Multiplex PCR assay using SCAR primers to detect *Eimeria* spp. in chicken

P. Venkateswara Rao · M. Raman ·
G. DhinakarRaj · S. Abdul Basith ·
S. Gomathinayagam

Received: 6 March 2012 / Accepted: 26 June 2012 / Published online: 17 July 2012
© Indian Society for Parasitology 2012

Abstract About 11 faecal samples from various regions of Tamil Nadu and Andhra Pradesh containing mixed spectrum of *Eimeria* species detected by morphometry viz. 15.4×11.2 , 28.5×20.3 , 31.1×18.5 , 13.2×12.4 , 20.8×17.5 and $22 \times 18 \mu\text{m}$ for *E. acervulina*, *E. brunetti*, *E. maxima*, *E. mitis*, *E. necatrix*, *E. praecox* and *E. tenella* respectively were taken for the study. The oocysts were concentrated and purified using faecal harvest protocol. The genomic DNA is extracted as per kit protocol. The amplicons of sizes 539 bp (*E. tenella*) and 460 bp (*E. mitis*) obtained could be visualised in a single lane for the multiplex PCR assay using sequence characterized amplified region primers.

Keywords Mixed spectrum · *Eimeria* · Morphometry · Faecal harvest · Multiplex PCR · SCAR

Introduction

Coccidiosis in chicken is caused by obligatory intracellular protozoa of the family Eimeriidae. Seven distinct *Eimeria* species can infect chickens, causing intestinal lesions of variable extent and severity, reducing the absorptive function of the mucosa and thus leading to weight loss, diarrhoea, poor FCR and higher mortality of the affected flocks (McDougald and Reid 1997). Species identification is classically performed using morphometry, pre-patent period, sporulation time, lesion scoring techniques based on severity, intestinal site and shape of the lesions and characteristics of the endogenous stages in the intestinal mucosa (Joyner and Long 1974; Long 1982). However, these techniques require highly trained personnel and are limited by the overlap of characteristics among different species (Long and Joyner 1984). Furthermore, mixed infections can pose a problem for the precise determination of the intestinal site of the lesions (Jeffers 1975; McDougald and Jeffers 1976; Shirley et al. 1984) has eliminated the pre-patent period as a good criterion for species identification. Initial efforts aiming at species identification through molecular polymorphism were based on isoenzyme analysis (Shirley 1975; Johnston and Fernando 1997). This method, however, requires large numbers of parasitic stages, is time consuming and cumbersome.

Another restriction was posed by the inherent inability of the method to deal with mixed samples, generating overlapping band profiles that are not informative. Several reports described the development of PCR assays for species discrimination of *Eimeria* spp. using as targets different regions of the ribosomal cistrons, including the 5S rRNA (Stucki et al. 1993), the small subunit rRNA (Tsuji et al. 1997), the ribosomal internal transcribed spacer 1

P. Venkateswara Rao · M. Raman · S. Abdul Basith ·
S. Gomathinayagam
Department of Veterinary Parasitology, Madras Veterinary
College, Tamil Nadu Veterinary & Animal Sciences University,
Vepery, Chennai 600 007, India

G. DhinakarRaj
Department of Animal Biotechnology, Madras Veterinary
College, Tamil Nadu Veterinary & Animal Sciences University,
Vepery, Chennai 600 007, India

Present Address:

P. Venkateswara Rao (✉)
Department of Veterinary Parasitology, College of Veterinary
Science, Sri Venkateswara Veterinary University, Korutla 505
326, Andhra Pradesh, India
e-mail: venkateshrao22@gmail.com

(ITS-1) (Schnitzler et al. 1998, 1999), and ITS-2 (Woods et al. 2000; Gasser et al. 2001).

Paran and Michelmore (1993) described a new marker, the sequence-characterised amplified region (SCAR), which is derived from RAPD fragments and can be amplified by a pair of specific primers under stringent conditions. Fernandez et al. (2003) developed a multiplex PCR assay using SCAR species specific markers for *Eimeria* spp. of domestic fowl for simultaneous discrimination of various *Eimeria* species that infect the domestic fowl.

Materials and methods

Mean morphometry of *Eimeria* oocysts

About 33 faecal samples from various poultry farms of Tamil Nadu and Andhra Pradesh were screened by morphometry for mixed *Eimeria* species infections.

Mixed sample source

The faecal samples from various regions of Tamil Nadu and Andhra Pradesh containing mixed spectrum of *Eimeria* species detected by morphometry (Levine 1985) were taken for the study.

Sample preparation

The oocysts from each mixed sample were taken as such without changing original species composition and proportion obtained from the poultry farms for purification. The oocysts were then concentrated and purified by faecal harvest protocol (Bumstead and Millard 1992).

Genomic DNA extraction from oocysts of *Eimeria*

The purified sample containing aliquots of $\sim 1 \times 10^6$ purified mixed species oocysts of various *Eimeria* species were taken and oocysts were pelleted as such at 10,000 rpm for 1 min. Equal volume of glass beads of 0.5 mm (Sigma, USA) were added and vortexed vigorously for 45 s. to break the oocyst and sporocyst walls. The genomic DNA extracted as per kit protocol (Kaya et al. 2007), is presumed to contain all the species present in that particular isolate.

Multiplex PCR using SCAR primers

Multiplex PCR was carried out in a Thermal cycler (Eppendorf, Germany) using SCAR primers. Table 1 for various mixed species containing isolates (Table 3). The reaction mix consisted of *Taq* DNA polymerase (Sigma, USA)-1.0 μ l (5 U/ μ l), 10 $\times$ buffer-3.5 μ l, dNTP mix-1.0 μ l forward primer-1.0 μ l $\times$ 6 (20 pmol of each of the 6 species), reverse primer-1.0 μ l $\times$ 6 (20 pmol of each of the 6 species), template DNA-3.0 μ l and made up to 35.0 μ l 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 obtained for various poultry *Eimeria* species are given in Table 2.

Table 1 Forward and reverse primers used in the PCR for 6 *Eimeria* spp. (Fernandez et al. 2003)

S.No	Species		Primer sequences 5'–3'	Expected amplicon size (bp)
1.	<i>E. acervulina</i>	F	AGTCAGCCACACAATAATGGCAAACATG	811
		R	AGTCAGCCACAGCGAAAGACGTATGTG	
2.	<i>E. tenella</i>	F	CCGCCCAAACCAGGTGTCACG	539
		R	CCGCCCAAACATGCAAGATGGC	
3.	<i>E. mitis</i>	F	AGTCAGCCACCAGTAGAGCCAATATTT	460
		R	AGTCAGCCACAAACAAATTCAAACCTCTAC	
4.	<i>E. maxima</i>	F	GGGTAACGCCAACTGCCGGGTATG	272
		R	AGCAAACCGTAAAGGCCGAAGTCCTAGA	
5.	<i>E. necatrix</i>	F	TTCATTTCGCTTAACAATATTTGGCCTCA	200
		R	ACAACGCCTCATAACCCCAAGAAATTTTG	
6.	<i>E. brunetti</i>	F	TGGTCGCAGAACCTACAGGGCTGT	626
		R	TGGTCGCAGACGTATATTAGGGGTCTG	

F forward primer, R reverse primer, bp base pairs

Table 2 Mean morphometry of *Eimeria* oocysts

S. No	<i>Eimeria</i> species	Mean oocyst morphometry (l × b) (μm)	Oocyst morphometry (l × b) (μm) Levine (1985)
1	<i>E. acervulina</i>	15.4 × 11.2	12–23 × 9–17
2	<i>E. brunetti</i>	28.5 × 20.3	14–34 × 12–26
3	<i>E. maxima</i>	31.1 × 18.5	21–42 × 16–30
4	<i>E. mitis</i>	13.2 × 12.4	10–21 × 9–18
5	<i>E. necatrix</i>	20.8 × 17.5	12–29 × 11–24
6	<i>E. tenella</i>	22 × 18	14–31 × 9–23

L length, B breadth

Mixed sample source

About 11 mixed species faecal isolates screened by morphometry were taken for further processing (Table 3).

More than one species of *Eimeria* were detected in samples obtained from Thiruchitrambalam, Cuddalore, Chinnakozhvari, Red hills, Coimbatore, Tiruppur, Nellore, Vellore, Thanjavur, Pakkam and Namakkal respectively by morphometry (Table 3).

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; SairaBanu et al. 2009).

The composition of species observed in mixed infection was of *E. tenella*, *E. maxima*, *E. acervulina* and *E. mitis*. The mixed infections of *E. tenella*, *E. acervulina* and *E. mitis* are more fatal among birds (Long 1973), he 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.

Multiplex PCR using SCAR primers

Multiplex PCR assay was carried out using samples with mixed infection to know the species contained in them. The amplicons of sizes 539 bp (*E. tenella*) and 460 bp (*E. mitis*) obtained could be visualised in a single lane (Fig. 1).

The multiplex PCR assay with other combinations of species occurring in mixed infections did not give amplicons since standardization of this assay was much more difficult than conventional individual PCR. As multiple primers were used in the same reaction, each one represents distinct binding efficiencies, different reaction kinetics occur simultaneously leading to heterogeneous amplification pattern. Further this aspect was incremented by the complex molecular cross interactions that took place during cycling altering the primer binding and contributing to different amplification rate of each product. The constraints due to samples with less numbers of oocysts of a particular species as observed in conventional PCR would also interfere with amplification process in multiplex PCR, all of which concurred with the opinion of Fernandez et al. (2003).

Multiplex PCR is a highly useful technique since mixed infections are more predominant than single species infection in natural conditions as reported by (Anna 2002; Raman et al. 2005; Antonik et al. 2008; SairaBanu et al. 2009). This assay greatly reduces the time and cost of chemicals avoiding PCR assays for individual species. In natural outbreaks of coccidiosis, mixed species infections with overlapping gross lesions in the intestines pose huge problems in the diagnosis and control of this economically important disease. In such circumstances, diagnostic multiplex PCR assay will be useful to devise control strategies to minimise the economic loss due to this disease. However

Table 3 Mixed *Eimeria* species isolates detected by morphometry

Farm	Geographical origin of isolate	Type of farm	Age (weeks)	OPG × 10 ³	Morphometry					
					ET	EMx	EA	EMi	EN	EB
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.	Coimbatore, T.N	B	4	78	+	–	+	–	–	–
6.	Tiruppur, T.N	B	6	350	+	+	+	+	–	+
7.	Nellore, A.P	B	5	24	+	–	–	–	–	–
8.	Vellore, T.N	L	5	130	+	+	–	–	–	+
9.	Thanjavur, T.N	L	6	15.5	+	+	–	–	–	+
10.	Pakkam, T.N	L	5	150	+	–	–	–	+	+
11.	Namakkal, T.N	L	6	235	+	+	+	+	+	+

T.N Tamil Nadu, A.P Andhra Pradesh, B broiler farm, L layer farm, OPG oocyst per gram, ET, *Eimeria tenella*; EMx, *Eimeria maxima*; EA, *Eimeria acervulina*; EMi, *Eimeria mitis*; EN, *Eimeria necatrix*; EB, *Eimeria brunetti*; +, positive for infection; –, negative for infection

Fig. 1 Lane 1 100 bp ladder, lane 2 460 bp *Eimeria mitis*; 539 bp *Eimeria tenella*

the low quantity of DNA of one species resulting from lower number of oocysts could not get amplified as seen with *E. brunetti* in this study and even is masked by the DNA of other predominant species in samples with mixed infections. This could be overcome by proper optimisation of DNA extraction and PCR protocols.

Acknowledgments The author wishes to thank the Tamil Nadu Veterinary and Animal Sciences University for funding the entire research project, Department of Veterinary Parasitology and Department of Animal Biotechnology for providing assistance with equipment.

References

- Anna T (2002) Antigenic profile of *Eimeria* spp. and detection of coproantigen in chicken coccidiosis. Ph.D. thesis submitted to Tamil Nadu Veterinary and Animal Sciences University
- Antonik SA, Okulewicz A, Gawel A, Mazurkiewicz M (2008) Prevalence of *Eimeria* species in hens in the Lower Silesia region. *Medycyna Wet* 64(7):886–888
- Bumstead N, Millard B (1992) Variation in susceptibility of inbred lines of chickens to seven species of *Eimeria*. *Parasitology* 104(3):407–413
- Fernandez S, Pagotto AH, Furtado MM, Katsuyama AM, Madeira AMBN, Gruber A (2003) A multiplex PCR assay for the simultaneous detection and discrimination of the seven *Eimeria* species that infect domestic fowl. *Parasitology* 127:317–325

- Gasser RB, Woods WG, Wood JM, Ashdown L, Richards G, Whithear KG (2001) Automated, fluorescence-based approach for the specific diagnosis of chicken coccidiosis. *Electrophoresis* 22:3546–3550
- Jeffers TK (1975) Attenuation of *Eimeria tenella* through selection for precociousness. *J Parasitol* 61:1083–1090
- Johnston DA, Fernando MA (1997) Isoenzymes of *Eimeria* from the domestic fowl: electrophoretic variants among species, strains and clones. *Parasitol Res* 83:464–470
- Joyner LP, Long PL (1974) The specific characters of *Eimeria*, with special reference to the coccidia of the fowl. *Avian Pathol* 3:145–157
- Kaya GC, Dale I, Maudlin I, Morgan K (2007) A novel procedure for total nucleic acid extraction from small numbers of *Eimeria* species oocysts. *Turkiye Parazit Derg* 31(3):180–183
- Levine ND (1985) Veterinary protozoology, 1st edn. Iowa State University Press, Ames, pp 178–188
- Long PL (1973) Pathology and pathogenicity of coccidial infections. In: Hammond DM, Long PL (eds) *The coccidia*. University Park Press, Baltimore, pp 183–252
- Long PL (1982) The biology of the coccidia. University Park Press, Baltimore, pp 169–178
- Long PL, Joyner LP (1984) Problems in the identification of species of *Eimeria*. *J Protozool* 31:535–541
- McDougald LR, Jeffers TK (1976) *Eimeria tenella* (Sporozoa; Coccidia): gametogony following a single asexual generation. *Science* 192:258–259
- McDougald LR, Reid WM (1997) Coccidiosis. In: Calnek BW, Barnes HJ, Beard CW, McDougald LR, Saif YM (eds) *Diseases of poultry*, 10th edn. Iowa State University Press, Ames, pp 865–883
- Paran I, Micheltore RW (1993) Development of reliable PCR-based markers linked to downy mildew resistance genes in lettuce. *Theor Appl Genet* 85:985–993
- Raman M, Gowthaman V, Mahaprabhu R, Anna T, Harikrishnan TJ (2005) Avian chorioallantoic membrane—an in-ovo model for detection of anti-coccidial drug resistance. *Ind J Anim Sci* 75(1):5–7
- Sairabanu S, Raman M, Gomathinayagam S, Dhinakar Raj G (2009) Preponderance of *Eimeria* species in commercial poultry in Tamil Nadu. *Ind J Anim Sci* 79(2):164–166
- Schnitzler BE, Thebo P, Mattson JG, Tomley F, Shirley MW (1998) Development of a diagnostic PCR assay for detection and discrimination of four pathogenic *Eimeria* species of the chicken. *Avian Pathol* 27:490–497
- Schnitzler BE, Thebo P, Tomley F, Ugglia A, Shirley MW (1999) PCR identification of chicken *Eimeria*: a simplified read-out. *Avian Pathol* 28:89–93
- Shirley MW (1975) Enzyme variation in *Eimeria* species of the chicken. *Parasitology* 71:369–376
- Shirley MW, McDonald V, Chapman HD, Millard BJ (1984) *Eimeria praecox*: selection and characteristics of precocious lines. *Avian Pathol* 13:669–682
- Stucki U, Braun R, Roditi I (1993) *Eimeria tenella*: characterization of a 5S ribosomal RNA repeat unit and its use as a species-specific probe. *Exp Parasitol* 76:68–75
- Tsuji N, Kawazu S, Ohta M, Kamio T, Isobe T, Shimura K, Fujisaki K (1997) Discrimination eight chicken *Eimeria* species using the two-step polymerase chain reaction. *J Parasitol* 83:966–970
- Woods WG, Whithear KG, Richards DG, Anderson GR, Jorgensen WK, Gasser RB (2000) Single-strand restriction fragment length polymorphism analysis of the second internal transcribed spacer (ribosomal DNA) for six species of *Eimeria* from chickens in Australia. *Int J Parasitol* 30:1019–1023