Haryana Vet. (June, 2018) **57 (1)**, 14-16

Research Article

MOLECULAR IDENTIFICATION OF *EIMERIA* SPECIES OF BUFFALO CALVES OF HARYANA USING ITS-1 GENE

NAVJOT NAIN[1], S.K GUPTA[1*], PIYUSH TOMAR[2], ARUN K. SANGWAN[1] and SNEHIL GUPTA[1]

[1]Department of Veterinary Parasitology; [2]Department of Veterinary Public Health and Epidemiology

College of Veterinary Sciences Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar-125004, India

ABSTRACT

Coccidiosis is one of the major problems in buffalo calves that is caused by protozoan parasite of genus *Eimeria.* In this study, PCR technique was standardized for rapid identification of genus *Eimeria* as well as different *Eimeria* species of buffalo calves. After detection of *Eimeria* species by conventional micrometry, a total of five common species of *Eimeria* namely *E. bovis*, *E. zuernii*, *E. alabamensis*, *E. cylindrica* and *E. auburnensis* were selected to standardize the species specific PCR. The PCR results were in concordance with the micrometry. Four species were also subjected to sequencing and phylogenetic analysis, which revealed that *E. bovis* and *E. zuernii* Indian isolates were closely related to Turkey and Japan isolates. *E. cylindrica* of Indian origin formed a separate clade with Japanese isolate, whereas, *E. alabamensis* isolate shared the same clade with Japanese isolate. The present study suggests that the PCR assays developed for different *Eimeria* spp. provide a simple, quick and precise tool to specifically detect these parasites from the field samples, which, otherwise, are difficult to be identified using routine parasitological techniques. It seems that this is the first study on molecular detection of *Eimeria* species prevalent in buffalo calves in India.

Keywords: Buffalo calf, Coccidiosis, *Eimeria* species, ITS-I region, India

Prevalence of coccidiosis in bovines has been reported from different parts of India; all these studies are based on morphological examination of oocysts and sporulation time. However, identification based on morphology may be unreliable particularly with mixed infections (Vladmir *et al*., 2010). Moreover, some species show morphological resemblance with each other (Kokuzawa *et al*., 2013). Therefore, molecular characterization is required to accurately know the epidemiology of different species and to infer phylogenetic relationship among different species. So far no study seems to have been carried out on molecular characterization of *Eimeria* species of buffaloes in India. Hence the present study was planned to standardize the PCR for *Eimeria* as well as molecular characterization of *Eimeria* species based on sequencing.

MATERIALS AND METHODS

Sample Collection:

A total of 427 faecal samples of buffalo calves from four districts of Haryana, namely, Fatehabad, Bhiwani, Sirsa and Hisar were collected and used. The samples were placed in a labelled clean polythene cover and transported to the laboratory on the same day of collection and were preserved at 4°C in refrigerator until processing. Standard floatation technique using saturated salt solution was carried out for the presence of coccidian oocysts under a microscope at 10x and 40x. Photographs of different *Eimeria* oocysts were taken and micrometry was done with the help of trinocular digital microscope (Carl Zeiss, Germany). Sporulation of oocysts was completed after 17 days using standard sporulation technique and various *Eimeria* oocysts (Nain *et al*., 2017) were identified with the help of standard taxonomic keys (Soulsby, 1982; Kennedy and Kalka, 1987; Sommer, 1998). Pooled faecal sample containing oocysts of five *Eimeria*s pecies *viz*. *E. bovis*, *E. zuernii*, *E. alabamensis*, *E. cylindrica* and *E. auburnensis* was used to standardize the PCR. The positive samples were preserved at - 20°C for molecular confirmation.

Molecular identification of different *Eimeria* species present in buffalo calves:

Extraction of genomic DNA from faeces was carried out by conventional phenol chloroform isoamyl alcohol (PCI) method with slight modification (Sambrook *et al.*,1989). Before extraction of DNA, faecal oocysts were concentrated using the standard floatation technique. The previously published primers (Kawahara *et al.,* 2010) were used to identify genus *Eimeria* and its five species, namely, *E. alabamensis, E. auburnensis, E. bovis, E. cylindrica* and *E. zuernii* (Table 1). A total of 25µl reaction mixture was used to amplify ITS-1 region containing 12.5 µl hot start master mix (Thermoscientific), 0.5µl of each primer set (10µM each) and 3µl template DNA (100ng/µl). The amplification was performed using a thermal cycler with the reaction cycle consisting of initial denaturation at 94°C for 5 min. followed by 35 cycles of denaturation at 94°C for 10 sec., annealing at 61°C for 30 sec. and extension at 72°C for 30 sec. followed by an elongation at 72°C for 2 min. and finally stored at 4°C for further use. After amplification, 5 µl of PCR product was loaded in 1.5% agarose gel. The amplicons were electrophoresed and visualised under UV gel documentation system Purified PCR products of four different *Eimeria* spp. were sequenced in both directions using Automated DNA Sequencer Applied Biosystem® 3130 XL Genetic Analyzer in the Department of Animal Biotechnology, LUVAS, Hisar. The nucleotide sequences

*Corresponding author: drsurengupta@gmail.com

Table 1

Genus and species-specific primer sets used for PCR amplification of ITS-1 region of *Eimeria*

Species	Forward Primer 5' to 3')	Reverse Primer (5' to 3')	Expected product size (bp)
Genus-common	gcaaaagtcgtaacacggtttccg	ctgcaattcacaatgcgtatcgc	348-546
E.alabamensis	cattcacacattgttctttcag	gcttccaaactaatgttctg	184
E. auburnensis	taaattggtgcgatgaggga	gcaatgagagaaagatttaata	295
E. bovis	tcataaaacatcacctccaa	ataattgcgataagggagaca	238
E. cylindrica	gacatttaaaaaaccgattggt	ggctgcaataagatagacata	304
E. zuernii	aacatgtttctacccactac	cgataaggaggaggacaac	344

of *E. alabamensis, E. bovis, E. zuernii, a*nd *E. cylindrica* were aligned using MEGA 7 (molecular evolutionary genetic analysis) software by Clustal W method. The evolutionary distances were computed by pair-wise distance method using the Maximum Composite Likelihood Model. Percentage identity was computed by MegAlign programme (Lasergene; DNASTAR, Madison,

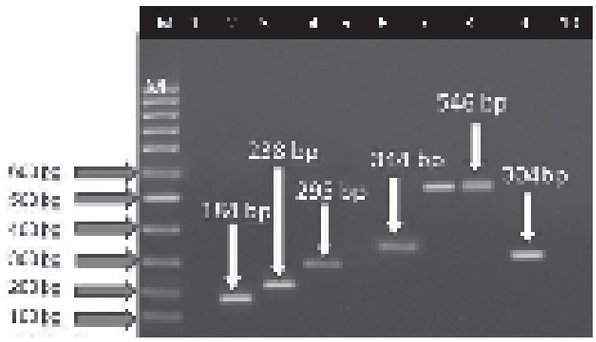

Fig.1: Agarose gel electrophoresis of PCR product of various *Eimeria* species: Lane 2*(E. alabamensis*), Lane 3 (*E. bovis*), Lane 4 (*E. auburnensis*), Lane 6 (*E. zuernii*), Lanes7 and 8 (Genus common) and Lane 9 (*E. cylindrica*). M: Marker ladder (100 base pair)

WI, USA). A phylogenetic tree of aligned sequences was constructed by Neighbor-Joining method (1000 replicates for bootstrap). Thirteen unrelated strains/isolates of different *Eimeria* species were downloaded and aligned with our *Eimeria* spp. isolates to construct phylogenetic tree.

RESULTS AND DISCUSSION

In the present study, PCR reaction was optimized for the common five *Eimeria* spp. of buffalo. The DNA sequences generated for ITS-1 region of five *Eimeria* spp. were used to study the prevailing genetic diversity. The ITS-1 region sequences showed marked differences in various species of *Eimeria* suggesting an evolutionary pattern of low intra-specific and high inter-specific variations in the DNA sequences as reported by Kawahara *et al* (2010). The conventional PCR using ITS-1 region for *Eimeria* genus and five *Eimeria* species revealed DNA bands of 546 bp for genus, 184 bp for *E. alabamensis*, 295 bp for *E. auburnensis*, 238 bp for *E. bovis*, 304 bp for *E. cylindrica* and 344 bp for *E.zuernii* (Fig.1). The phylogenetic tree constructed based on ITS-1 region sequence revealed that *E. bovis* and *E. zuernii* Indian isolates are closely related to Turkey and Japan isolates, whereas, *E. cylindrica* of Indian origin formed a different clade with Japanese isolate (AB557616.1) suggesting strain variation with geographical isolation. *E. alabamensis* isolate shared the same clade with Japanese isolate (AB557607.1) showing evolutionary relationship (Fig. 2). Similarly, Kawahara *et al.* (2010) analysed their generated sequences to investigate the homology between *Eimeria* isolates of Japan.

Although classical approach of morphometry is less expensive as no highly technical instrument and facility is required, the sensitivity of the test is compromised and there might be more false positive cases. The present study proved superior to conventional morphometry and might be helpful for conducting molecular epidemiological investigations of coccidiosis in the entire country. A key step towards the use of *Eimeria* species-specific PCR can serve as a sensitive and

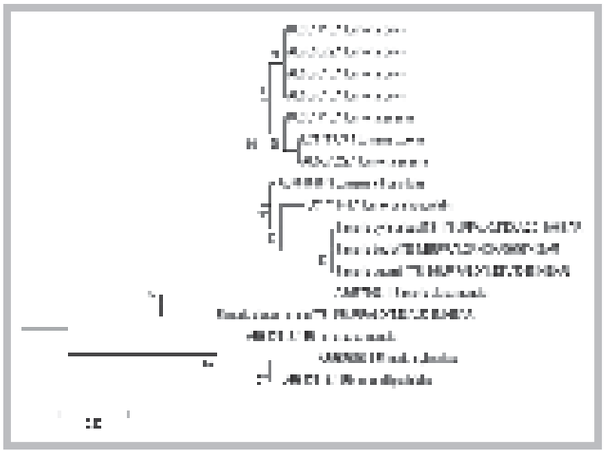

Fig.2: Phylogenetic tree based on partial nucleotide sequences of ITS-1 region of rRNA of four species of *Eimeria.* Phylogenetic tree was constructed by the neighbour joining method using 1000 bootstrap replicates value in Mega7 software. Sequences indicated by arrows are from the present study and others are previously published sequences.

reproducible discriminatory tool under field conditions. At many occasions, the size of some *Eimeria* spp. oocysts coincides with that of other *Eimeria* oocysts, which leads to unreliable identification. So, molecular diagnosis is required to overcome this and to correctly identify the species. In conclusion, our study suggests that the DNA sequence variation in ITS-1 region of *Eimeria* spp. could be correlated with the morphological characteristics of oocysts. The PCR seems superior to conventional faecal examination of oocysts in view of sensitivity, rapidity, specificity and reliability.

REFERENCES

Kawahara, F., Zhang, G., Mingala, C. N., Tamura, Y., Koiwa, M., Onuma, M. and Nunoya, T. (2010). Genetic analysis and development of species-specific PCR assays based on ITS-1 region of rRNA in bovine *Eimeria* parasites. *Vet. Parasitol.* **174(1)**: 49-57.

Kennedy, M.J. and Kalka, R.A. (1987). A survey of *Eimeria* species in cattle in central Alberta. *Can. Vet. J.* **28**: 124-125.

Kokuzawa, T., Ichikawa-Seki, M. and Itagaki, T. (2013). Determination of phylogenetic relationships among *Eimeria* species, which parasitize cattle, on basis of 18S rDNA sequence. *J.Vet. Med. Sci*. **75 (11):** 1427-1431.

Nain, N., Gupta, S.K.,Sangwan, A.K. and Gupta S. (2017). Prevalence of *Eimeria* species in buffalo calves of Haryana. *Haryana Vet*. **56(1)**: 5-8.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989). In: Molecular cloning, a laboratory manual. Second edition. Cold Spring Harbor Laboratory Press, New York.

Sommer, C. (1998). Quantitative characterization, classification and reconstruction of oocyst shapes of *Eimeria* species from Cattle. *Parasitol.* **116:**21-28.

Soulsby, E.J.L. (1982). Helminths, Arthropods and Protozoa of Domesticated Animals. 7th ed. Bailliere, Tindall and Cassell, London.

Vladmir, V., Blake, D.P. and Poplstein, M. (2010). Quantitative real-time PCR assays for detection and quantification of all seven *Eimeria* species that infect the chicken. *Vet. Parasitol.* **174(3-4):**183-190.