RESEARCH ARTICLE

Molecular Detection of Non-Tuberculous *Mycobacterium* and *Cryptosporidium* Co-Infections in Cattle

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Abstract

Infectious diarrhea in young ruminants is a major problem, often involving co-infections with multiple pathogens like *Cryptosporidium*, non-tuberculous mycobacteria (NTM), and others. This study aims to investigate the prevalence of co-infections with NTM and *Cryptosporidium* in cattle using molecular techniques, acknowledging the potential for these pathogens to interact and modulate the course of diarrhea in cattle on an organized dairy farm in Bengaluru, Karnataka during 2019-20. A total of 17 crossbred cattle suffering with chronic diarrhea out of 82 cattle were screened for *Mycobacterium* and *Cryptosporidium* infections by conventional staining protocols and PCR. DNA extracted from fecal samples and rectal pinch was subjected to PCR targeting *hsp65* gene (441 bp) specific to *Mycobacterium*. The multiplex PCR was also performed to identify species of *Mycobacterium* under MTB Complex (MTBC) using two pairs of primers (*Rv1506c*) that gave positive result to *hsp65* gene. Further, *Mycobacterium* spp. was differentiated as MTBC or Non-Tuberculous *Mycobacterium* (NTM) by using a commercial qPCR kit. A two-step nested PCR protocol was followed to amplify ~ 830 bp fragment of the 18S rRNA gene of *Cryptosporidium*. Out of 17 DNA samples, *hsp65* gene (441 bp) specific to *Mycobacterium* genus was amplified in 6 samples, but none of them belonged to MTB complex. All the 6 DNA samples were identified as NTM by qPCR. Subsequently, all the 17 DNA samples were subjected to a two-step nested PCR protocol. Five samples out of them amplified ~ 830 bp fragment of 18S rRNA gene of *Cryptosporidium* spp., which were also positive for NTM. This study signified the possibility of co-infection with *Mycobacterium* spp. and *Cryptosporidium* spp. in immuno-compromised chronic diarrheic crossbred cattle.

Key words: *Cryptosporidium*, Non-tuberculous *Mycobacterium*, Multiplex Nested PCR, qPCR

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Introduction

Infectious diarrhea in young ruminants is one of the biggest challenges facing economic productivity and animal welfare, leading to increased mortality rates. The protozoan *Cryptosporidium* is known as one of the major pathogens causing diarrhea in young livestock, especially in calves. *Cryptosporidium* is an intracellular extra cytoplasmic protozoan parasite which infects all domestic and wild animals including human beings. Cryptosporidiosis caused by members of *Cryptosporidium* spp. is a debilitating diarrheal disease which is life threatening to the immunocompromised individuals (Kaduková, *et al.,* 2024). Non-tuberculous mycobacteria (NTM) co-infections with *Cryptosporidium* in cattle can occur, but the specific prevalence and impact of these co-infections are not well-defined (Biet and Boschiroli, 2014). Some studies have shown that, NTM present in lymph nodes of cattle can also be found in the faeces of same cattle (Ghielmetti *et al*., 2018).

Furthermore, infection with other pathogens may be modulated by active *Cryptosporidium* infection. In fact, studies conducted on young, diarrheic livestock demonstrated that other enteropathogenic agents can be found in affected animals (Mor *et al.,* 2024). It has been suggested that calves and small ruminants may play an important role as zoonotic reservoirs for these human pathogens (Alberto *et al.,* 2024). Although co-infections thus seem to be common in diarrheic calves, lambs or goat kids, information about interactions between various pathogens and the pathophysiology of co-infection is sparse. While the exact prevalence and impact of NTM and *Cryptosporidium* co-infections in cattle are not fully understood, studies suggest that they can occur (Zeng *et al*., 2013). NTM and *Cryptosporidium* can be found in the same animal, particularly in calves. The presence of NTM might be

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a factor in the severity or persistence of cryptosporidiosis, although more research is needed. NTM and *Cryptosporidium* co-infections can have a significant impact on cattle health, productivity, and economic losses. Proper diagnosis and treatment of both infections are crucial for animal health and management. Hence, this study was planned to detect the co-infections of non-tuberculous *mycobacterium* and *cryptosporidium* in cattle by molecular techniques.

Materials and Methods

A total of 17 crossbred cattle suffering with chronic diarrhea out of 82 cattle in an organized dairy farm in Bengaluru, Karnataka (India) during 2019-20, were screened for *Mycobacterium* and *Cryptosporidium* co-infections by conventional staining techniques followed by multiplex and nested PCR, respectively. All the 17 samples were used to prepare thin faecal smear on a clean glass slide and were subjected to Ziehl-Neelsen (Z-N) Acid Fast staining (Murray *et al.,* 2007) and modified cold strong Ziehl-Neelsen (mZ-N) staining method as per the descriptions of Fayer and Xiao (2008) with slight modification in the smear preparation. The DNA was extracted from fecal samples and rectal pinch using HiMedia Stool DNA Extraction kit and subjected to polymerase chain reaction targeting *hsp65* gene (441 bp) specific to *Mycobacterium* (Telenti *et al.,* 1993). The multiplex PCR was also performed to identify species of *Mycobacterium* under MTB Complex (MTBC) using two pairs of primers (*Rv1506c*) as described by Bakshi *et al.* (2005) on DNA samples that amplified *hsp65* gene. Further, *Mycobacterium* spp. was differentiated as MTBC or Non-Tuberculous *Mycobacterium* (NTM) by using a commercial qPCR kit. A two-step nested PCR protocol was performed to amplify ~ 830 bp fragment of the 18S rRNA gene of *Cryptosporidium* as described by Xiao *et al.* (1999, 2001) for detection of *Cryptosporidium* spp. Finally, the PCR products were analysed by agarose gel electrophoresis in a submarine horizontal gel electrophoresis apparatus as described by Sambrook and Russel (2001).

Results and Discussion

Identification of *Mycobacterium* spp. by Ziehl-Neelsen (Z-N) Acid-Fast Staining

In Z-N acid fast staining, *Mycobacterium* spp. stained bright pink in colour and appeared as long slender bacilli in small clusters as acid fast organisms in five out of 17 samples tested (Fig. 1). Weber *et al.* (2009) evaluated Ziehl-Neelsen stained faecal smear and ELISA as tools for surveillance of clinical paratuberculosis in cattle in the Netherlands and observed substantial agreement between the two tests. However, Vilchèze and Kremer (2017) reported both acid-fast positive and acid-fast negative *Mycobacterium tuberculosis* as "The Koch Paradox" which has also been reported by several researchers.

Identification of *Cryptosporidium* Oocysts by Modified Z-N (mZ-N) Staining Method

In mZ-N staining, *Cryptosporidium* oocysts stained red and appeared as spherules on pale green back ground, but the degree and proportion of staining varied with individual oocysts (Fig. 2). Out of 17 dung samples, five samples showed positive for *Cryptosporidium* oocysts in mZ-N staining technique in this study. Similar findings were reported by Sateesh *et al.* (2022) in and around Bidar, where they have

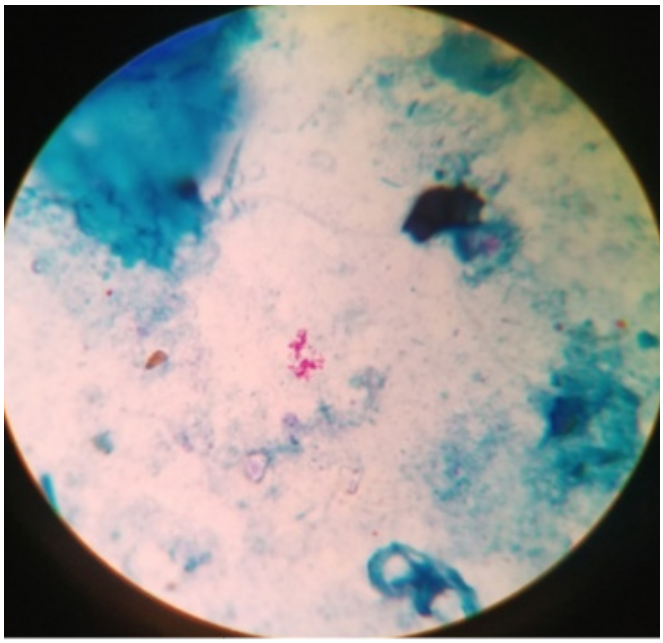

Fig. 1: *Mycobacterium* spp. in rectal pinch by Z-N acid fast staining (x1000)

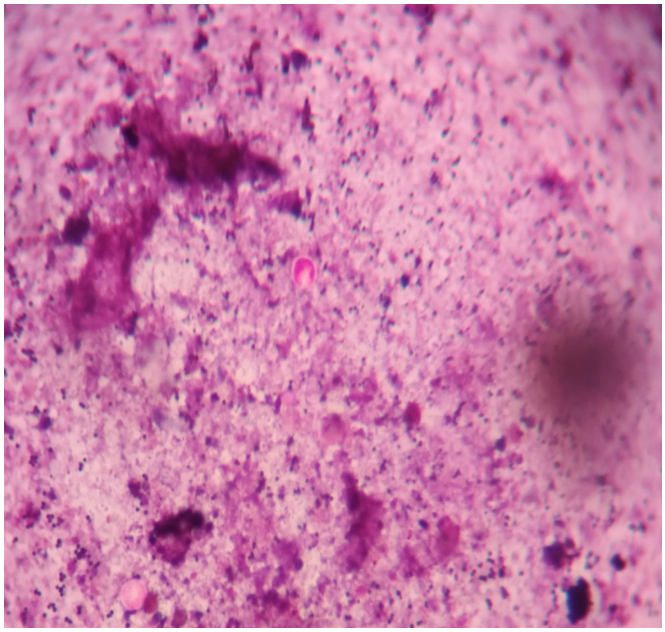

Fig. 2: *Cryptosporidium* oocyst in modified Z-N staining technique (x1000)

Table 1: PCR based detection of *Mycobacterium* targeting *hsp65* gene from rectal pinch samples and determination of *Mycobacterium* species by multiplex PCR

Sample No.	Target gene and Amplicon size			Species of *Mycobacterium* by qCR
	hsp65 gene (441 bp)	*M.tuberculosis Rv1506c* (262 bp)	*M. bovis Rv1506c* (168 bp)	
591	+	-	-	NTM
642	+	-	-	NTM
P-34	+	-	-	NTM
P-30	+	-	-	NTM
20324	+	-	-	NTM
HF-X	+	-	-	NTM

reported 14.63% overall prevalence using mZ-N staining method. Comparable findings were also reported from southern states of India by Venu *et al.* (2013) and parts of Mizoram by Das *et al*. (2020).

Detection of Genus *Mycobacterium* by PCR

In the present study, a total of 17 fecal samples with rectal pinches of chronic diarrheic cattle from dairy farm were subjected to DNA extraction and amplification of *hsp65* gene by PCR, which is considered to be specific to genus *Mycobacterium.* The results of PCR revealed that, five rectal pinch samples from diarrheic cattle which were positive for acid-fast organisms showed amplification of *hsp65* gene (440 bp) (Table 1; Fig. 3). The results of PCR indicated that, PCR could detect mycobacteriosis only if the animal had active infection and the organisms were found in circulation or excreted through milk, nasal discharge and feces in this study. Srivastava *et al.* (2008) found that pre-scapular lymph gland was found to be most suitable specimen for detection of *M. tuberculosis* complex from cattle and is thus of diagnostic importance. Romero *et al.* (1999) concluded in their study that no single infected animal was positive for all the samples collected from that animal. Therefore, multiples relevant samples from the same animal should be collected in different frequencies to detect tuberculosis in infected animals. Different DNA sequences specific to *Mycobacterium tuberculosis* complex (MTBC) including insertion sequence (*IS6110, IS1081*) and heat shock protein (*hsp65*) have been evaluated in many laboratories for detection of *Mycobacterium* spp. in clinical specimens (Azar *et al.,* 2017). Among these genes, the highly conserved *IS6110* and *hsp65* have been widely targeted in several studies (Bannalikar and Verma, 2006; Gopinath and Singh, 2009; Bhanu Rekha *et al.,* 2015; Priyadarshini *et al.,* 2017).

Determination of *Mycobacterium* spp. by Multiplex PCR

The multiplex PCR was carried out on positive DNA samples that amplified *hsp65* gene. Two pairs of primers were used to differentiate species of *Mycobacterium* corresponding to *M. tuberculosis* and *M. bovis*. The results of PCR showed that, none of the samples amplified species specific product for MTBC (Table 1). Further, they were identified as Non-Tuberculous *Mycobacterium* (NTM) by using a commercial qPCR kit by a private laboratory.

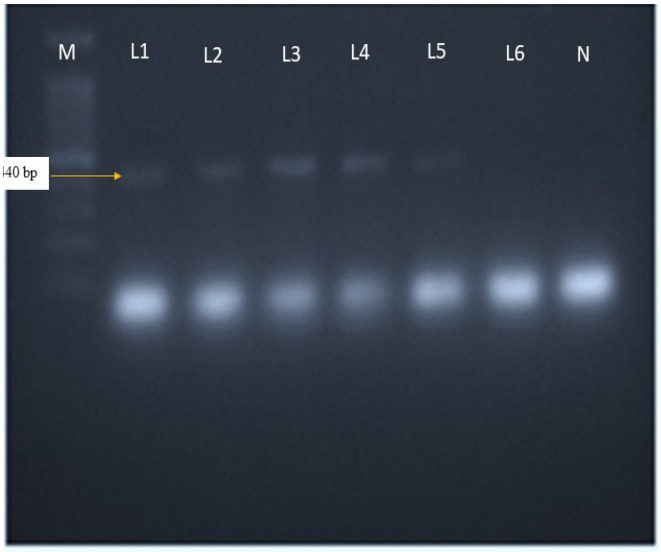

Fig. 3: PCR amplification of *hsp65* gene (441 bp) of *Mycobacterium* from rectal samples of cattle. Lane M: 100-bp DNA ladder, L1-L5: Positive samples, L6: Negative sample, Lane N: NTC

In this study, PCR could not detect any MTBC from SIT and IFN-γ ELISA positive animals which indicated that SIT and IFN-γ ELISA have cross reactivity with non-tuberculous *Mycobacteria* as reported by Norby *et al.* (2004) and Jenkins *et al.*(2018). Therefore, the utility of SIT and IFN-γ assay in diagnosis of tuberculosis in bovines need to be re-evaluated as a results of these tests in this study falsely attributed non-tuberculous mycobacteriosis as tuberculosis. This could lead to misrepresentation of the zoonotic tuberculosis burden in cattle as most of the species of NTM are non-zoonotic. This study supported the earlier findings of Arshad (2019) and Sripad *et al.* (2019), who also reported the decreasing trend of occurrence of MTBC and occurrence of NTM in cattle over the years.

Therefore, qPCR could be the test of choice for screening and confirmation of tuberculosis in cattle where the facilities are available owing to the high cost and false positive results of IFN-γ assay and low specificity of SIT. But the cost of initial investments is also high and requires technical expertise for conducting qPCR test. Further studies are required to improve the accuracy of test results and reduction in the cost of IFN-γ assay. Lateral flow assay could be an alternative to IFN-γ assay for screening of tuberculosis in bovines due to its low cost, rapidity in performance and simplicity in interpretation of results.

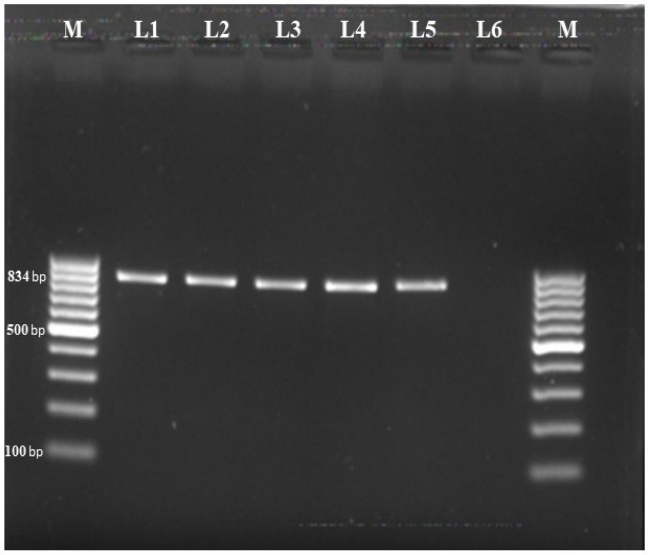

Fig. 4: Agarose gel electrophoresis of nested PCR amplified products of 834 bp in size of 18S rRNA gene of *Cryptosporidium*. M: 100 bp DNA ladder; L1- L5: DNA samples showing amplification for 18S rRNA gene of *Cryptosporidium* by nPCR; L6: Non-template control

Detection of *Cryptosporidium* in Fecal Samples by Nested PCR

All the 17 DNA samples extracted from fecal samples of chronic diarrheic cattle were subjected to nPCR. Agarose gel electrophoresis of nested PCR amplified products of 834 bp in size of 18S rRNA gene of *Cryptosporidium* were amplified in five dung samples of cattle out of 17 samples tested (Fig. 4), which were also positive for *Mycobacterium*. These findings were in accordance with earlier studies of Venu *et al.* (2012) and Bhat *et al.* (2014), where they also recorded prevalence of *Cryptosporidium* spp by nPCR in different parts of India.

The use of genetic tools like molecular tests has led to clear understanding of the epidemiology and population genetics of *Cryptosporidium* species in livestock. A higher degree of sensitivity of nested PCR assay has revolutionized the field of diagnosis in *Cryptosporidium* spp. in comparison to conventional method like by microscopy after staining feacal smears with Modified Ziehl-Neelsen stain for detection of round, sporulated oocysts of 4 to 5 µm in size.

Conclusion

This study signified the possibility of co-infection with *Mycobacterium* spp. and *Cryptosporidium* spp. in immuno-compromised chronic diarrheic crossbred cattle. This warrants the zoonotic significance of both the diseases in humans. PCR was found to be better test in rapid detection of mycobacterial and cryptosporidial co-infections which usually go unnoticed in regular conventional detection methods. This study highlighted the scope of research in identification of new species of NTM infecting domestic animals. More research is needed on molecular mechanisms to better understand the prevalence, impact, and interactions between NTM and *Cryptosporidium* co-infections in cattle, and to determine the specific mechanisms by which these co-infections affect cattle health, so as to develop effective management strategies of these co-infections of zoonotic pathogens.

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