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QUANTITATIVE REAL TIME PCR FOR DETECTION AND QUANTIFICATION OF COCCIDIOSIS IN CHICKEN INTESTINAL TISSUE#

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ABSTRACT

A SYBR® Green based quantitative real-time PCR (qPCR) technique for detection and quantification of *Eimeria* species during its *in vivo* developmental phase in the intestinal tissue of broilers was standardized. Sixty intestinal tissue samples were collected from suspected, ailing and dead birds in commercial broiler farms in Tamil Nadu and Andhra Pradesh. All the intestinal samples were screened by a generic ITS-1 based conventional PCR out of which 29 (48.3 percent) were found positive for *Eimeria* species. A qPCR assay was standardized for detection of genus *Eimeria* in the intestinal tissue using the 5S ribosomal RNA as the target of amplification. The parasite genome numbers in tissue were estimated and comparisons drawn between generic *Eimeria* counts and species-specific *E. tenella* counts indicated presence of species other than *E. tenella* in caecal tissue. Quantitative real-time PCR was found to be a sensitive quantitative technique to detect *Eimeria* infection in broilers during the pre-patent and sub-clinical phase of infection in commercial broilers when compared to genus specific conventional PCR.

Keywords: *Eimeria*, qPCR, absolute quantification

INTRODUCTION

Enteric protozoa belonging to the genus *Eimeria* (Protozoa: Apicomplexa: Eucoccidia: Eimeriidae) unquestionably crown the list of most ruinous parasites affecting livestock and poultry. Coccidiosis has remained the focus of anxiety for commercial poultry producers not only due to the losses incurred as a result of mortality in acute infections or lowered production, but also due to the cost input required for effective chemoprophylaxis and immunoprophylaxis. Avian coccidiosis has resulted in losses exceeding US$ 3

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billion annually to the poultry industry worldwide (Blake *et al.,* 2008). Over the past few decades, the Indian scenario of poultry rearing has transformed from a mere leisurely backyard activity to an insatiable profit seeking industry. Pervasiveness of the pathogen cannot be ignored when studies indicate that coccidiosis accounts for 95 percent of the total economic losses in commercial broiler industry and 3 percent loss in commercial layer industry, mainly due to cost of chemoprophylaxis and drop in meat and egg production. Total annual losses are estimated to be around Rs. 1.14 billion in India (Bera *et al*., 2010).

The oocysts which are passed out in the droppings of an infected bird are the most accessible stage in the lifecycle of *Eimeria* and thus most widely used for diagnosis of infection in birds. However, considerable overlapping of dimensions of oocysts of different species exists and this makes it difficult, even for an expert to identify with certainty the species involved in the disease. Investigation of *in vivo* development of the parasite requires histopathological examination of tissue sections which again is a skill demanding and time consuming affair. Thus, considering reliability and time and labour effectiveness, molecular diagnosis and speciation methods are increasingly being used worldwide. In the present study, a qPCR technique was employed for detection and quantification of *Eimeria* infection using intestinal tissue samples. In addition, a similar assay was standardized for species-specific detection of *E. tenella* known to affect the caecal tissue.

MATERIALS AND METHODS

Collection of field samples

Intestinal tissue samples (n=60) were collected from suspected, ailing and dead birds from commercial broiler farms in and around Chennai, Palladam and Coimbatore in Tamil Nadu and Chittoor in Andhra Pradesh. The entire intestinal tract was examined for lesions consistent with coccidial infections. Intestinal tract was divided into 4 regions *viz*. upper intestine (U), middle intestine (M), lower small intestine and rectum (L) and caeca (C). Representative samples (~ 2 cm long pieces of intestinal tract) were collected from these four regions in separate 1.5ml collection tubes. They were preserved in RNAlater (Qiagen, Germany) and stored at -20°C until further processing for DNA extraction.

DNA extraction from tissues

The intestinal tissue samples were thawed at room temperature and rinsed

with tap water in order to remove adhering faecal debris. Thirty milligrams of each tissue sample were weighed out and transferred to 2 ml microcentrifuge tubes. DNeasy tissue kit (Qiagen, Germany) was used as per manufacturer's instructions. The DNA concentration was measured in a biospectrophotometer.

ITS-1 based PCR

DNA extracted from all the intestinal tissue samples underwent preliminary screening for *Eimeria* using ITS-1 genus-specific primers (Lew *et al*., 2003). 2X Amplicon Taq DNA polymerase Master mix with dNTPs containing 1.5mM $MgCl_2$ was used. The cycle conditions employed included an initial denaturation at 94ºC for 3 min; 30 cycles involving denaturation at 94ºC for 30 sec, annealing at 56ºC for 30 sec and elongation at 72ºC for 1 min 30 sec; followed by a final elongation at 72ºC for 7 min. The PCR products were analysed by an agarose gel electrophoresis.

Quantitative Real time PCR

Primers: The genus-specific primers based on the 5S ribosomal subunit, *E. tenella* specific primers based on the sequence characterized amplified regions (SCAR) of *E. tenella*, and primers for endogenous control *viz.*, chicken glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene are included in Table-1.

PCR amplification of *Eimeria* 5S rRNA: The target 5S rRNA region of *Eimeria* was amplified by conventional PCR. Cycling conditions of an initial denaturation at 94ºC for 1 min, followed by 30 cycles (94ºC for 1 min, 60ºC for 1 min and 72ºC for 1 min) and a final extension at 72ºC for 10 min was followed. The products were run on an agarose gel and the desired band (~119 bp) was purified using the QIAquick® PCR purification system (Qiagen, Germany) following the manufacturer's instructions for sequencing and confirmation. The purified product

Table 1. Primer details for *Eimeria* spp. and GAPDH

Genus		Sequence 5'-3'	Reference
Eimeria spp.	EF1	AAGTTGCGTAAATAGAGCCCTC	Lew *et al*. (2003)
	ER1	AGACATCCATTGCTGAAAG	
5S rRNA	For	TCATCACCCAAAGGGATT	Blake *et al.* (2006)
	Rev	TTCATACTGCGTCTAATGCAC	
E. tenella	TEN- F	TCGTCTTTGGCTGGCTATTC	Vrba *et al*. (2010)
	TEN- R	CAGAGAGTCGCCGTCACAGT	
GAPDH	For	CGCAAGGGCTAGGACGG	Myers *et al*. (2003)
	Rev	GCGCTCTTGCGGGTACC	

was ligated into a TA vector (Promega, U.S.A). The transformed *E. coli* colonies were chosen based on blue-white colony selection and by a colony PCR. Plasmids were extracted using Fast plasmid kit (Eppendorf, Germany) following manufacturer's instructions. Plasmid DNA was also subjected to qPCR to generate standard curves.

DNA standards: Tenfold dilutions of a known concentration of DNA were used as standards for *Eimeria*, GAPDH and *E. tenella* (Pure DNA of *E. tenella* reference strain, IAH, Compton, U.K) was used). The standards were subjected to qPCR and the standard curve was obtained. Efficiency of the reaction was calculated as $10^{(-1/slope)}$.

qPCR protocol: All the qPCR was performed using 7500 FAST Real time PCR system (Applied Biosystems, U.S.A). A reaction volume of 20 µl comprised of 10 µl of SYBR Green master mix (Sigma, U.S.A), 500nM each of forward and reverse primers, 6 µl of nuclease free water and 1 µl of undiluted DNA template. A passive reference dye ROX (Sigma, U.S.A) was added as per manufacturer's recommendations. The reaction conditions were the same for all the 3 real time PCR assays (5S rRNA, chicken GAPDH and *E. tenella* SCAR). Cycle conditions were 95ºC for 2 min (1 cycle); 95ºC for 15 sec, 60ºC for 1 min (40 cycles). After completion of 40 cycles, melting curve analysis of the products was done in order to rule out non-specific amplification. The dissociation stage involved 1 cycle of 95ºC for 15 sec followed by 60ºC for 1 min and 95ºC for 1 min. The threshold fluorescence level was set to 0.02 for all the 3 assays.

Quantification of genome: Genome numbers of *Eimeria* present in 30 mg of intestinal tissue was calculated based on the 5S rRNA based assay as described by Morgan *et al.* (2009). Similarly, *E. tenella* genomes present in the caecal tissue was determined and compared with the *Eimeria* genome counts in the same samples.

RESULTS AND DISCUSSION

A total of 60 intestinal tissue samples (10 from upper intestine, 15 from middle intestine, 17 from lower intestine and rectum and 18 from caecum) were subjected to conventional PCR based on ITS-1 spacer for generic detection of *Eimeria*. Twenty-nine samples (48.3 percent) showed amplification for the ITS-1 region in which distinct bands of desired size (~600 – 800 bp) were observed (Fig. 1). Of the 11 samples collected from Chennai, five (45.5 percent) were found positive. Likewise, 10 (55.5 percent) out of 18 samples collected from broiler breeder farms in Coimbatore and 14 (51.8

percent) out of 27 samples collected from commercial broiler farms in and around Chittoor were found positive for *Eimeria*. None of the intestinal tissue samples collected from Palladam showed positive results. Out of 18 caecal samples that were subjected to PCR, 9 were detected positive by the ITS based PCR. ITS based assays have been described previously by several workers (Lew *et al*., 2003; Aarthi *et al*., 2010). Being a multicopy genomic target, the assay was found to be very sensitive in detection of infection from intestinal tissue samples. The theoretical detection limit was reported to be as low as 0.4 to 2 oocysts of *Eimeria* per PCR (Haug *et al*., 2007). In the present study, ITS-1 based generic PCR was found to detect infections in samples containing > 8 genomes/µl. Since 2 µl of DNA template was used, 16 genomes (= 2 sporulated oocysts) per PCR could be detected. The conventional PCR based on 5S rRNA yielded specific amplicons (Fig. 2). The *Eimeria* 5S ribosomal repeat region exhibits a high degree of conservation among *Eimeria* species, making it an attractive target for development of sensitive qPCR (Blake *et. al*., 2006).

All the samples (n=60) which had amplified with 5S rRNA based primers exhibited a mean melting temperature of 84.7ºC ± 0.30 (Fig. 3). A single peak was observed for the dissociation curve for all

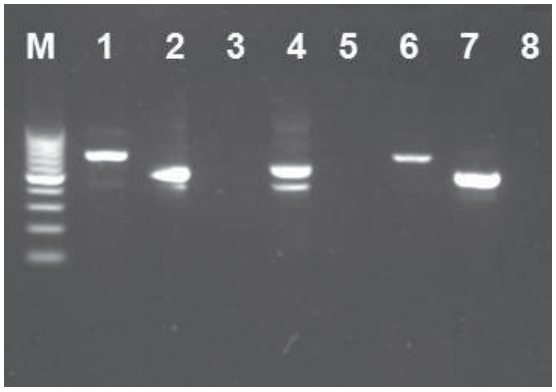

Fig. 1. ITS-1 based genus specific PCR for *Eimeria*
Lane M: 100 bp DNA ladder, Lane 1, 2, 4, 6: *Eimeria* genus specific amplicon, Lane 7: positive control

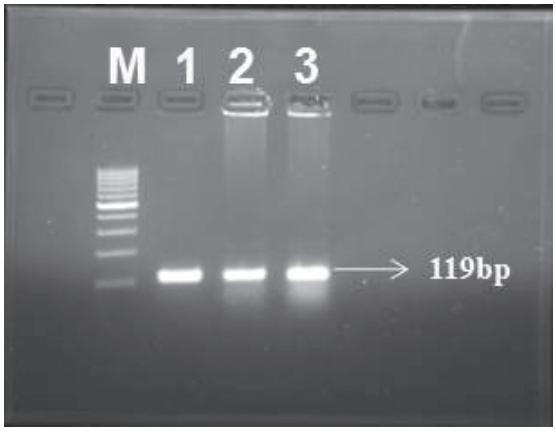

Fig. 2. PCR for 5S rRNA
Lane M: 100 bp DNA ladder, Lane 1, 2 and 3: 119 bp band specific to 5S rRNA

positive amplifications. A C_t was obtained for 46 samples and it was undetermined for 14 samples. The negative controls showed a much lower melting temperature and no peak was seen in the dissociation curve. All the sixty samples were assayed simultaneously with the chicken GAPDH gene endogenous control. All the samples showed positive amplification for GAPDH.

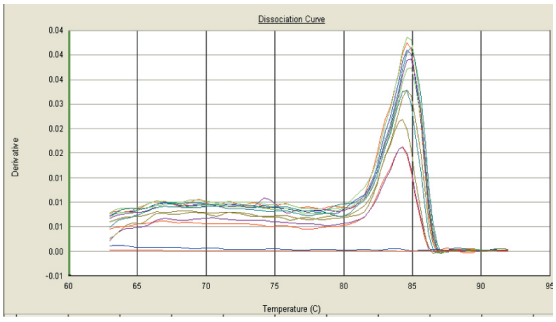

Fig. 3. Dissociation curve for 5S rRNA based qPCR (genomic DNA)

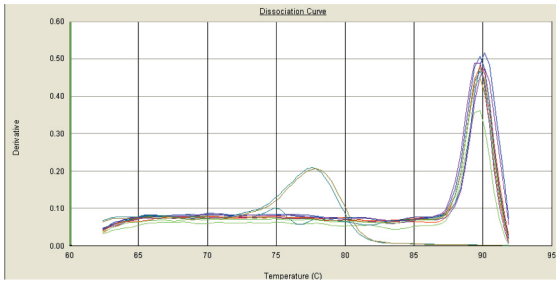

Fig. 4. Dissociation curve for chicken GAPDH based qPCR

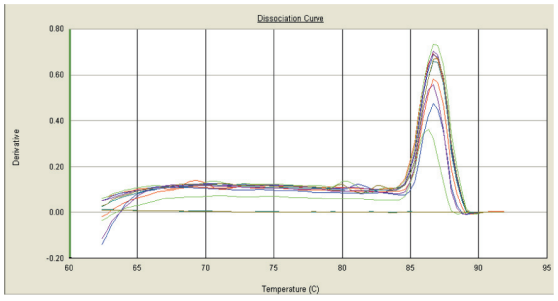

Fig. 5. Dissociation curve for SCAR *E. tenella* based qPCR

A melting curve analysis was done to rule out any non-specific amplification. The mean melting temperature was noted to be 88.8ºC ± 0.18 (n=60) with a single peak of dissociation (Fig. 4). DNA from 18 caecal samples were subjected to an additional *E. tenella* specific qPCR. An *E. tenella* specific amplification was detected in 11 samples. The target specificity of amplification was indicated by a single peak of the dissociation curve and a mean melting temperature of 86.16ºC ± 0.5 (n=18) (Fig. 5).

For 10-fold dilution series with genomic *Eimeria* DNA efficiency of PCR was calculated as 2.15 whereas for GAPDH the PCR efficiency turned out to be 2.106. Theoretically a "perfect" PCR has an efficiency of 2.0. An efficiency inferred >2 can reflect non-target fluorescence or may be due to DNA saturation that causes less of a change in C_t scores at higher sample concentrations, causing a slope compression that inflates efficiency (Morgan *et al.*, 2009). Though an alternative real-time PCR method for species specific detection of *E. tenella* exists using the ribosomal ITS-2 DNA sequences (Morgan *et al.*, 2009), the non-polymorphic, single-copy SCAR target was considered better suited for specific detection and precise quantification of the parasite. An efficiency of 1.903 was obtained for the assay and GAPDH gene was used as an endogenous control while absolute quantification. The melting temperature recorded was different from that recorded for the 5S rRNA assay. Kawahara *et al.* (2008) have reported a SYBR Green based qPCR targeting the ITS-1 region wherein different species of *Eimeria* present in a mixture of oocysts could be differentiated

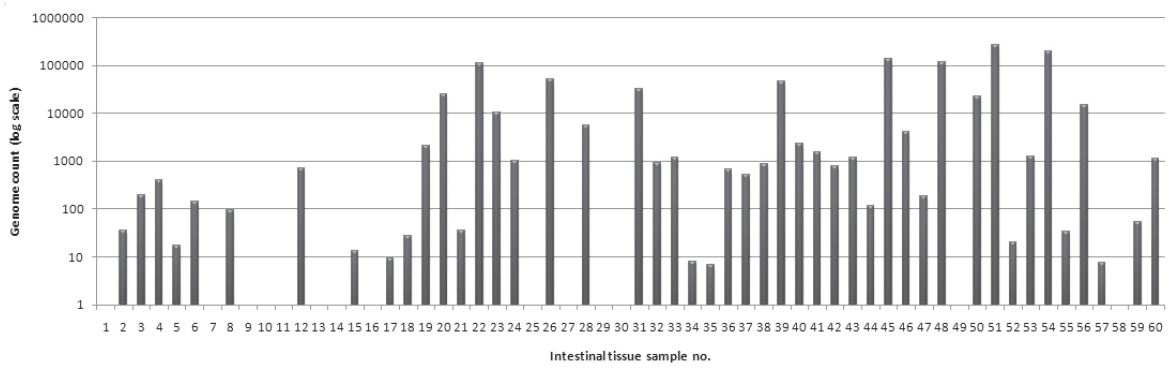

Fig. 6. *Eimeria* genome counts in different samples

using the melting temperatures which were unique to each species. Whether such a possibility exists in a SCAR based assay needs to be investigated. This can be done through developing similar assays for other *Eimeria* species.

Mean genome counts in different tissues were calculated. Mean *Eimeria* count was 87 ± 129 in upper intestine, 10213± 29433 in middle intestine, 13629 ± 34987 in lower intestine and rectum and 37169 ±75799 in caecum. Due to a very high variation in the genome counts between samples, the data was not subjected to statistical analysis (Fig. 6).

Proportion of *E. tenella* genome (assessed by SCAR based qPCR) among total *Eimeria* genome (derived by 5S rRNA based qPCR) in the respective caecal tissue samples was assessed. Caecal tissue was found to harbour single infection with *E. tenella* in two cases. Other caecal tissue samples indicated the presence of other species of *Eimeria* in different proportions (Fig. 7). On comparison, generic *Eimeria* count was higher in most of the cases than the SCAR based *E. tenella* counts. This could be due to presence of other species of *Eimeria* in the sample tested or due to presence of an *E. tenella* population with an unlikely loss or rearrangement of the target region escaping detection (Vrba *et al*., 2010). The proportion of *E. tenella* out of the total *Eimeria* count ranged between zero to 99.9 percent. The complexity of *Eimeria* lifecycle, variable generations of schizogony in different species and difference in biotic potential of different species are some of the factors that need to be considered while making assumptions on the parasite multiplication *in vivo*. Sampling a larger section of host intestine would lessen inter-sample variation (Blake *et al*., 2006) and increase reproducibility of quantification data but at the cost of sensitivity. SYBR Green based qPCR was thus found to be a very sensitive technique

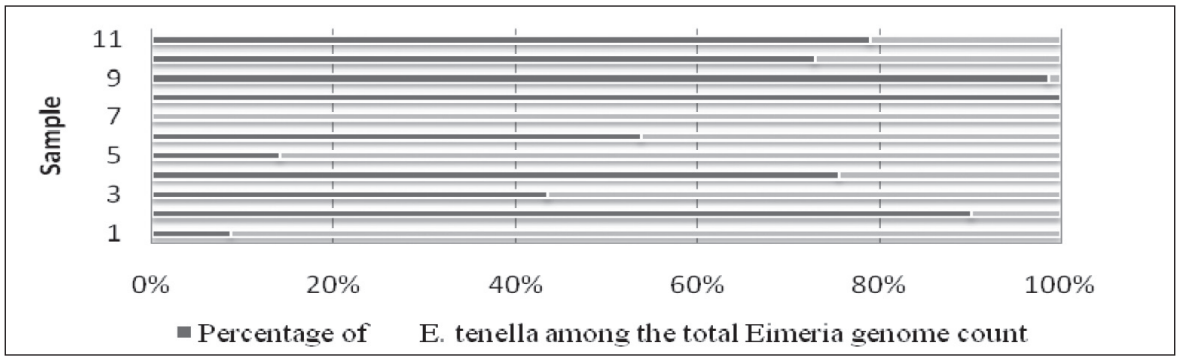

Fig. 7. Proportion of *E. tenella* among total *Eimeria* genome

for detection of *Eimeria* in intestinal tissue samples. The generic qPCR can be effectively supplemented with species-specific qPCR assays for speciation and preponderance studies.

SUMMARY

In the present study, generic PCR was employed with the intention of screening the samples for *Eimeria* prior to subjecting them to quantitative real-time PCR. The ITS-1 based generic PCR assay was standardized to detect coccidiosis in birds using intestinal samples. A SYBR Green based quantitative PCR was developed and standardized for detection of genus *Eimeria* as well as species-specific detection of *E. tenella*. The technique enabled highly sensitive detection of *Eimeria* in 46 samples as against 29 samples by a genus specific conventional PCR. A SCAR based species specific qPCR assay was developed for detection and quantification of *E. tenella*, the most pathogenic of all the seven poultry *Eimeria*. Though, majority of *Eimeria* genome present in caecum was confirmed to be *E. tenella*, a possibility of the presence of other *Eimeria* species in this location was identified.

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