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Diagnosis of cryptosporidiosis in neonatal calf diarrhoea using nested PCR technique

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Abstract
Present study was done for the detection of *Cryptosporidium* induced diarrhoea in neonatal calves using Nested PCR technique. Neonatal calf diarrhoea is an economically important disease in dairy sector which is present all over the world. Both infectious and non-infectious etiologies are reported. Major infectious etiological agents include rota virus, corona virus, *E. coli* and *Cryptosporidium parvum*. Using staining techniques it was able to detect the presence of *Cryptosporidium* in five out of 50 animals with diarrhoea but the Nested PCR technique was helped in diagnosing 19 positive cases among the same group of 50 animals. Hence this is a more sensitive technique for the advanced diagnosis of Cryptosporidiosis.

Keywords: Cryptosporidiosis, nested PCR, neonatal calves

1. Introduction

Neonatal calf diarrhoea (NCD) has a worldwide occurrence and considering the etiology, bacteria, virus and protozoa are the most important disease causing organisms [1]. Major etiological agents responsible for NCD include rota virus, corona virus, enterotoxigenic *E coli* and *Cryptosporidium parvum,* which are collectively responsible for 75- 95 percent of infection worldwide [2]. The protozoa, Cryptosporidia are one of the most important and endemic disease causing organism which leads to neonatal enteritis worldwide mainly in one to four week old calves [3]. All over the world, in many countries cryptosporidia is reported as the major cause of enteritis in neonatal calves which leads to calf mortality in untreated cases.

All over Kerala, prevalence of Cryptosporidiosis with emphasis on Thrissur district was 77.18 percent, with the prevalence in cattle being 84.32 percent. They identified five species of *Cryptosporidium*, *viz*., *C. andersoni*, *C. pestis*, *C. parvum*, *C. bovis* and *C. ryanae* by morphology, morphometry and molecular methods [4]. Disease is generally transmitted by thick-walled sporulated oocysts of *Cryptosporidium spp*. that are excreted by infected hosts. They are extremely resistant to many environmental conditions. The tough outer wall of the oocysts helps to survive under cool, moist temperate conditions, but by desiccation, the oocysts can be inactivated [5].

There are different species of *Cryptosporidium* and *Cryptosporidium parvum* is often associated with gastrointestinal tract pathogen in humans and neonatal calves. The four important species of *Cryptosporidium* in cattle are *C. parvum*, *C. bovis*, *C. ryanae* and *C. andersoni*. Among all the *Cryptosporidium* Spp, the clinical manifestation of the disease condition in neonatal calves is associated with *C. parvum.* Asymptomatic shedding of oocysts is frequently seen in the claves above 6 weeks of age [6]. Generally the calves infected with the protozoa exhibit clinical symptoms such as lethargy, tenesmus and watery diarrhoea with undigested milk, blood, mucus or bile in faeces.

The identification of high numbers of *C. bovis* oocysts in diarrheic samples indicates that this species could be associated with NCD and these were indistinguishable morphologically by Ziehl Neelsen acid fast staining [7]. Even though PCR is slightly expensive and time consuming diagnostic tool, it is having better sensitivity and specificity than other coprological and serological methods. Also it has an added advantage of differentiating the species of *Cryptosporidium* [4].

2. Materials and Methods
2.1 Selection of animals
Area of investigation was in Thrissur district of Kerala. Around fifty neonatal calves i.e under 30 days of age with diarrhoea were subjected to study and those are collected from different private and government farms.

2.2 Collection of samples
Faecal samples were collected from animals with diarrhoea, directly from the rectum. Each samples were transported to the laboratory under refrigeration.

2.3 Processing of sample
Thin faecal smears were prepared from the collected samples. Duplicate smears were prepared for each sample. They were air-dried and fixed with absolute methyl alcohol and allowed to dry. Commercially available ZN Acid Fast staining kit (HIMEDIA, India) was used for staining those smears.
All fifty collected faecal samples were subjected to DNA extraction using commercially available DNA extraction kits (QIAamp® DNA Stool Mini Kit, QIAGEN, Germany).

2.4 Detection of *Cryptosporidium*
DNA that extracted from each sample was subjected to pathogen specific PCR. PCR of an 18S rRNA gene of *Cryptosporidium* was carried out using genus specific primers. Nested PCR was done following the procedure of Sreekrishnan, (2013) with minor modifications [4].

Primers
External Forward primer: SSU-F2
5'TTCTAGAGCTAATACATGCG- 3'
External Reverse primer: SSU-R2
5'- CCCATTTCCTTCGAAACAGGA- 3'
Internal Forward primer: SSU-F3
5'GGAAGGGTTGTATTTATTAGATAAAG- 3'
Internal Reverse primer: SSU-R4
5'-CTCATAAGGTGCTGAAGGAGTA-3'

Product was subjected to electrophoresis and imaged using Gel doc. This was done for observing amplified products with corresponding product size. The products were further subjected to sequencing and homology. The documented genotypes were confirmed by using BLAST software of NCBI.

3. Results and Discussion
Neonatal calf diarrhoea is having a multifactorial etiology [1]. Various aetiological agents have been identified for neonatal calf diarrhoea like bacteria, protozoa, virus and parasite. The predominant aetiological agent in our study was *C. bovis*. In most of the studies *C. parvum* was considered as the major etiological agent but *C. bovis* also can be considered as pathogenic [1]. Out of 50 faecal smears, five were positive for *Cryptosporidium* spp. (Fig: 1). Modified Ziehl Neelsen acid fast staining was used to identify organism in faecal smears but it was unable to distinguish the species morphologically [7].
DNA extracted from 70 animals including normal animals were subjected to nested PCR technique. Among that 19 samples were positive for *Cryptosporidium* spp. using species specific primers with amplicon size 840bp [4]. A BLAST search against Genbank revealed one of the sample showing 100 percent similarity to *Cryptosporidium bovis* isolate ECUST23057 small subunit ribosomal RNA gene with sample id: MF074602.1.That was deposited in Genbank and got ID as *Cryptosporidium bovis* isolate Mannuthy small subunit ribosomal RNA gene, partial sequence, Sequence ID: MH166335.1, Length: 811. Other 18 samples were showing 100 percent similarity with *Cryptosporodium bovis* Mannuthy isolate. Polymerase Chain Reaction is the best method to identify the Cryptosporidial organism in faecal sample and it is having better sensitivity than other diagnostic methods [4, 7].

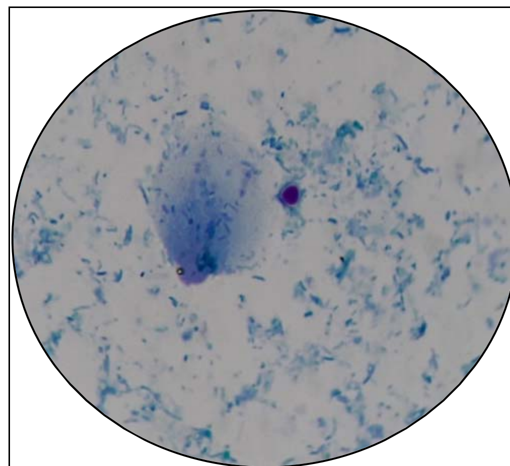

Fig 1: Stained Image of *Cryptosporidium* Spp.

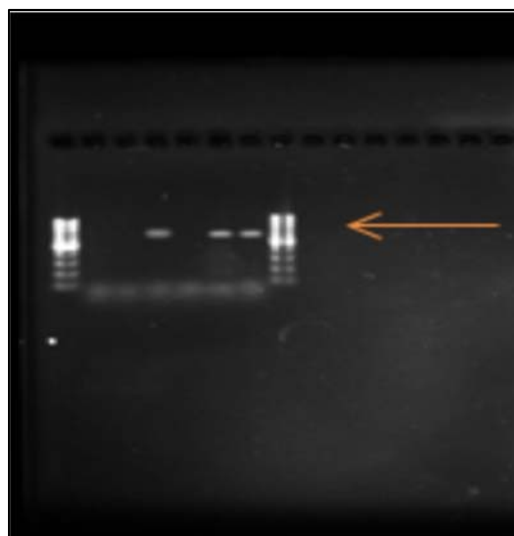

Fig 2: The amplicon of test sample with 840 bp

4. Conclusion
Present study concludes that molecular techniques could help to diagnose more than other conventional methods. Here the *Cryptosporidium Spp.* Causing Neonatal calf diarrhoea could better diagnosed using Nested PCR technique rather than staining methods. Among 50 samples only 5 samples identified as positive using staining technique whereas it had been found as 19 positive samples when used Nested PCR technique.

5. Conflict of interest
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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