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# Prevalence of *Eimeria bovis* in cattles of Cholistan desert, Pakistan

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**Abstract**

Protozoan parasites are responsible for a number of diseases in livestock thus affecting a country economy. The coccidiosis is very common parasitic disease in livestock of Pakistan especially in desert animals. A valid procedure was used to detect *E. bovis*in fecal samples of cholistanicow. PCR reaction was performed using species specific primer for amplification of ITS-1 region of rRNA gene. *E. bovis* was confirmed in fecal samples obtained cholistani cow while *E.* zuernii was absent. The PCR based detection of *Eimeria* parasites seemed highly sensitive when compared with traditional morphological examination of oocytes.

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## Introduction

Protozoan parasites cause a number of diseases in dairy animals all over the world especially in developing countries, therefore considered as a major constraint on dairy farming (Om *et al.,* 2010). Coccidiosis, an important intestinal disease responsible for mortality and morbidity in cattles caused by different species of genus *Eimeria*. The clinical signs for coccidiosis include bloody diarrhea, straining, appetite loss, fever, debility, weight loss and death in severe case (Nalbantoglu *et al.,* 2008). The incidence of *Eimeria* species including *E. bovis, E. auburnesis, E. canadensis, E. ellipsoidalis/ alabamensis, E. brasiliensis, E. cylindrica, E. zuernii, E. wyomingensis, E. subspherica* and *E. pellita*. In naturally infected Calves in north wales (Kind *et al.,* 2007).

Scientific information on cattle parasites particularly on coccidiosis epidemiology is limited with reference to desert. A lot of literature is available on coccidiosis in poultry (Akhtar *et al., 2012)* but little information is available on coccidiosis in ruminants. The prevalence and types of coccidia species in slaughter goats and sheep has been found in Tanzania (Kusiluka *et al.,* 1996). Different molecular techniques involve already available sequence of many invertebrate species, accessibility of molecular probe in the form of commercial kits and species specific primers can easily be manufactured. Techniques like SDS-PAGE, RAPD, SSR and other considered to be efficient for molecular analyses. Among them studies on sequence or identification of rRNA subunits demonstrate ancestral or detection studies efficiently (Olsen and Woese, 1993).

The studies related to variations in 18S rRNA gene sequence in bovine coccidia are still lacking (Li *et al.,* 2007). Different methods for molecular identification of apicomplexa using PCR are in progress but an attractive genomic DNA target is the internal transcribed spacer (ITS-1) derived from rRNA gene and results devired are not available (Schnitzler *et al.,* 1998, 1999). Due to heterogeneity of both sequence compositions (5'-3') and lengths among different species, the ITS-1 region is a promising target to design the specific primers (Kawahara *et al.,* 2008).

Keeping in view the reliability and applicability of PCR assays depending on the specific ITS-1 region for identification of bovine Eimeria, present project was performed to confirm the presence of *Eimeria* spp. in fecal samples of Cholistani cattle.

## Materials and methods

*Field data*

A total of 200fecal samples were randomly collected from Cholistan desert (n=145 Lesser Cholistan) while (n=55 Greater Cholistan). A research proforma was also filled at sampling site to record the field data. Fecal samples were examined for the presence of oocyte using a microscope. Different species of *Eimeria* (*E. bovis*and *E.* zuernii) were identified morphologically with 25 oocyte in each sample according to protocol given by (Levine and Ivens, 1970). The oocytes were separated from fecal debris and concentrated by flotation technique method using saturated sodium chloride solution. The whole procedure and laboratory protocol were approved by ethical committee of Dept. of Life Sciences, The Islamia University of Bahawalpur, Pakistan.

*Laboratory work*

The suspended acolytes were sedimented and later resuspended in lysis buffer. The suspension was transferred to round bottom flask and vortexed to disrupt the oocytes. DNA was extracted from lysate using commercial DNA kit. Genus common primer with Forward GCAAAAGTCGTAACACGGTTTCC Gand Reverse CTGCAATTCACAATGCGTATCGC sequence containing whole ITS-1 region were used. A total of 20µl reaction mixture was used to amplify ITS-1 region containing 10µl *Taq*, 1µl of 10 µM primer set (0.5 µM each), 1µl of genomic DNA, dNTPs and 3.5mM magnesium chloride. PCR cycles consisted of an initial denaturing step at 94°C for 30 s followed by 35 cycles at 94°C for 10 s, 55°C for 30 s, 72°C for 30 with final extension at 72°C for 2min using Thermal Cycler (Life Technologies Corporation, CA, USA). The PCR products were run on agarose gel electrophoresis for their size analysis.

Species specific primer for *E. bovis*with the sequence Forward TCATAAAACATCACCTCCAA and Reverse ATAATTGCGATAAGGGAGACA while for *E. zunerii* primer with the sequence Forward AACATGTTTC-TACCCACTAC and Reverse CGATAAGGAGG-AGGACAAC were used for parasitic species detection in the sample (Kawahara *et al.,* 2010). The reaction mixture and PCR condition used were the same as mentioned above except for the 35 cycles which at 94°C for 10 s,55°C for 20 s, 72°C for 20 s. After amplification, 10µl of PCR product was electrophoresed on a 1.5% agarose gel with 100 bp DNA ladder (Fermantas, Japan). DNA fragments were revealed visually with ethidium bromidestainin gunder UV illumination. Moreover, DNA extracted from a mixed isolates containing 5000-10000 oocysts/ml was used in PCR assays with genus common and species specific primer sets.

**Results**

Fecal samples were separated to isolate the bovine coccidiaoocysts. Fecal isolates were separated in a way that each isolate contain single *Eimeria* spp. The protocols for design of ITS-1 region of rRNA in *bovine*. *Eimeria* for two parasite species were used as given by (Kawahara *et al.,* 2010) for forward and reverse primers. The PCR products were separated on 1.3% agarose gel, later stained with ethidium bromide and photographs were taken under ultra violet light. All the fecal sample isolates separated on morphological basis for parasitic presence were positive for *E. bovis while* no sample was positive for *E. zunerii*. The absence of a parasitic species from fecal sample isolate indicates its absence.

In the field isolates, ITS-1 region based PCR products were successfully amplified with genus common primers. The obtained ITS-1 region based products were compared with morphologically separated oocysts to determine the fragment size of *Eimeria* spp. Genus common primer separate the products between 350-550 bps. Later the species specific primers were applied and the obtained *E. bovis* fragments were consistent in all isolates without any variation, composed of 238 bps fragment (Fig. 1).

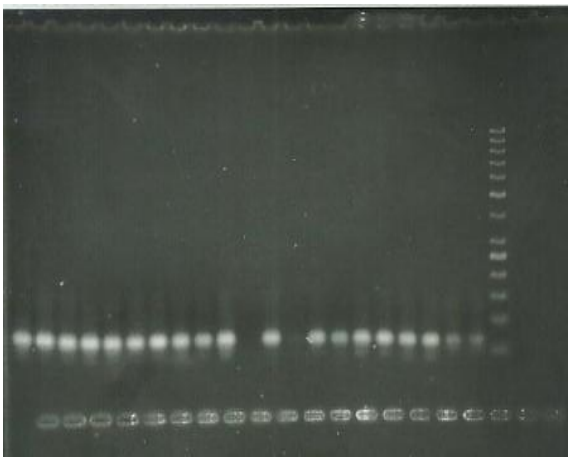

**Fig. 1.** Gel showing species specific primer based amplification from *E. bovis* DNA.

(Alphabets represent amplified PCR products obtained from available *E. bovis*oocyte DNA samples).

The mixed oocysts samples were further evaluated with species specific primer through PCR assay. Genomic DNA obtained from a mixture of sample containing 5000 oocysts/ml each of two species was prepared. It was assumed that the amount of DNA in reactive solution contained approximately 10-15 oocysts and there is no loss during the PCR processing. So the species specific primer amplified a specific parasitic species with expected fragment size. *E. bovis* produce a single band in each sample obtained from an isolate.

**Discussion**

In the present research work, inter species specific DNA sequence in ITS-1 region of the rRNA genes of two *Eimeria spp*. was done to analyze their presence in fecal samples. The ITS-1 region when compared with rRNA genes, have low intra specific and high inter specific variations in DNA sequence, and they also minimize the risk of cross reaction with other species (Kawahara *et al.,* 2010).

The complete amplification of ITS-1 fragment from *E. bovis* confirmed the success of PCR assay. There was no cross amplification of DNA samples in all isolates. In mixed species template *E. bovis* primer amplified the specific fragment indicating the success ratio of primer used for amplification. The ITS fragment is a direct tool for differential diagnosis even it also check the differences within the genus (Costa *et al.,* 2001).

Further the DNA fragment corresponding to 15 or less oocysts of each species proved the efficiency of PCR assay. It can be assumed that PCR assay is more sensitive when compared with oocysts count method i.e. McMaster's method which can quantify only 100 oocysts in 1g of fecal sample (Martynova Van Kley, 2008).

Highly variable DNA bases sequence was observed at ITS fragments. This was very helpful in identifying *E. bovis* from *E. zuernii*. These findings are in line with the results given by Schnitzler *et al.*, (1999). The amplification of PCR products through ITS-1 rRNA gene is similar to the findings given by Woods *et al.,* (2000) where he used RFLP-PCR to amplify ITS-2 by specific primers and obtained DNA products of six other species belonged to genus *Eimeria*.

The data compiled from field proforma showed while considering different species belonged to genus Eimeria, *E. bovis*is critically pathogenic among bovine coccidian (Hoge *et al.,* 1995). Young stock seemed more susceptible to parasitic infections as compared to mature individuals due to lack of immunity. Similarly females were badly affected with parasitic diseases as compared to males in stock. High parasitic prevalence was observed during rainy and post rainy season (Ali *et al.,* 2009).

One probable reason for this may be the high rainfall, humidity and low temperature which favored the development of oocysts (Raza *et al.,* 2014). An unhygienic condition also serves as an important factor for prevalence of enteric protozoan sin the livestock.

## Conclusion

PCR products obtained from ITS-1 region (rRNA) based species specific primer revealed that *E. bovis* was detected in cholistani cattle fecal oocytes. Here the PCR method proved extremely specific and sensitive. Such studies will be helpful to treat these livestock animals to get better productivity.

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