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Identification of *Eimeria mitis* and *Eimeria praecox* in Broiler Feces Using Polymerase Chain Reaction

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ABSTRACT

There are few reports concerning the epidemiology of *Eimeria praecox* and *Eimeria mitis* in Brazil. In the present experiment, the polymerase chain reaction (PCR) was used to identify these species in 156 samples of broiler chicken feces from several Brazilian states and the Federal District. Oocysts present in feces samples were purified by sodium chloride flotation followed by addition of DNAzol reagent (Invitrogen®) for extraction of genomic DNA. DNA was precipitated and stored following DNAzol reagent manufacture's instructions. The primers and PCR conditions were as described by Schnitzler *et al.* (1999). In the 156 field samples analyzed by PCR, 70 and 45 were positive for *E. praecox* and *E. mitis*, respectively. In this study we have shown that DNA extraction using DNAzol followed by PCR can be a useful tool in epidemiological studies, since it provides fast and reliable detection of *Eimeria* sp. in field samples.

INTRODUCTION

Eimeriosis is the most important protozoan disease to the world poultry industry. In spite of some controversy (Shirley *et al.*, 1983; Barta *et al.*, 1997), domestic chickens are considered susceptible to seven species of *Eimeria*: *E. acervulina, E. brunetti, E. maxima, E. mitis, E. necatrix, E. praecox* and *E. tenella.*

The identification of *Eimeria* species is commonly accomplished through the analysis of some characteristics such as pre-patent period, morphology and morphometry of oocysts and other stages of the life cycle, site of development in the host, macroscopic lesions and isoenzyme analysis by electrophoresis (Joyner & Long, 1974; Chapman, 1982).

The analysis of these characteristics is labor intensive to be used as routine diagnostic procedure and does not provide enough data for identification to the species level. Oocysts morphology and morphometry may differ a little within a specie. In addition, characteristics may be similar among different species. The analysis of macroscopic lesions and parasite site of development in the host requires the availability of birds for necropsy and generally does not provide conclusive data, mainly in infections caused by *E. mitis* and *E. praecox*.

Pre-patent period data may be used for *E. praecox* identification, but the procedure demands birds and facilities for experimental inoculation. Furthermore, this characteristic must be cautiously interpreted due to the introduction of precocious vaccine strains with shorter pre-patent period in broiler flocks (Shirley & Bedrnik, 1997).

Isoenzyme analysis can also be used for identification of *Eimeria* species. This technique is efficient but laborious, demands a great number of oocysts and is not conclusive for all field isolates (Chapman, 1982; Kucera, 1990).

Schnitzler *et al.* (1998) and Schnitzler *et al.* (1999) designed species-specific primer*s* to be used in the polymerase chain reaction (PCR) to identify the seven *Eimeria* species of domestic chickens.

There are few reports related to the occurrence of the less pathogenic *Eimeria* species of domestic chickens, such as *E. mitis* and *E. praecox,* mainly in Brazil (Kawazoe & Figueiredo, 1990; Santos *et al.*, 2003). Despite the current belief that these species have low pathogenicity, they can be responsible for decreased weight gain, poorer feed efficiency and shank pigmentation in broiler chickens (Gore & Long, 1982; Ruff & Edgar, 1982; Fitz-Coy & Edgar, 1992; Jorgensen *et al.*, 1997).

Owing to the lack of information related to domestic chicken eimeriosis in Brazil and to its recognized economic importance to the poultry industry, this work was carried out to determine whether *E. mitis* and *E. praecox* are present in some Brazilian states and the Federal District through the utilization of PCR, a highly sensitive and specific technique.

MATERIAL AND METHODS

Samples

One hundred and fifty six samples of feces were collected from broiler chickens as follows: 18 samples from the Federal District, 24 from Goiás State, 23 from Mato Grosso do Sul, 2 from Minas Gerais, 21 from Paraná, 42 from Rio Grande do Sul, 5 from Santa Catarina and 21 from São Paulo.

Each sample was a pool of many feces samples taken at different locations at one or more poultry houses belonging to one broiler integration and preserved in 2.5% potassium dichromate at +4° C.

DNA extracted from oocysts of *E. mitis* and *E. praecox* from the Czech Republic were kindly supplied by Dr. Petr Bedrník (Biopharm) and Prof. Arthur Gruber (São Paulo State University) and used as positive control. Ultra-pure autoclaved water served as negative control.

Genomic DNA Extraction

Oocysts present in 10 g of feces were purified in saturated saline solution and washed by centrifugation at 1,000 x g for 5 min with distilled water. The sediment containing oocysts was resuspended in phosphated buffered saline (PBS) pH 7.4 added with 0.1% Tween 80, homogenized by vortexing and centrifuged at 800 x g for 5 min. Sodium hypochlorite 5-6% was added and after 20 minutes at 4° C (Fernando & Pasternak, 1991; Procunier *et al.*, 1993), the sediment was washed with distilled water 4 times by centrifugation at 800 x g for 5 min.

One mL of DNAzol reagent (Invitrogen®) was added to the sediment containing oocyst, transferred to a 1.5 ml microcentrifuge tube with 0.5 volume of 2-mm glass beads, and vortexed continuously for 10 minutes at maximum speed. The tubes were centrifuged for 10 min at 10,000 x g and the supernatant was transferred to a fresh 1.5-ml microcentrifuge tube. The DNA was precipitated by adding 0.5 volume of 100% cold ethanol and mixing by inversion. The tubes were centrifuged at 4,000 x g for 3 min and the supernatant was discarded. The DNA pellet was washed twice with 1 mL of 70% cold ethanol, mixed by inversion and centrifuged at 4,000 x g for 3 min at 4°C. The supernatant was discarded and the DNA sediment was eluted with 50 mL NaOH 8 mM and 5.05 mL of 0.1 M HEPES buffer. Extracted DNA was purified using Prep-A Gene DNA purification system (Bio-Rad®) and stored at -20°C.

Polymerase Chain Reaction (PCR)

Samples were analyzed by PCR with the primers designed by Schnitzler *et al.* (1999) (Table 1).

The amplification reaction mixture consisted of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 200 mM of each deoxynucleoside triphosphate (dNTP), 10 pmol of each primer, 1 U of platinum *Taq* DNA polymerase (Invitrogen®) and 10 µL of target DNA in a 50 mL reaction volume.

PCR cycling conditions were: denaturation at 95° C for 2 min, followed by 29 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 60 s and a last extension step at 72°C for 7 min. Amplifications were carried out in 0.2-mL polypropylene tubes using a MJ Research® PTC 100 termocycler. Ten µL of the amplified products were analyzed by electrophoresis on a 1.5 % agarose gel stained with ethidium bromide and examined under UV light.

Determination of PCR Sensitivity

PCR sensitivity was determined using serial dilutions of suspensions containing 100, 500, 1,000, 5,000, 10,000, 25,000, 50,000 and 100,000 oocysts of *E. mitis* or *E. praecox*. The suspension corresponding to each dilution was spiked in one sample of 10 g of feces of chickens free of infection with *Eimeria* spp. Inoculated feces were homogenized and submitted to oocyst purification and DNA extraction as described above, followed by PCR for each dilution, in order to determine

Table 1 - Primers used in the Polymerase Chain Reaction (Schnitzler *et al.* 1999).

Primer	Species	Sequence (5´-3´)	Annealing position*	Amplification product size
EPF	*E. praecox*	CATCATCGGAATGGCTTTTTGA	151 bp	391 bp
EPR	*E. praecox*	AATAAATAGCGCAAAATTAAGCA	541 bp	
EmiF	*E. mitis*	TATTTCCTGTCGTCGTCTCGC	158 bp	327 bp
EmiR	*E. mitis*	GTATGCAAGAGAGAATCGGGA	484 bp	

*Primers anneal to the ITS1 gene of *E. praecox* (GenBank accession number AF065092) and *E. mitis* (GenBank accession number AF065093).

the minimum detectable oocyst number. Feces samples were collected from birds reared in an isolator with positive pressure (ALESCO®) since the first day of life and were shown to be negative for *Eimeria* spp. oocysts by flotation in saturated saline solution and PCR.

RESULTS AND DISCUSSION

The primers used (Schnitzler *et al.*, 1999) amplified as expected species-specific DNA bands of 327 bp for *E. mitis* and 391 bp for *E. praecox* (Figure 1).

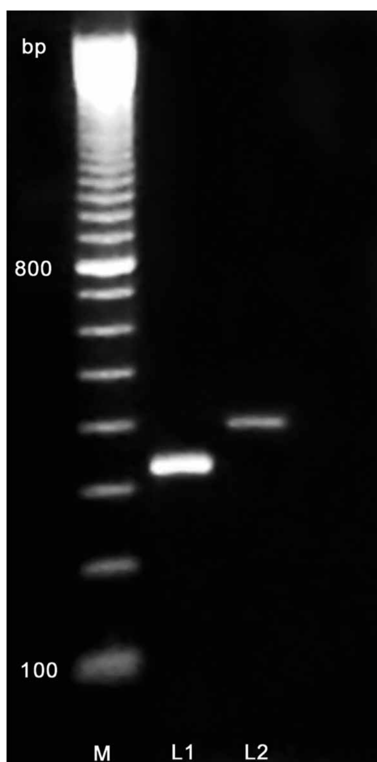

Figure 1 - Agarose gel electrophoresis. Polymerase Chain Reaction for *E. mitis and E. praecox*. M: 100 bp molecular weight marker; L1: Positive sample for *E. mitis* (327 bp). L2: Positive sample for *E. praecox* (391 bp).

The sensitivity of PCR was determined by spiking increasing oocyst numbers of *E. mitis* or *E. praecox* in the feces of birds free of *Eimeria* spp. infection. For both species, amplification of a specie-specific DNA band was observed in samples spiked with a minimum of 50 oocysts per gram of feces.

The analysis of 156 samples of feces collected in the different states showed that both species were present in almost every state, but *E. mitis* could not be detected in the states of GO and MG (Table 2). No DNA amplification band was observed in the negative controls, demonstrating absence of contamination in the PCR assay.

Table 2 - Results of Polymerase Chain Reaction for *E. praecox* and *E. mitis* in feces samples (n=156) collected from broiler chickens in the different Brazilian States and the Federal District.

		DF	GO	MG	MS	PR	RS	SC	SP	Total
E. mitis	Positive	1	0	0	8	7	16	5	8	45
	Negative	17	24	2	15	14	26	0	13	111
E. praecox	Positive	7	6	1	12	11	18	4	11	70
	Negative	11	18	1	11	10	24	1	10	86

In Brazil, studies regarding avian eimeriosis epidemiology have already been carried out in the State of São Paulo, but with a restricted number of samples. The occurrence of *E. mitis* and *E. praecox* was detected in these reports (Kawazoe & Figueiredo, 1990; Santos *et al.*, 2003). In the present experiment, *E. mitis* was detected in five out of seven Brazilian states, besides the Federal District. A high occurrence of *E. praecox* was also observed in most samples.

The identification of *Eimeria* species in birds by PCR has the advantages of greater specificity and sensitivity in comparison to the conventional identification techniques (Lew *et al.*, 2003). Recently, the simultaneous identification of 7 species of *Eimeria* using a multiplex PCR was reported in Brazil (Fernandez *et al.*, 2003).

PCR is commonly used for the identification of *Eimeria* sp. in samples purified and containing a great amount of oocysts. This is the first report of

determination of sensitivity of this technique and its use for identification of avian *Eimeria* species in field samples, in which the number of oocysts is generally small and therefore, makes diagnosis by the use of conventional techniques difficult and sometimes unviable.

It is worth noting that, in spite of the high sensitivity of PCR, the occurrence of false-negative results is possible, since in the conditions of the present study at least 50 oocysts per gram of feces were necessary to obtain positive results. It was also observed in this experiment that PCR coupled to DNA extraction directly from feces using DNAzol requires a posterior DNA purification to avoid the occurrence of false-negative results due to presence of PCR inhibitory substances in the feces (Lawson *et al.*, 1997).

The available information on avian eimeriosis are related to the species of *Eimeria* that cause more evident macroscopic lesions or are more economically relevant, as *E. acervulina*, *E. brunetti*, *E. maxima*, *E. necatrix* and *E. tenella*.

The presence of *E. mitis* and *E. praecox* in broiler flocks in several states demonstrates the need of including these two species in studies involving the economic impact of *Eimeria* sp. infection in the Brazilian poultry industry.

REFERENCES

Barta JR, Martin ED, Liberator PA, Dashkevicz M, Anderson JW, Feighner SD, Elbrecht A, Perkins-Barow A, Jenkins MC, Danforth HD, Ruff MD, Profous-Juchelka H. Phylogenetic relationships among eight Eimeria species infecting domestic fowl inferred using complete small subunit ribosomal DNA sequences. Journal of Parasitology 1997; 83:262-271.

Chapman HD. The use of enzyme electrophoresis for the identification of the species of Eimeria present in field isolates of coccidia. Parasitology 1982; 85:437-442.

Fernandez S, Pagotto AH, Furtado MM, Katsuyama AM, Madeira AM, Gruber A. A multiplex PCR assay for the simultaneous detection and discrimination of the seven *Eimeria* species that infect domestic fowl. Parasitology 2003; 127:317-325.

Fernando MA, Pasternak JJ. Eimeria spp. of the domestic fowl: resolution of chromosomes by field inversion gel electrophoresis. Experimental Parasitology 1991; 72:306-310.

Fitz-Coy SH, Edgar SA. Pathogenicity and control of Eimeria mitis infections in broiler chickens. Avian Diseases 1992; 36:44-48.

Gore TC, Long PL. The biology and pathogenicity of a recent field isolate of Eimeria praecox Johnson, 1930. Journal of Protozoology 1982; 29:82-85.

Jorgensen WK, Stewart NP, Jeston PJ, Molloy JB, Blight GW, Dalgliesh RJ. Isolation and pathogenicity of Australian strains of Eimeria praecox and Eimeria mitis. Australian Veterinary Journal 1997; 75:592-595.

Joyner LP, Long P. The specific characters of the Eimeria, with special reference to the coccidian of the fowl. Avian Pathology 1974; 3: 145-157.

Kawazoe U, Figueiredo AC. Levantamento de coccidiose aviária em três granjas de frangos de corte da região de Campinas – São Paulo – Brasil. Arquivos Brasileiros de Medicina Veterinária e Zootecnia 1990; 42:317-336.

Kucera J. Identification of Eimeria species in Czechoslovakia. Avian Pathology 1990; 19:59-66.

Lawson AJ, Linton D, Stanley J, Owen RJ. Polymerase chain reaction detection and speciation of Campylobacter upsaliensis and C. helveticus in human faeces and comparison with culture techniques. Journal of Applied Microbiology 1997; 83:375-380.

Lew AE, Anderson GR, Minchin CM, Jeston PJ, Jorgensen WK. Inter- and intra-strain variation and PCR detection of the internal transcribed spacer 1 (ITS-1) sequences of Australian isolates of Eimeria species from chickens. Veterinary Parasitology 2003; 112: 33-50.

Procunier JD, Fernando MA, Barta JR. Species and strain differentiation of Eimeria spp. of the domestic fowl using DNA polymorphisms amplified by arbitrary primers. Parasitology Research 1993; 79:98-102.

Ruff MD, Edgar SA. Reduced intestinal absorption in broilers during Eimeria mitis infection. American Journal of Veterinary Research 1982; 43:507-509.

Santos RFS, Kavavata GM, Almeida SM, Hisano M, Calixto LFL, Meireles MV. Ocorrência de Eimeria sp. em frangos de corte no Estado de São Paulo. Ars Veterinaria 2003; 19:230-234.

Shirley MW, Bedrnik P. Live attenuated vaccines against avian coccidiosis: success with precocious and egg-adapted lines of Eimeria. Parasitology Today 1997; 13:481-484.

Shirley MW, Jeffers TK, Long PL. Studies to determine the taxonomic status of Eimeria mitis, Tyzzer 1929 and E. mivati, Edgar and Seibold 1964. Parasitology 1983; 87:185-198.

Schnitzler BE, Thebo PL, Mattsson JG, Tomley FM, Shirley MW. Development of a diagnostic PCR assay for the detection and discrimination of four pathogenic Eimeria species of the chicken. Avian Pathology 1998; 27:490-497.

Schnitzler BE, Thebo PL, Tomley FM, Uggala A, Shirley MW. PCR identification of chicken Eimeria: a simplified read-out. Avian Pathology 1999; 28:89-93.