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Isolation of mycobacterium from milk samples of cattle

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Abstract
Bovine tuberculosis is a chronic debilitating disease of cattle caused by organisms of Mycobacterium tuberculosis complex (MTBC). Hence, the present study was carried out to isolate the organisms of MTBC from milk samples. However, out of 17 milk samples inoculated, only one isolate was obtained, which was later identified as non tuberculus mycobacterium (NTM) using IS*6110* PCR.

Keywords: Bovine tuberculosis, MTBC, NTM, milk, isolation

Introduction
Bovine tuberculosis (bTB) is a chronic disease of cattle. Though, *Mycobacterium bovis* (*M. bovis*) is identified as the predominant cause of the disease, other mycobacterial organisms have also been reported as potential cause of bTB (OIE, 2009) [1]. The genus *Mycobacteria* comprise of a wide range of organisms *viz*., pathogens belonging to MTBC, opportunistic and potential pathogens of Mycobacterium avium complex (MAC) and saprophytic species (Rastogi *et al*., 2001) [2]. The bTB infected animals exhibit varying symptoms like respiratory distress, light fever, reduced appetite, weakness and emaciation (OIE, 2009) [1]. The present study was carried out to isolate mycobacterium from bovine milk samples of emaciated animals.

2. Materials and methods
2.1 Sample collection
Seventeen milk samples were collected from cattle showing emaciation with or without respiratory signs.

2.2 Decontamination
The samples were initially centrifuged at 1500 xg for 20 minutes. The sediment obtained was reconstituted in 2mL normal saline and decontaminated using modified Petroff's method with four per cent sodium hydroxide, strictly following the guidelines by CTD (2009) [3] and ECDC (2012) [4]. Smears prepared from decontaminated milk samples were used for Ziehl- Neelsen staining. The pellet obtained was used for inoculation.

2.3 Isolation
The samples were inoculated onto Lowenstein-Jensen medium with glycerol and Stone brink media and incubated at 37 ˚C for eight weeks and inspected weekly for visible growth. The samples were considered negative, if no visible growth was detected after eight weeks of incubation. The cells from colonies that suggested the growth of mycobacteria were examined microscopically after ZN staining.

2.4 Extraction of genomic DNA
Presumptive mycobacterial colonies were inactivated as described earlier by Bemer- Melchior and Drugeon, (1999) [5]. The genomic DNA was extracted using QIAamp® DNA mini kit (Qiagen, Germany) following manufacturer's instructions. The concentration and purity of the extracted DNA were checked by Nanodrop 2000 spectrophotometer (Thermo Scientific, USA) and those samples with an OD value (260/280) of 1.8 or above were used for PCR.

2.5 Genus specific PCR
The PCR amplification was carried out using primers as described by Pinsky and Banaei

(2008) [6] for amplification of a 78 bp product specific for *16S rRNA*. The PCR reactions were performed using 2X Emerald Amp PCR master mix (Takara Bio, USA) as per the manufacturer's instructions. In brief, the reaction mix comprised of 12.5 µL of master mix, 1 µL each of forward and reverse primers (10 p*M*/µL), 1 µL of DNA template and nuclease free water to make a total volume of 25 µL. The reaction cycle involved initial denaturation of 96˚C for 5 min followed by 30 cycles of amplification consisting of denaturation at 96 ˚C for 30 sec, annealing at 60 ˚C for 30 sec. and extension at 72 ˚C for 30 sec with a final extension at 72˚C for 5 minutes. The final amplified product was identified using submarine agarose gel electrophoresis system. The gel was visualised and documented using an automatic gel documentation system (Bio-Rad, USA).

2.6 IS6110 PCR

Primers used for the PCR, were based on previously described sequences (Collins *et al.*, 1999) [16], specific for an insertion element, IS*6110*. The PCR was carried out at an annealing temperature of 58 ˚C for 1 min to amplify a 445 bp region.
The primers used in the study are depicted in Table 1.

Table 1: Primers

Primer	Sequence (5'-3')
Myc16SrRNA F	CAACGCGAAGAACCTTACCT
Myc16SrRNA R	TGCACACAGGCCACAAGGGA
IS6110 F	GACCACGACCGAAGAATCCGCTG
IS6110 R	CGGACAGGCCGAGTTTGGTCATC

3. Results and discussions

Bovine tuberculosis is a chronic debilitating disease of cattle caused predominantly by *M. bovis*. The involvement of other members of MTBC have also been reported (Srivastava *et al.*, 2008) [8]. Milk has been reported to be a good sample for detection of pathogenic mycobacterium (Vitale *et al.*, 1998) [9]. Hence, the present study was conducted to detect mycobacterium from bovine milk from emaciated animals by isolation and amplification of genus specific PCR.
Mycobacterium was isolated from only one milk sample. The isolate revealed acid fast organism on ZN staining. The colony yielded an amplicon of 78 bp confirming the *Mycobacteria* genus (Fig.1). However, the sample was negative for IS6110 PCR, confirming it as NTM. Isolation of mycobacterium was not reported from milk samples by Neeraja *et al*. (2014) [10] which is in agreement with the results of the present study. Palmer and Waters (2006) [11] reported that only five per cent animal's positive for bTB secrete the organism in milk. In a study conducted in Argentinian dairy herds with high prevalence of bTB, MTBC was not detected in milk samples (Perez *et al*., 2002) [12]. Hence the absence of isolation may be attributed either to absence of disease condition, or absence of shedding of organism in milk.
Non tuberculous mycobacterium was reported to be predominantly isolated from milk samples of cattle (Bolanos *et al.*, 2018) [17]. The NTM organisms are widely distributed in the environment (Chang *et al*., 2002) [14]. However, there is an increase in reports of different diseases caused by NTM in humans (Neonakis *et al*., 2015) [15]. The reports of isolation of NTM from immune competent and immunosuppressed individuals highlights the need of further studies of involvement of NTM in causing disease in animals. The excretion of these organisms in milk poses a risk to public health. Also, these organisms may cause cross reactivity and interfere with the routine screening test for bTB (Bolanos *et al*., 2018) [17]. Hence, further investigations are to be carried out to rule out the involvement of NTM in causing disease in cattle, as well as its transmission.

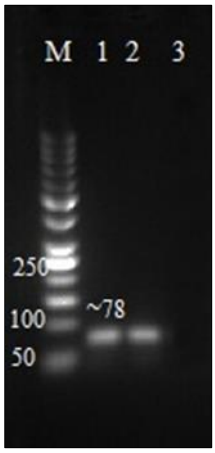

Fig 1: Mycobacterium genus specific PCR

Lane M: 50 bp Molecular marker
L 1: Positive control
L 2: Positive isolates
L3: No template control

4. Conclusion

Mycobacterium was isolated from one sample of milk in the present study from culture and isolation however, was confirmed as NTM by PCR. Despite the absence of MTBC, the detection of NTM from milk, high lights the risk of pathogen transmission to humans, since many of the NTM organisms have been reported to cause disease in both immune-competent and immuno- compromised individuals.

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