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Identification of *Mycobacterium tuberculosis* Complex by Culture and Duplex Polymerase Chain Reaction in Bovines

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ABSTRACT

Bovine tuberculosis (bTB) is an economically important zoonotic disease (can spread to human through inhalation or ingestion) caused by *Mycobacterium bovis* which belongs to *Mycobacterium tuberculosis* complex (MTC). Control and eradication of infection is difficult even in organized dairy farms. So combinations of tests like culturing and nucleic acid-based diagnostics are used for the isolation and identification of mycobacterial infections in cattle. Even though, there are many advances in diagnosis of bovine TB infection in cattle but till now, isolation identification of the etiological agent from clinical samples stands as a definitive and gold standard test. Nucleic acid based methods like Polymerase Chain Reaction (PCR) which have advantages of speed, sensitivity and specificity can be used for diagnosis of tuberculosis along with isolation. In the present study, isolation of *Mycobacterium tuberculosis* complex organisms was attempted from nasal swabs and milk of cattle using Lowenstein-Jensen (LJ) media without glycerol. Cattle which were positive for tuberculosis either by skin test or gamma interferon test were selected. Two of the twelve nasal swabs and none of the seven milk samples showed typical mycobacterial colonies on LJ media after 8 weeks of incubation. Ziehl-Neelsen staining of colonies showed slender, rod shaped acid fast organisms suggestive of *Mycobacterium*. Deoxy ribo nucleic acid (DNA) was extracted by boiling method and amplified by duplex PCR for 245 and 500 bp amplicons specific for MTC (*IS6110*) and *M. bovis* (RvD1Rv2031c), respectively. Electrophoresis revealed 245 bp product but not 500 bp which confirmed the identity and relatedness of the isolated mycobacterium to *Mycobacterium tuberculosis* complex.

Key words: Bovine tuberculosis, *Mycobacterium tuberculosis* complex, culture, isolation, diagnosis, duplex PCR

INTRODUCTION

Bovine tuberculosis (bTB) is an economically important zoonotic disease caused by *Mycobacterium bovis* which belongs to *Mycobacterium tuberculosis* complex. The complex also

includes *Mycobacterium bovis*, *M. tuberculosis*, *M. africanum*, *M. microti*, *M. canetti* and *M. caprae* (Cousins *et al.*, 2003; Good and Duignan, 2011; Atkins and Robinson, 2013). The disease can spread to humans, typically by the inhalation of aerosols or the ingestion of unpasteurized milk (Phillips *et al.*, 2003; Evans *et al.*, 2007; Verhagen *et al.*, 2011). Office International des Epizooties (OIE) classified bTB as a list B transmissible disease of public health importance and is of high significance to the international trade of animals and animal products (OIE, 1999) and presently is a listed disease. Bovine tuberculosis occurs in almost every country of the world and is of major importance in dairy cattle due to high morbidity and loss of production as infected animals lose 10-25% of their productive efficiency (Cousins, 2001). It also has high economic impact on animal industry causing annual loss of three billion dollars worldwide affecting 50 million cattle (Cousins, 2001; Challu, 2007). Prevalence of bTB in Indian cattle and buffaloes was 1.6-16 and 3-25%, respectively (Challu, 2007). High prevalence of tuberculosis was detected in some South Indian states like Karnataka (30-35%) and Tamil Nadu (34.58%) (Mukherjee, 2006).

Control and eradication of infection is difficult even in organized dairy farms for sensitive, specific and reliable diagnostic tests for the detection of pre-clinical infections. Accurate diagnosis of disease in live animals is of paramount importance for an effective disease control and eradication program. So combinations of tests like culturing and nucleic acid-based diagnostics are used for the isolation and identification of mycobacterial infections in cattle. Even though, there are many advances in diagnosis of bovine TB infection in cattle but till now, isolation and identification of the etiological agent from clinical samples stands as a definitive and gold standard test. However isolation is slow, cumbersome and requires atleast 8 weeks (Rohonczy *et al.*, 1996; Collins, 2011; Figueiredo *et al.*, 2012).

The Ziehl-Neelsen acid fast staining is a simple and rapid method but cannot detect the species and lacks specificity and sensitivity (Barouni *et al.*, 2004). Nucleic acid based methods like Polymerase Chain Reaction (PCR) which have advantages of speed, sensitivity and specificity can be used for diagnosis of tuberculosis along with isolation (Lermo *et al.*, 2010; Collins, 2011; Gan *et al.*, 2013). IS6110 and RvD1Rv2031c sequences are detected in *M. tuberculosis* complex and *M. bovis*, respectively which yield 245 and 500 bp fragment on amplification in a single tube reaction.

MATERIALS AND METHODS

Culturing

Samples: Ante mortem samples like milk samples (n = 7) and nasal swabs (n = 12) were collected from twelve cattle which were positive for skin or gamma interferon test.

Cultural isolation: The samples for isolation of mycobacteria were processed by decontaminating with 4% NaOH (modified Petroff's method). Nasal swabs were agitated in 1 mL of Phosphate Buffered Saline (PBS) and 5 mL of milk taken in to sterile centrifuge tubes separately. Double volume of sterile NaOH (4%) was added to the sample, mixed and kept on shaker for 10-15 min, centrifuged at 3,000× g for 15 min and supernatant was discarded. Sterile distilled water was added up to neck of the bottle, mixed thoroughly and centrifuged at 3000× g for 15 min. The supernatant

was discarded and about 50 μ L of sediment was inoculated in to Lowenstein-Jensen (LJ) medium without glycerol and incubated at 37°C for 8 weeks in CO₂ incubator (Srivastava *et al.*, 2008).

Duplex-Polymerase Chain Reaction (PCR): The DNA was extracted from colonies on LJ slants by boiling method (Hosek *et al.*, 2006). A loop full of inoculum was added to 30 μ L of nuclease free water and boiled at 95°C for 15 min and kept at -20°C for 10 min. It was then re-centrifuged for 15 min at 13,000 rpm and 5-10 μ L supernatant was used as a template. Duplex PCR was performed in a reaction mix (50 μ L) containing 5 μ L of 10X PCR buffer (Invitrogen®), 200 μ M dNTP, 2.5 U of recombinant Taq polymerase (Invitrogen®), 0.2 μ M of each primer (Figueiredo *et al.*, 2009) -JB21(5'-TCG TCC GCT GAT GCA AGT GC-3') and JB22 (5'-CGT CCG CTG ACC TCA AGA AAG-3') for amplifying RvD1Rv2031c genes specific to *M. bovis* and INS1 (5'-CGT GAG GGC ATC GAG GTG GC-3') and INS2 (5'-GCG TAG GCG TCG GTG ACA AA-3') for amplifying *IS6110* gene specific to *M. tuberculosis* complex, 2.0 mM MgCl₂ and 5 μ L of purified DNA template. Amplification was carried out in a Palm cycler™ (Corbett Research, Australia) with the cycling conditions of 94°C for 5 min, followed by 30 cycles of 1 min at 94°C, 1 min at 68 and 72°C with a final extension at 72°C for 7 min. PCR products were checked by electrophoresis on 1.75% agarose gel containing ethidium bromide (10 μ g mL⁻¹) (Figueiredo *et al.*, 2009).

RESULTS AND DISCUSSION

Two of the twelve nasal swabs and none of the milk samples showed characteristic colonies suggestive of *Mycobacterium* spp. (Fig. 1) after 8th week of incubation at 37°C on LJ medium without glycerol. Ziehl-Neelsen staining of colonies showed slender, rod shaped acid fast organisms



Fig. 1: Lowenstein Jensen's medium with mycobacterial colonies (8 weeks old)

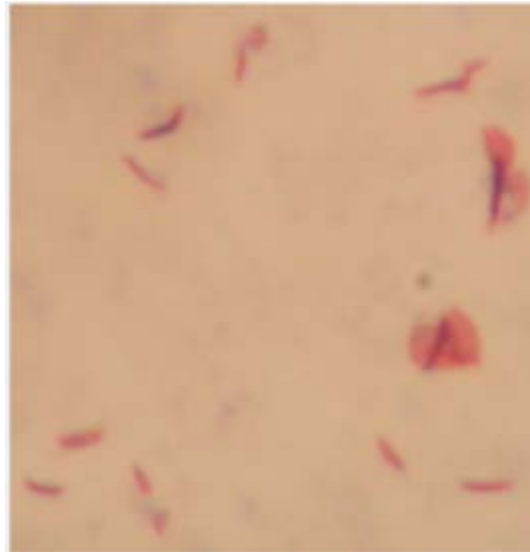


Fig. 2: Ziehl-Neelsen staining technique showing acid fast organisms from 8 week old colonies (1000X)

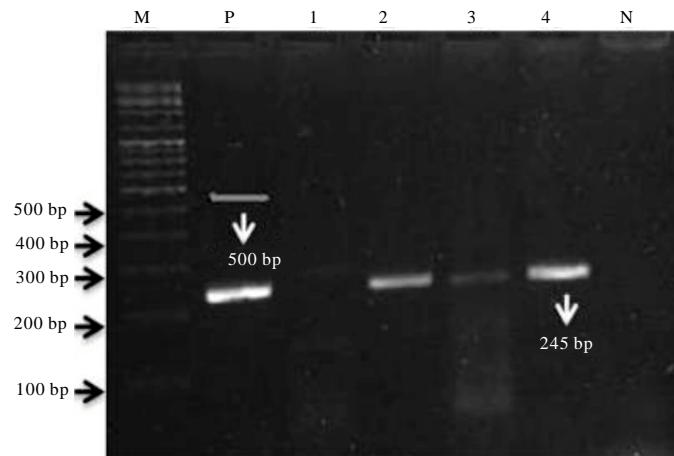


Fig. 3: Agarose gel electrophoresis of duplex PCR amplified products of *IS6110* (245 bp specific for *M. tuberculosis* complex) and *RvD1Rv2031c* genes (500 bp specific for *M. bovis*), Lane M:100 bp ladder, P: Positive control, 1, 2, 3 and 4: Sample numbers and N: Negative control

(Fig. 2) suggestive of *Mycobacterium* spp. In duplex PCR, the two isolates produced specific amplicons of 245 bp for *IS6110* gene specific for *M. tuberculosis* complex (Fig. 3) but not 500 bp amplicon which is specific for *M. bovis*.

Culturing, though considered as definitive test, is limited to post mortem samples than ante mortem samples due to intermittent shedding of the organism. After 8 weeks of inoculation,

isolation could be made from only two nasal swabs (16.67%) and none from milk samples. The results are in agreement with Figueiredo *et al.* (2012) who opined that nasal mucus samples are better than milk samples because in milk, mycobacterium could be killed by the action of macrophages and it is reported that only 5% of tuberculin reactors would excrete mycobacterium in milk (Palmer and Waters, 2006). However, Srivastava *et al.* (2008) in a study isolated both *M. bovis* (6/40; 15%) and *M. tuberculosis* (4/14; 28.5%) from the milk samples.

Definitive diagnosis of tuberculosis is carried out by isolation, biochemical identification and species level differentiation to MTC by culturing from post mortem samples. However, isolation from milk, nasal swabs and other such ante mortem samples is difficult because of varied factors. Those include, (a) Animals positive for Cell Mediated Immunity (CMI) based tests may not excrete organisms always in milk and nasal secretions because of intermittent shedding, (b) Clinical samples with less mycobacterial load might lose the bacteria while sample preparation (decontamination and antibiotic treatment), there is a loss of 5-10% viable bacteria (Stavri *et al.*, 2003) and decontaminants like 4% NaOH are more toxic compared to others (Corner *et al.*, 2012), (c) Mycobacterium being fastidious and slow growing in nature (Adams, 2001) requires at least six weeks for visible growth, (d) There are reports that only about 5% of Tuberculin-Reacting Cattle (TRC) can eliminate *M. bovis*. Because of all these factors, isolation from milk and other ante mortem samples became difficult and it is, therefore, restricted to post mortem samples.

M. bovis isolation from nasal swabs indicated that aerosol transmission of disease to humans and other animals is evident and isolation of *Mycobacterium* from healthy cattle indicate sub-clinical infection/latent infection in the herd (Srivastava *et al.*, 2008). Further, isolation is laborious, time consuming, less sensitive and more specific method. Presence of some positive cultures is sufficient to declare the outbreak of tuberculosis in the herd (Figueiredo *et al.*, 2010).

Duplex PCR was applied to two isolates obtained from nasal swabs to differentiate MTC and *M. bovis* in single tube reaction. Two isolates were confirmed as MTC as they produced 245 bp size amplicon but not as *M. bovis*, since it did not yield 500 bp amplicon. The results are not in agreement with Figueiredo *et al.* (2010) who could differentiate *M. bovis* from MTC in single-step multiplex PCR and Figueiredo *et al.* (2009) conducted multiplex PCR for the identification of *M. bovis* isolates having 100% concordance with the conventional culturing and biochemical reactions.

The isolates obtained in this study were confirmed as MTC but not *M. bovis*. There are chances that it may be *M. bovis*, since 13.3% of *M. bovis* failed to produce 500 bp fragment in multiplex PCR (mPCR) as reported by Figueiredo *et al.* (2010) and they may be *M. caprae* also, as it is naturally infecting the cattle. Alvarez *et al.* (2012) confirmed the presence of bTB in 42 herds by isolation of *M. bovis*/*M. caprae*. Good and Duignan (2011) reported bTB as a chronic bacterial disease caused by *M. bovis* and *M. caprae*, both cause infectious disease in cattle and the diagnostic tests used for detection of disease caused by *M. caprae* is not considered to be substantially different from that caused by *M. bovis*.

CONCLUSION

Confirmation of the identity and relatedness of the isolated mycobacterium to *Mycobacterium Tuberculosis* Complex (MTC) can be done exclusively based on molecular diagnostic tools like the duplex PCR which can be applied for differentiation of MTC and *M. bovis* in single tube reaction to further strengthens the diagnosis.

REFERENCES

- Adams, L.G., 2001. *In vivo* and *in vitro* diagnosis of *Mycobacterium bovis* infection. Rev. Scient. Tech. (Int. Office Epizootics), 20: 304-324.
- Alvarez, J.A., A. Perez, J. Bezos, A. Marques and S. Grau *et al.*, 2012. Evaluation of the sensitivity and specificity of bovine tuberculosis diagnostic tests in naturally infected cattle herds using a Bayesian approach. Vet. Microbiol., 155: 38-43.
- Atkins, P.J. and P.A. Robinson, 2013. Bovine tuberculosis and badgers in Britain: Relevance of the past. Epidemiol. Infect., 141: 1437-1444.
- Barouni, A.S., C.J. Augusto, M.T.P. Lopes, M.S. Zanini and C.E. Salas, 2004. A *pncA* polymorphism to differentiate between *Mycobacterium bovis* and *Mycobacterium tuberculosis*. Mol. Cell. Probes, 18: 167-170.
- Challu, V.K., 2007. Zoonotic importance of tuberculosis. NTI Bull., 43: 37-40.
- Collins, D.M., 2011. Advances in molecular diagnostics for *Mycobacterium bovis*. Vet. Microbiol., 151: 2-7.
- Corner, L.A.L., E. Gormley and D.U. Pfeiffer, 2012. Primary isolation of *Mycobacterium bovis* from bovine tissues: Conditions for maximising the number of positive cultures. Vet. Microbiol., 156: 162-171.
- Cousins, D.V., 2001. *Mycobacterium bovis* infection and control in domestic livestock. Rev. Sci. Tech., 20: 71-85.
- Cousins, D.V., R. Bastida, A. Cataldi, V. Quse and S. Redrobe *et al.*, 2003. Tuberculosis in seals caused by a novel member of the *Mycobacterium tuberculosis* complex: *Mycobacterium pinnipedii* sp. nov. Int. J. Syst. Evol. Microbiol., 53: 1305-1314.
- Evans, J.T., E.G. Smith, A. Banerjee, R.M.M. Smith and J. Dale *et al.*, 2007. Cluster of human tuberculosis caused by *Mycobacterium bovis*: Evidence for person-to-person transmission in the UK. Lancet, 369: 1270-1276.
- Figueiredo, E.F.S., C.A.C. Junior, L.V. Furlanetto, F.G.S. Silva and R.S. Duarte *et al.*, 2012. Molecular Techniques for Identification of species of the *Mycobacterium tuberculosis* Complex: The Use of Multiplex PCR and an Adapted HPLC Method for Identification of *Mycobacterium bovis* and Diagnosis of Bovine Tuberculosis. In: Understanding Tuberculosis-Global Experiences and Innovative Approaches to the Diagnosis, Cardona, P.J. (Ed.). InTech Publisher, USA., ISBN: 978-953-307-938-7, pp: 411-432.
- Figueiredo, E.F.S., F.G. Silvestre, W.N. Campos, L.V. Furlanetto and L. Medeiros *et al.*, 2009. Identification of *Mycobacterium bovis* isolates by a multiplex PCR. Braz. J. Microbiol., 40: 231-233.
- Figueiredo, E.F.S., R.C.T. Carvalho, F.G. Silvestre, W. Lilenbaum, L.S. Fonseca, J.T. Silva and V.M.F. Paschoalin, 2010. Detection of *Mycobacterium bovis* DNA in nasal swabs from tuberculous cattle by a multiplex PCR. Braz. J. Microbiol., 41: 386-390.
- Gan, W., X. Zhou, H. Yang, H. Chen and J. Qiao *et al.*, 2013. Development of a test for bovine tuberculosis in cattle based on measurement of gamma interferon mRNA by real-time PCR. Vet. Rec., 173: 117-117.
- Good, M. and A. Duignan, 2011. Perspectives on the history of bovine TB and the role of tuberculin in bovine TB eradication. Vet. Med. Int. 10.4061/2011/410470
- Hosek, J., P. Svastova, M. Moravkova, I. Pavlik and M. Bartos, 2006. Methods of mycobacterial DNA isolation from different biological material: A review. Vet. Med., 51: 180-192.

- Lermo, A., S. Liebana, S. Campoy, S. Fabiano and I.M. Garcia *et al.*, 2010. A novel strategy for screening-out raw milk contaminated with *Mycobacterium bovis* on dairy farms by double-tagging PCR and electrochemical genosensing. *Int. J. Microbiol.*, 13: 91-97.
- Mukherjee, F., 2006. Comparative prevalence of tuberculosis in two dairy herds in India. *Rev. Sci. Tech.*, 25: 1125-1130.
- OIE, 1999. Bovine Tuberculosis. In: Manual of Standards: List B Diseases, OIE (Ed.). Office International des Epizooties, Paris, France, pp: 1-9.
- Palmer, M.V. and W.R. Waters, 2006. Advances in bovine tuberculosis diagnosis and pathogenesis: What policy makers need to know. *Vet. Microbiol.*, 112: 181-190.
- Phillips, C.J.C., C.R.W. Foster, P.A. Morris and R. Teverson, 2003. The transmission of *Mycobacterium bovis* infection to cattle. *Res. Vet. Sci.*, 74: 1-15.
- Rohonczy, E.B., A.V. Balachandran, T.W. Dukes, J.B. Payeur and J.C. Rhyan *et al.*, 1996. A comparison of gross pathology, histopathology and mycobacterial culture for the diagnosis of tuberculosis in elk (*Cervus elaphus*). *Can. J. Vet. Res.*, 60: 108-114.
- Srivastava, K., D.S. Chauhan, P. Gupta, H.B. Singh and V.D. Sharma *et al.*, 2008. Isolation of *Mycobacterium bovis* and *M. tuberculosis* from cattle of some farms in north India-Possible relevance in human health. *Indian J. Med. Res.*, 128: 26-31.
- Stavri, H., O. Moldovan, F. Mihaltan, D. Banica and R.J. Doyle, 2003. Rapid dot sputum and serum assay in pulmonary tuberculosis. *J. Microbiol. Meth.*, 52: 285-296.
- Verhagen, L.M., S. van den Hof, H. van Deutekom, P.W. Hermans, K. Kremer, M.W. Borgdorff and D. van Soolingen, 2011. Mycobacterial factors relevant for transmission of tuberculosis. *J. Infect. Dis.*, 203: 1249-1255.