Screening Milk for Bovine Tuberculosis in Dairy Farms in Central Province, Sri Lanka

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ABSTRACT: *Bovine tuberculosis is a chronic bacterial disease caused by Mycobacterium bovis. It can be transmitted to humans through direct contact with infected cattle or consumption of unpasteurized milk and milk products. The current study was performed to assess whether raw milk produced in three large scale dairy farms in Central Province of Sri Lanka contain any M. bovis. Raw milk samples were collected from 330 cows representing 230 single intradermal comparative cervical tuberculin test reactors and 100 none-reactors. All milk samples were cultured on Lowenstein Jensen medium with/ without sodium pyruvate for the isolation of Mycobacterium spp., and slopes were observed for the occurrence of growth daily for the first week and then at weekly intervals for 8 weeks. Direct Polymerase Chain Reaction (PCR) was performed simultaneously on all milk samples to detect M. bovis after extracting DNA with a commercial kit. The minimum detection level of M. bovis for PCR in milk was 200 CFU/mL. Only two milk samples from reactive cows were positive for acid fast bacilli. However, their cultures were confirmed as non-tuberculous mycobacteria by PCR. Consequently, all milk samples were confirmed negative for M. bovis according to direct PCR. It was concluded that the milk samples from three large scale dairy farms in Central Province of Sri Lanka did not contain M. bovis.*

Keywords: *Bovine tuberculosis, M. bovis, Milk, PCR*

INTRODUCTION

Bovine tuberculosis is an emerging zoonotic disease caused by *M. bovis*, a member of the *Mycobacterium tuberculosis* complex (Van Soolingen *et al*., 1997). Most mammals are susceptible to *M. bovis* due to its broad host spectrum whereas, cattle are the primary host among the domesticated animals (Schmitt *et al*., 2002). Although *Mycobacterium tuberculosis* act as the causative organism in majority of the human tuberculosis cases, a considerable number of cases have been caused by *M. bovis* in humans (De la Rua-Domenech, 2006). In addition to the public health risk, bovine tuberculosis imparts great economic losses in livestock industry due to the trade restrictions, reduced milk production and reproductive losses (Boland *et al*., 2010). The prevalence of bovine tuberculosis is higher in developing countries than in most of the industrialized countries due to lack of efficient

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control programs and consumption of raw milk or unpasteurized dairy products (Ashford *et al*., 2001).

Bovine tuberculosis can be transmitted to human through inhalation of aerosols, consumption of contaminated food such as milk and meat products or direct contact with infected animals (Une and Mori, 2007). Consumption of unpasteurized milk and dairy products, especially soft and hard cheeses has been identified as the primary risk factor of *M. bovis* infection in humans (Bolaños *et al*., 2017).

Mycobacterium bovis and atypical mycobacteria have been isolated from raw cow milk in many studies conducted in several countries such as Tanzania, Turkey, Zambia, Tunisia and Brazil (Bolaños *et al*., 2017). Bovine tuberculosis has been reported and confirmed in cattle in Sri Lanka since 2012 as reported by Department of Animal Production and Health (Anonymous, 2014; Kumara *et al*., 2014; Kumara *et al*., 2015). During the last few years there had been several deaths of cattle due to tuberculosis in few farms in Central Province of Sri Lanka (Kumara *et al*., 2014; Kumara *et al*., 2015). Therefore, it is essential to ensure that the milk and milk products are free from *M. bovis* in order to minimize the public health concerns.

Bovine tuberculosis in live cattle is usually diagnosed on the basis of delayed hypersensitivity reaction in single intradermal comparative cervical tuberculin test (SCITT). Tuberculin test which can be easily performed on large number of cattle has been traditionally used worldwide for the detection of bovine tuberculosis. However, there are issues associated with this test due to the broad range of sensitivity and specificity those arise from skin reactive common antigens of all mycobacteria (Figueiredo *et al*., 2010). Culture and identification of the organism is the gold standard of diagnosis for *Mycobacterium tuberculosis* complex. However, *M. tuberculosis* and *M. bovis* take several weeks for growth causing considerable delays in decision making regarding bovine tuberculosis outbreaks and on suspected meat inspection cases. Therefore the use of molecular techniques, such as Polymerase Chain Reaction (PCR) provide rapid, safe and reliable means for the diagnosis of bovine tuberculosis where *Mycobacterium* spp. can be identified directly from samples without culturing (Brosch *et al*., 2002; Nahar *et al*., 2011). The objective of the current study was to assess raw milk produced in three large scale dairy farms in Central Province of Sri Lanka for the presence of *M. bovis* using both conventional culture methods and PCR.

MATERIALS AND METHODS

Sample collection

Milk samples were collected from three large scale dairy farms in Central Province of Sri Lanka from May 2016 to October 2017. Altogether 330 milk samples were collected including 230 SICCT positive cows and 100 negative cows. The SICCT was previously performed on these cows as a routine screening test. During sampling, udder was disinfected using 70% alcohol and a pooled milk samples from all quarters were collected in to sterile containers. Fifty milliliters of milk was collected from each cow and stored in ice until delivered to the lab.

Culture procedure of milk samples

Ten milliliter of whole milk from each sample was centrifuged at 40000 g for 15 minutes and the supernatant was discarded. The sediment was suspended in sterilized physiological saline solution and equal volume of 4% sterile sodium hydroxide was added for decontamination. Suspension was incubated at 37 ℃ for 30 minutes and centrifuged at 40000 g for 30 minutes. The sediment was neutralized with 6% hydrochloric acid and re-centrifuged before inoculation on Lowenstein Jensen medium (LJ) and LJ with sodium pyruvate (LJP), and incubated at 37 ºC (Kahla *et al*., 2011; Franco *et al*., 2013). Culture bottles were observed for occurrence of growth daily for first week and thereafter at weekly intervals for 8 weeks.

DNA extraction and PCR amplification

DNA was extracted from whole milk using ReliaprepTM gDNATissue Miniprep kit (Promega, Madison, USA) according to the manufacturer's instructions. The specific PCR amplification for *M. bovis* was performed with the RD4 and RD9 flanking primers according to previously described protocols with modifications (Brosch et al., 2002). The reaction mixture for PCR contained 10 µl of 2x PCR master mix (Promega, Hilden, USA), 0.5 µl of 10 pmol/µl forward and reverse primer, 7 µl of nuclease free water, 2 µl of DNA template in a volume of 20 µl. The reaction was performed in a thermal cycler (Veriti, Thermo Fisher Scientific, USA). The cycling conditions were an initial denaturation at 95 ℃ for 15 minutes followed by a 30 cycles of denaturation at 94 ℃ for 1 minutes, annealing at 61 ℃ for 1minute and primer extension at 72 ℃ for 1 minutes, with a final extension at 72 ℃ for 10 minutes (Brosch et al., 2002; Warren et al., 2006). Positive control *M. bovis* and *M. tuberculosis* reference strain H37Rv DNA was obtained from collaborator's laboratory in UK.

Sensitivity and specificity assays

Sensitivity assay was performed by spiking whole milk from an SICCT negative cow with *M. bovis* (10^{-1}-10^{6} CFU/mL). A standard stock suspension prepared using *M. bovis* Bacillus Calmette-Guérin (BCG) vaccine strain was used for spiking due to safety reasons. DNA was extracted from spiked milk samples and PCR was performed using identical protocols as described above. The PCR for the specificity assay was carried out using DNA from *M. fortuitum*, *M. chelonae*, *M. gordonae*, *Staphylococcus* spp., *Streptococcus* spp., *Bacillus* spp., *Klebsiella* spp. and *M. tuberculosis* reference strain H37Rv DNA.

RESULTS AND DISCUSSION

According to the current study, only two milk samples (0.8%) were positive for acid fast bacilli. These two samples were originating from SICCT reactors. However, the PCR results of the two samples were negative (Figure 1), confirming them as non-tuberculous mycobacteria (NTM). Species level identification of the NTM was not attempted. Direct PCR results for all milk samples were also negative, and thus confirmed that none of the milk samples contained *M. bovis*. Different target regions including RvD1-Rv2031c, MPB70, RD4/RD9 and IS6110 have been used in the previously described PCR assays for detecting *M. bovis* in milk (Romero *et al*., 1999; Perez *et al*., 2002). The PCR assay employed in the current study used RD4 and RD9 deletion typing (Brosch *et al*., 2002; Warren *et al*., 2006). Cadmus *et al.* (2010) have reported 1% positivity for *M. bovis* in milk samples by PCR using RD4 and RD9 deletion typing. However, molecular studies conducted

in different countries have shown widely different results for the presence of *M. bovis* in SICCT positive cow milk varying from 0 to 50% (Romero *et al*., 1999; Perez *et al*., 2002; Zumárraga *et al*., 2005).

Figure 1. Representative agarose gel electrophoresis of PCR products from milk samples. Lane M: 100 bp DNA ladder, 1 – 11: DNA from milk samples, 12: *M. bovis* positive control DNA

For example, IS6110 PCR has detected 28% of milk samples positive for *M. bovis* in one study while all milk samples were negative for *M. bovis* in another study (Perez et al., 2002; Zumarraga *et al*., 2005). It implies that the presence of *M. bovis* in milk is highly heterogeneous and these variable results could be attributed to the variation of shedding of *M. bovis* with the progression of the disease, cell mediated immunity and epidemiological factors such as metabolic imbalances and peripartum conditions resulting different levels of pathogens in body fluids such as milk (Pollock *et al*., 2005; Piccinini *et al*., 2006).

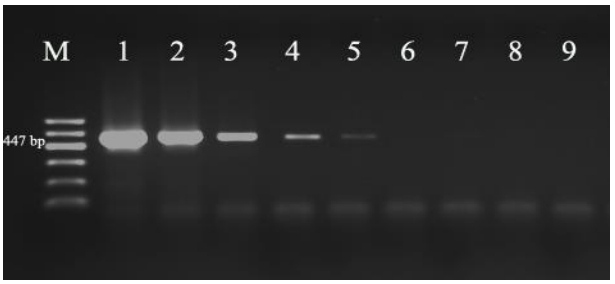

Figure 2. Agarose gel electrophoresis of PCR products from 10-fold serial dilutions of *M. bovis* BCG in whole milk. Lane M: 100bp DNA ladder, Lane 1 to 6: Milk samples added with *M. bovis* ($2*10^6$ to $2*10^{-1}$ CFU/mL), 9: Negative control

The identification of NTM including *M. flavescens*, *M. terrae*, *M. smegmatis*, *M. fortuitum* and *M. gordonae* in milk has been described in several previous studies conducted in Tanzania, Turkey and Brazil (Kazwala *et al*., 1998; Konuk *et al*., 2007; Franco *et al*., 2013). Similarly, saprophytic mycobacteria were recovered from 13% out of 285 milk samples in a study conducted in Michigan (Sanchez and Rosell, 1983). Although *M. tuberculosis* and *M. bovis* act as the major pathogenic species, NTM have become increasingly important in causing human infections in recent years (Bolaños *et al*., 2017). According to World Health Organization estimates, cow milk may be the source of approximately 15 foodborne bacterial infections including *M. bovis* (Abrahão *et al*., 2005).The sensitivity of the PCR assay was confirmed by using DNA extracted from whole milk added with *M. bovis* BCG vaccine

strain at concentrations of 10^1 to 10^6 CFU/mL (Figure 2). The limit of detection of the PCR assay for *M. bovis* in whole milk was 200 CFU/mL where the specific amplification of the fragment for *M. bovis* (447 bp) was clearly detected (Figure 3). According to the previous studies, the limit of detection of the PCR for *M. bovis* in milk varies from 80-1000 CFU/mL (Junior *et al*., 2005; Zumárraga *et al*., 2005). It has been reported that an udder infection can result in the shedding of *Tuberculosis bacilli* ranging from 500 to 500,000 CFU/mL of milk (Zanini *et al*., 1998). Therefore, the estimated limit of detection of 200 CFU/mL can be expressed as an acceptable level for the diagnosis of *M. bovis* in milk. No amplifications were observed for *M. fortuitum*, *M. chelonae*, *M. gordonae*, *Staphylococcus* spp., *Streptococcus* spp., *Bacillus* spp. and *Klebsiella* spp. or *M. tuberculosis* H37Rv in the specificity assay (Figure 3).

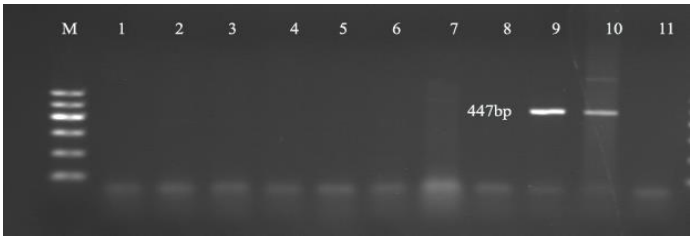

Figure 3. Specificity of the PCR. Agarose gel electrophoresis of PCR fragments of specificity assay
Lane M-100bp DNA ladder, 1: *M. fortuitum* 2: *M. chelonae*, 3: *M. gordonae*, 4: *Staphylococcus* spp. 5: *Streptococcus* spp. 6: *Bacillus* spp. 7: *Klebsiella* spp. 8: *M. tuberculosis* H37Rv, 9: *M. bovis* positive control, 10: *M. bovis* BCG 11: Negative control

CONCLUSIONS

Microbiological culture enumeration methods together with PCR based identification could be used as possible tools for the identification of *M. bovis* in whole milk. The PCR assay allowed the detection of *M. bovis* DNA in whole milk, with a detection limit of 200 CFU/mL and this can be used as a direct detection method for the diagnosis of *M. bovis* in whole milk. According to the present study, none of the whole milk samples collected in three large scale dairy farms in Sri Lanka was contaminated with *M. bovis*.

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