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Research Paper

Occurrence of *Mycobacterium bovis* and non-tuberculous mycobacteria (NTM) in raw and pasteurized milk in the northwestern region of Paraná, Brazil

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Abstract

Milk is widely consumed in Brazil and can be the vehicle of agent transmission. In this study, was evaluated the occurrence of *Mycobacterium bovis* and non-tuberculous mycobacteria (NTM) in raw and pasteurized milk consumed in the northwestern region of Paraná, Brazil. Fifty-two milk samples (20 pasteurized and 32 raw) from dairy farms near the municipality of Maringa, Parana State, Brazil were collected. Milk samples were decontaminated using 5% oxalic acid method and cultured on Lowenstein-Jensen and Stonebrink media at 35 °C and 30 °C, with and without 5-10% CO_2. Mycobacteria isolates were identified by morphological features, PCR-Restriction Fragment Length Polymorphism Analysis (PCR-PRA) and Mycolic acids analysis. Thirteen (25%) raw and 2 (4%) pasteurized milk samples were positive for acid fast bacilli growth. Nine different species of NTM were isolated (*M. nonchromogenicum*, *M. peregrinum*, *M. smegmatis*, *M. neoaurum*, *M. fortuitum*, *M. chelonae*, *M. flavescens*, *M. kansasii* and *M. scrofulaceum*). *M. bovis* was not detected. Raw and pasteurized milk may be considered one source for NTM human infection. The paper reinforces the need for intensification of measures in order to avoid the milk contamination and consequently prevent diseases in the south of Brazil.

Key words: non-tuberculous mycobacteria, milk, *Mycobacterium,* PCR-PRA, mycolic acids analysis.

Introduction

Milk is considered a potential vehicle for transmission of some organisms which may be pathogenic for humans. External interferences in the temperature of pasteurization, extreme bacterial load contamination during milking and bottling process may favor the survival of some species of bacteria including pathogenic or facultative pathogenic mycobacteria (Donaghy *et al.*, 2007; Leite *et al.*, 2003).

Some species of mycobacteria, such as *Mycobacterium bovis* and non-tuberculosus mycobacteria (NTM), are zoonotic agents with a wide range of mammalian hosts (Konuk *et al.*, 2007). In the past, *M. bovis* was a significant cause of human tuberculosis (TB) mainly in children (Thoen *et al.*, 2006). Pasteurization of milk and dairy products dropped the disease rate drastically. Although, in developed countries, bovine tuberculosis has been considered

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under control, the re-emergence of the disease has been reported (Rowe and Donaghy, 2008; Thoen *et al.*, 2006).

According to Cosivi *et al.* (1998), *M. bovis* should be considered a problem of human public health considering its involvement in 2% pulmonary and 8% extrapulmonary TB cases in Latin America. Meantime, TB incidence caused by *M. bovis* in humans is difficult to investigate due to the TB laboratory diagnosis is based on acid-fast staining and culture in Lowenstein-Jensen medium (LJ), which does not promote *M. bovis* growth (Leite *et al.*, 2003).

NTM are ubiquitous organisms and were believed to represent environmental contamination. Recently, these organisms have been recognized a significant cause of infection in both immunocompetent (Bodle *et al.*, 2008; Dailloux *et al.*, 2006) and immunocompromised humans mainly with the emergence of HIV/AIDS (Falkinham, 1996; Konuk *et al.*, 2007). In AIDS patients, the NTM had a direct impact on the picture of the mycobacterial disease. In these patients and other immunodeficient individuals mycobacterial disease is usually disseminated (Falkinham, 1996) and may cause death in few weeks.

NTM species may be transmitted to humans from the environment, such as ingestion of contaminated food (Pardo *et al.*, 2001), water, fruit, vegetable and milk (Konuk *et al.*, 2007). There is no evidence that these organisms are transmitted person-to-person (Schlossberg, 2006).

Several recent reports have suggested the incidence of NTM disease is increasing in many countries, as in Taiwan (Chih-Cheng *et al.*, 2010), Canada (Marras *et al.*, 2007), England, Wales and Northern Ireland (Moore *et al.*, 2010). In 2009, an outbreak of subcutaneous abscesses due to *M. abscessus* was detected in Spain which affected healthy women who had undergone mesotherapy procedures in an aesthetic clinic (Galmés-Truyols *et al.*, 2011). In Brazil, outbreak of postoperative infections by NTM have been recognized in some regions (Macedo and Henriques, 2009) and characterized as a public health problem (Fontana, 2008).

In south of Brazil, the state of Parana had an increased number of notified cases of rapidly growing mycobacteria in the period 1998-2009, especially the species *M. abscessus* subsp *bolletti* which represented 19.8% of cases of infection associated with invasive procedures (Brasil, 2011).

The emergence of NTM, as significant environmental pathogens, has attracted more attention (Brasil, 2011; Moore *et al.*, 2010). Investigations on NTM transmission sources and mechanisms would contribute for better epidemiological understanding of disease caused by these mycobacteria. Some works show that animal products such as milk, seem to be reservoirs of mycobacteria and may pose a risk to the public (Carvalho *et al.*, 2009).

In Brazil, *M. kansasii*, *M. simiae* and *M. lentiflavum* has been isolated from buffalo raw milk (Jordão Jr. *et al.*, 2009) and *M. avium* subspecies *paratuberculosis* was detected in 3.6% of the bovine milk in the Minas Gerais State, Brazil (Carvalho *et al.*, 2009).

The objective of the present study was to detect the occurrence of *M. bovis* and NTM in raw and pasteurized milk consumed in the northwestern region of Paraná, Brazil and to identify them by morphological features, mycolic acid analysis and PCR-Restriction Fragment Length Polymorphism Analysis (PCR-PRA) of *hsp*65 gene.

Materials and Methods

Milk samples collection

A total of 52 milk samples (32 raw and 20 pasteurized) were cultured for *M. bovis* and non-tuberculous mycobacteria. *M. avium* subspecie *paratuberculosis* was not included in the research. The raw milk samples were collected directly from different dairy farms and the pasteurized milk, belonging to eighteen commercial brands was randomly sampled in supermarket chains. The milks were collected in autumn months (from April to May) in the region of Maringa, state of Parana, Brazil. Samples were obtained under aseptic conditions. All samples were transported to the laboratory, in ice box, for pretreatment and culture on the same day of sampling.

Milk samples cultures

After homogenization, milk samples (5 mL) were submitted to 5% oxalic acid decontamination process (Leite *et al.*, 2003) and centrifuged at 3.000 g for 10 min at 4 °C. A 200 μL aliquot of each decontaminated sample were seeded onto LJ (Becton, Dickinson and Company, Sparks, MD, USA) and Stonebrink medium (Stonebrink *et al.*, 1969). Cultures were incubated at 35 °C with and without 5-10% CO_2 and at 30 °C in normal atmosphere for up to 3 months, and inspected weekly for mycobacterial growth Stonebrink and LJ (Kent and Kubica, 1985; Leite *et al.*, 1998). All colonies that suggested growth of mycobacteria were stained for acid-fast bacilli (AFB) and examined under an optic microscope.

Identification of mycobacterial species

All AFB colonies were initially identified by conventional methods (rate growth, colonial morphology, pigment production) (Kent and Kubica, 1985). Identification by mycolic acid analysis was carried out by one dimensional thin layer chromatography (TLC) according to Leite *et al.* (1998).

Molecular identification of the mycobateria isolates was carried out by PCR-Restriction Fragment Length Polymorphism Analysis (PCR-PRA) (Telenti *et al.*, 1993). Mycobacteria DNA was extracted as described by Bollela *et al.* (1999). Briefly, a loop full of fresh LJ-culture was suspended in 1 mL of distilled water, boiled for 10 min and placed at -20 °C for 10 min. This procedure was repeated

three times and then centrifuged 5 min at 12,000 *g*. The supernatant was used for PCR amplification.

PCR was based on the amplification of a 439 bp segment of the *hsp65* gene using the primers Tb11 (5'-ACC AAC GAT GGT GTG TCC AT-3') and Tb12 (5'-CTT GTC GAA CCG CAT ACC CT-3'). PCR assays used 5 μL of DNA in 20 μL of the reaction mixture containing 0.5 μM of each primer (Integrated DNA Technologies, Inc. Coralville, USA) and PCR Master Mix (Promega Corporation, Madison, Wisconsin, USA), according to manufacturer's instruction. DNA amplification was carried out in an Eppendorf thermocycler (Mastercycler® gradient PCR, Hamburg, Germany) using conventional amplification with an initial cycle of 5 min at 94 °C, followed by 45 cycles of 1 min at 94 °C, 1 min at 60 °C and 1 min at 72 °C, and a final extension of 10 min at 72 °C (Telenti *et al.*, 1993).

PCR product was digested with 10 U *Bst*EII (Boehringer Mannhein., Germany) and *Hae*III (Invitrogen TM, CA, the USA) endonucleases. Restriction fragments were separated by 3% agarose gel electrophoresis in TBE buffer (0.45 mM Tris-HCl, 0.45 mM boric acid, 2.5 mM EDTA, pH 8.0) for 1 hour at 100 V. DNA ladders (50 bp and 25 bp) (Invitrogen life technologies, São Paulo, Brazil) were used as molecular markers. Gels were stained with ethidium bromide, visualized under ultraviolet light and photodocumented with a Power Shot S215 Digital camera (Cannon, NY, USA). Restriction fragments were analyzed using the PRA site (http://app.chuv.ch/prasite/index.html) and results were compared with those previously described (Brunello *et al.*, 2001; Devallois *et al.*, 1997; Telenti *et al.*, 1993).

Statistical analysis

Occurrence of mycobacteria in raw and pasteurized milk samples was compared with chi-square test. Differences with p-value lower than 0.05 were considered significant.

Results

A total of 15 milk samples (28.8%) were contaminated with NTM (Table 1). The occurrence of mycobacteria in raw samples (25.0%) was higher than in pasteurized ones (3.8%, p = 0.039).

Nine different NTM were isolated and identified by rate growth, colonial morphology, pigment production, mycolic acid analysis and PCR-PRA from 15 milk samples. Five milk samples (four raw and one pasteurized) were positive for two different species of NTM in each sample (Table 2). No slow-grower mycobacteria from the *Mycobacterium tuberculosis* complex (*M. bovis*) were isolated.

The most frequent NTM found in milk samples were *M. nonchromogenicum* (25.0%, 5/20 isolates), *M. peregrinum* (20.0%, 4/20 isolates) and *Mycobacterium smegmatis* (15.0%, 3/20 isolates) (Table 2). *M. nonchromogenicum* and *M. smegmatis* were isolated only from raw samples, while *M. peregrinum* was isolated from two raw and two pasteurized samples.

Discussion

The current study contributed towards demonstrating the diversity of mycobacteria species in milk from farms in Maringa, state of Parana, southern Brazil. The results are relevant since approximately 50% or more of all milk consumed in Brazil is not pasteurized (Leite *et al.*, 2003) and specifically a section of the population of the town around Maringa drinks raw milk and uses raw dairy products.

Considering the importance of bovine tuberculosis for the public health and that no data is available about the prevalence of *M. bovis* in local cattle herds or disease caused by this *Mycobacterium* species in human, the milk samples were cultured for *M. bovis* in Stonebrink medium. *M. bovis* was not detected in the present study; however, we consider the number of samples is too few to draw a conclusion about tuberculosis in local dairy cows. Meantime, considerable number of NTM was cultured from raw and pasteurized milk samples in LJ and Stonebrink media at 30 ºC and 35 °C.

NTM were detected in raw and pasteurized milk in another study undertaken in the State of São Paulo, Brazil (Leite *et al.*, 2003) and included *M. fortuitum*, *M. marinum*, *M. kansasii*, *M. gordonae* and some unidentified rapidly growing mycobacteria. In a similar study, in Turkey, four NTM species (*M. terrae*, *M. kansasii*, *M. haemophilum* and *M. agri*) were isolated from raw milk (Konukt *et al.*, 2007).

It should be emphasized in the present study that some of NTM isolated in milk samples (*M. fortuitum, M. chelonae, M. kansasii, M. scrofulaceum*) are potential pathogens and may cause a variety of manifestation in hu-

Table 1 - Occurence of non-tuberculous mycobacteria in milk samples in south of Brazil.

Samples	Positive	Negative	Total	p
Raw	40.6% (13/32)	59.4% (19/32)	615% (32/52)	
Pasteurized	10% (2/20)	90% (18/20)	385% (20/52)	p = 0039[a]
Total	288% (15/52)	712% (37/52)	1000% (52)	

[a] p < 005, chi-square test.

Table 2 - Identification of non-tuberculous mycobacteria isolated from milk samples according to Mycolic acid analysis and PCR-PRA patterns.

Samples	Tests	Species		
	Mycolic acid	PRA[a] BstEII	HaeIII	
Raw	I, III, IV	235/130/85	130/105	*M kansasii* type2
Raw	I, IV, VI	235/115/85	145/60/55	*M nonchromogenicum* type 1
Raw	I, IV, VI	320/115	145/60/55	*M nonchromogenicum* type 2
Raw	I, IV, VI	440	150/85/55	*M flavescens* type 3
Raw	I, IV, VI	440	150/90/60	*M flavescens* type 3
Raw	I, IV, VI	320/115	170/140	*M neoaurum* type 1
	I, IV, VI	320/115	145/60/55	*M nonchromogenicum* type 2
Raw	I, IV, VI	440	150/90/60	*M flavescens* type 3
	I, IV, VI	235/210	145/130/95	*M scrofulaceum* type 1
Raw	I, V	235/210	145/140/100/55	*M peregrinum* type 1
Raw	I, V	235/130/85	145/125/60	*M smegmatis* type 1
Raw	I, V	235/130/85	145/125/60	*M smegmatis* type 1
Raw	I, V	235/130/85	145/125/60	*M smegmatis* type 1
	I, IV, VI	320/115	145/60/55	*M nonchromogenicum* type 2
Raw	I, V	235/115/85	145/120/60/55	*M fortuitum* type 1
	I, V	235/210	145/120/100/55	*M peregrinum* type 2
Raw	I, VI	320/115	145/60/55	*M nonchromogenicum* type 2
Pasteurized	I, V	235/210	140/120/100	*M peregrinum* type 2
Pasteurized	I, V	235/210	145/120/100/55	*M peregrinum* type 2
	I, II	320/130	200/60/55	*M chelonae* type 1

[a]PRA, PCR-restriction fragment length polymorphism analysis.

mans undergoing immune system suppression (Saad *et al.*, 1997, Wolinsky *et al.*, 1992). In a National surveillance, *M. kansasii* (13.7%) and *M. fortuitum* (10.8%) together with *M. avium* (44.1%) were the mainly NTM isolated in culture from patients with mycobacteriosis (Barreto and Campos, 2000).

Although it has been established that pasteurization kills *M. tuberculosis* in milk, survival of some nontuberculous *Mycobacterium* species after simulated laboratory pasteurization has been reported (Grant *et al.*, 1996). For example, *M. kansasii* isolated in raw milk, in this study, are among those mycobacteria extensively found in Brazilian environment, including water systems (Falcão *et al.*, 1993) and can survive the pasteurization (63.5 °C for 30 min) (Grant *et al.*, 1996).

M. peregrinum and *M. chelonae* were the two NTM detected in pasteurized milk. The detection of the mycobacteria in pasteurized milk may be explained by extreme bacterial load contamination during milking and bottling process (Grant *et al.*, 1996; Leite *et al.*, 2003) or the formation of mycobactera biofilm by incorrect maintenance of the equipment used in pasteurization process. According to Grant *et al.* (1996), clumping of the mycobacteria cells during heating or an increase of resistance by a non understood mechanism, may be responsible for surviving the pasteurization.

In conclusion, consumption of raw milk remains a risk factor for exposure to the NTM. The implementation of measures that prevent milk contamination during and post milking with NTM are needed to avoid diseases, which is relevant mainly for patients with immunological disorders.

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