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Short communication

Detection of nontuberculous mycobacteria from water buffalo raw milk in Brazil

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ABSTRACT

Milk is an important nutritional source to man and water buffalo raw milk is used to produce mozzarella cheese. Products from unpasteurized milk have been associated with certain infectious diseases and can carry pathogenic mycobacteria. Nontuberculous mycobacteria (NTM) are emerging pathogens causing opportunistic infections in humans and animals. The objectives of this study were to demonstrate the presence of mycobacteria in water buffaloes' milk and to determine their role as possible sources of NTM infections. In this study, raw milk samples from dairy water buffaloes (*Bubalus bubalis*) ($N = 23$) were decontaminated by Petroff method and inoculated on to Löwenstein–Jensen and Stonebrink medium. After confirming positive colonies for acid fast bacilli (AFB) by Ziehl-Neelsen technique, the isolated mycobacteria were identified by PCR-Restriction Enzyme Analysis (PRA) and mycolic acids analysis by thin-layer chromatography (TLC). *Mycobacterium simiae* (2 isolates), *Mycobacterium kansasii* (2 isolates), *Mycobacterium flavescens* (2 isolates), *Mycobacterium gordonae* (3 isolates) and *Mycobacterium lentiflavum* (1 isolate) were identified by these techniques. The isolation of opportunistic pathogens such as *M. kansasii*, *M. simiae* and *M. lentiflavum* from raw milk represent a risk for the consumers of mozzarella cheese made by this milk.

1. Introduction

Milk is an important source of protein and other nutrients, but it can also be contaminated by pathogenic agents. The transmission of environmental mycobacteria from infected animals to humans is real, most likely by ingestion of infected milk and raw (unpasteurized) dairy products, meaning a risk factor for foodborne diseases (Leite et al., 2003).

Nontuberculous mycobacteria (NTM) have been variously described by the adjectives as "atypical", "anonymous", "mycobacteria other than TB (MOTT)", "environmental", "environmental opportunistic", and, seemingly most commonly in the medical literature, "nontuberculous" or "NTM" (Falkinham, 2002). From a total of 120 mycobacterial species recognized in the world, 50 species are considered opportunistic NTM. They are aerobic and rod type bacteria and have an acid-fast dying features (Tortoli, 2006). NTM are normal inhabitants of natural waters, drinking waters, animals, birds and soils. Thus, the environment is most important source of infections of those mycobacteria to humans, mainly entering the human body through gastrointestinal mucosal membrane by the ingestion of food. They have even been recovered from meat, fish, dairy products, fruits, vegetables and specially milk (Leite et al., 2003; Konuk et al., 2007; Norby et al., 2007).

In many countries, unpasteurized milk has historically been and is still being used for making cheese, especially water buffalo mozzarella cheese (Mauriello et al., 2003). In contrast of the high demand of water buffalo mozzarella cheese, there are just a few papers regarding water buffalo milk and mycobacterial contamination (Konuk et al., 2007). Jha et al. (2007) isolated. *Mycobacterium bovis* and other seven species of environmental mycobacteria in Nepal, from feces and raw milk of positive single intradermal tail caudal fold test (SIDt) milking buffaloes and cattle.

Although NTMs are widespread in the environment, there is no evidence that these organisms are transmitted person-to-person (Schlossberg, 2006). The incidence of human diseases caused by opportunistic NTM has increased as a result of various forms of immunosuppression, notably HIV infection and transplants (Zumla and Grange, 2002). The situation of nontuberculous mycobacterial disease has been drastically changed by emergence of the AIDS epidemic throughout the world and 25–50% of immunocompromised hosts in the United States and Europe eventually become infected by NTM (Donnabella et al., 2000). Many pathogenic NTM have been identified and they are associated with a wide variety of localized, organ-specific, and systemic infections and because of

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that, there is an urgent need for better understanding of the sources and routes of NTM transmission (Konuk et al., 2007).

Procedures for the isolation of NTM by conventional methods are fastidious, requiring several weeks for adequate growth and sometimes, accurate identification by biochemical tests is not possible. For this reason, genotypic methods for the identification of mycobacteria have been developed and used in recent years (Leite et al., 2005).

Telenti et al. (1993) described a method of mycobacterial differential diagnosis, named PRA (PCR-Restriction Enzyme Analysis), based on the amplification of 441 bp fragment of the *hsp65* gene by PCR and followed by digestion of the amplified product with BstEII and HaeIII restriction enzymes. Approaches based on PRA demonstrate an easy, rapid and inexpensive way to identify several mycobacterial species (Seok et al., 2006).

Thin-layer chromatography (TLC) has been used by several laboratories for fast confirmation of mycobacterial identification according to their mycolic acid profiles. TLC is a useful technique due to its simplicity, length of the procedure and requirement for small quantities of material (Leite et al., 2005).

The objectives of this study were to demonstrate the presence of *Mycobacterium* spp. in whole raw water buffalo milk, which is used exclusively for making mozzarella cheese and also determine if this microorganisms are possible sources of human infection or colonization.

2. Materials and methods

2.1. Milk samples

Between January and December of 2004, raw milk samples were obtained from a model farm producer of buffalo's milk in Brazil where animals are breed in a semi-extensive way. In this research, 23 randomly Murrah water buffaloes (*Bubalus bubalis*) were selected and one sample of each animal was collected and studied. Seven animals had a test positive to bovine tuberculosis, SIDt (single intradermal tail caudal fold test), and 16 had a negative SIDt. This important dairy farm in State of Sao Paulo has a herd of 72 water buffaloes, but only 40 were at lactation period, producing 200 L milk per day. The samples were brought to the Faculty of Pharmacy for processing.

2.2. Isolation procedures

Before processing, special care was taken to avoid contamination with environmental bacteria or fungi: all the collecting material was decontaminated with 0.5% sodium hypochlorite. The samples were processed in a biological safety cabinet class II B2 which was cleaned with 0.5% sodium hypochlorite and 70% alcohol followed by UV light for 30 min with the air circulating. Only heat sterilized centrifuge tubes with screw tap were used. The milk samples (5 ml) were decontaminated by the Petroff method (Palomino and Portaels, 1998) and centrifuged at 3000 × g for 20 min. The sediment and the milk fat were inoculated onto Löwenstein–Jensen (LJ) and Stonebrink Media. Cultures were incubated in the presence of 5–10% CO_2 at 30 °C and 37 °C for 90 days and inspected weekly for bacterial growth. Colonies positive for Acid fast bacilli (AFB) by Ziehl-Neelsen technique were submitted to a molecular identification by PRA (Silva et al., 2001) and also using TLC methodology (Leite et al., 2005).

2.3. PRA

DNA extraction was performed by adding a loopful of mycobacteria into 400 μl TE buffer (10 mM Tris–HCl, 1 mM EDTA-pH 8, 1% Triton X-100), heated at 95 °C for 10 min, and stored at −20 °C. Amplification of a 441 bp fragment from the *hsp65* gene with primers Tb11 (5′-ACCAACGATGGTGTGTCCAT-3′) and Tb12 (5′-CTT GTCGAACCGCATACCCT-3′) was performed according to the protocol described by Telenti et al. (1993). Reaction mix contained 20 mM Tris, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 10% glycerol, 200 μM dNTP, 25 pmol of each primer and 1U Taq polymerase (Gibco/BRL). DNA was submitted to denaturation at 95 °C for 10 min, followed by 45 cycles at 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min and a final extension step at 72 °C for 7 min. Ten to fifteen microliters of amplified product were digested with BstEII and HaeIII (Gibco/BRL) and loaded on 4% agarose gel (Gibco/BRL) and analyzed on the internet Prasite (2007).

2.4. Mycolic acids by TLC

Mycolic acid extraction and methylation procedures were carried out according to the methodology proposed by Leite et al. (2005). Approximately, 25–50 mg of mycobacteria derived from both media were dispersed into a 5% potassium hydroxide solution in 2-methoxyethanol. The mixture was maintained at 110 °C for 2 h, cooled, and subsequently acidified with 1 ml of sulfuric acid solution (20%, w/w), after which the mycolic acids were released from their potassium salts by the sulfuric acid treatment. The mycolic acids were then extracted by shaking the mixture twice with diethyl ether (5 ml). The ether phase was decanted and washed three times with 2 ml of water. Ether was removed in a water bath, leaving a residue of mycolic acids that was methylated by adding 1 ml of a diazomethane ether solution. This reagent was prepared with nitrosomethylurea–potassium hydroxide. To carry out TLC, the methyl esters of the mycolic acids were spotted onto silica gel G (20 × 20 cm × 0.25 mm plates). Mycolic acids of the reference strains spotted together served as a standard for the proper identification of the mycolic acids extracted from the strains under study. Using two different solvent systems, one-dimensional analysis was performed: diethyl ether/petroleum ether (12:88, v/v) with three chromatogram developments; and dichloromethane with a single development. The visualization of spots was achieved by spraying the chromatograms with 0.01% (w/v) rhodamine in phosphate buffer.

3. Results and discussion

From 23 raw milk samples analyzed, nontuberculous mycobacteria were found in five (21.7%) samples, two of them (8.7%) derived from two positive animals and three (13.1%) from three negative animals. No *M. bovis* were found in all samples, even in the seven samples derived from SIDt positive animals. These results support the general belief that the reaction to tuberculin in water buffaloes is often pronounced, and that there are more doubtful and nonspecific reactions in buffaloes than in cattle. In view of their predilection to wallowing in mud and water, buffaloes may have widespread exposure to environmental and saprophytic mycobacteria, which may cause a high level of false positive cross-reactivity to bovine tuberculin PPD (Protein Purified Derivative) (Kanameda et al., 1999).

A total of 10 AFB were isolated and identified at species level using PRA and mycolic acids analysis by TLC. Table 1 shows samples with positive cultures and Fig. 1 shows PRA results based on the digestion of amplified fragments of 441-bp from *hsp65* gene. The results according both methodologies were: *Mycobacterium simiae* (2 isolates), *Mycobacterium kansassii* (2 isolates), *Mycobacterium flavescens* (2 isolates), *Mycobacterium gordonae* (3 isolates) and *Mycobacterium lentiflavum* (1 isolate). The mycolate profiles were respectively I, II, IV for *M. simiae* and *M. lentiflavum*, I, III, IV for