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Speciation of poultry *Eimeria* by morphometry and SCAR PCR in Southern India

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ABSTRACT

The percentage prevalence of *Eimeria tenella, E. maxima, E. acervulina, E. mitis, E. necatrix* and *E. brunetti* were 48.48, 42.42, 12.12, 9.09, 9.09 and 21.21%, respectively, based on morphometric observations. An overall prevalence of different species of *Eimeria* was recorded as 57.57% in various agroclimatic zones of southern India. Of the 21 samples collected from broiler farms, 13 samples were positive (61.9%) for either single or mixed infection of *Eimeria* species. Of the 12 samples collected from layer farms, 6 samples (50%) were positive for either single or mixed infection of *Eimeria* species. PCR using SCAR (sequence characterized amplified region) primers showed amplicons of sizes 811 bp (*E. acervulina*), 539 bp (*E. tenella*), 460 bp (*E. mitis*), 272 bp (*E. maxima*) and 200 bp (*E. necatrix*) and their percentage prevalence were 6.06, 27.27, 6.06, 15.15 and 3.03%, respectively.

Key words: Broiler farms, *Eimeria*, Layer farms, Morphometry, Prevalence,Southern India, SCAR PCR

Coccidiosis, a serious intestinal disease of intensively reared chicken, is caused by *Eimeria,* an enteric-protozoan. Economically, the most important of the genus *Eimeria* are the 7 species viz. *E. acervulina, E. brunetti, E. maxima, E. mitis, E. necatrix, E. praecox* and *E. tenella*. Differentiating various *Eimeria* species could be difficult due to their similar clinical signs and developmental stages (Joyner and Long 1974). Furthermore, mixed infections add difficulty in identifying the species when overlapping intestinal lesions (Chapman 2002).

Variants of PCR assay or PCR-based capillary electrophoresis techniques (Gasser *et al.* 2005, Morris *et al.* 2007) are being successfully used for species specific detection of avian *Eimeria* — internal transcribed spacer ITS-1 (Schnitzler *et al.* 1999, Meireles *et al.* 2004, Jenkins *et al.* 2006, Haug *et al.* 2007) and ITS–2 region (Schnitzler *et al.* 1999, Meireles *et al.*2004, Jenkins *et al*. 2006, Haug *et al.* 2007) were used efficiently as target sites (Su *et al.* 2003, Aarthi *et al.* 2010). A diagnostic multiplex PCR assay was developed for simultaneous detection of 7 *Eimeria* species (Fernandez *et al.* 2003, Kutkat *et al.* 2009) to detect multiple infection under field condition. Extending to recent findings, the study was conducted on the faecal and intestinal samples of chicken collected from different locations in Southern India to estimate the prevalence of various *Eimeria* species using morphometry and PCR.

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MATERIALS AND METHODS

Sample source: Faecal and intestinal samples were collected randomly from organized broiler and layer farms covering 33 locations of various agro-climatic zones of Tamil Nadu, Puducherry and Andhra Pradesh. Of the above, 21 samples were from broiler farms and 12 samples were from layer farms. The mucosanguinous and watery faecal samples were collected. The mucosal scrapings of the intestinal lesions were also collected.

Sample processing protocols: Faecal and intestinal harvest protocols are done to obtain the clean well sporulated oocysts free from unwanted feacal debris. Debris interfere with DNA extraction and PCR technique.

Faecal harvest protocol- The poultry faecal samples collected from various farms were processed immediately after collection. The sample is thoroughly homogenised in 2.5% potassium dichromate solution by pressing the hard faecal pellets between 2 glass slides and by constant stirring. After the sample has reached the desirable emulsion consistency, it was sieved through a nylon sieve with a pore size of ~1mm. The filterate thus obtained was transferred to wide mouth plastic 500 ml capacity beaker and the sample was kept for sporulation at room temperatue of 37° to 40°C

with constant stirring that hastened the sporulation process by providing oxygen to oocysts. The oocyst harvest (90% sporulated oocysts) was enumerated (Bumstead and Millard 1992).

Intestinal harvest protocol- Intestinal harvest (90% sporulated oocysts) was done as per the IAH (2007).

Mean morphometry of Eimeria *oocysts:* The morphometry was done using a calibrated binocular research microscope by taking the mean values of measurements of at least 100 oocysts of each species from different field samples.

Purification of Eimeria *oocysts:* The purification of *Eimeria* oocysts was done as per Cantacessi *et al.* (2008) with slight modifications (the density gradient step using sheather's sugar solution given in the protocol was not done). The final pellet suspension was aliquoted to contain ~ 1×10^6 purified oocysts in 100 µl distilled water and stored at 4°C for further processing.

Genomic DNA extraction from oocysts of Eimeria*:* The purified sample containing aliquots of approximately 1×10^6 purified oocysts were taken and oocysts were pelleted at 10 000 rpm for 1 min. Equal volume of glass beads of 0.5 mm were added and vortexed vigorously for 45 sec to break the oocyst and sporocyst walls. The genomic DNA is extracted as per kit protocol (Kaya *et al.* 2007).

PCR using SCAR primers: PCR was carried out in a thermal cycler using SCAR primers for individual species for all the 33 isolates. SCAR primers (Fernandez *et al*. 2003) developed by using RAPD markers previously characterized were used in the PCR reaction (Table 1). The reaction mix consisted of 2X RBC *Taq* polymerase master mix, 12.5 µl; Primer forward 1.0 µl (20 p mol) primer reverse-1.0 µl (20 p mol), template DNA 0 µl and made up to 25.0 µl with nuclease free water. Standardized cycling conditions consisted of initial denaturation: 96°C for 5 min (denaturation, 94°C for 1 min; annealing, 62°C for 2 min; extension, 72°C for 1 min) for 30 cycles and final extension: 72°C for 7 min.

RESULTS AND DISCUSSION

Mean morphometry of Eimeria *oocysts:* The mean oocysts morphometric values are given in Table 2. The prevalence of *E. tenella, E. maxima, E. acervulina, E. mitis, E. necatrix* and *E. brunetti* were 48.48, 42.42, 12.12, 9.09, 9.09 and 21.21%, respectively, based on morphometric observations.

Morphometry of oocysts was in coherence with previous studies. Oocyst morphometry was described based on the shape and size of the *Eimeria* oocysts by earlier workers (Levine 1985 and Long 1982). Thebo *et al*. (1998) identified 7 species of *Eimeria* in Swedish chickens based on the criteria of oocyst morphology.

Al-Natour *et al*. (2002) conducted a flock level prevalence study according to the site of infection and oocyst morphology including size, shape and colour after sporulation according to (MAFF 1984). Khan *et al*. (2006) examined oocysts microscopically and the species were identified on the basis of shape and size of sporocysts and sporozoites (Levine 1961).

Species spectra: An overall prevalence of different species of *Eimeria* was recorded as 57.57% from 33 locations in various agro-climatic zones of Tamil Nadu, Puducherry and Andhra Pradesh of Southern India. Of the 21 samples collected from broiler farms, 13 samples were positive (61.9%) for either single or mixed infection of *Eimeria* species. Of the 12 samples collected from layer farms, 6 samples (50%) were positive for either single or mixed

Table 1. Forward and reverse primers used in the PCR reaction for 5 *Eimeria* spp (Fernandez *et al.* 2003)

Species		Primer sequences 5'- 3'	Expected amplicon size (bp)
E. acervulina	F	AGTCAGCCACACAATAATGGCAAACATG	811
	R	AGTCAGCCACAGCGAAAGACGTATGTG	
E. tenella	F	CCGCCCAAACCAGGTGTCACG	539
	R	CCGCCCAAACATGCAAGATGGC	
E. mitis	F	AGTCAGCCACCAGTAGAGCCAATATTT	460
	R	AGTCAGCCACAAACAAATTCAAACTCTAC	
E. maxima	F	GGGTAACGCCAACTGCCGGGTATG	272
	R	AGCAAACCGTAAAGGCCGAAGTCCTAGA	
E. necatrix	F	TTCATTTCGCTTAACAATATTTGGCCTCA	200
	R	ACAACGCCTCATAACCCCAAGAAATTTTG	

Table 2. Mean morphometry of *Eimeria* oocysts

Eimeria species	Mean oocyst morphometry(l×b) (in microns)	Oocyst morphometry (l×b) (in microns) Levine (1985)
E. acervulina	15.4 × 11.2	12–23 × 9–17
E. brunetti	28.5 × 20.3	14–34 × 12–26
E. maxima	31.1 × 18.5	21–42 × 16–30
E. mitis	13.2 × 12.4	10–21 × 9–18
E. necatrix	20.8 × 17.5	12–29 × 11–24
E. tenella	22 × 18	14–31 × 9–23

infection of *Eimeria* species. Single species isolates were obtained for *E. maxima* for isolate 4 and *E. tenella* for isolates 18, 19 and 20.

The highest OPG (oocyst per gram) was recorded from isolate 10 with 350,000 oocysts followed by isolate 31 with 235,000 oocysts (Table 3). Coccidiosis is still more of a problem of broilers than of layers (Anna 2002, Raman *et al.* 2005). Our results are not in agreement with SairaBanu *et al.* (2009) who observed more prevalence (55.7%) in layers.

The present study indicated that *E. tenella* was the predominant species in commercial chicken especially in the age group of 3–6 weeks (Table 3). This is in agreement with the previous prevalence studies in Tamil Nadu (Anna 2002,Raman *et al.* 2005, SairaBanu *et al.* 2009) and elsewhere (Al-Natour *et al.* 2002), but contrary to the reports by few workers (Abdul Basith 1995, Lobago *et al.* 2005, Khan *et al.* 2006, Nematollahi *et al.* 2008) who indicated the predominance of other *Eimeria* species.

In the present study, *E. maxima* is the second most prevalent species which is in contrary to the reports of Anna (2002), Raman *et al.* (2005), SairaBanu *et al.* (2009) who found *E. acervulina* to be the second most common species.

The species prevalence indicated that mixed infection was a common factor with more than one species of *Eimeria* detected in samples obtained from Palladam, Cuddalore, Thiruchitrambalam, Vellore, Kanchipuram, Thiruvallur, Tiruppur, Namakkal and Thanjavur. This is also in agreement with the reports by previous workers on the prevalence of coccidiosis as a mixed infection (Raman *et al.* 2005, Antonik *et al.* 2008, Saira Banu *et al.* 2009).

The spectra of species observed in mixed infection consisted of *E. tenella, E. maxima, E. acervulina* and *E. mitis*.

Table 3. Species detection by morphometry and SCAR from various poultry farms

Farm	Geographical origin of isolate	Type of farm	Age (weeks)	OPG $\times 10^3$	Morphometry						SCAR PCR				
					ET	EMx	EA	EMi	EN	EB	ET	EMx	EA	EMi	EN
1.	Thiruchitrambalam,T N	B	6	47.5	+	+	-	-	-	-	+	+	-	-	-
2.	Cuddalore, T N	B	4	9.3	+	+	-	-	-	-	+	+	-	-	-
3.	Chinnakozhvari, T N	B	5	58.6	+	+	-	-	-	-	+	+	-	-	-
4.	Red hills, T N	B	5	200	-	+	-	-	-	-	-	+	-	-	-
5.	Krishnagiri, T N	B	3	0.6	-	+	-	-	-	-	-	-	-	-	-
6.	Coimbatore, T N	B	4	78	+	-	+	-	-	-	+	-	-	-	-
7.	Nilgiris, T N	B	4	-	-	-	-	-	-	-	-	-	-	-	-
8.	Palladam, T N	B	4	11	-	+	-	-	-	+	-	-	-	-	-
9.	Kanchipuram, T N	B	5	7.4	+	+	-	-	-	-	-	-	-	-	-
10.	Tiruppur, T N	B	6	350	+	+	+	+	-	+	+	-	+	+	-
11.	Ariyankuppam, P Y	B	5	-	-	-	-	-	-	-	-	-	-	-	-
12.	Nonnankuppam, P Y	B	4	-	-	-	-	-	-	-	-	-	-	-	-
13.	Muthialpet, P Y	B	4	-	-	-	-	-	-	-	-	-	-	-	-
14.	Mudaliarpet, P Y	B	6	-	-	-	-	-	-	-	-	-	-	-	-
15.	Reddiarpalayam, P Y	B	6	-	-	-	-	-	-	-	-	-	-	-	-
16.	Murungapakkam, P Y	B	6	-	-	-	-	-	-	-	-	-	-	-	-
17.	Lawspet, P Y	B	5	-	-	-	-	-	-	-	-	-	-	-	-
18.	Nellore, A P	B	5	24	+	-	-	-	-	-	+	-	-	-	-
19.	Chittor, A P	B	6	3.7	+	-	-	-	-	-	-	-	-	-	-
20.	V Kota, A P	B	6	1.5	+	-	-	-	-	-	-	-	-	-	-
21.	Vellore, T N	B	5	130	+	+	-	-	-	+	+	+	-	-	-
22.	Dharmapuri, T N	L	5	-	-	-	-	-	-	-	-	-	-	-	-
23.	Thirupathur, T N	L	6	-	-	-	-	-	-	-	-	-	-	-	-
24.	Tiruvannamalai, T N	L	7	-	-	-	-	-	-	-	-	-	-	-	-
25.	Madurai, T N	L	7	-	-	-	-	-	-	-	-	-	-	-	-
26.	Ulundurpettai, T N	L	4	-	-	-	-	-	-	-	-	-	-	-	-
27.	Villupuram, T N	L	5	-	-	-	-	-	-	-	-	-	-	-	-
28.	Thiruvallur, T N	L	3	6.6	+	+	+	+	+	-	-	-	-	-	-
29.	Thanjavur, T N	L	6	15.5	+	+	-	-	-	+	+	-	-	-	-
30.	Pakkam, T N	L	5	150	+	-	-	-	+	+	+	-	-	-	+
31.	Namakkal, T N	L	6	235	+	+	+	+	+	+	-	-	+	+	
32.	Karur, T N	L	6	0.35	+	+	-	-	-	+	-	-	-	-	-
33.	Salem, T N	L	5	1.3	+	+	-	-	-	-	-	-	-	-	-

B, Broiler farm; L, layer farm;TN, Tamil Nadu; PY, Puducherry;AP, Andhra Pradesh; ET, *Eimeria tenella;* EMx, *Eimeria maxima;* EA, *Eimeria acervulina;* EMi, *Eimeria mitis;* EN, *Eimeria necatrix;* EB, *Eimeria brunette;*+, positive for infection, -, negative for infection; OPG, oocysts per gram.

Long (1973) found that both *E. tenella* and *E. acervulina* were having the ability to produce a higher mortality in chicken during 4–6 weeks of age.

The isolates collected from the broiler farms of Chittoor, Nellore and V. Kota in Andhra Pradesh had monospecific infection with *E. tenella* indicating the predominance of caecal coccidiosis in broilers in those regions.

E. brunetti, a less prevalent but significantly pathogenic species was surprisingly found in broiler and layer farms as a mixed infection causing high morbidity with reduction in FCR that adversely affects broiler production in Palladam, Tiruppur, Coimbatore, Karur and Namakkal.

Abdul Basith *et al*. (1995) also recorded a higher incidence of *Eimeria brunetti* (44.69%) in Namakkal and adjoining areas over a period of 12 months.

PCR using SCAR primers: The results showed amplicons of sizes 811 bp (*E. acervulina*), 539 bp (*E. tenella*), 460 bp

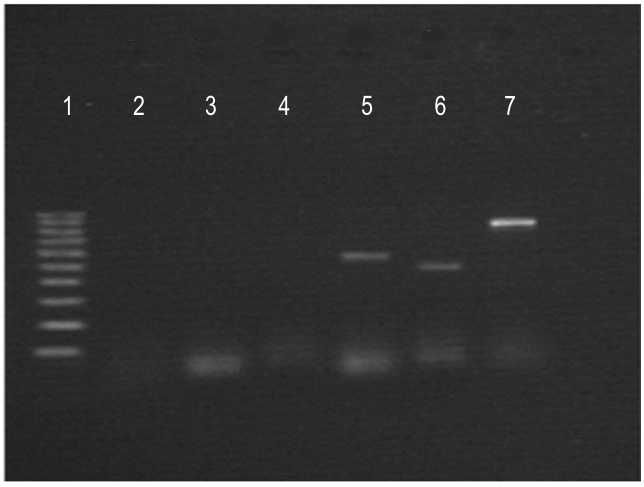

Fig. 1. Amplification of *Eimeria* species by PCR. Lane 1-100 bp marker; lane 5-539 bp, E. t enella, lane 6-460 bp, lane 7-811 bp, *E. acervulina* - Tiruppur isolate.

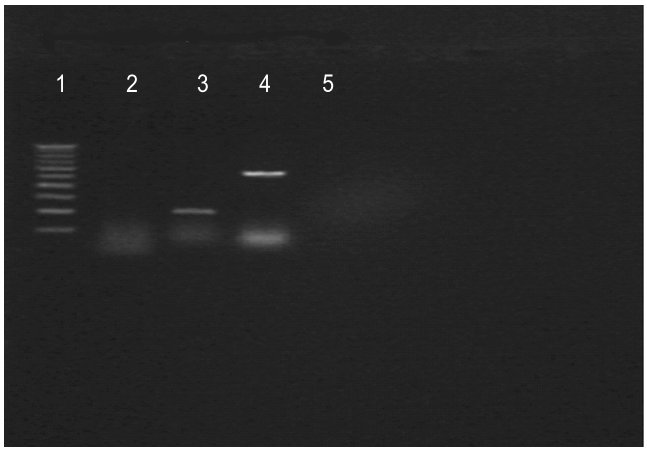

Fig. 2. Amplification of *Eimeria* species by PCR Lane 1-100 bp marker; Lane 3- 200 bp, *E. necatrix*, lane 4- 539 bp, E, tenella-Pakkam isolate.

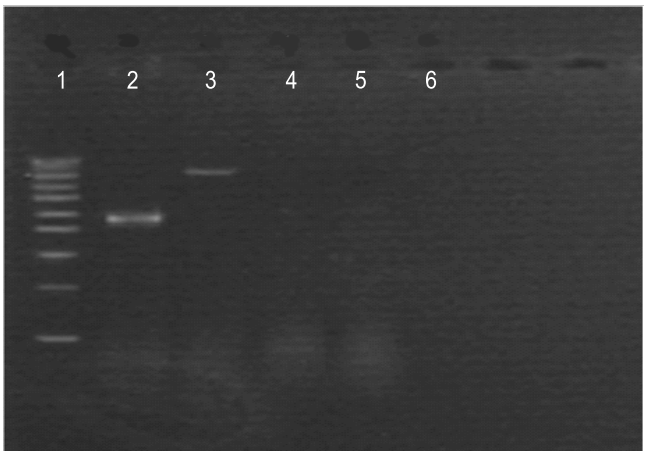

Fig. 3. Amplification of *Eimeria* species by PCR. Lane 1- 100 bp marker, lane 2- 460 bp, *E. mithis*, lane 3- 811 bp, E. acervulina-Namakhal isolate.

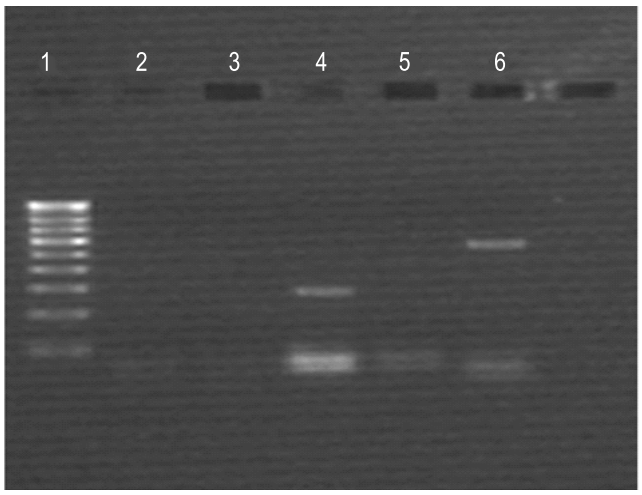

Fig. 4. Amplification of *Eimeria* species by PCR. Lane 1- 100 bp marker; Lane 4-272 bp, *E. maxima*, lane 6- 539 bp, *E. tenella*-Thiruchitrambalam isolate.

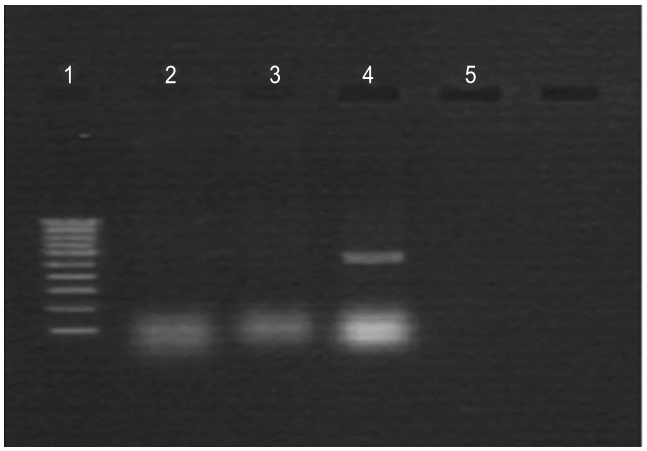

Fig. 5. Amplification of *Eimeria* species by PCR. Lane 1-100 bp marker; Lane 4-539 bp, *E. tenella*–Nellore isolate.

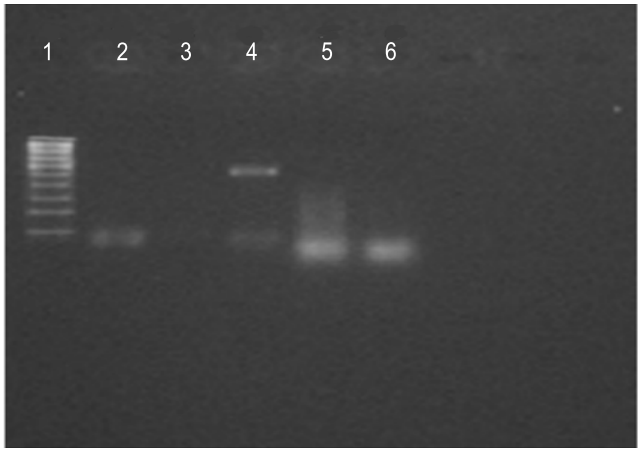

Fig. 6. Amplification of *Eimeria* species by PCR. Lane 1-100 bp marker; Lane 4-539 bp, *E. tenella*–Coimbatore isolate.

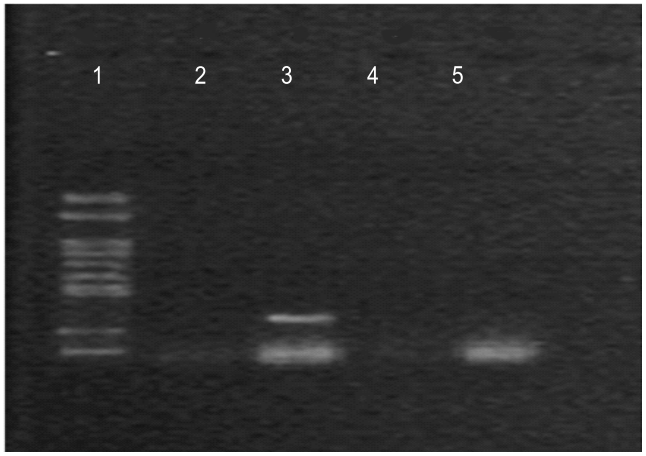

Fig. 7. Amplification of *Eimeria* species by PCR. Lane 1: 100 bp marker; lane 3: 272 bp *Eimeria maxima*-vollore isolate

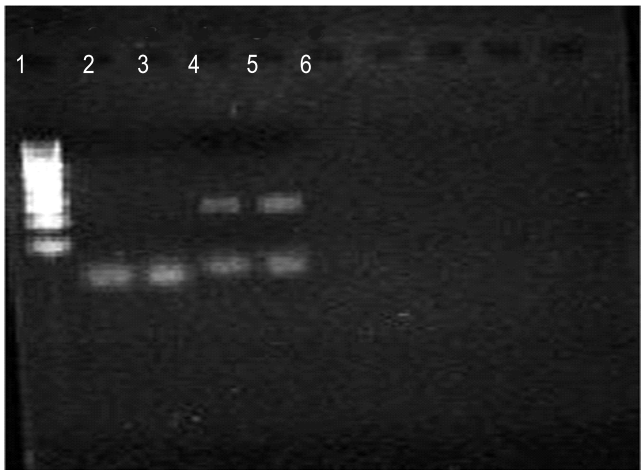

Fig. 8. Amplification of *Eimeria* species by PCR. Lane 1- 100 bp marker; Lane 4,5 - 272 bp, *E. maxima*- Red hills Chennai isolate.

(*E. mitis*), 272 bp (*E. maxima*) and 200 bp (*E. necatrix*) and their percentage prevalence were found to be 6.06, 27.27, 6.06, 15.15 and 3.03% respectively (Figs 1–8). Fernandez *et al.* (2003) obtained amplicons of sizes 811 bp (*E. acervulina*), 539 bp (*E. tenella*), 460 bp (*E. mitis*), 272 bp (*E. maxima*), 200 bp (*E. necatrix*), 626 bp (*E. brunetti*) and 354 bp (*E. praecox*).

Kutkat *et al*. (2009) obtained the amplicons for only 6 species of *Eimeria*, viz. *E. acervulina*, *E. tenella*, *E. mitis*, *E. maxima*, *E. necatrix* and *E. praecox* from faecal and intestinal samples.The PCR carried out using SCAR primers was specificis of great value as it could detect multiple species under the same cycling conditions and even in a single tube reaction. However, the assay was standardized using a minimum of 50 000 oocysts to obtain good amplification.

The prevalence of *Eimeria* species as detected by morphometry of oocysts was confirmed in SCAR PCRs in the samples collected from various farms, viz. 6, 18, 29 (*E. tenella* only detected by PCR), 1, 2, 3, 21 (*E. tenella* and *E. maxima* detected by PCR), 4 (*E. maxima* only detected by PCR), 10 (*E. tenella*, *E. acervulina* and *E. mitis* detected by PCR), 30 (*E. tenella* and *E. necatrix* detected by PCR) and 31 (*E. acervulina* and *E. mitis* detected by PCR).

However, there is no correlation in the prevalence data obtained by morphometry of oocysts and PCR using SCAR primers in the samples collected from the remaining farms (Table 3). Identification of species in these farms was possible more with morphometric studies than SCAR PCR.

The above results indicated that the present study detected with certainty the 5 species of *Eimeria* namely *E. acervulina*, *E. maxima*, *E. mitis*, *E. necatrix* and *E. tenella* with a minimum of 10 000 oocysts in contrast to 5 000 oocysts used by Fernandez *et al*. (2003) for the sensitivity assay. The reason for the different results in morphometry and SCAR PCR (Table 3) could be attributed to less number of oocysts harvested from the samples. Eventhough the species identification was possible by morphometry in certain farms, we could not get amplification of DNA. Low concentration suspensions of oocysts led to a decreased oocyst disruption as opined by Fernandez *et al*. (2003) for the inconsistent DNA yields from samples. Further, they indicated a detection threshold of 50 oocysts of *E. acervulina,* 500 oocysts of *E. maxima* and 100 oocysts of *E. tenella* and inconsistent DNA yields from samples having 10 to 5000 oocysts.

The results obtained by using both morphometry and PCR were coherent with the previous studies. The species identification in Lower Silesia region was carried out by the classical method (location of anatomicropathologic changes in the alimentary tract, the morphology and size of oocysts, time of sporulation) and by PCR. The most frequently occuring species were *E. acervulina, E. tenella, E. maxima* and *E. necatrix* (Shirley 1986). A prevalence of 69% of coccidiosis was by at least 2 species of coccidia, and only 31% of the cases by 1 species of coccidia. The results of the PCR analysis confirmed the species composition of *Eimeria* species determined by the classical method (Antonik *et al.*2008).

As there exists a lot of variation in morphometry due to absolute requirement of skilled personnel in identifying and measuring the dimensions of various oocysts, molecular technique such as PCR with very high sensitivity and specificity eliminates these variations and provides a reliable method to confirm the morphometry results and minimizes the individual variations in morphometry.

The current SCAR PCR technique carried out in faecal samples is comparatively less efficient than a nested PCR technique involving ITS-2 region from tissue samples (Aarthi *et al.*2010). However, SCAR PCR from faecal samples has the advantages over the nested PCR in that it eliminates the use of animal tissues. Collection of tissue samples absolutely requires a dead bird at the time of sampling or sacrifice of a few affected birds which might require approval from the animal ethical committee. But in the field conditions, sub clinical coccidiosis is more prevalent which need not be fatal often. The SCAR PCR standardized in this study will be useful in the detection of subclinical infections from faecal samples without slaughtering the affected birds.

Molecular speciation of local isolates would help in systematic documentation of different strains of various *Eimeria* species. It would facilitate a deeper understanding to the strain variations and help in phylogenetic resolutions (genetic diversity). Identification of novel protein coding genes from *E.brunetti* has been done by analyzing the ESTs because sequence homology will help in better understanding of the biology of the parasite (Aarthi *et al.*2011).

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