Isolation and Identification of Mycobacteria from Livestock Specimens and Milk Obtained in Brazil

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The prevalence of Mycobacterium bovis *and other mycobacterial species in livestock specimens and milk was evaluated. An emphasis was placed upon the distribution of these organisms in milk that is readily available to the public that was either untreated, pasteurized, or treated using ultra high temperature. Twenty-two pathologic specimens from livestock (bovine, swine and bubaline) in five Brazilian states and 128 bovine milk samples from retail markets in the State of São Paulo were examined for mycobacteria. Identification was made by classical biochemical tests, thin layer chromatography of mycolic acids and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis. Mycobacteria were isolated from 15 (68.2%) caseous lesions and from 23 (18%) milk samples. Eleven isolates were identified as* M. bovis*, and the remaining 27 nontuberculous mycobacterial isolates were represented by five species and six unidentified rapidly growing mycobacterial strains. The data demonstrate that animal products in Brazil are frequent reservoirs of mycobacteria and may pose a risk to the public.*

Key words: *Mycobacterium* - livestock - bovine - polymerase chain reaction (PCR) - restriction fragment length polymorphism (RFLP) - mycolic acid analysis - Brazil

Milk and meat are important sources of protein and other nutrients but can be contaminated by pathogenic agents. The possibility exists for the transmission of tuberculosis and other mycobacterial infections from animals to humans, most likely by ingestion of infected meat or raw (unpasteurized) dairy products (Chapman & Speight 1968, Bryan 1969, Sweeney et al. 1992). In developed countries, the introductions of milk pasteurization and tuberculin (ppd) positive cattle eradication programs have greatly reduced the incidence of human and bovine diseases caused by *Mycobacterium bovis* (Caffrey 1994). In Brazil, however, where bovine tuberculosis control and eradication programs have only recently been implemented, *M. bovis* disease remains an important veterinary disease, with almost 200 000 infected cows among a total cattle population of ~ 170 million (Kantor & Ritacco 1994). This organism also poses human public health problems as well because it is suspected that *M. bovis* infection is responsible for approximately 4 000 of the approximately 80 000 cases of human tuberculosis reported each year in Brazil (WHO 1993). Johne's disease, another important veterinary disease caused by *M. paratuberculosis* infection in cattle, has not been reported in Brazil, with rare exceptions among imported animals (Roxo 1997).

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In areas of endemicity where bovine and human tuberculosis coexist, the differentiation of *M. bovis* from *M. tuberculosis* is important for monitoring the spread of *M. bovis* among cows and from them to humans. Distinguishing these two species by conventional (e.g. biochemical) tests is typically more time consuming than by molecular methods (Scorpio et al. 1997). The genotypic detection of insertion element IS*6110* is a reliable assay to identify species that belong to the *M. tuberculosis* complex (MTC) and has been applied to studies of MTC infections in cattle (Gutiérrez et al. 1995, Vitale et al. 1998), although the identification of IS*6110* by polymerase chain reaction (PCR) fails to differentiate *M. bovis* from *M. tuberculosis*. The polymorphisms in the two genes, *oxyR* and *pncA,* appear to be stable features of these two species, and identification of these genes by restriction analysis of PCR products or by allelic-specific PCR amplification has proven useful toward for this end (Sreevatsan et al. 1996, De los Monteros et al. 1998). In addition, the mycobacterial species other than those, belonging to the MTC, have been recovered from environmental sources and from livestock; a considerable number have been isolated from raw and pasteurized milk (Chapman & Speight 1968, Hosty & McDurmont 1975).

MATERIALS AND METHODS

We evaluated the distribution of *M. bovis* and other mycobacterial species in livestock specimens, particularly pathologic specimens, in five geographic areas of Brazil (São Paulo, Southeast region; Paraná and Santa Catarina, South region; Goiás, West central region; and Pará, North region), and milk from retail markets in the State of São Paulo. Livestock samples examined included pathologic specimens from 22 animals (10 water buffalo lymph nodes,

4 swine lymph nodes, 3 bovine lung fragments and 5 bovine lymph nodes). Official investigators of the Inspection Service of the Ministry of Agriculture (Brazilian Federal Government) selected samples that showed caseous lesions suggestive of tuberculosis. All samples were frozen and maintained in ice during transportation to the laboratory. Milk samples were obtained in various markets in the State of São Paulo and included 78 raw samples, 40 pasteurized samples, and 10 samples sterilized by ultra-high temperature (UHT) treatment. Pasteurization was performed by heating samples to 71,7°C for 15 s and UHT was performed by heating samples to 150ºC for 2 s, followed by cooling to 4°C. Both of these treatment processes are used among commercial dairies in Brazil. These procedures were performed commercially prior to shipment of milk samples to the laboratory.

Culture and identification - Milk samples (5 ml) were decontaminated using 5% oxalic acid, concentrated by centrifugation (10 min, 1200 x g, 4°C) and cultured on Löwenstein-Jensen and Stonebrink Media (Stonebrink et al. 1969). Cultures were incubated in the presence of 5-10% CO_2 at 30ºC and 37ºC for 90 days and inspected weekly for bacterial growth. Pathologic samples were treated by Petroff method (Brasil 1994) and after centrifugation (10 min, 1200 x g, 4°C), they were cultivated as milk samples. The cells from eugonic and dysgonic colonies that suggest the growth of mycobacteria were examined microscopically after Ziehl-Neelsen staining for acid-fast bacilli. Eugonic mycobacterial isolates were identified by conventional methods (rate of growth, colonial morphology and pigmentation, and biochemical properties) (Brasil 1994), and analysis of mycolic acid profiles using thin-layer chromatography (TLC) (Leite et al. 1998). Unusually slowly growing dysgonic cultures, that required 50-60 days for the appearance of colonies, limited to one or two in each Stonebrink medium tube, were submitted to PCR analysis. Initially they were examined for the presence of IS*6110* (Eisenach et al. 1992) and, if positive, they were characterized by PCR-RFLP analysis of a region of *oxyR* (Sreevatsan et al. 1996).

Mycolic acids analysis - Extraction and identification of mycolic acids from reference and isolated strains were performed according to Leite et al. (1998). The identification of mycolic acids, as methyl esters, was done by one-dimensional TLC, with silica gel as the stationary phase and two different elution systems: diethyl ether/petroleum ether 12:88 v/v (three developments), and dichloromethane (single development). The visualization of spots was achieved by spraying the chromatograms with 0.01% (w/v) rhodamine in phosphate buffer.

PCR analysis - For mycobacterial DNA extraction, the cells from Löwenstein-Jensen medium cultures were suspended in 1 ml of distilled water in microcentrifuge tubes. DNA was liberated by boiling the suspensions for 10 min, performed according to Eisenach et al (1993). The primer sequences were internal to the insertion sequence IS*6110* (5'- CCTGCGAGCGTAGGCGTCGG-3' and 5'- CTCGTCCA GCGCCGCTTCGG-3'), product length 123 base pairs (bp). Reagents were added to a 0.2 ml microcentrifuge tube: 2.5 µl 10 X PCR buffer, 4 µl pooled dNTPs (1.25 mM each), 0.6 µl of 5 µM stocks of each primer, 0.125 µl Taq polymerase 2.5 U/µl, 1 µl DNA template and water to a final volume of 25 µl. Cycle conditions were as follows: 95° C for 5 min, 35 cycles of amplification (94°C for 30 s followed by 58°C for 30 s and 72°C for 30 s) and 72°C for 10 min. Five µl of the amplified product was subjected to electrophoresis on a 2% (w/v) agarose gel with 0.03% of ethidium bromide. After electrophoresis DNA was visualized by UV transilluminator.

PCR-RFLP analysis - This method was performed as (Sreevatsan et al. 1996) with some modification. A 548 bp fragment of *oxy* R contained nucleotide 285 was amplified by PCR. The following oligonucleotide primers were used: 5'- GGTGATATATCACACCATA- 3' and 5'- CTATGCGX GATCAGGCGTACTTG-3'. Cycle conditions were as follows: 94° C for 60 s, 30 cycles of amplification (94°C for 21 s followed by 55°C for 21 s and 72°C for 22 s) and 72°C for 5 min. The PCR product (10 µl) was digested with 4 U of *Alu*I (New England Biolabs). Digestion was carried out at 37°C for 90 min, and the resulting DNA fragments were eletrophoretically separated with agarose a 1.8% containing 0.03% ethidium bromide. After electrophoresis DNA was visualized with a UV transilluminator.

RESULTS

Mycobacteria were isolated from 15 of 22 (68.2%) caseous lesions from livestock and from 23 of 128 (18%) milk samples (Table I). Table II shows the differential characteristics used to identify the environmental mycobacteria isolated from pathologic specimens and milk samples. Eleven isolates were identified as *M. tuberculosis* complex by PCR identification of IS6110 and were subsequently determined to be *M. bovis* by PCR-RFLP identification of *oxyR* (Figure). The amplification of a 548-bp region of *oxyR* by PCR, followed by digestion with *Alu*I resulted in the generation of 5 DNA bands for *M. bovis* compared to 4 bands for *M. tuberculosis*. No *M. tuberculosis* isolates were found in any sample tested. Of the 11 *M. bovis* isolates, 3 were found among the 5 bovine lymph nodes and 10 water-buffalo lymph nodes examined, 2 were found among 4 porcine lymph nodes, 2 were found among 3 bovine lung fragments, and 1 was found among the 128 milk samples tested (Table I). Four *M. avium* isolates and 1 *M. fortuitum* isolate were also found in pathologic specimens. Nontuberculous mycobacteria were found in 9 (22.5%) of the pasteurized milk samples and 14 (16.7%) of the raw milk samples. No mycobacteria were found in the 10 UHT milk samples tested. Nontuberculous *Mycobacterium* species isolated from milk samples included *M. fortuitum* (6 isolates), *M. marinum* (3 isolates), *M. kansasii* (1 isolate), *M. gordonae* (6 isolates), and 6 unidentified rapidly growing *Mycobacterium* isolates.

DISCUSSION

These results demonstrate the diversity of *Mycobacterium* species that may be found among livestock samples particularly in raw and pasteurized milk. Mycolic acid analysis and the classical biochemical testing determined the identification of species in this study, with the exception of *M. tuberculosis* complex isolates, which were iden-

TABLE I

Distribution of 38 *Mycobacterium* isolates from 22 pathologic specimens and 128 milk samples

	No. isolates						
Sample (No.)	*M. bovis*	*M. avium*	*M. fortuitum*	RGM	*M. marinum*	*M. kansasii*	*M. gordonae*
Milk							
Raw (78)	1	-	4	3	1	-	5
UHT (10)	-	-	-	-	-	-	-
Past (40)	-	-	2	3	2	1	1
Specimens							
Bovine LN (5)	3	1	-	-	-	-	-
Buffalo LN (10)	3	-	1	-	-	-	-
Swine LN (4)	2	3	-	-	-	-	-
Bovine lung (3)	2	-	-	-	-	-	-
Total	11	4	7	6	3	1	6

RGM: unidentified rapidly growing *Mycobacterium* species; UHT: ultra-high temperature treatment; LN: lymph node; Past: pasteurized

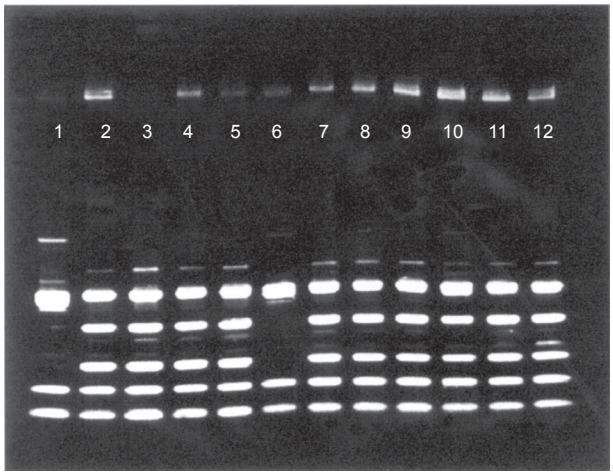
TABLE II

Differential characteristics used to identify mycobacteria from milk and pathology specimens

Test or property	*Mycobacterium fortuitum*	RGM	*M. tuberculosis* complex	*M.gordonae*	*M. marinum*	*M. kansasii*	*M. avium*
Number of isolates	7	6	11	6	3	1	4
Mycolic acids	I.V	I.VI	I.III.IV	I.III.IV	I.III.IV	I.III.IV	I.IV.VI
Growth in less than 7 days	+	+	-	-	-	-	-
Growth at 25°C	+	+	-	+	+	-	-
Growth at 37°C	+	+	+	+	-	+	+
Photoreative pigment	-	-	-	-	+	+	-
Pigment in dark	-	-	-	+	-	-	-
Growth in presence of:							
PNB (500 µg/ml)	+	+	-	+	+	+	+
TCH (2 µg/ml)	+	+	-	+	+	+	+
NaCl (5%)	+	+	-	-	-	-	-
Growth on:							
McConkey agar	+	-	ND	ND	ND	ND	ND
Nutrient agar	+	+	-	-	+	-	-
Niacin production	-	-	-	-	+	-	-
Nitrate reduction	+	+	-	-	-	+	-
Arylsulfatase (3 days)	+	-	-	-	+	-	-

I: long no oxygenated mycolates; II: short no oxygenated mycolates; III: methoxymycolates; IV: ketomycolates; V: epoxymycolates; VI: dicarboxylic mycolates; RGM: unidentified rapidly growing *Mycobacterium* species; PNB: p-nitrobenzoic acid; TCH, thiophen-2-carboxylic acid hydrazide; ND: not determined

tified by PCR and RFLP analysis. These molecular methods should be particularly useful for dysgonic slowly growing mycobacterial strains for which biochemical and growth data are difficult to obtain but for which even minimal growth can yield sufficient DNA for PCR amplification. The difference among the growth rates of mycobacteria species may be justified by differences in key respiratory pathways or energy production, in the velocity of oxygen and nutrients diffusion across the cell envelope, in the rate of lipid synthesis and assimilation by cell walls, or finally in the number of rRNA operon (Goodfellow & Magee 1977). There is clear correlation between slowly growing capacity with disease chronicity and the ability of survive of mycobacteria in a latent state in the host (Goodfellow & Magee 1977). By PCR-RFLP method, we could differentiate *M. bovis* from *M. tuberculosis* using specific *oxy*R nucleotide 285. This method showed rapid, highly sensitive and specific. Algorithms based on restriction analyses of additional polymorphic gene regions have facilitated the identification of at least 48 *Mycobac-*



Differentiation of *Mycobacterium tuberculosis* from *M. bovis* by polymerase chain reaction-restriction fragment lenght polymorphism analysis of *oxy R*. Lanes - 1, 6: *M. tuberculosis* $H_{37}Rv$; 2: *M. bovis* AN_5; 3, 4, 5: bovine lymph nodes; 7, 8: buffalo lymph nodes; 9, 10: bovine lung fragments; 11, 12: porcine lymph nodes

terium species (Hernandez et al. 1999, Roth et al. 2000), including those found in our study.

Although *M. tuberculosis* was not isolated from any sample, *M. bovis* was isolated from all types of pathologic samples evaluated and from raw milk. These data support the premise that livestock tissue and milk samples may be a reservoir for *M. bovis* transmission in these regions of Brazil. Concerning the importance of this for the public health, it is necessary and urgent to check if *M. bovis is* also prevalent in the other regions. Because *M. bovis* is a primary pathogen causing bovine tuberculosis infections among livestock, it is of particular concern that this species was obtained in 11 of 38 positive cultures (28.9%). Of even greater concern is the presence of *M. bovis* in one raw milk sample. Since approximately 50% of all milk consumed in Brazil is not pasteurized, consumers are at risk for *M. bovis* infection.

The additional *Mycobacterium* species identified from pathologic specimens and milk samples, including *M. avium*, *M. fortuitum*, *M. marinum* and *M. kansasii*, are considered potentially pathogenic and cause a variety of clinical manifestation in humans (Wolinsky 1992). In a national surveillance of mycobacteriosis, from 500 cultures of nontuberculous mycobacteria, M. a*vium* was the major isolates with 44.1% followed by M. *kansasii* with 13.7% and *M. fortuitum* with 10.8% (Barreto & Campos 2002). Particularly in immunocompromised patients, the mycobacterial infection caused by *M. avium* is second only to tuberculosis in prevalence in Brazil (Saad et al. 1997). Although it has been established that pasteurization kills *M. tuberculosis* in milk (Hosty & McDurmont 1975), survival of some nontuberculous *Mycobacterium* species after simulated laboratory pasteurization has been reported (Harrington & Karison 1965, Grant et al. 1996, Stabel et al. 1997). The organisms found among our pasteurized samples are among the species known to survive pasteurization. But this does not exclude the possibility of contamination during the bottling process. Our finding, that none of the milk subject to UHT yielded any microorganisms, supports the former possibility. Other mycobacterial species found in this study, particularly *M. gordonae* and the unidentified rapidly growing organisms, have been previously found extensively in the Brazilian environment, including natural water systems (Falcão et al. 1993).

The presence of *M. bovis,* and other potentially pathogenic mycobacteria in livestock tissue and milk suggests that humans may be exposed to these organisms as the result of ingestion. Decreases in food associated mycobacterial disease in Brazil may be achieved through increased surveillance of livestock products, particularly milk, for the presence of mycobacteria and intensification of measures to avoid food contamination.

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