Isolation and identification of bovine tuberculosis in a Brazilian herd (São Paulo)

AFC Nassar/+, S Miyashiro, CG Oliveira*, WA Pacheco, RA Ogata**

Instituto Biológico, Av. Conselheiro Rodrigues Alves 1252 04014-002 São Paulo, SP, Brasil *Coordenadoria de Assistência Técnica Integral, São Paulo, SP, Brasil **Universidade de São Paulo, São Paulo, SP, Brasil

Mycobacterium *was verified in animals from a Brazilian dairy herd, a total of 42 samples from 30 cows were submitted to culture and the isolated strains were analyzed by two polymerase chain reaction (PCR), the first specific for species belonging to the* Mycobacterium *complex (MTBC) and the other for differentiating* M. tuberculosis *from* M. bovis*. Twenty seven samples (64.3%) from 18 animals (60%) were positive for mycobacteria by culture, including samples from 15 retrofaryngeal lymphnodes (55.5%), 9 prescapular lymphnodes (33.3%), 2 lungs (7.4%), and 1 liver (3.7%). All isolated colonies were confirmed by PCR to contain MTBC organisms, and were identified as* M. bovis *by the same methodology.*

Key words: tuberculosis - bovine - *Mycobacterium bovis* - intradermal tuberculin test - isolation - polymerase chain reaction

Bovine tuberculosis (TB), caused mainly by *Mycobacterium bovis* is a disease causing considerable economic losses related to affected herds (Abrahão 1999). While most cases of human tuberculosis are caused by *M. tuberculosis*, infections caused by *M. bovis* are being increasingly documented as being the cause of epidemic nosocomial bursts in Paris (Bouvet et al. 1993). From a clinical and epidemiological perspective, a rapid method to differentiate both species is important for timely diagnosis, effective treatment (due to the intrinsic resistance of *M. bovis* to pirazynamide) and epidemiology of TB (Shah et al. 2002).

The bovine population in Brazil consists of about 180 million animals, with prevalence of TB ranging between 0.9 and 2.9% depending on the region and kind of production (Kantor & Ritacco 1994). Between 1989 and 1998, official notification data indicated a national prevalence of 1.3% of infected animals (Brasil 2005). Due to disgonic and very slow growth, the identification of *M. bovis* by conventional biochemical methods is cumbersome and time-consuming. Polymerase chain reaction (PCR) is a sensitive and fast diagnostic tool that can be used to detect the agent in clinical samples in 48 h, but the presence of inhibitors in samples can interfere with its performance (Haddad et al. 2004, Singh et al. 2004, Brasil 2005).

Among the several PCR systems developed for detection of species composing the *M.* tuberculosis complex (MTBC) (Wards et al. 1995, Brasil 2005), we focused on two. One was described by Niyaz Ahmed et al. (1999) and based on specific primers (NZ1 and NZ2) for the insertion sequence IS 1081, selectively present in organisms of the MTBC. A multiplex-PCR with primers JB and specific primers for the gene encoding for pyrazinimidase (*pnc*A) was reported by Shah et al. (2002) to differentiate between *M. bovis* and *M. tuberculosis* on colonies in culture and this method could detect less than 20 pg of DNA, and this last reaction was the second applied reaction in the present work for differentiation between *M. bovis* and *M. tuberculosis*.

We here present our work aiming at the detection of *M. bovis* mainly in retrofaryngeal lymphnode samples of bovines from a herd of Tatuí (São Paulo, Brazil) by microbiological culture, and MTBC and *M. bovis* specific PCR systems, and comparing the microbiological results with the positive or inconclusive results at single intradermal comparative tuberculin test (SICTT) presented by these animals.

Thirty bovines from a Tatuí herd in São Paulo, Southeast Brazil and with positive (> 4 mm) or inconclusive (2 to 3.9 mm) response to SICTT were taken to sanitary slaughter. We collected 42 samples composed of the retrofaryngeal lymphnodes independent of the presence or not of suggestive lesions for TB while this was perfomed for prescapular lylmphnodes, lung, and liver only when presenting characteristic lesions of TB (Table). Samples were maintained in sterile bags at 4ºC for transport to the Laboratory of Tuberculosis of Instituto Biológico, São Paulo, Brazil until futher analysis.

For culturing, samples were decontaminated by means of the Petroff method (Kantor 1979) and each one was inoculated in two tubes with Stonebrink and Petragnani medium, incubated at 37ºC, and examined for presence of micobacteria growth weekly, during 90 days.

One or two colonies from each positive culture were suspended in 200 µl sterile saline solution (0.85% NaCl) and boiled for 5 min for DNA liberation as described by Bermer-Melchior and Drugeon (1999). Ten microliter of each of these were submitted to the PCR reactions for detection of species of the MTBC and for differentiation of *M. tuberculosis* and *M. bovis*.

+Corresponding author: nassar@biologico.sp.gov.br
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TABLE
Samples collected at slaughterhouse from Tatui, SP, Brazil - 2006

					PCR		
Animal identification	SICTT (mm)	Collected samples	Lesion	Microbiological culture	NZ	pncA- *M. bovis*	pncA- *M. tuberculosis*
1	Inconclusive (2.7)	A	AL	Positive	Positive	Positive	Negative
2	Positive (8.2)	A	PL	Negative	-	-	-
3	Positive (8.4)	A	PL	Negative	-	-	-
4	Positive (5.0)	A	PL	Positive	Positive	Positive	Negative
5	Positive (8.4)	A	PL	Positive	Positive	Positive	Negative
		B	PL	Positive	Positive	Positive	Negative
		C	PL	Positive	Positive	Positive	Negative
		D	PL	Positive	Positive	Positive	Negative
6	Positive (12.8)	A	AL	Positive	Positive	Positive	Negative
7	Positive (8.3)	A	PL	Positive	Positive	Positive	Negative
		B	PL	Positive	Positive	Positive	Negative
8	Positive (15.9)	A	PL	Negative	-	-	-
9	Positive (5.4)	A	PL	Positive	Positive	Positive	Negative
10	Inconclusive (3.2)	A	AL	Positive	Positive	Positive	Negative
		B	PL	Positive	Positive	Positive	Negative
11	Positive (6.5)	A	AL	Negative	-	-	-
		B	PL	Positive	Positive	Positive	Negative
12	Positive (8.8)	A	AL	Negative	-	-	-
		B	PL	Positive	Positive	Positive	Negative
13	Positive (5.6)	A	AL	Positive	Positive	Positive	Negative
		B	PL	Positive	Positive	Positive	Negative
14	Positive (5.1)	A	PL	Positive	Positive	Positive	Negative
15	Positive (7.5)	A	PL	Positive	Positive	Positive	Negative
16	Positive (5.1)	A	AL	Negative	-	-	-
17	Positive (18.5)	A	PL	Negative	-	-	-
18	Positive (5.6)	A	AL	Negative	-	-	-
19	Positive (4.7)	A	AL	Negative	-	-	-
20	Inconclusive (3.8)	A	PL	Negative	-	-	-
21	Inconclusive(3.75)	A	PL	Negative	-	-	-
22	Inconclusive (3.5)	A	PL	Positive	Positive	Positive	Negative
23	Positive (25.9)	A	PL	Positive	Positive	Positive	Negative
24	Positive (4.3)	A	AL	Negative	-	-	-
25	Positive (9.9)	A	PL	Positive	Positive	Positive	Negative
26	Inconclusive (3.1)	A	AL	Positive	Positive	Positive	Negative
		B	PL	Positive	Positive	Positive	Negative
27	Positive (6.0)	A	AL	Negative	-	-	-
28	Positive (5.2)	A	AL	Negative	-	-	-
			B	PL	Positive	Positive	Negative
29	Positive (8.1)	A	AL	Negative	-	-	-
30	Positive (8.1)	A	AL	Positive	Positive	Positive	Negative
		B	PL	Positive	Positive	Positive	Negative
		C	PL	Positive	Positive	Positive	Negative

A: retrofaryngeal lymphnode; B: prescapular lymphnode; C: lung; D: liver; PL: caseous lesion presence; AL: caseous lesion absence; SICTT: single intradermal comparative tuberculin test; PCR: polymerase chain reaction; *M*: *Mycobacterium*.

The PCR for MTBC detection was described by Niyaz Ahmed et al. (1999) and uses primers NZ1 (5'CGACAGCGAGCAGCTTCTCGCTG 3') and NZ2 (5'GTCGCCACCACGCTGCTGGTCAGTG 3') specific for IS 1081, only present in MTBC.

Amplification was performed in a total volume of 50 µl, with 200 µM of each dNTP, 1X PCR buffer (10 mM Tris-HCL, pH 8.0; 50 mM KCL), 2 mM $MgCl_2$, 25 pmol of each primer (NZ1 and NZ2), 1.25 U Taq DNA polymerase and 10 µl of DNA. The PCR reaction consisted of an initial denaturation of 95ºC for 4 min, 30 cycles including 94ºC for 1 min, 63ºC for 90 s and 72ºC for 1 min followed by final extension at 72ºC for 10 min. The expected size of the amplified product was 306 bp.

For differentiation between *M. bovis* and *M. tuberculosis* we used the system described by De Los Monteros et al. (2002), adding 10 µl of bacterial lysate to a PCR mix of 50 µl containing dNTP, buffer, $MgCl_2$ and Taq DNA polymerase as described above and 30 pmol of the forward primer *pnc*ATB-1.2 (ATGCGGGCGTTGATCTCGTC) complementary to part of *pnc*A that is shared both species, and species-specific reverse prim-

ers *pnc*AMB-2 (CGGTGTGCCGGA-GAAGCCG) and *pnc*AMT-2 (CGGTGTGCCGGAGAAG-CGG), specific for *M. bovis* and *M. tuberculosis* respectively. After initial denaturation at 95ºC for 10 min, amplification was obtained with 30 cycles of 94ºC for 1 min, 67ºC for 1 min, and 72ºC for 1 min was carried out, followed by a final extension step.

Positive PCR controls were *M. bovis* AN5 (ATCC15755) and *M. tuberculosis* H_{37}RV (ATCC 27294), and ultra pure water was used as negative control. Amplification reactions were carried out in a Peltier Thermal Cycler-100 (MJ Research).

Analysis of the amplified products was performed by means of electrophoresis in 1.3% agarose gel with TBE 0.5 X running buffer (0.045 M TRIS-Borate and 1 mM of EDTA pH 8.0). Gel was stained with ethidium bromide, visualized with a UV transiluminator (300-320 nm) and photographed by a fotodocumentation system (Kodak Digital Camera DC/120 Zoom) and analyzed with software 1D Image Analysis (Kodak Digital Science).

Fisher and Mann-Whitney tests were performed using MedCalc® software version 8.2.0.2 and Minitab® version 14.1.

Among the 30 animals submitted to sanitary slaughter, all presented pathologic signs of TB: 28 (93.3%) presenting localized TB lesions at retrofaryngeal lymphnode, 1 animal (3.3%) with generalized TB including caseous lesions in retrofaryngeal and prescapular lymphnodes, lung, and liver, and another animal presenting respiratory TB with lung lesion. Eighteen animals (60%) had a culture positive sample (27 of 42, 64.3%) from at least one of the forementioned tissues, including 15 retrofaryngeal lymphnodes (55.5%), 9 prescapular lymphnodes (33.3%), 2 lungs (7.4%), and 1 liver (3.7%) (Table).

Bacteria present in every analyzed culture belonged to MTBC as determined by PCR with primers NZ1 and NZ2. Additionally, using the *pncA* based PCR, all were identified as *M. bovis* as shown in the Table.

There was no statistically significant association (Fisher's exact test, $p = 0.7152$) between the presence of lesion at retrofaryngeal lymphnodes and positive culture. There was no statiscally significant association (Mann-Whitney test, $p = 0.9325$) between SICTT value and at least one analyzed positive sample for microbiological culture.

Of the 30 animals studied here, 24 (80%) were positive (> 4 mm) to SICTT, and 6 (20%) were inconclusive (2-3.9 mm). Inconclusive results can occur due to recent infection (30 to 50 days), end of pregnancy, malnutrition, and anergy in advanced cases of infection (Monaghan et al. 1994, Brasil 2005). Other factors are test-related and include dose of application and tuberculin conservation, both of which influence the test result (Monaghan et al. 1994). Estimates of the sensitivity of tuberculin test range from 68-95% while specificity is estimated to be 96-99% (Monaghan et al. 1994), and in the present study the sensitivity of SICTT was 24/30 (80%). Silva et al. (2006) compared the seric levels of adenosine diaminase (ADA) to SICTT results during diagnosis of bovine TB, but the authors reported the impossibility to establish a meaningful cutoff for ADA seric values, concluding that the latter in its present form is of no use as an auxiliary test for diagnosis of bovine TB.

Among the 30 animals with samples submitted to microbiological culture, 18/30 (60%) had some *Mycobacterium* sp. culture positive sample. When analyzing culture positivity among animals with SICTT positive our inconclusive outcome, in 6 of 24 (25%) positives and 2 out of 6 (33.3%) inconclusives, no positive cultures were obtained, demonstrating the absence of relation between SICTT and cultures results ($p = 0.9325$). In a study performed on 2500 bovines from six slaughterhouses in Mexico, 400 (16%) animals presented typical TB lesions during routine inspection of carcasses at abbatoirs between July 1996 and January 1997, and 336 (84%) presented lymphnode lesions (Milian-Suago et al. 2000) and 308 (77%) samples were positive for *Mycobacterium* sp. upon culturing and histologic examination. In another Brazilian study, peformed by Zanini et al. (2001), 42.6% of 54 lymphnode samples from slaughterhouses in the Southeast region of the country were culture-positive for *Mycobacterium* sp. and identified by biochemical testing and Ziehl-Neelsen (ZN), as *M. bovis*. In contrast (Araújo et al. 2005) 72 samples were selected during slaugther inspection in abattoirs in the state of Mato Grosso do Sul, Brazil, obtained only 23.6% of samples showed colonies suggestive of mycobacteria that were confirmed to be acid-fast bacilli by ZN. In the present study, mycobacteria were isolated in 64.3% of the samples, including half of the evaluated retrofaryngeal lymphnodes samples and all prescapular lymphnodes (9), lungs (2), and liver (1) samples. When analyzing culture efficacy among animals, 60% were positive for mycobacteria. There was no significant association between presence of retrofaryngeal lymphnode lesions and positivity at microbiological culture ($p > 0.05$). This absence of association may be due to sampling bias, as no negative animal at SICTT was evaluated.

In the present study, all isolated mycobacteria were characterized as *M. bovis* by PCR analysis. Araújo et al. (2005) reported that among 72 bovine lymphnode slaughterhouse samples, 17 were positive for mycobacteria, and 13 were confirmed by PCR with primers JB21 and JB22 as *M. bovis*. Two of the four remaining samples were identified as belonging to MTBC while the other two did not belong to the MTBC, as measured by restriction enzyme analysis (PRA) of the hsp65 gene.

Additionally, Shah et al. (2002) reported genetic analysis of isolates from human, bovine, swine, wild mammals, and guinea pigs sources that had been submitted to conventional biochemical test. Using the *pncA* direct PCR system, we demonstrated a confirmed 100% specifity of the genotyping procedure when compared to conventional procedures.

We conclude from our data, that all animals that presented positive or inconclusive SICTT were infected with *M. bovis* as evidenced by PCR based analysis. However, due to lack of conventional identification procedures and animals with negative SICTT and without infection with *M. tuberculosis*, no conclusions on accuracy of the PCR based procedures can be drawn presently, requiring additional studies.

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