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# Isolation, Identification and Molecular Characterization of *Eimeria* spp Infecting Chicken in Khartoum State, Sudan Using *ITS1* Gene

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**Abstract:** Coccidiosis in broiler chickens highly affects the economy for both producers and consumers. The later get a low quality meat due to anaemia induced by the parasite. The disease is characterized by lesions caused by seven host specific members of the family Eimeriidae of the phylum Apicomplexa. Collected positive samples of *Eimeria species* from broiler chickens were identified by measuring the dimensions of the sporulated oocyst. Seven species of *Eimeria* were detected using this method. When DNA was extracted and species specific primers were used to amplify *ITS1* gene using single specific primer PCR and multiplex PCR, only six species were identified. The accuracy of identification of broiler chicken *Eimeria* species using PCR is more reliable than the conventional methods like öocyst measurements or histopathology of the affected intestinal regions. This is indicated by the finding that *E. brunetti* which was identified morphologically by öocyst measurement, could not be identified molecularly. The mitochondrial genome sequences (*ITS1)* are highly suited for molecular diagnostics of coccidia and may be a potential genetic marker for molecular epidemiology of broiler chicken coccidiosis in the future in Sudan. The aim of this study is to determine and identify the species causing poultry coccidiosis in broiler chicken by traditional methods and molecular characterization using *ITS*.

**Keywords:** Apicomplexa, Broiler Chicken, *Eimeria*, *ITS1*, Poultry Coccidiosis

## 1. Introduction

Eimeriasis in chickens is generally characterized by parasite replication in the intestinal mucosa leading to extreme intestinal damage, depending on the number of ingested sporulated öocyst, resulting in diarrhea, morbidity and mortality with consequent serious economic losses. It is the most prevalent poultry disease worldwide.

The lesions are caused by seven host specific members of the family Eimeriidae of the phylum Apicomplexa. These are *Eimeria tenella* (caecum), *E. acervulina* (upper half of the small intestine), *E. brunetti* (small intestine, rectum, caecum and cloaca), *E. maxima* (small intestine), *E. mitis* (lower small intestine) and *E. praecox* (no distinct lesions but decreased growth rate). Among these species, the most prevalent and worldwide are *E. tenella* followed by *E. maxima* and *E. acervulina* [l]. Identification of Eimeria species is central for control strategies.

The aim of this study is to determine and identify the species causing poultry coccidiosis in broiler chicken by traditional methods and molecular characterization using the mitochondrial gene *ITS1* for each species.

# 2. Material and Methods

### *2.1. Collection and Processing of Chickens Guts*

A total of 100 gut samples of broiler chickens suspected for coccidiosis from different farm systems located in Khartoum State were microscopically examined for *Eimeria* species öocysts. Guts were opened by longitudinal incision and contents were thus collected and examined microscopically [2]. A total of 50 positive samples were detected in these samples. Flotation technique using saturated sodium chloride was done. Öocysts were aspirated using disposable pasture pipette and their number was counted using cell McMaster chamber. The mixture was diluted with distilled water (4:1) to remove sodium chloride and the sediment obtained was subjected to sporulation [3, 4]. They were sporulated and purified using standard techniques of Shirley [5]. Purified sporulated öocysts were stored in 2.5% potassium dichromate ($K_2Cr_2O_7$) prior to nucleic acid preparation. The sporulation was confirmed by examination under the microscope.

### *2.2. Purification of Sporulated Oocysts*

The suspension of the sporulated öocyst was mixed with an equal amount of 2.5 per cent potassium dichromate aqueous solution. After thorough mixing, the mixture was filtered through sieve followed by muslin cloth. The filtrate was then centrifuged at 1500 rpm for 10 minutes. About 50 percent of the supernatant was discarded and the remaining portion of the supernatant was poured in a fresh tube and mixed with an equal amount of sodium chloride aqueous solution for flotation as described by Levine [6]. The mixture was the centrifuged at 1500 rpm for 10 minutes; and the supernatant of the sporulated öocyst was aspirated and collected in a clean tube. The sediment was processed in the same way until no sporulated öocysts remain in the supernatant. The collected supernatant was mixed with water (1:4) in a falcon tube and kept overnight undisturbed at 4°C. The sporulated oocysts, settled at the bottom, were collected by removing all water to about 2.5 cm above the bottom of the tube. The remaining solution was centrifuged at 1500 rpm for 10 minutes. The clean sporulated öocysts were further used for öocysts measurement and DNA extraction.

### *2.3. Parasitological Examination*

A calibrated ocular micrometer was used to determine the length and width the sporulated and un-sporulated öocysts at a magnification of 400X. Thirty random öocysts from different samples were measured and their shapes were recorded as well. The index (Length/Width) was noted, and the different species of *Eimeria* were identified following the keys given by Soulsby [2] and Conway & McKenzie [7].

### *2.4. Molecular Analysis*

DNA extraction was performed according to Guven [8] and the modification of Gadelhag [9]. Fifty cycles of freezing and thawing, using liquid nitrogen and a water path at 50°C were carried out for complete rupturing of öocyst walls. During this process, 10µl were taken and examined under the microscope (40 X) to ensure complete öocyst wall destruction. For protease digestion 300µL of TE buffer (10mMTris-HCl, 0.1Mm EDTA pH 8), 3µL of 10% (W/V) SDS and 10µL of proteinase K (20mg /ml) were added to the öocyst suspension. After one hour incubation at 37°C, öocyst DNA was extracted.

#### *2.4.1. DNA Extraction*

DNA was extracted using Cetyl–Trimethyl Ammonium Bromide (CTAB) method as described by Zhao [10]. The (CTAB) buffer (2% weight/volume CTAB, 1.4 M NaCl, 0.2% [beta]-mercapto-ethanol, 20 mM EDTA, 100 mM TRI), protocol of Murray and Thomson [11] was used. DNA extraction buffer (lysis) was prepared using 1% SDS, 50 mM Tris/HCl (pH 8.0), 50 mM NaCl and 25 mM EDTA (pH 8.0). The samples were placed in a falcon tubes, then 100µl of previously made extraction lysis buffer were added to each tube and after 10 min, the tissue inside the tube was homogenized gently and thoroughly. Another 100 µL of extraction buffer were added. 350 µL of hot CTAB and incubated at 65°C water bath for 10 minutes. The suspension was extracted using equal volume of chloroform and centrifuged at 6000 rpm. The aqueous upper layer was transferred to a fresh tube. Double volume of absolute ethanol (MG) was added and left at -20°C overnight, and then the mixture was centrifuged at 6000 rpm for 10 minutes, washed twice with 70% ethanol at 6000 rpm for 5 minutes. The tubes then dried at air drift for ~30 minutes and re-suspended in ($ddH_2O$), left overnight at 4°C. DNA quality and quantity were then determined by Nano-drop Spectrophotometer (ND1000 Spectrophotometer, Nano-drop Technologies, Tnc.) and Agarose Gel Electrophoresis.

#### *2.4.2. DNA Amplification by PCR*

Sequences were amplified from genomic DNA as described by Schnitzle [12]. Primers specific for *Eimeria* species, namely: *E. tenella, E. acervulina, E. maxima, E. necatrix E, praecox, E. mitis* and *E. brunetti* (Table 1).

***Table 1.*** *Set of DNA primers used in the Polymerase Chain Reaction (PCR) to amplify the ITS1 region gene for different Eimeria. Species [12].*

*Tm = melting temperature*

| Species | Primer sequence | Product size (bp) | Tm (°C) |
|---|---|---|---|
| *E. maxima* | 5'GGGTAACGCCAACTGCCGGGTATG AGCAAACCGTAAAGGCCGAAGTCCTAGA3' | 350 bp | 51°C |
| *E. tenella* | 5'AATTTAGTCCATCGCAACCCT 3'CGAGCGCTCTGCATACGACA | 123 bp | 57°C |

| Species | Primer sequence | Product size (bp) | Tm (°C) |
|---|---|---|---|
| *E. necatrix* | 5'TACATCCCAATCTTTGAATCG<br>3'GGCATACTAGCTTCGAGCAAC | 285 bp | 57°C |
| *E. acervulina* | 5'GGCTTGGATGATGTTTGCTG<br>3'CGAACGCAATAACACACGCT | 321 bp | 51°C |
| *E. praecox* | 5'CATCATCGGAATGGCTTTTTGA<br>3'AATAAATAGCGCAAAATTAAGCA | 368 bp | 52°C |
| *E. mitis* | 5'TATTTCCTGTCGTCGTCTCGC<br>3'GTATGCAAGAGAGAATCGGGA | 306 bp | 51°C |
| *E. brunetti* | 5'GATCAGTTTGAGCAAACCTTCG<br>3'TGGTCTTCCGTACGTCGGAT | | |

*Eimeria* template DNA was added to Maxime PCR Premix kit (i-taq-iNtRon biotechnology. Korea) with 1 µL forward primer and 1 µL reverse primer. The total volume was adjusted to 20µL using deionized water. A control reaction containing sterile deionized water, but without template DNA was always performed to confirm the absence of contamination.

# 3. Results and Discussion

### *3.1. Öocyst Measurements*

The mean values of *Eimeria tenella* öocyst shape index is 1.15 µm, *E. necatrix* 1.16 µm, *E. acervulina* 1.25 µm *E. praecox* 1.20 µm, *E. mitis* 1.04 µm, *E. maxima* 1.41 µm, and *E. brunetti* 1.33 µm. The calculated shape index is also shown as compared to previous references [7]. The percentages of the species in the measured samples are shown in Table 2.

The öocyst measurement revealed the presence of all the seven species of *Eimeria* in Khartoum province with dominance of *Eimeria tenella* (34.3%). Followed by *E. necatrix* (17.4%), *E. acervulina* (14.4%), *E. praecox* (11.4%), *E. mitis* (11.4%), *E. maxima* (5.7%), and *E. brunetti* (5.7%). The species percentage in the measured samples is shown in Table 3.

***Table 2.*** *The average shape index for the seven Eimeria species.*

| Eimeria sp. | Average shape index in µm |
|---|---|
| E. tenella | 1.15 |
| E. necatrix | 1.16 |
| E. acervulina | 1.25 |
| E. praecox | 1.20 |
| E. mitis | 1.04 |
| E. maxima | 1.41 |
| E. brunetti | 1.33 |

***Table 3.*** *The species percentage in the measured samples.*

| Species | Percentage |
|---|---|
| *E. tenella* | 34.3% |
| *E. necatrix* | 17.4% |
| *E. acervulina* | 14.1% |
| *E. praecox* | 11.4% |
| *E. mitis* | 11.4% |
| *E. maxima* | 5.7% |
| *E. brunetti* | 5.7% |

### *3.2. Un-sporulated and Sporulated Öocyst*

In the present study un-sporulated and sporulated öocysts isolated from infected chickens are shown in figure 1 and figure 2.

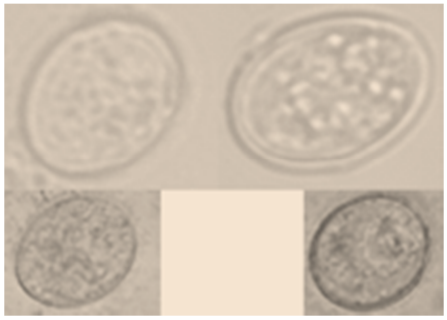


***Figure 1.*** *Unsporulated öocyst from different regions of the chicken intestine. 400 x.*

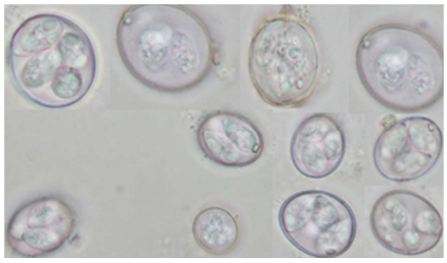


***Figure 2.*** *Sporulated öocyst at different stages of sprulation.*

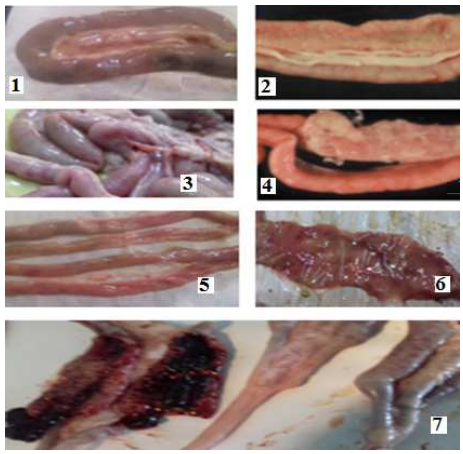



***Figure 3.*** *Showing different regions of the intestine from outside and inside.*

1: the upper intestine showing white spots on the outer surface,
2: upper intestines when infected showing watery white spots, is shown.
3: the middle intestine showing spots on the outer surface.
4: a change in colour of intestinal content when opened.
5: the infected lower intestine showing colour change in the outer surface.
6: an increase in secretion of mucus may be apparent in the intestinal contents.
7: unopened caecum when infected showing scattered petechiae, which are reddish or purple in colour and in opened caecum with bloody contents.

### *3.3. Molecular Results*

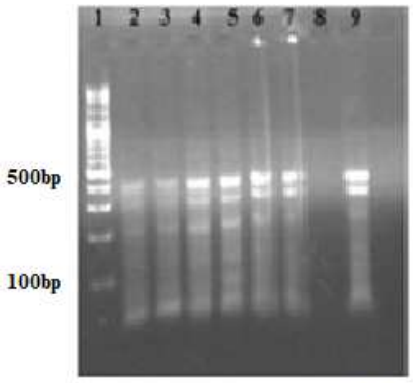
#### *3.3.1. DNA Quantification*

Using Nano-drop spectrophotometer (ND-1000), the mean DNA quantity was 30-120ng/µl and DNA quality was 1.5-2.3 for a single *Eimeria* species sample. The species specific (*ITS1*) diagnostic PCR amplifications worked successfully within the described range.

#### *3.3.2. PCR Results*

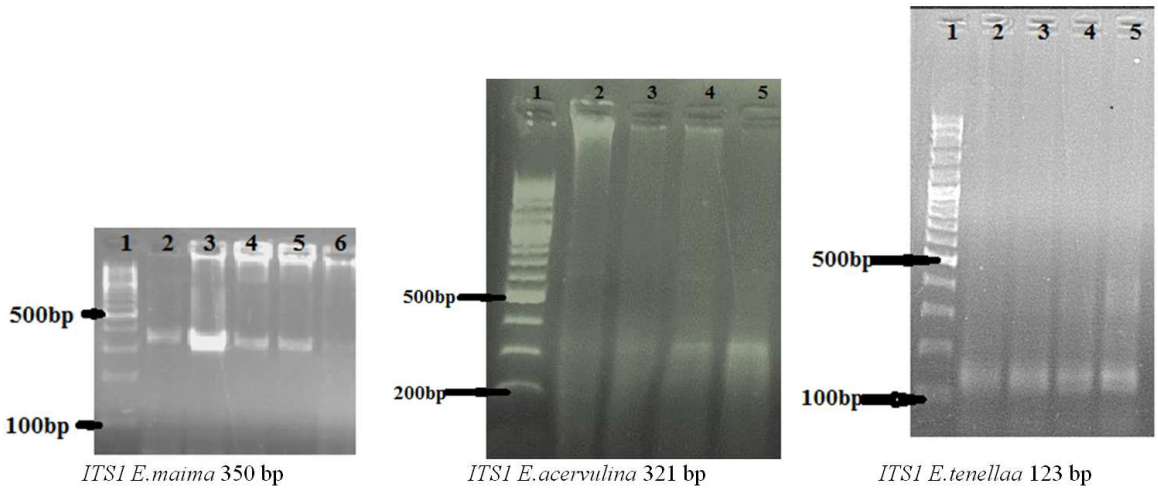
A multiplex PCR for *ITS1* primer specific reveal three *Eimeria* species, *E. praecox, E. mitis* and *E. necatrix* with band size 368 bp, 306 bp and 285 bp respectively.

A single PCR *ITS1* specific primer for *E. maxima*, *E. acervulina* and *E. tenella* was successfully amplified a 350 bp, 321 bp and 123 bp respectively. *E. brunetti* amplification was negative.




multiplex for *E. praecox* 368bp Lane 1 - 7 and 9; *E. mitis* 306 bp. Lane 1 - 7 and 9, *E. necatrix* 285 bp.

***Figure 4.*** *Agrose gel electrophoresis showing multiplex PCR ITS1 for 3 different Eimeria sp.*




*ITS1 E.maima* 350 bp *ITS1 E.acervulina* 321 bp *ITS1 E.tenellaa* 123 bp

***Figure 5.*** *Agrose gel electrophoresis showing specific PCR for 3 different Eimeria spp.*

The specific diagnosis of *Eimeria* infections in chickens is clearly central to a better understanding of epidemiology and dynamics of the disease in intensive and extensive chicken establishments. *Eimeria* species, molecular methods can be helpful in epidemiological studies of the parasite, an aspect that has been less investigated to date. This is particularly important for planning an effective prevention and control program of coccidiosis. Traditionally, diagnosis has been achieved by detecting *Eimeria* öocysts excreted in the faeces of chickens by measuring öocyst and sporocyst dimensions or assessing the site and extent of the pathological lesions in the intestine of chickens. Although the microscopic examination can absolutely show the negative faecal samples, such traditional methods have generally had major limitations in the specific diagnosis of coccidiosis and identification of *Eimeria* species. As yet, there has not been any documentary report related to the occurrence and epidemiological pattern of the pathogenic *Eimeria* species of domestic chickens, in Sudan. In the present study, the mean values of öocyst shape index for *E. necatrix*, *Eimeria tenella, E. acervulina, E. parecox*, *E. mitis, E. maxima*, and *E. brunetti* are in agreement with William [13], Salim [14] and Amer [15] findings for the same species. As seen in the results, the four infected chicken intestine parts have distinct features related to the presence of the parasite ranging from white spots, watery white content, increase in secretion of mucus, and colour change. The effect of the parasite extended to the caecum where scattered reddish or purple petechiae marked the region.

The results of the present study are the first on the molecular characterization of *Eimeria* species in Sudan. The molecular investigation in this study resulted in six species of *Eimeria* genus, *E. mitis*, *E. praecox*, *E. necatrix*, *E. tenella*, *E. maxima* and *E. acervulina*. This result is in agreement with Morris [16], who indicated the absence of the *E. brunetti* species in their amplified samples.

## 4. Conclusions

1. Understanding of the genetic structure of *Eimeria* spp. populations is critical for addressing important biological and control issues such as spread of anti-coccidial resistance alleles and developing strategies for control.
2. The accuracy of identification of poultry *Eimeria* species using PCR is more reliable than the conventional methods like öocyst measurements or

histopathology of the affected intestinal regions. This is indicated by the finding that *E. brunetti* identified morphologically by öocyst measurement is not detected molecularly.

3. The mitochondrial genome sequences (*ITS1)* are highly suited for molecular diagnostics of coccidia and may be a potential genetic marker for molecular epidemiology in the future for poultry coccidiosis in Sudan.

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