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Evaluation of conventional and computational image analysis (COCCIMORPH) methods towards identification of molecular defined species of *Eimeria* from broiler chicken

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Abstract

The present communication reports the results of comparative evaluation of three methods viz. Conventional, COCCIMORPH tool and PCR analysis in identification of *Eimeria* species circulating in the broiler chicken flocks of Assam. A total of 109 carcasses of dead broilers obtained from 45 small scale farms were examined at post-mortem for recovery of oocysts and lesion study in different portions of intestines affected with *Eimeria* infection. Micrometry of sporulated oocysts and the intestinal lesions were considered for identification of *Eimeria* species by the conventional method. Photomicrographs of sporulated oocyst types identified by the conventional method were analysed by digital COCCIMORPH tool for further interpretation on *Eimeria* species. PCR analysis was done in infected gut tissues of birds for amplification of DNA using species specific primers and confirmation of *Eimeria* species.

The study confirmed identification of four species of *Eimeria* - *E. tenella*, *E. acervulina*, *E. mitis* and *E. maxima* infecting broiler chickens of the study area. Identification of former three species was found to be uniform in all the tests. Considering the PCR result as 100% accurate, species identification by the conventional method in the present study was found 60% and 80% in agreement with those of COCCIMORPH and PCR respectively while identification by COCCIMORPH was 75% accurate with the PCR result. The two tests, although showed to be less efficient than the PCR method, may prove valuable when used together in the identification process in absence of a well equipped PCR laboratory.

Keywords: Chicken, *Eimeria*, conventional method, COCCIMORPH, PCR

1. Introduction

Coccidiosis in chicken is a major parasitic disease caused by atleast 7 species of *Eimeria* which includes *Eimeria tenella*, *Eimeria acervulina*, *Eimeria necatrix*, *Eimeria brunetti*, *Eimeria maxima*, *Eimeria praecox* and *Eimeria mitis* [1]. The infection in a susceptible bird is initiated through ingestion of sporulated oocyst of *Eimeria* species and entry of sporozoite stages into the intestinal epithelial cells. Infected birds suffer from varying degree of intestinal damage which results into nutrient malabsorption, inefficient feed utilization and predisposition to clostridial infection and necrotic enteritis [2]. Affected birds exhibit depression, ruffled feather, anorexia, diarrhoea with or without blood, weight loss and high mortality. Under field condition, multiple species infection causing subacute, acute clinical or chronic morbid process is a common occurrence. The disease has been known for many years and still considered as the most economical one affecting poultry production worldwide [3]. Major loss in broiler industry is due to subclinical coccidiosis by impact on the body weight gain and feed conversion ratio [4].

Identification of *Eimeria* spp. circulating in the chicken flocks of a geographical region is important for adoption of prevention and control measures. Conventionally, identification of different *Eimeria* spp. involved in the disease process is performed on the basis of their biology (sporulation time, pre patent period, patent period), morphology of oocysts and the lesions produced in different portions of the intestine [5]. There is considerable overlapping in the morphology of the oocysts, biology and intestinal lesions produced by different species [6]. Moreover, the oocysts lack a differentiating morphological feature for which a single criterion is usually insufficient for species identification. Kucera and Reznicky [7] introduced a computerized image analysis method based on the length and width measurement of the oocysts for species differentiation of fowl coccidia. Later on Castanon *et al.* [8] developed a

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computerized oocyst image analysis software called COCCIMORPH for identification of chicken *Eimeria* oocysts. This involved uploading of digital images of unidentified sporulated oocysts in the software and obtain automated species assignment. Recently, molecular techniques have been proved to be very useful and recognized as gold standard for accurate identification of different *Eimeria* spp from the oocysts [9, 10] and/or infected intestinal tissues [11]. Depending on the facilities available the three methods have been employed either individually or in combination for species identification of poultry coccidian [1, 12]. The present investigation was undertaken with an objective to evaluate the conventional, image analysis (COCCIMORPH) and PCR methods on a comparative basis in determination of different species of *Eimeria* prevalent in broiler chickens raised in Assam (India).

2. Materials and Methods

During an epidemiological study of coccidia and coccidiosis in broiler chickens of Assam, 109 carcasses of dead broilers at different ages up to 6 weeks were collected from a total of 45 small scale farms for post-mortem examination and detection of lesions due to coccidia infection in the intestine. Portions of the intestine (duodenum, jejunum, ileum, caecum and colon) with gross lesions visible from their serosal surface and suggestive of coccidia infection were separated out, cut open longitudinally and the contents adhering to the mucosal surface were removed gently by washing with cold normal saline solution. Mucosal scrapings were obtained and examined under microscope for detection of oocysts of *Eimeria*. Scrapings found positive to only one type oocysts were homogenized individually in normal saline solution and divided into two parts, the major part mixed with 2.5% potassium dichromate solution and kept for sporulation of oocysts at room temperature while the remaining part put into properly labelled 2-ml PCR tubes and kept at -20°C for PCR analysis.

2.1 Micrometry and image analysis

Sporulated oocysts were made potassium dichromate free by repeated changing with water and sedimentation by centrifugation. The oocysts thus collected from designated

samples were at first measured using micrometry scales under 400 X magnification. Calculated average size based on measurements of 20 oocysts and shape index were considered along with corresponding lesions in the affected intestine portion for identification of *Eimeria* species by the conventional method [6].

Photomicrographs of the measured oocysts were taken with a photo micrographic camera for image analysis. The COCCIMORPH software tool (<http://www.coccidia.icb.usp.br/coccimorph>) was downloaded from the internet and the digital images of each conventionally identified oocyst types were uploaded for their interpretation [8].

2.2 Polymerase Chain Reaction (PCR) analysis

PCR analysis was done for amplification of DNA of *Eimeria* species from the infected gut tissues [11]. DNA extraction from the designated tissue homogenates corresponding to the conventionally identified oocyst types was done with D'Neasy blood and tissue kit (Qiagen) as per manufacturer's protocol. Amplification of template DNA in PCR was done separately for each tentatively identified species using primers specific for *E. tenella*, *E. acervulina*, *E. necatrix*, *E. brunetti*, *E. maxima*, *E. praecox* and *E. mitis* [1] with pre standardized thermocyclic conditions of initial denaturation at 94°C for 3 minutes followed by 30 cycles of 30 sec at 94°C, annealing at 62°C for 30 sec, extension at 72°C for 1 min and final extension at 72°C for 7 min. Amplified PCR products were checked by gel electrophoresis in 1.5% agarose gel prestained with ethidium bromide (0.5 µg/ml), visualisation of the product in gel documentation system and identification by size using a 100bp ladder.

2.3 Data analysis

The performance of Conventional and COCCIMORPH tools in identification of *Eimeria* species present in the study area was estimated by comparison of their respective results with that of PCR analysis.

3. Results and Discussion

The speciation of *Eimeria* by the Conventional method, COCCIMORPH tool and PCR analysis is presented in table 1.

Table 1: Speciation of *Eimeria* by Morphometry, COCCIMORPH tool and PCR assay

Isolates	Criteria		Micrometry of sporulated oocysts			Coccimorph identification	PCR identification
	Shape	Location	Mean oocyst size (µm)	Mean shape index	Likely species		
1	Large ovoid	Small intestine	26.6 X 20.02	1.4	<i>E. maxima</i>	<i>E. acervulina</i>	<i>E. maxima</i>
2	Medium ovoid	Caecum	22.88 X 20.02	1.14	<i>E. tenella</i>	<i>E. tenella</i>	<i>E. tenella</i>
3	Medium ovoid	Colon	18.79 X 14.3	1.31	<i>E. brunetti</i>	<i>E. tenella</i>	<i>E. tenella</i>
4	Small ovoid	Small intestine	17.16 X 14.3	1.2	<i>E. acervulina</i>	<i>E. acervulina</i>	<i>E. acervulina</i>
5	Small spherical	Small intestine	8.58 X 8.58	1	<i>E. mitis</i>	<i>E. mitis</i>	<i>E. mitis</i>

Based on the shape and size of the oocysts and from the intestinal lesion, the conventional method identified 5 types of *Eimeria* species affecting broiler chickens of the study area. These included *E. tenella*, *E. acervulina*, *E. maxima*, *E. mitis* and *E. brunetti*. Application of the COCCIMORPH tool showed their identification into 3 species viz. *E. tenella*, *E. acervulina* and *E. mitis*. PCR assay confirmed identity of 4 out of 5 conventionally identified *Eimeria* species which excluded *E. brunetti*.

Comparing the results, identification of *E. tenella*, *E. acervulina* and *E. mitis* was found to be uniform in all the three tests employed in the present investigation.

Identification of species by the conventional method was found 60% in agreement with that of COCCIMORPH tool and 80% in agreement with the PCR results. COCCIMORPH tool identified species conformed to 3 out of 4 species identified in PCR and the uniformity was calculated as 75%. *E. maxima* identification was found to be uniform in Conventional and PCR methods only. In contrast, it was misidentified as *E. acervulina* in COCCIMORPH tool application. *E. brunetti* identified by conventional method was found to be *E. tenella* in both COCCIMORPH and PCR analysis.

Determination of *Eimeria* species prevalent in a geographical

location is important in epidemiological investigation and also for development of an area specific vaccine to control coccidiosis [12]. Conventional method based identification of *Eimeria* species has been employed for long and is being most frequently applied till today. In the present study, identification of *E. tenella* and *E. acervulina* could be accomplished easily on the basis of the lesions produced in the caecum and the anterior most (duodenum) portion of the intestine respectively. *E. maxima* was identified on the basis of oocyst size which was the largest of all species and developed in the mid small intestine. Likewise, *E. mitis* oocyst being the smallest and consistently spherical in shape could be identified easily by the Conventional method. Tentative identification of these species conformed to the reports published elsewhere [13, 14]. However, oocysts obtained from the lesion in the colon of a bird and tentatively identified as *E. brunetti* was in turn confirmed as *E. tenella* in COCCIMORPH and PCR applications. From the present findings it appears that micrometry or lesion study can't be considered alone for species identification by the conventional method. This agrees to the earlier reports that the difficulties in proper identification is due to variation as high as upto 40% in the size and shape of the oocysts [15]. Similarly, the lesions produced by different species of *Eimeria* in the intestine may extend beyond their specific site and cause overlapping lesions in heavy infection [16]. Additionally, mixed infection might hamper accurate identification of species in field situation by the conventional method when used in isolation [1, 5, 16].

In the present study, identification of *E. tenella*, *E. acervulina* and *E. mitis* was found to be uniform by all the three methods. The findings are in agreement with Kumar *et al.* [1] and Pant *et al.* [17] who reported COCCIMORPH to be the most effective tool for identification of *E. acervulina* and *E. mitis*. Siddiki *et al.* [18] also documented identical results. Considering PCR as 100% accurate, species identification by the conventional method in the present study was found 60% and 80% in agreement with those of COCCIMORPH and PCR respectively while identification by COCCIMORPH tool was found to be 75% accurate with the PCR result. The correctness of species assignment by Conventional or COCCIMORPH tool was estimated to be 80% of the PCR confirmed result which agreed to the earlier reports [1, 18] that the former methods are less efficient than the PCR method.

4. Conclusion

Conclusively, the study identified *E. tenella*, *E. acervulina*, *E. mitis* and *E. maxima* in the broiler chickens of the study area. Although Conventional method and the COCCIMORPH tool could not prove their efficiency in identification of chicken *Eimeria* species at par with the PCR result, it is suggested that these two methods in combination and with little expertise could be valuable in the identification process in absence of a well equipped PCR laboratory.

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