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Research Article

ISOLATION AND MOLECULAR IDENTIFICATION OF *EIMERIA* SPECIES CIRCULATING IN BROILER CHICKEN FLOCKS OF ASSAM (INDIA)

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ABSTRACT

The present study was conducted in a total of 45 small scale broiler chicken farms covering three districts of Assam in order to identify different species of *Eimeria* circulating in broiler birds. Examination of intestines obtained from 109 carcasses of deceased broiler upto 6 weeks of age showed 88.88% of the farms to be coccidia positive. On the basis of conventional lesion study and morphometry of oocysts, five species of *Eimeria* were identified which included *E. tenella*, *E. acervulina*, *E. mitis*, *E. maxima* and *E. brunetti*. Molecular identification using nested PCR confirmed the presence of former four species. Farm-wise species involvement analysis revealed presence of *E. tenella* in 62.5% farms as mono species infection while the rest 37.5% coccidia positive farms were having mixed infection of *E. tenella* with *E. acervulina*, *E. mitis* and *E. maxima*. *E. tenella* was the most predominant species affecting the farms with 62.5% prevalence score followed by *E. acervulina* (23.43%), *E. mitis* (12.50%) and *E. maxima* (1.56%). Successful result of PCR analysis performed in gut tissues of experimentally infected broiler chickens indicated gut tissue as a better option to oocysts for molecular identification of *Eimeria* species from field cases.

Keywords: Assam, Broiler chicken, *Eimeria*, India, Nested PCR

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Coccidiosis in chicken, clinical or subclinical is an economically important enteric disease caused by the protozoan parasites under the genus *Eimeria*. It continues to be one of the most commonly occurring disease recognized worldwide in the poultry industry. There are seven recognized species of *Eimeria* (*E. acervulina*, *E. brunetti*, *E. maxima*, *E. mitis*, *E. necatrix*, *E. praecox* and *E. tenella*) which under field condition commonly occur as mixed infection in different parts of the intestine to cause high morbidity and mortality in the affected flocks round the year (Hooda *et al.*, 2009). Diagnosis of the disease and accurate identification of *Eimeria* species is not only important to understand the epidemiology of infection but also to formulate effective control strategies using different managemental, chemical and immunological methods (Tewari and Maharana, 2011). Recently, Polymerase Chain Reaction (PCR) based amplification of DNA has been used in accurate identification of *Eimeria* species involved in single or mixed infection and use of PCR assay has gained popularity over the traditional methods for their reliance on genomic sequence of *Eimeria* species (Guven *et al.*, 2013). Perusal of published literature reveals ample epidemiological information based on conventional methodology on coccidia and coccidiosis in chickens from different parts of India (Jadhav *et al.*, 2012). However, these conventional approaches are time consuming and the overlapping of variable morphological and biological features of different species limit their accurate identification in field condition where mixed infection is the rule (Siddiki *et al.*, 2014). Accurate identification of different *Eimeria* species through use of molecular tools has also appeared in a few reports published from the northern and southern regions of India (Kumar *et al.*, 2014, Prakashbabu *et al.*, 2017). The present study reports isolation and molecular identification of different *Eimeria* species circulating in the broiler chickens of Assam which is situated in the north eastern region of India.

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MATERIALS AND METHODS

The study was conducted for one-year period in a total of 45 small scale farms which raised broiler chicken flocks on deep litter for commercial purpose upto 6 weeks of age. Overall, intestines from 109 carcasses of deceased broilers at different ages were collected farm wise during post mortem examinations conducted at the farm premises in the study period. Intestines brought to the laboratory were examined for the detection of Coccidia infection. At first, a small amount of content from the terminal part of each well tied intestine was processed by routine sodium chloride salt floatation method and examined under microscope. Oocysts positive intestines were visually examined for gross lesions, cut open and luminal contents were taken out separately from each portion of intestines (small intestine and large intestine) and put in 2.5% potassium dichromate solution in water at room temperature for 7 days to complete sporulation. Sporulated oocysts suspension was washed several time with distilled water by repeated centrifugation to remove potassium

dichromate, concentrated by salt floatation and stored salt free at 4 ºC until use. Micrometry was performed on sporulated oocysts from each isolate and tentative identification of *Eimeria* species was done by considering the calculated average size (length×width) and shape index (length/ width) of the oocysts.

Experimental infection: Propagation of tentatively identified *Eimeria* species were done in broiler chicks raised from day old in coccidia free environment and supplied with coccidiostat free feed and water *ad libitum*. The oocysts were cleaned with sodium hypochlorite solution (4-6% active chlorine) for 2 minutes and washed in distilled water. Concentration of oocysts/ml of water was determined using neubauer counting chamber. Birds when 2 weeks old were inoculated orally with 2×10^4 sporulated oocysts taking one bird for each tentatively identified species. At the end of 96 hours of oral infection, birds were sacrificed by cervical dislocation and necropsy performed. A portion from each of the five locations (duodenum, jejunum, ileum, caecum and colon) of individual intestines was excised out after gross observation and put separately in Petri dishes. After thorough washing of the cut open pieces, deep mucosal scrapings were obtained and homogenized in 3 ml of ice-cold saline. The homogenates thus obtained bird wise and intestinal portion wise were kept in properly labeled tubes and kept frozen at -20 ºC until further use.

Molecular identification: DNA extraction from each sample was done with DNeasy blood and tissue kit (Qiagen) as per kit protocol. Nested PCR based on the amplification of internal transcribed spacer-I (ITS-I) regions of ribosomal DNA were standardized using genus and species specific primers of *Eimeria* (Table 1) as per Kumar *et al.* (2014). PCR was performed in all DNA extracts with initial denaturation at 94ºC for 2 minute, 30 cycles of denaturation at 94 ºC for 30 second, annealing at 61.8 ºC for 30 second, extension at 72 ºC for 7 minute in techne 500 thermal cycler. PCR product in 5 µl volume mixed with 1 µl bromophenol blue, 6X loading dye and 2.5 µl of 100bp DNA ladder as marker was run in 1.5% agarose gel pre-stained with ethidium bromide (0.5µg/ml) for gel electrophoresis and thereafter visualization of expected product size in a gel documentation system.

RESULTS AND DISCUSSION

All oocysts isolated from the intestines of broiler chickens were tentatively categorized on the basis of lesion study and morphometry into 5 species which included *E. tenella*, *E. acervulina*, *E. mitis*, *E. maxima* and *E. brunetti*. PCR amplification confirmed presence of first 4 species (Fig. 1). Some oocysts recovered from terminal part of intestine and morphologically identified to be *E. brunette*; however, belonged to *E. tenella* in PCR. This reflected morphological variability and spread of *E. tenella* beyond their specific site in heavily infected intestine (Raman *et al.,* 2011). Molecular confirmation of 4 species found to be prevalent in the north east region of India agreed to Prakashbabu *et al.* (2017) except their additional record of *E. necatrix*, *E. brunetti* and *E. praecox*

Table 1

Genus- specific and species-specific primers of *Eimeria*

Parasite	Primer sequence	Product size	Reference
Eimeria genus	EF1: AAGTTGCGTAAATAGAGCCCTC ER1: AGACATCCATTGCTGAAAG	400-600bp	Kumar *et al.* (2014)
Eimeria acervulina (AC)	EAF: GGCTTGGATGATGTTTGCTG EAR: CGAACGCAATAACACACGCT	321bp	
Eimeria maxima (MA)	EMFA1: CT/ACACCACTCACAATGAGGCAC EMR1: GTGAT/ATCGTTC/TGG/AG/AAGTTTGC	145bp	
Eimeria mitis (MI)	EMiiFA : GGGTTTATTTCCTGTCC/GTCGTCTC EMiiRA : GCAAGAGAGAATCGGAATGCC	328bp	
Eimeria brunette (BR)	EBF : GATCAGTTTGAGCAAACCTTCG EBR : TGGTCTTCCGTACGTCGGAT	311bp	
Eimeia necatrix (NE)	ENF : TACATCCCAATCTTTGAATCG ENR : GGCATACTAGCTTCGAGCAAC	383bp	
Eimeria praecox (PR)	EPFA : AAAA/GCAA/CAGCGATTCAAG EPRA : CCAAGCGATTTCATCATT/CGGGGA/G	116 bp	
Eimeria tenella (TE)	ETF : AATTTAGTCCATCGCAACCCT ETR : CGAGCGCTCTGCATACGACA	278 bp	

Table 2

Isolation and identification of different Eimeria species circulating in broiler chicken farms (N=45) of rural Assam

Study District	No. of farms		No. of bird carcasses		Coccidia Prevalence score							
					Farm wise				Species wise			
	Visited	Coccidia positive	Examined	Coccidia positive	TE	TE+AC	TE+AC +MI	TE+AC+ MI+MA	TE	AC	MI	MA
Kamrup	31	27	71	48 (67.61)	18	5	3	1	27	9	4	1
Darrang	7	6	24	13 (54.71)	3	1	2	-	6	3	2	-
Morigaon	7	7	14	7 (50.00)	4	1	2	-	7	3	2	-
Total	45	40	109	68	25	7	7	1	40	15	8	1
		(88.88%)		(62.39%)	(62.50%)	(17.50%)	(17.50%)	(2.50)	(62.50%)	(23.43%)	(12.50%)	(1.56%)

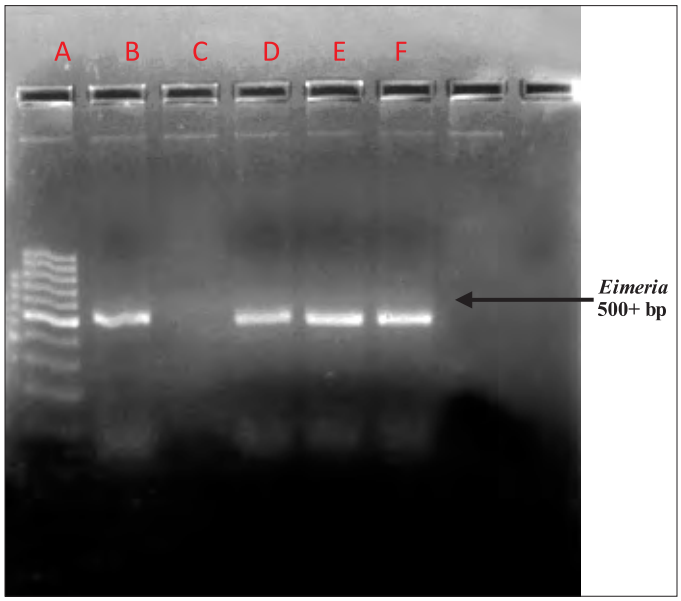

Fig.1. (a) Showing 500+bp *Eimeria* genus DNA in 1.5% agarose gel
LANE A: 100 bp Plus DNA LADDER,
LANE B: Positive control for *Eimeria* genus.
LANE C: Negative control (Nuclease free water)
LANE D, E and F: Positive samples

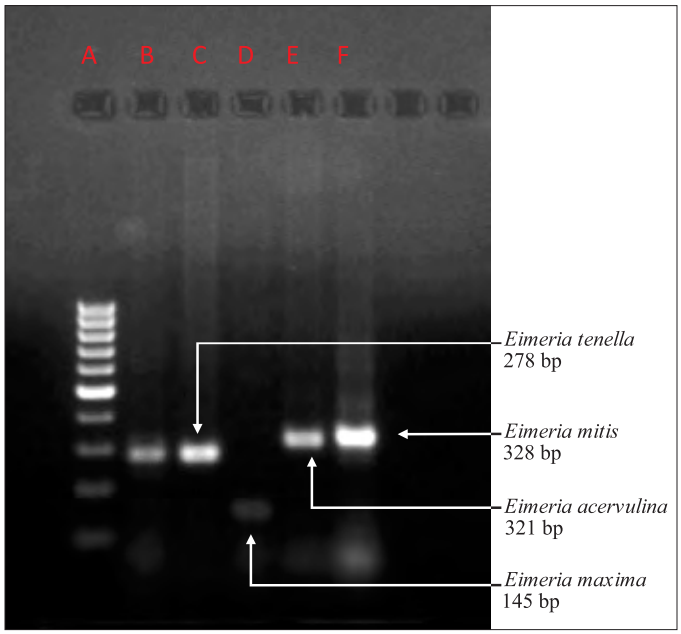

Fig. 1. (b) Showing four *Eimeria* species positive DNA carried out by standardising a same annealing temperature of 61.8 degree centigrade using gradient PCR, viz., *E. tenella* (278bp), *E. acervulina* (321bp), *E. maxima* (145 bp) and *E. mitis* (328 bp) in 1.5 % agarose gel pre-stained with ETBR
LANE A: 100 bp plus DNA LADDER
LANE B: Positive control for *E. tenella*
LANE C: *E. tenella*
LANE D: *E. maxima* LANE E: *E. acervulina* LANE F: *E. mitis*

from the north Indian states and former two species from the south Indian states. In the present study, 88.88% of the broiler farms were found positive for coccidia infection (Table 2) similar to the very high prevalence reports from other parts of India (Thenmozi *et al.*, 2014, Prakashbabu *et al.*, 2017). Farm wise species involvement revealed presence of *E. tenella* in 62.5% farms as mono-species infection while the rest 37.5% coccidia positive farms were having mixed infection of *E. tenella*, with *E. acervulina*, *E. mitis* and *E. maxima* in 2, 3 or 4 species combinations. *E. tenella* was the most predominant species affecting the farms with 62.5% prevalence score followed by *E. acervulina* (23.43%), *E. mitis* (12.5%) and *E. maxima* (1.56%). The present record of *E. tenella* as the most predominant species agreed to the reports made by several workers from the neighbouring geographical regions of India (Jadhav *et al.*, 2012; Swetha *et al.*, 2016; Prakashbabu *et al.*, 2017) and abroad (Haugh *et al.*, 2008 and Iqbal *et al.*, 2017). Most prevalent combination of *E. tenella* and *E. acervulina* observed in the present study also agreed to the reports of Haugh *et al.* (2008) and Prakashbabu *et al.* (2017). The common occurrence of *E. tenella* might exert a greater impact on broiler production due to highest pathogenic potential as opined by many workers (Long *et al.*, 1976; Blake *et al.*, 2015).

Molecular methods have proved to be very useful for accurate identification of *Eimeria* species infecting chickens in different geographical regions (Carvalho *et al.*,

2011). Approaches have been made using PCR in DNAs extracted by various ways from the passed out faecal oocysts which claimed to be sensitive but with a highly variable results (Zhao *et al.*, 2001) due to several limiting factors like low concentration of oocysts, infective DNA extraction during disruption of resistant oocyst wall and faecal inhibition in PCR (Farnandez *et al.*, 2003 and Kumar *et al.*, 2014). Thus, the bottleneck of an effective molecular diagnostic procedure is stated not to be the PCR amplification of genomic coccidia DNA but rather the preparation of DNA from the oocysts samples (Farnandez *et al.*, 2003). In the present study, PCR based test, for identification of *Eimeria* species was performed in gut tissue samples of experimentally infected chickens. The experiment was successful as reported by Nolan *et al.* (2015). Positive PCR indicated presence of developing stages of parasites in gut tissues which could help early detection and discrimination of *Eimeria* spp. in clinical as well as subclinical field cases even before the shedding of oocysts in faeces as suggested by Hassan *et al.* (2016).

Conclusively, the present study confirms presence of *E. tenella*, *E. acervulina*, *E. mitis* and *E. maxima* circulating in the broiler chicken farms of the study area and gut tissue is suggested to be better option for use in molecular discrimination of *Eimeria* spp. when faecal oocysts fail to yield optimum result.

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