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# Diagnosis of *Eimeria* species using traditional and molecular methods in field studies

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ABSTRACT

The objective of this study was to identify and characterize species of *Eimeria* in broiler chickens using traditional morphological and pathological plus molecular (DNA amplification) diagnostic methodologies. Using a combination of those techniques it was possible to identify the presence of multiple circulating species in the flock as well as higher frequencies for some of them, especially *Eimeria praecox* and *Eimeria maxima*, which were identified in 100% of the flocks. The frequencies of the other species were *Eimeria mitis* and *Eimeria necatrix* (93.3%), *Eimeria tenella* (76,7%), *Eimeria acervulina* (56.7%) and *Eimeria brunetti* (16.7%). However using the lesion score, the most common species were *E. maxima* (46.7%), *E. acervulina* (30%), *E. tenella* (23.3%), and *E. necatrix* (10%). *E. brunetti* and *E. praecox* were not identified by using lesion score. DNA amplification had detection sensitivity for *Eimeria* species in the field samples of at least 20 oocysts. The implementation of DNA amplification as a routine diagnostic technique in aviaries can assist *Eimeria* population.

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## 1. Introduction

Brazilian poultry industry leads the world ranking of chicken meat for exportation (ABEF, 2008) but costs with some diseases are still high. Avian Coccidiosis is ubiquitous and the disease is presented at all poultry branches with most diverse farming systems. There are seven different *Eimeria* species in chicken, with different pathological potential. The parasites undergo a faecal:oral type of life cycle. Coccidia of the genus *Eimeria* are very common in poultry flocks all over the world, but there is limited information on the occurrence of the different *Eimeria* species. This is due to the fact that traditional species differentiation is complicated, time-consuming, and expensive and claims the use of animal experiments (Shirley et al., 2005; Williams, 2005).

The accurate identification of *Eimeria* species has important implications for diagnosis and disease control, but also to the epidemiology and biology studies, creation of new vaccines and selection of anticoccidial drugs (Tsuji et al., 1997; Woods et al., 2000; Morris and Gasser, 2006; Sun et al., 2009; Lee et al., 2010).

Different methodologies are available for specific diagnosis of *Eimeria*. Traditional methods are based on the oocysts morphological characteristics, the parasite biology, the clinical signs of the affected animals, and the typical macroscopic lesions that are assessed by the role of lesion score during necropsy (Long and Joyner, 1984).

However, natural infections by *Eimeria* are generally mixed with more than one species, whose morphological characteristics and pathological changes may be similar, hampering the accurate diagnosis of the species (Reid, 1973; Williams, 2001). Therefore, these methods should not be used as isolated criterion for differentiation of species (Long and Joyner, 1984; Woods et al., 2000; López et al., 2007). Moreover, the molecular techniques have

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gained importance in specific diagnosis of *Eimeria* (Allen and Fetterer, 2002).

The development of molecular tools has allowed not only the diagnoses, but also the study on genetic variability of pathogens based on small quantities of oocysts through molecular markers (Schnitzler et al., 1998; Costa et al., 2001). Fernandez et al. (2003) identified species-specific markers for *Eimeria* spp. from a group of SCAR markers (Sequence-Characterized Amplified Region). This enabled the use of the Polymerase Chain Reaction (PCR) technique, constituting an effective and integrated diagnosis method, which is able to detect the seven *Eimeria* species individually or simultaneously in a single reaction. The use of this technique has allowed the rapid and efficient diagnosis of species of poultry coccidia (Fernandez et al., 2003; Lien et al., 2007).

This study was carried out to evaluate the infection and perform specific diagnosis using traditional and molecular methods during a field trial.

## 2. Materials and methods

### 2.1. Farms

The study was conducted on broiler farms at the production complex of Feira de Santana, micro region in the North-central region of Bahia state. The area is composed by 24 municipalities and has a total area of 12,602,610 $km^2$. The climate is tropical humid and the rainy season lasts from March to September, with annual rainfall ranging from 848 to 1200 mm, mean temperature of 26.5 °C and relative humidity ranging from 70% to 75%.

Thirty broiler farms were selected for their suitability, pertaining to integrated companies in the region, as well as independent producers. Young birds come from different hatcheries housed in the farms up to one-day-old. Meaning the best homogeneity poultry flocks aged between 3 and 6 weeks where choose. During the visits, technicians, veterinarians, or owners participated in the activities providing health and performance information recorded as a questionnaire.

### 2.2. Samples

Fresh fecal samples were collected at different houses on each farm, following a straight line from one end to another with approximate distance of 50–70 m. Along this path, portions of feces were manually collected and placed in plastic bags. Next, all feces content was homogenized for the removal of approximately 200 g from the shed. Finally, the sub-samples of all sheds were put together for new homogenization and removal of 200 g of sample representative of that property. The samples were kept in plastic bags and transported under refrigeration to the Laboratory of Veterinary Parasitology, Universidade Estadual de Santa Cruz.

### 2.3. Lesion scores

During the sampling, from two to four birds were randomly separated in each house, sacrificed by cervical dislocation (CFMV, 2002), and necropsied for lesion scoring according to Johnson and Reid (1970).

### 2.4. Laboratory processing

The samples were initially filtered through sieves covered with folded gauze and centrifuged at 3000 rpm (250 rounds) for 10 min. Then, all material was suspended into a solution of potassium dichromate ($K_2Cr_2O_7$) at 2.5% for sporulation and placed into *Petri* dishes at room temperature for seven days. The oocysts were recovered by centrifugation in saturated NaCl solution at 3000 rpm (250 rounds) for 5 min followed by washing with distilled water. At the end, the material was concentrated by centrifugation at 3000 rpm (250 rounds) for 10 min, stored in potassium dichromate solution, quantified in *Newbauer* chamber (Teixeira, 2007) and stored at 4 °C.

### 2.5. Oocysts morphology

For measurement purposes, 100 oocysts from each fecal sample were randomly photographed using a microscope Olympus BX 51 coupled Olympus DP71 camera and subsequently measured with the assistance of software Image-Pro Express 6.0. The parameters used in the morphological identification were length, width and shape index.

### 2.6. DNA extraction

A 6 mL volume of each sample was twice washed with distilled water and centrifuged for 10 min at 14,000 × *g* to remove the potassium dichromate solution. The *pellet* was subsequently washed in a 5–6% sodium hypochlorite solution and left for 10 min at 4 °C, followed by two washes in distilled water. Then, the *pellet* was eluted in TE (10 mM Tris–HCl, pH 8.0, 200 mM EDTA, pH 8.0). In a way to break the outer membrane of the oocysts, approximately 0.35 g of glass beads of 425–600 μm (Sigma Aldrich Corp.®) was added to the tubes, stirred in vortex QL-9001 (Biomixer®) 2800 rpm for 5 min, and followed by centrifugation at 11,500 × *g* for 5 min for waste disposal. Beads were washed again with TE, followed by agitation and centrifugation. Digestion was conducted with RNase A (20 μg/mL) at 37 °C for 1 h, followed by digestion with Proteinase K (120 μg/mL) plus SDS (0.5%) 50 °C for 1 h. DNA was extracted with phenol/chloroform/isoamylic alcohol and chloroform, and precipitated with 100% ethanol and ammonium acetate (5 M) in the ratio 1/10. The *pellet* was washed with 85% ethanol and suspended in 10 mM Tris–HCl, pH 8.0, and quantified by spectrophotometry at absorbance of 260 nm and 280 nm.

### 2.7. Polymerase chain reaction (PCR)

PCR amplifications were individually made for each *primer* pair using 200 μM dNTP, 5.0 mM $MgCl_2$, 2 U of Taq DNA polymerase (Invitrogen®), and 1.6× amplification buffer (supplied by the manufacturer) in a final volume of 25 μL. The *primers* were used in different concentrations: 0.85 mM for Br-01 *primers*, 0.70 mM *primers* for Ac-01,

**Table 1**
List of farms and species identified according to the technique applied to 30 farms.

| | Lesion score | | | | | | | Morphology | | | | | | | PCR | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Species[a] Farms | Ac | Br | Te | Mi | Pr | Ma | Ne | Ac | Br | Te | Mi | Pr | Ma | Ne | Ac | Br | Te | Mi | Pr | Ma | Ne |
| 1 | | | | | | + | | | + | + | + | + | + | + | | | + | + | + | + | + |
| 2 | | | | | | | | + | + | + | + | + | + | + | | | | + | + | + | + |
| 3 | | | | | | | | | + | + | + | + | + | + | + | | + | + | + | + | + |
| 4 | + | | | | | + | | | + | + | + | + | + | + | + | | | + | + | + | + |
| 5 | | | | | | + | | + | + | + | + | + | + | + | | | | + | + | + | + |
| 6 | + | | + | | | + | | + | + | + | + | + | + | + | + | | + | + | + | + | + |
| 7 | + | | | | | + | | + | + | + | + | + | + | + | | | | + | + | + | |
| 8 | | | | | | + | | + | + | + | | + | + | | + | + | + | + | + | + | + |
| 9 | | | | | | | | + | + | + | + | + | + | + | + | | + | + | + | + | + |
| 10 | | | + | | | + | | + | + | + | + | + | + | + | | | + | + | + | + | + |
| 11 | + | | | | | + | | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 12 | | | | | | + | | | + | + | | + | + | + | | | + | + | + | + | + |
| 13 | | | | | | + | | | + | + | + | + | + | + | + | | + | + | + | + | + |
| 14 | | | | | | | | | + | + | + | + | + | + | | | + | + | + | + | + |
| 15 | | | | | | | | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 16 | + | | | | | | | + | + | + | + | + | + | + | + | | | + | + | + | + |
| 17 | + | | | | | | | | + | + | + | + | + | + | | | + | | + | + | |
| 18 | | | | | | | | | + | + | + | + | + | + | + | | + | + | + | + | + |
| 19 | | | | | | | + | + | + | + | + | + | + | + | + | | + | + | + | + | + |
| 20 | | | + | | | | | + | + | + | + | + | + | + | | + | | + | + | + | + |
| 21 | | | | | | | + | + | + | + | + | + | + | + | + | | + | + | + | + | + |
| 22 | | | | | | + | | | + | + | + | + | + | + | | | + | + | + | + | + |
| 23 | | | + | | | | | + | + | + | + | + | + | + | + | | + | + | + | + | + |
| 24 | | | | | | | | | + | + | + | + | + | + | + | | + | + | + | + | + |
| 25 | + | | + | | | + | | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 26 | + | | | | | + | | + | + | + | + | + | + | + | | | + | + | + | + | + |
| 27 | | | + | | | | | + | + | + | + | + | + | + | + | | + | + | + | + | + |
| 28 | | | | | | | | | + | + | + | + | + | + | | | | | + | + | + |
| 29 | + | | | | | + | | + | + | + | + | + | + | + | | | + | + | + | + | + |
| 30 | | | + | | | | + | + | + | + | + | + | + | + | + | | + | + | + | + | + |

[a] Ac – *E. acervulina*, Br – *E. brunetti*, Te – *E. tenella*, Mi – *E. mitis*, Pr – *E. praecox*, Ma – *E. maxima*, Ne – *E. necatrix*.

Pr-01 and NC-01 and 0.55 mM for *primers* Tn-01, Mt-01 and Mx-01 (Fernandez et al., 2003). Thermocycled conditions consisted of an initial denaturation at 95 °C for 5 min and 30 cycles of 1 min at 94 °C and 2 min at 65 °C with a final extension step at 72 °C for 5 min in the thermocycler MJ96G (Biocycle®). All amplification products were analyzed by separation on 3% agarose gel followed by staining with ethidium bromide, and examined under UV light. Two positive controls were used: pure liofilized DNA from seven species of *Eimeria* provided by Biovet Laboratory and another isolated directly from the commercial vaccine Bio-Coccivet R® (Biovet Laboratories) composed of all seven *Eimeria* species.

## 3. Results

Data from *Eimeria* species diagnosis with different methods are shown in Table 1. The seven species of the genus *Eimeria* were identified in all broiler farms in the micro region of Feira de Santana using the PCR technique. The less frequent specie found was *Eimeria brunetti* with 16.7% frequency while all farms (100%) were positive for both *Eimeria maxima* and *Eimeria praecox*. Differently, the most common species found using the lesion score were *E. maxima* (46.7%) followed by *Eimeria acervulina* (30.0%), *Eimeria tenella* (23.3%) and *Eimeria necatrix* (10.0%). However, *Eimeria mitis*, *E. brunetti* and *E. preacox* were not found. It was observed in the morphological analysis that farms presented 100% positivity for *E. brunetti*, *E. tenella* and *E. praecox* but *E. acervulina* was less frequent with 63.3%.

Considering the number of oocysts for DNA extraction, samples containing at least 20 oocysts of each species were necessary to amplification trough PCR. The *primers* were sufficiently sensitive and specific enabling the discrimination of seven *Eimeria* species. The amplified fragments presented different sizes: *E. acervulina* (811 bp), *E. brunetti* (626 bp), *E. tenella* (539 bp), *E. mitis* (460 bp), *E. praecox* (354 bp), *E. maxima* (272 bp) and *E. necatrix* (200 bp) (Fig. 1).

Using PCR five farms (13.7%) were positive for all species of *Eimeria*. Differently, using morphology, all seven species were observed in 60% of the farms.

## 4. Discussion

According to the present data there is difference in the field diagnosis of *Eimeria* species using different methods. These changes can be explained by the specificity and sensitivity that each technique have.

It was possible to see a high frequency of *Eimeria* species through the application of PCR, showing that coccidia are widely distributed across the poultry producing area of Bahia state, whereas many factors may be contributing to this fact. At first, the climatic characteristics of the region include temperature conditions and high humidity all the way the year, which are favorable to sporulation and survival of viable oocysts in the environment for long

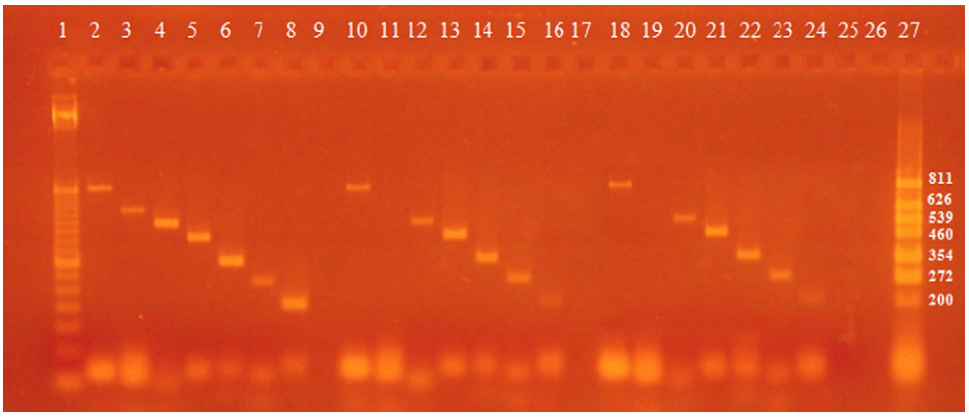


**Fig. 1.** Resolution in 3% agarose gel showing amplifications from *Eimeria* species. Column 1 molecular weight marker 50 bp; columns 2–8 results from farm 8 with positive samples of seven species; column 9 empty; columns 10–16 results from farm 3 with positive samples of six species; column 17 empty; columns 18–24 results from farm 9 with positive samples of six species; columns 11 and 19 negative samples; column 25 and 26 negative control; column 27 positive control, *E. acervulina* (811 bp), *E. brunetti* (626 bp), *E. tenella* (539 bp), *E. mitis* (460 bp), *E. praecox* (354 bp), *E. maxima* (272 bp) and *E. necatrix* (200 bp).

periods (Williams, 1999). Another factor is related to location and distribution of poultry farms. Most farms emerged from different agricultural activities changing for the poultry business without experience and ignoring the basic aspects of preventive health. Their structures were built very close to each other, as well as busy access lanes. Still, there is no sanitary measure for visitants to avoid the introduction of pathogens. During visits, a large number of people not involved with the job were constantly observed in the farms. Such people could be carrying oocysts from other farms, stuck on their clothes and vehicles. The reuse of bed without proper management and dirt floor in some sheds has directly contributed to the proliferation and maintenance of *Eimeria* oocysts in the environment. The lack of adequate pest control and maintenance of other animals near the aviaries also favor the dissemination of protozoa in the sheds. All these conditions favorable to the dispersion of oocysts explain the high frequencies of species occurring in properties of the micro region of Feira de Santana, since the oocysts are extremely resistant and can remain viable for long periods in environments (Berchieri Júnior and Macari, 2000; Shirley et al., 2005).

It is know that minor differences in this management could affect the shedding and surveillance of oocysts and this could explain differences in species variation or different contamination levels between farms. In fact, all farms are very straight in the manner that preventive hygiene methods are ignored. Then, significant differences in the microenvironments in which the oocysts were found could not be observed. Moreover, is quite clear that the anticoccidial program is used as the main preventive measure for the control of coccidiosis and this is common for all farms. Thus, we suspect that variations in the *Eimeria* species found were mostly caused by drug management. Unfortunately, we had not access to this anticoccidial program since they strongly protect their diet formulations as commercial secret. Also, there is no significant difference in microenvironments in which oocysts were found because farmers visited have all similar management.

Many observations can be made regarding the frequencies of species. Regarding most pathogenic species, it is remarkable that some of them were quite frequent in the properties, indicating a potential impact on poultry production. *E. tenella* is considered the most pathogenic specie, present in 23 of the 30 farms investigated. This indicates need for constant monitoring, since it has a great potential to cause injury to birds, even with reduced number of oocysts. According to Conway et al. (1993), *E. tenella* and *E. acervulina* (which has moderated pathogenic) are able to provoke changes in birds starting from 100 oocysts, and are associated to large economic losses. The frequency of *E. brunetti* (16.7%) observed in this work represents a major risk since this is a kind of moderate pathogenicity associated to damage and hemorrhagic cases in birds (Costa, 2000). Less pathogenic species such as *E. mitis* and *E. praecox* are not commonly related to clinical cases, but in major infections they can increase feed conversion or even lead young animals to death (Berchieri Júnior and Macari, 2000).

The results obtained in this study differ from those found by Prado (2005) in Santa Catarina State, which identified greater frequencies of *E. acervulina* (90%), *E. maxima* (60%), *E. tenella* (60%) through the PCR technique. However, all properties were negative for *E. mitis*. Meireles et al. (2004) using *primers* specific to *E. mitis* and *E. praecox* found frequencies of 28.8% and 44.9% in poultry farms of central southern Brazil, respectively. Differences among studies may be due to changes in the *Eimeira population*, based on the climatic characteristics of the region (Nowzari et al., 2005), or even, be associated with different management practices, level of mechanization of production, control of parasites, and also the anticoccidial softwares used.

Small quantities of oocysts were sufficient to correctly detect and discriminate species using PCR. Fernandez et al. (2003) identified *Eimeria* species in samples containing from two to eight oocysts originated from experimental infections, using the same methodology. Yet, Haug et al. (2007) observed that there is a variation in the detection of each *Eimeria* species, depending on the number of oocysts used and the technique adopted for DNA extraction.

In field samples, many factors may interfere in the success and effectiveness of diagnosis by PCR, especially in regards to the presence of contamination. According to Haug et al. (2007) the DNA extraction process in stool samples is influenced by the formation of inhibitors of Taq DNA polymerase that affect the reaction.

At least three *Eimeria* species were simultaneously identified using a multiplex PCR. These data are consistent with literature that reveals the occurrence of mixed infections in animals, thus hindering the accurate diagnosis of the species applying techniques traditionally used (Long and Joyner, 1984; Shirley, 1995; Williams, 2001).

In Japan, Kawahara et al. (2008) identified *E. brunette* 65.6%, *E. maxima* and *E. necatrix* 50%, *E. tenella* 37.5%, and *E. acervulina* at 25% in the properties evaluated, using real time PCR for the detection of ITS-1. In China Sun et al. (2009) identified *E. tenella*, *E. praecox* and *E. acervulina* in more than 70% of the properties. Yet in South Korea, Lee et al. (2010) identified a high prevalence of the seven species emphasizing *E. acervulina*, *E. tenella* and *E. brunetti*. These data reinforce the idea that several factors influence the presence and prevalence of *Eimeria* species in each region.

The morphological evaluation of oocysts also resulted in the diagnosis of a wide variety of species in the farms. Luchese et al. (2007) also identified frequencies ranging from 45.52%, 18%, 14% and 12.32% for *E. maxima*, *E. brunetti*, *E. tenella* and *E. acervulina*, respectively, through the morphology. Terra et al. (2001), evaluated 60 carcasses of slaughtered broiler chickens in the city of Monte Alegre do Sul, Brazil, and identified frequencies of 90% for *E. maxima*, 86.4% for *E. tenella*, 86% for *E. mitis*, and 25% for *E. acervulina* and *E. necatrix*, through the morphology. However, Santos et al. (2003) failed to identify the occurrence of *Eimeria* species in farms of São Paulo state using morphology, considering the observed overlap in the measurements of oocysts among species and the occurrence of infections caused by mixed infections, preventing diagnosis. According to this data (Table 1), morphology could be a sensitive method for the discrimination of *Eimeria* species in field trials. Nevertheless, morphology is a technique with A sort of limitations to be used as a single tool for diagnosis of *Eimeria* species. It means that results obtained with this method should be carefully interpreted (Woods et al., 2000; López et al., 2007). 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 (Costa, 2000; Sun et al., 2009). Then, despite the results observed we suggested that morphology is not efficient as the PCR in the discrimination of *Eimeria* species.

The analysis of the lesion score shown to be the less effective method for the diagnosis of *Eimeria* species in the study conditions. First, before the advent of anticoccidial drugs clinical coccidiosis are quite scarce in field operations and clear pathological lesions are hard to found. Second, different species parasitize the same or very close regions along the intestinal tract of birds could be overlapped with another. Also, when observing pathological macroscopic injuries changes are possible according to the life stage in which animals are evaluated or due to the use of anticoccidial drugs into the diet (Prado, 2005). Later, even the characteristics of lesions may help in diagnosis, the differences are subtle and require technical training and much experience to provide reliable results.

## 5. Conclusion

The implementation of PCR as a routine diagnostic technique in poultry flocks could enhance the monitoring of fluctuations in *Eimeria* populations, helping in the adoption of specific measures against the parasite without the need to plus unnecessary work, thereby reducing production costs.

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