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Optimization of single step multiplex PCR for detection of *Eimeria tenella* and *Eimeria necatrix* from commercial broilers

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Coccidiosis is an enteric parasitic disease of chicken caused by protozoan parasites of genus *Eimeria* (Apicomplexa: Eucoccidia: Eimeriidae). *E. tenella, E. necatrix, E. acervulina, E. maxima, E. brunetti, E. mitis,* and *E. praecox* are the 7 species of *Eimeria* which are considered as main causative agents of coccidiosis in chicken (Kumar *et al*. 2015). Coccidiosis causes production losses and high morbidity due to acute bloody enteritis. The timely and specific diagnosis of coccidiosis plays a key role in prevention, surveillance and control of disease. Traditionally, diagnosis was achieved by enumerating *Eimeria* oocysts excreted in feces of infected chickens, measuring oocyst/sporocyst dimensions and assessing the site and extent of the pathological lesions caused by *Eimeria* in the intestine of chickens (Yang *et al*. 2015). But the unsporulated oocysts of *Eimeria* species are difficult to differentiate (Godwin and Morgan 2014). Furthermore, the mixed infections can pose a problem for the precise determination of the type of coccidiosis and the intestinal site of the lesions (You 2014). Therefore, these approaches can be unreliable, particularly given that multiple species of *Eimeria* can simultaneously infect the host and because there can be an "overlap" in the sizes of oocysts and the sites of infection in the intestines for some species. Increasingly, molecular tools were developed and relied upon for the diagnosis of coccidiosis. Diagnostic laboratories are increasingly utilizing DNA based technologies for identification of *Eimeria* species due to difficulties in morphological identification. In order to overcome these diagnostic difficulties the present study was designed with an aim to detect *Eimeria tenella* and *Eimeria necatrix* by targeting ITS–2 gene in a single step multiplex PCR.

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One hundred fecal samples of broiler birds from 10 commercial broiler farms were collected from Lahore. The samples were collected in zipper bags and placed in ice jars. The samples were collected carefully to avoid any contamination with soil or other contaminants and stored at 4°C till processing. Firstly, simple microscopy was performed to observe the Eimerial oocysts. Slides were examined at 40X. Secondly, microscopy and micrometry was performed on fecal samples after flotation and sedimentation technique. Micrometry by simple microscopy was performed for Eimerial oocysts as shown in Fig. 1 and Table 1, but it was not concluded which *Eimeria* are there by comparing with the keys from parasitology manuals.

DNA extraction from fecal samples and CocciVac D vaccine was performed by using Axygen DNA extraction kit. The protocol was carried out according to the manufacturer's instructions with little modification that the lysis of the oocysts was performed over-night at 58°C. Primers of *E. tenella* and *E. necatrix* were designed with help of MPprimer tool and dimerization of two pairs of primers were checked by MFEprimer-2.0 which is a fast thermodynamics-based program for checking PCR primer specificity. The primers were thus designed as shown below:

Primer	Primer sequence	Product size
E. necatrix Forward	AAAAGCATGCCGCGTCTG AGGG	643bp
E. necatrix Reverse	ATATGCGGTCCCGCCCGA AAGA	
E. tenella Forward	TCGCTCATGCGGCAGTACG TTG	261bp
E. tenella Reverse	CATTCCGCATCGGCAGCT CAGT	

The PCR reaction was performed in a total volume of 20µl reaction mixture containing 10 pM of each primer, Master Mix (2X) containing nTaq DNA Polymerase 0.2 units/µl, nTaq buffer (containing 3 mM Mg^{2+}), dNTP mixture: 0.4 mM each. The PCR was employed for five min at 95°C followed by 35 cycles of 95°C for 30 sec, the annealing temperature 58°C for 30 sec, and 72°C for one

Table 1. Micrometry of Eimerial oocysts of CocciVac D vaccine

1.	35×27.5 µm
2.	15×17.5 µm
3.	20×12.5 µm
4.	30×27.5 µm
5.	25×25 µm
6.	20×15 µm
7.	40×35 µm
8.	45×35 µm

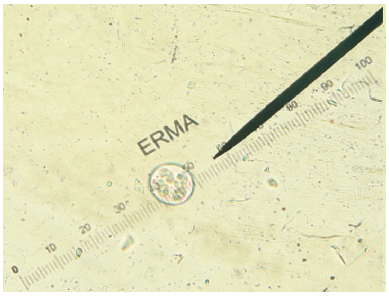

Fig. 1. Micrometry of Eimerial oocysts of CocciVac D vaccine.

minute. Commercially prepared vaccine CocciVac D was used as positive control.

The optimized multiplex PCR was employed for detection of *E. tenella* and *E. necatrix* in the field conditions from the commercial broilers feces. Out of 100 samples we found six *E. tenella* and only one *E. necatrix* positive by this method. The detection limit of oocysts by this method was as least as 16 oocysts. These results are in contrary with Awais *et al*. (2012) that revealed 27.4% prevalence of *E. tennela* and 4% *E. nectarix* in Faisalabad, Punjab. The high prevalence in Faisalabad may also be due to non-premixing of anticoccial drugs in commercial broilers feed in Faisalabad.

There has not been much work on coccidiosis in Pakistan. The exact detection of *Eimeria* species by using modern molecular techniques is therefore essential for disease surveillance and eradication. This technique is novel as we ourselves designed its primers for the first time. These primers were specific and sensitive and allowed amplification of samples containing as few as 16 oocysts as compare to the previous studies of Carvalho *et al*. (2011) who had a detection limit of 20 oocysts. However, our oocyst detection limit was low in comparison to the multiplex PCR proposed by Fernandez *et al*. (2003) who showed a threshold of 1–5 pg, which corresponded approximately to 2–8 sporulated oocysts.

We concluded that this newly optimized multiplex PCR can serve as a rapid and cost-effective diagnostic method for the detection and discrimination of *Eimeria* species in commercial broilers. Moreover, this technique can be extended upto the detection of 7 species of *Eimeria* that will be used to detect multiple coccidian infections in poultry. Thus, this research would be reference test in Pakistan to detect coccidiosis.

SUMMARY

Multiplex polymerase chain reaction (PCR) was optimized for detection of 2 important species of *Eimeria* (*Eimeria tenella* and *E. necatrix*). This optimized protocol was used to screen 100 fecal samples collected from broiler birds in Lahore periphery. Out of 100 samples we found 6 *E. tenella* and only 1 *E. necatrix* positive by this method. The detection limit of oocysts by this method was as least as 16 oocysts. This optimized multiplex PCR method can be used as routine diagnostic tool for detection of *E. tenella* and *E. necatrix* and can be extended up to the detection of 7 *Eimeria* species in future.

REFERENCES

Awais M M, Akhtar A, Iqbal Z, Muhammad F and Anwar M I. 2012. Seasonal prevalence of coccidiosis in industrial broiler chickens in Faisalabad, Punjab, Pakistan. *Tropical Animal Health and Production* **44**: 323–28.

Carvalho S, Wenceslau A A, Teixeira M, Carneiro J A M, Melo A D B and Albuquerque G R. 2011. Diagnosis of *Eimeria* species using traditional and molecular methods in field studies. *Veterinary Parasitology* **176**: 95–100.

Fernandez S, Pagotto A H, Furtado M M, Katsuyama A M, Madeira A M B N and Gruber A. 2003. A multiplex PCR assay for the simultaneous detection and discrimination of the seven *Eimeria* species that infect domestic fowl. *Parasitology* **127**: 317–25.

Godwin R M and Morgan J A T. 2014. A simple, one-tube assay for the simultaneous detection and diagnosis of ten Australian poultry *Eimeria*. *Electrophoresis* **4**: 494–502.

Kumar S, Garg R, Banerjee P S, Ram H, Kundu K, Kumar S and Mandal M. 2015. Genetic diversity within ITS–1 region of *Eimeria* species infecting chickens of north India. *Infection, Genetics and Evolution* **36**: 262–67.

Yang W C, Tien Y J, Chung C Y, Chen Y C, Chiou W H, Hsu S Y, Liu H Y, Liang C L and Chang C L. 2015. Effect of ***Bidens pilosa*** on infection and drug resistance of ***Eimeria*** in chickens. *Research in Veterinary Sciences* **98**: 74–81.

You M J. 2014. Detection of four important *Eimeria* species by multiplex PCR in a single assay. *Parasitology International* **63**: 527–32.