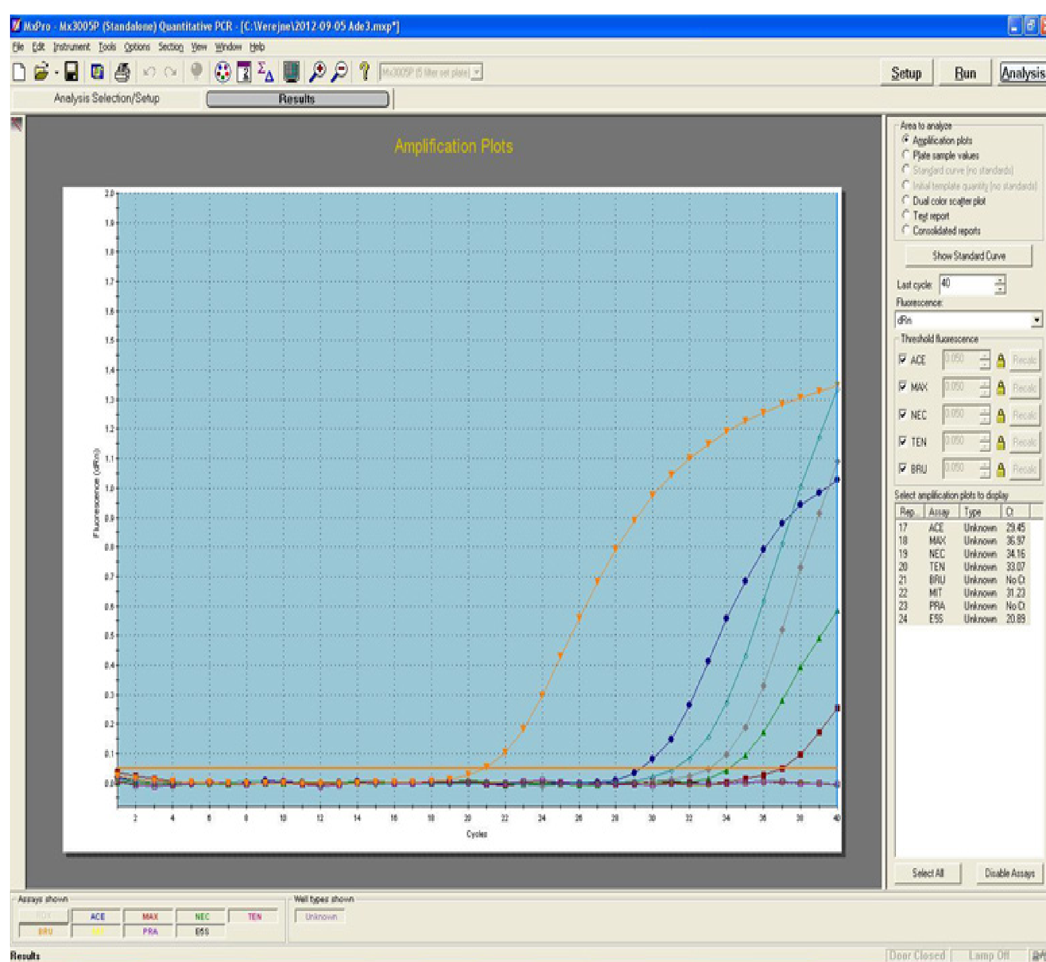

Figure 1 Real-time qPCR plots for the five (5) Eimeria species of poultry in pooled fecal samples taken from commercial chicken farms in Nigeria. Blue, E. acervulina; Light green, E. mitis; Ash colour, E. tenella; Deep green, E. necatrix; Magenta, E. maxima.

Results

Morphometric of Eimeria species

The NTP group made up of medium sized oocysts of *E. necatrix, E. tenella* and *E. praecox* has a relative abundance of 52%. This was followed by the small sized oocysts of the AM group comprising of *E. acervulina* and *E. mitis* with a relative abundance of 45% while the big sized oocysts of the BM group comprising of *E. brunetti* and *E. maxima* was only 3%.

Molecular results of Eimeria species

The results qPCR of the pooled samples are shown in table 2. Only five *Eimeria* species namely: *E. acervulina*, *E. tenella*, *E. mitis*, *E. necatrix* and *E. maxima* were detected. *Eimeria brunetti* and *E. praecox* were not detected in the samples. *E. mitis* has the highest abundance at 50.9% followed by *E. acervulina* at 37.8%. Others were *E. necatrix,* 7.1%, *E. tenella*, 3.1% and *E. maxima* 1.1%. The first four are known to be pathogenic while *E. mitis* although less pathogenic. The primers were sufficiently sensitive and specie specific to enable the discrimination of the seven *Eimeria* species of chicken. The qPCR used in this study was fully quantitative and removed the requirement for gel electrophoresis. This method was able to measure the precise gene copy numbers by comparison with a standard curve as well as the Ct values between the samples.

Table 2 Real-time quantitative polymerase chain reaction (qPCR) results for the seven (7) *Eimeria* species of poultry in pooled fecal samples taken from commercial chicken farms in Nigeria

Species	qPCR Ct Cycle	Abundance
E. acervulina	29.45	37.83%
E. mitis	31.2	50.91%
E. brunetti	No Ct	Not detected
E. maxima	36.97	1.06%
E. necatrix	34.16	7.10%
E. tenella	33.07	3.11%
E. praecox	No Ct	Not detected

Discussion

The identification of *Eimeria* species based on traditional morphometric method has been the gold standard in Nigeria until the introduction of molecular diagnostic method which is yet to be fully embraced. Both the morphometric and molecular methods indicated a low relative abundance of the BM group of 3% and 1.1% respectively. Their low abundance in the environment may either due to the efficacy of control measures or their innate inability to flourish under the existing environmental conditions. The real-time qPCR detected *E. maxima* but not *E. brunetti*. The morphometric method indicated an abundance of 45% for the AM group while the real-time qPCR reported 88.7% made up of 50.9% for *E. mitis* and 37.8% for *E. acervulina.* Similarly, morphometry method indicated an abundance of 52% for the TNP group while the real-time qPCR indicated 10.2% abundance made up of 7.1% for *E. necatrix* and 3.1% for *E. tenella. E. praecox* was not detected**.** The huge disparity in the figures reported for the morphometric and molecular methods is probably an indication that some *Eimeria* species were either undetected or wrongly categorized due essentially to the differences in the specificity and sensitivity of the two different techniques. Majaro,[12] reported that oocyst size and shapes do vary as the duration and severity of infection increases and this might be partly contributory to the observed disparities. It has also been reported that the measurements of the oocysts undergo variations due to changes in metabolism of parasites or birds, and even in the value of the shape morphometric indices that may overlap and lead to misleading conclusions regarding the species.[13] Jatau et al.,[14] reported that comparison of microscopic observation of oocysts with PCR-based detection of *Eimeria* genomic DNA yielded variable results. The identification of five *Eimeria* species in this study namely *E. acervulina*, *E. tenella*, *E. necatrix*, *E. mitis* and *E. maxima* is in agreement with those reported by other researchers [15,16,17] who also reported the five species without *E. praecox* and *E. brunetti*. Ogedengbe et al.,[18] using a sequenced characterized multiplex PCR method reported the characterization of 4 *Eimeria* species from samples taken from Nigeria, namely; *Eimeria tenella*, *E. acervulina*, *E. mitis* and *E. brunetti*. This perhaps should have confirmed the presence of E. *brunetti* in Nigeria but the 2 samples labelled D6 subjected to Agar gel electrophoresis gave conflicting results. One indicated the presence of *E. brunetti* and *E. tenella* while the other indicated *E. tenella* and *E acervulina*. Moreover, none of the other 8 samples from Nigeria contained *E. brunetti*. Further works in this area is needed to fully elucidate the identity of circulating *Eimeria* species in different parts of Nigeria. The result of the present study probably represents an improvement over the molecular identification of the *Eimeria* species in Nigeria carried out by Jatau and Ojimelukwe et al.[15,19] which reported only four of the seven *Eimeria* species each namely *E. acervulina, E tenella, E. necatrix*, and *E mitis* but not *E. maxima*. The non detection of *E. maxima* in those two earlier molecular studies was probably due to the very low relative abundance of *E. maxima* (about 1.1% in the present study) and the lower sensitivity of the conventional PCR used in those studies as against the real-time qPCR (used for the present study) which can consistently detect DNA equivalent to a single sporulated oocyst as against the Agarose gel resolution that required about 10 folds.[5] This is probably the first real-time qPCR study to confirm the presence of circulating *Eimeria maxima* in Nigeria.

The present result is at variance with those of Carvalho et al.,[20] Sun et al.,[13] and Al-Natour et al.,[21] who all reported the presence of the seven *Eimeria* species. Although Schwarz et al.[23] reported six *Eimeria* species; *E. brunetti* and *E. praecox* were present. The non-detection of *E. brunetti* in this study contradicted the assertion of Majaro[13] that the species of *Eimeria* causing coccidial infection in Nigeria were *E. tenella, E. necatrix*, *E. brunetti* and *E. acervulina*. However, the unreliability of morphological *Eimeria* species identification used in those studies could be responsible for this. Precise identification and genetic characterisation of different species of *Eimeria* are central to surveillance, prevention and control of coccidial infection.[23] The development of molecular tools has allowed not only the diagnosis but also the study on genetic variability of pathogens based on small quantities of oocysts through molecular markers.[8,23]

Conclusion

Five species of Eimeria identified and confirmed in this study provide useful epizootiological information on chicken coccodiosis in Nigeria. This will help in discrimination and selection of a cost effective and efficacious anti coccidial vaccine for use in the area. The preliminary data generated could serve as a template for future baseline epizootiological studies leading to better and more effective treatment, control and preventive strategies in the area.

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Conflicts of interest

We hereby declare that there is no conflict of interest.

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