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Detection of *Mycobacterium bovis*–Infected Dairy Herds Using PCR in Bulk Tank Milk Samples

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Abstract

Bovine tuberculosis (bTB) is a chronic and zoonotic disease due to *Mycobacterium bovis.* The tuberculosis eradication campaign carried out in Argentina has considerably improved the health situation of the herds. Here we evaluated a strategy to detect *M. bovis*–infected herds by *Touch-Down* IS*6110* polymerase chain reaction (PCR) in bulk tank raw milk from dairy farms. We evaluated 177 samples from herds with the official tuberculosis free certificate (TFC) and 80 from herds without the certificate, non-tuberculosis-free certificate (NTFC), from 10 departments of Santa Fe province, Argentina. To avoid the effect of Taq polymerase inhibitors, a dilution of DNA template was performed. Positive PCR results were obtained in 102 (40%) of the samples, whereas negative ones were obtained in 155 (60%) of the samples. Importantly, 44% of NTFC and 38% of TFC samples were positive. All samples were subjected to culture in Löwenstein Jensen and Stonebrink media with no positive isolation. The negative predictive value (NPV) of PCR in the TFC group was 95%, while the positive predictive value (PPV) of PCR in the NTFC group was 51%. Based on these results, this work proposes a method that should be applied regularly to detect *M. bovis*--infected dairy herds, complementary to the official test of tuberculin, or purifed protein derivative (PPD), to control dairy herds, especially those free of tuberculosis.

Introduction

Mycobacterium bovis, the cause of tuberculosis in cattle, is also pathogenic for humans and a large number of other animals (wild, farmed, feral, and domestic animals).

In Argentina, the National Program for Control and Eradication of tuberculosis from bovines and pigs establishes the use of tuberculin purifed protein derivative (PPD) for diagnosis of bovine tuberculosis (bTB) in these animals. The PPD-positive animals should be sacrificed, and herds that are PPD-negative twice a year are considered free of infection (SENASA, 2009a). Although PPD is a good tool for the eradication and control of bTB, it is known to lack sufficient sensitivity and specificity, and to have economic disadvantages related to the immobilization of animals and the frequency of application (Michel *et al*., 2010; Bennett, 2009; Torgerson and Torgerson, 2009; de la Rua-Domenech *et al*., 2006; Vitale *et al*., 1998; Gardner and Hird, 1989; Neill *et al*., 1994; Seiler, 1979; Francis *et al*., 1978). Therefore, it is a priority to improve and simplify diagnosis of bTB in the Control and Eradication campaign to prevent this situation.

In Argentina, the prevalence of bTB detected at slaughter fell from 6.7% in 1969 to 0.9% in 2009, and the number of farms officially certified as free of bTB has increased from 44 in 1995, to 7507 in 2009 (SENASA, 2009b). The excretion of mycobacteria in milk is intermittent, and up to 30% of infected cows eliminate mycobacteria by milk (Perez *et al*., 2002). In 2009, Santa Fe province, which is located in the center region of Argentina, produced 199.4 million liters of milk, which represents 41% of the total milk production of Argentina (Infortambo, 2009). Between 2001 and 2006, a total of 590 tuberculosis cases were reported in this province, and only six (1.02%) were due to *M. bovis* (de Kantor *et al*., 2008). Recently, the first multi-drug-resistant *M. bovis* transmission between

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humans in Argentina has been discovered, showing the importance of the application of molecular strategies to identify patients infected with *M. bovis*, especially in regions with high prevalence of bTB (Etchechoury *et al*., 2010).

Although there are numerous articles about the detection of *M. bovis* in bovine milk published in peer review journals (Serrano-Moreno *et al*., 2008; Di Pinto *et al*., 2006; Zumárraga *et al*., 1999, 2005; Pérez *et al*., 2002; Antognoli *et al*., 2001; Sreevatsan *et al*., 2000; Cornejo *et al*., 1998; Vitale *et al.,* 1998; Zanini *et al*., 1998), a diagnostic methodology has not yet been implemented in bulk tank milk samples.

Previously, we have demonstrated that using a *Touch-Down* (TD) polymerase chairn reaction (PCR) modification to amplify IS*6110,* the sensitivity is increased by 2 logs, as compared with conventional PCR, in milk and nasal swabs of individual samples (Zumárraga *et al*., 2005).

The aim of this work was to evaluate the usefulness of TD-IS*6110* PCR in bulk tank raw milk to detect *Mycobacterium bovis*–infected herds.

Methods

Milk samples and dairy herds studied

A total of 257 samples of raw milk (400 mL) were collected from different dairy farm bulk tanks from 10 departments of Santa Fe province, Argentina, between 2003 and 2008. Samples were chilled and transported to the laboratory. Half of these samples (200 mL) were used for DNA extraction, and the other half (200 mL) were frozen. The number of samples taken from each department was as follows: 55 from Castellanos, one from Caseros, one from General López, one from General Obligado, 13 from La Capital, 115 from Las Colonias, 11 from San Cristóbal, four from San Jerónimo, 27 from San Martín, and 29 from San Justo (Fig. 1).

Classification of dairy herds

The herds were classified in two categories, based on whether they had or did not have the official certificate extended to dairy herds by the National Program, which certifies that they are PPD-negative twice a year. The first group was referred to as tuberculosis-free certificate (TFC) and the other as non-tuberculosis-free certificate (NTFC) (177 and 80, respectively).

Additional epidemiological data about the number of animals, the volume of milk in the bulk tank, as well as the results and date of the tuberculin test were obtained from each dairy farm, according to that reported by the owners and/or their private veterinary doctor in the NTFC group or by the official inspections in the TFC group. Indeed, we recovered information regarding the percentages of TFC dairy herds and the percentages of slaughtered animals with typical macroscopic lesions of tuberculosis from TFC dairy herds from each department of the area studied.

DNA extraction

A volume of 200 mL of raw milk was centrifuged at 3,000 rpm for 15 min, and the cell layer obtained was washed twice with sterile PBS to remove milk fat from the supernatant. The pellet was suspended in 4 mL of sterile PBS, and then 2 mL was cultured; in the other, 2 mL was heated at 70°C for 70 min to kill the microorganisms and then stored at −20°C

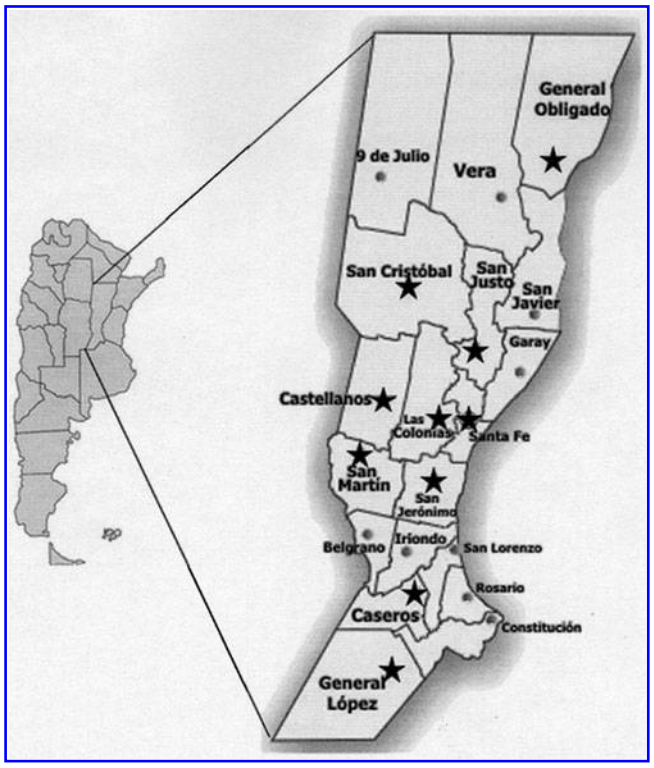

FIG. 1. Maps of Argentina and Santa Fe province. The 10 departments of Santa Fe province sampled are denoted with a star.

until genomic DNA extraction. This aliquot of milk pellet was divided into two portions of 1 mL each to follow DNA extraction and PCR simultaneously in two different laboratories.

DNA extraction from the milk pellet was performed according to that previously described (Zumárraga *et al*., 2005), with minor modifications. The pellet was diluted 1:2 in NTE buffer (10 mM NaCl; 20 mM Tris-hydrochloride, pH 7.4; 1 mM EDTA) with 10% sodium dodecyl sulfate (SDS) and incubated at 37°C for 1 h. Then, the mixture was incubated with 20 μL proteinase K (10 mg/mL) at 65°C for 40 min.

The treated pellet was boiled for 10 min, treated twice with phenol/chloroform/isoamyl alcohol (25:24:1, vol/vol) and once with chloroform/isoamyl alcohol (24:1); 100 mL of 5 M NaCl was then added, and DNA was precipitated with 0.6 volumes of isopropanol at −20°C overnight. The sample was centrifuged for 15 min at 12,000 × *g*. The precipitated DNA was washed with 1 mL of ethanol 70%, dried, and suspended in 40 μL of RNAse-free water. An additional 1/20 and 1/40 dilutions in water were made, and 2 μL was used for PCR.

Touch-Down *IS*6110 *PCR*

The IS*6110* PCR was performed using the TD modification according to that previously described (Zumárraga *et al*., 2005).

The amplification mix consisted of Taq buffer (10 mM Tris–HCl, pH 9.0; 50 mM KCl and 0.1% Triton X-100), 2.5 mM, $MgCl_2$, 0.2 mM of each deoxynucleotide triphosphate, 1 mM of each primer INS1 and INS2 (Hermans *et al*., 1990), 2 μL template, and 1.25 U of Taq DNA polymerase, in a final

volume of 50 μL. One volume of mineral oil was used to cover the mix. All the products were supplied by Promega Corporation (USA).

TD amplification was performed with an initial denaturing step at 96°C for 3 min, followed by 8 cycles of 96°C for 1 min, annealing temperatures starting at 72°C for 1 min (decreasing by 1°C/cycle), and extension at 72°C for 1 min. This step was followed by 30 cycles of 96°C for 1 min, 65°C for 1 min, 72°C for 2 min, and a final extension at 72°C for 8 min. The reaction was performed in a Programmable Thermal Controller (PTC100; M.J. Research, Inc., Boston, MA). A 245-bp amplified product was obtained.

PCR results were considered only when the results between the two different laboratories were concordant.

Bacteriological procedures

The pellets of all milk samples were microscopically examined after staining using the Ziehl Neelsen method. The pellets were also incubated in Löwenstein Jensen and Stonebrink media at 37°C for 60 days (de Kantor *et al*., 1986).

Statistical analysis

The kappa index, and the positive predictive value (PPV) and negative predictive value (NPV) of the PPD and TD-IS*6110* PCR tests were compared between the TFC and NTFC dairy herds.

The Pearson $\chi 2$ test was used to compare TD-IS*6110* PCR versus status of dairy herds.

Results

A total of 257 dairy herds (177 TFC and 80 NTFC) were examined by bacteriological procedures, PPD, and TD-IS*6110* PCR using bulk tank raw milk samples during a 6-year period (2003–2008).

Globally, positive and negative TD-IS*6110* PCR results were obtained in 102 (40%) and 155 (60%) of total samples respectively. In NTFC herds, 35 (44%) samples were positive and 45 (56%) were negative, whereas in TFC herds 110 (62%) were negative and 67 (38%) were positive by TD-IS*6110* PCR, respectively. The concordance between TD-IS*6110* PCR and the official tuberculosis status of herds was 56% ($\chi 2 = 0.80$; $p = 0.3710$; kappa index, 0.05).

To evaluate the efficiency of the National Program for Control and Eradication of bTB in Santa Fe province, we compared PPD results and the official sanitary status (TFC) or lack of it (NTFC), and found that the concordance was 80% (207/257; $p < 0.0001$).

Then, we analyzed the agreement between TD-IS*6110* PCR and PPD results in each dairy herd group (Table 1). The concordance between both diagnostic tests was 59.4% (123/207; $\chi 2 = 1.17$, $p = 0.2794$; kappa index, 0.28). The NPV of TD-IS*6110* PCR in the TFC group was 95%, whereas the PPV of PCR in the NTFC group was much lower (51%).

We obtained interesting results when analyzing the prevalence of *M. bovis* infection by PPD and TD-IS*6110* PCR (Table 1). A similar prevalence was shown by TD-IS*6110* PCR and PPD in NTFC and by TD-IS*6110* PCR in TFC dairy herds. By contrast, the prevalence by PPD in the TFC group was 10 times lower.

Most of the samples (45%) were taken from dairy herds from Las Colonias department (Table 2), where 55% of the dairy farms had the official certificate (TFC) and 11% had bTB reported in at least one bovine at slaughter. The prevalence by TD-IS*6110* PCR in the TFC group was similar to that observed in the NTFC group, obtained by PPD or TD-IS*6110* PCR. Similar results were observed in Castellanos department. The highest prevalence was observed in San Cristóbal department, irrespective of the group studied and the diagnostic test applied. Here, although 50% of the dairy farms had the official certificate, four of them had at least one bovine with macroscopic tuberculosis lesion observed during slaughter (Table 2). A similar situation was observed in dairy herds from San Martín department. The same prevalence of bTB was observed in NTFC herds from San Justo department by the PPD test and TD-IS*6110* PCR.

Finally, the prevalences by TD-IS*6110* PCR in TFC dairy farms from La Capital and San Justo were 17% and 25%, respectively. Nevertheless, there were no reports of tuberculosis lesions in abbatoirs from La Capital or San Justo farms.

There were no isolates of mycobacteria among the 257 milk samples cultured. In contrast, positive Ziehl Neelsen stain was observed in only two samples, taken from TFC herds; one yielded a positive TD-IS*6110* PCR result.

Table 1. PPD and TD-IS*6110* PCR Results, in NTFC and TFC Dairy Herds

Dairy herds	*PPD*	*TD IS*6110 *PCR+*	*TD IS*6110 *PCR−*	*Total*
NTFC (n = 80)	Positive	18	20	38[a]
	Negative	17	25	42
TFC (n = 177)	Positive	3	5	8[a]
	Negative	64	105	169
Total		102	155	257

[a]Number of dairy herd with at least one animal with positive skin reaction.

PPD, purifed protein derivative; PCR, polymerase chain reaction; TFC, tuberculosis-free certificate; NTFC, non-tuberculosis-free certificate; TD, *Touch-Down.*

Discussion

The data presented here represent a successful attempt to obtain a more sensitive and specific test for the diagnosis of tuberculosis in TFC dairy herds. In particular, we studied the usefulness of PCR as a tool to test infection in bulk tank raw milk from TFC and NTFC dairy herds.

Although the culture is the method of reference to confirm the infection by *M. bovis*, we did not detect growth of mycobacteria in any of the culture media used. Indeed, this circumstance affects the confirmation of PCR-positive results.

Therefore, we compared TD-IS*6110* PCR with PPD and found that 25 of the dairy farms (20 NTFC and five TFC; 9.7%) considered infected by the PPD test showed negative TD-IS*6110* PCR results in raw milk (Table 1), suggesting the false positive results of PPD testing or the presence of Taq polymerase inhibitors in these samples. In milk, calcium is one of the main inhibitors of polymerase probably because of its ability to compete with the Mg ion (Abu Al-Soud and Râdström, 1998). We observed inhibition of polymerase when using the DNA extracted directly for the PCR. However, when we performed 1/20 and 1/40 dilutions of the DNA in water, we were able to amplify the milk samples.

Table 2. Bovine Tuberculosis (bTB) Prevalence by PPD and TD-IS*6110* PCR, in TFC and NTFC Dairy Herds

		bTB prevalence in dairy herds (%)					
		NTFC dairy herds		*TFC dairy herds*			
Department	*No. of samples*	*TD-IS*6110 *PCR*	*PPD*	*TD-IS*6110 *PCR*	*PPD*	*TFC dairy farms*[a] *(%) (year 2007)*	*TFC dairy herds, with bTB reported in slaughter ([a]) (%) (year 2008)*
Caseros	1	—	—	0 (0/1)	0 (0/1)	59 (13/22)	0 (0/3)
Castellanos	55	40 (10/25)	40 (10/25)	53 (16/30)	0 (0/30)	16 (448/1279)	11 (13/120)
General López	1	—	—	0 (0/1)	0 (0/1)	46 (49/106)	16 (5/32)
General Obligado	1	0 (0/1)	0 (0/1)	—	—	61 (28/46)	0 (0/5)
La Capital	13	0 (0/1)	0 (0/1)	17 (2/12)	0 (0/12)	69 (87/127)	0 (0/15)
Las Colonias	115	38 (8/21)	29 (6/21)	39 (37/94)	2 (2/94)	55 (547/1168)	11 (13/120)
San Cristóbal	11	71 (5/7)	71 (5/7)	75 (3/4)	50 (2/4)	50 (375/749)	6 (4/70)
San Jerónimo	4	0 (0/2)	0 (0/2)	50 (1/2)	0 (0/2)	19 (28/149)	0 (0/1)
San Justo	29	62 (8/13)	62 (8/13)	25 (4/16)	6 (1/16)	24 (24/99)	0 (0/5)
San Martin	27	40 (4/10)	90 (9/10)	24 (4/17)	18 (3/17)	38 (133/345)	7 (1/15)
Total	257	44 (35/80)	48 (38/80)	38 (67/177)	4.5 (8/177)	45 (1832/4090)	10 (35/363)

[a]Information provided by Production Ministry of Santa Fe province.

PPD, purifed protein derivative; PCR, polymerase chain reaction; TFC, tuberculosis-free certificate; NTFC, non-tuberculosis-free certificate; TD, *Touch-Down.*

Thus, we revealed the presence of *M. bovis* in milk by TD-IS*6110* PCR from 81 dairy farms in which no cattle had positive PPD skin reaction. When Paolicchi *et al.* (2003) performed IS*900* PCR to detect *M. avium* subsp. *paratuberculosis* directly in feces and milk samples, only one sample was positive, probably due to the presence of polymerase inhibitors (Paolicchi *et al.*, 2003). Other authors performed IS*900* PCR using bulk tank milk to detect *M. avium* subsp. *paratuberculosis* and suggested that this assay may not be useful for screening infected herds (Jayarao *et al.*, 2004). Nevertheless, the detection of *M. avium* subsp. *paratuberculosis* in retail pasteurized whole milk by PCR and culture should be considered, because this mycobacteria can resist thermal treatment (Ellingson *et al.*, 2005).

In previous work, the detection limit of TD-IS*6110* PCR using the INS1/INS2 primer set in *M. bovis*—spiked raw milk was calculated as three colony-forming units (CFU) (Zumárraga *et al.*, 2005). Taking into account that the mean number of dairy cows in those 25 farms was 148 and that the corresponding volume of milk in the bulk tank was 2,000 liters, the volume of the milk sample analyzed in this study (200 mL) represents 0.01% of the total volume. This could contribute to the low sensitivity in the detection of *M. bovis*.

In another study performed in Argentina, where milk samples from individual animals were studied using PPD as the gold standard of diagnosis of bTB, TD-IS*6110* PCR sensitivity was 55%, specificity 96%, agreement 73%, and kappa 0.49 (Bernardelli *et al.*, 2005).

It is important to point out that there were 64 farms in TFC dairy herds that were negative by PPD but positive by TD-IS*6110* PCR. This could be very good evidence that some farmers sacrificed PPD-positive bovines from the herd previous to the official inspection to be able to acquire the free-of-tuberculosis certification. On the other hand, the false-negative results by PPD observed may also convert to a positive skin test. This situation could explain the 38% positive TD-IS*6110* PCR results from TFC dairy herd samples. However, since we did not take other samples from the same farm, we could not confirm this. There is evidence of isolation of *M. bovis* from respiratory tracts and lymph nodes from samples negative to PPD (Vitale *et al.*, 1998) but positive to the interferon-gamma test (Neill *et al.*, 1994; Neill *et al.*, 1992). Early tuberculosis infection must be considered, especially when the skin test could not detect it. Furthermore, Vitale *et al.* (1998) found that 39% of milk samples from skin test-negative animals were positive by PCR, suggesting that 52% of the skin test-negative animals tested in their study may be false negatives.

We analyzed whether it was possible to use this test in epidemiological studies by comparing the prevalence between NTFC and TFC dairy herds, and the prevalences were similar by both diagnostic tests in NTFC. By contrast and due to the high number of negative PPD in TFC dairy herds (169/177), the prevalence was 10 times lower by PPD.

According to the information provided by the Production Ministry of Santa Fe Province, the rate of dairy farms free of bTB in each department sampled in this work was 16–69% (Table 2) (Ministerio de la Producción de Santa Fe, 2008). The rate was higher than 55% only in three departments: Caseros, General Obligado, and La Capital (Table 2), but only 15 samples were taken from farms localized in these areas, mainly in La Capital department. Nevertheless, the prevalence by TD-IS*6110* PCR in this department was the lowest, and none of the TFC dairy herds of La Capital had bTB reported at slaughter (Ministerio de la Producción de Santa Fe, 2008).

The department with the highest number of TFC herds was Las Colonias, with 55% TFC dairy farms. However, 13 of these TFC dairy herds had bTB reported at slaughter (Table 2). A similar prevalence of 15.6% was estimated by Abdala *et al.* (2002) from dairy herds with macroscopic TB lesions reported at slaughter. On the other hand, the highest prevalence of bTB infection was observed in San Cristóbal department irrespective of the methodology used. The prevalences by TD-IS*6110* PCR and PPD were similar only in this department (Table 2).

The data reported here suggest that the strategy of TD-IS*6110* PCR in bulk tank raw milk could be used as vigilance for the negative skin test herds with official free-of-tuberculosis certification since the NPV was 95%. Moreover, this strategy should be applied in official inspection of dairy herds, which would increase diagnostic efficacy, not only because the sample is very easy to collect but also because the results are more specific and sensitive than the traditional PPD, which is carried out only in a small number of cows (10%). After a positive result of TD-IS*6110* PCR in bulk tank, bTB diagnosis should be confirmed by testing individual animals in the herd.

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Disclosure Statement

No competing financial interests exist.

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