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Use of touch-down polymerase chain reaction to enhance the sensitivity of *Mycobacterium bovis* detection

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Abstract. The confirmatory diagnosis of *Mycobacterium bovis* (*M. bovis*) in animal samples is carried out by culture in Stonebrink media. However, culture is very slow because of the extremely long duplication time of the bacillus and difficult because of the scarcity of bacilli in diagnostic samples. This study describes the development of a single-tube touch-down polymerase chain reaction (PCR) protocol for the detection of *M. bovis* using primers that target the IS*6110* element. Spiked water and milk as well as routine diagnostic samples (milk and nasal swabs) from *M. bovis*–positive cattle were tested. This protocol allows the rapid and sensitive detection of *M. bovis* in bovine samples by enhancing the sensitivity of standard PCR amplification.

Key words: Mycobacteria; *Mycobacterium bovis*; touch-down PCR.

Introduction

Bovine tuberculosis is an important zoonosis worldwide. *Mycobacterium bovis*, the causative agent of this disease in cattle, is also a pathogen for humans and several economically important animals. *Mycobacterium bovis* is a member of the *M. tuberculosis* complex, a group that also includes *M. tuberculosis, M. africanum, M. canetti*, and *M. microti*, and the recently described *M. caprae* and *M. pinnipedii*.[5] In Argentina, a 3–5% prevalence rate (about 4 million cattle) has been estimated in the cattle population.[12] Diagnosis of human tuberculosis relies on clinical manifestations, skin testing, and subsequent identification of the bacteria by Ziehl–Neelsen (ZN) stain and bacterial culture. Unfortunately, culturing the organism requires 4–6 weeks to attain the desired cell growth for identification. Moreover, although the ZN procedure is fast, it lacks specificity and sensitivity. It has been reported that upon detection of tuberculin-positive animals, tuberculous lung lesions were evident in only 70% of reactive cattle, whereas *M. bovis* was isolated from nasal or tracheal swabs in just 19% of confirmed cases.[17] These discrepancies underscore the need for more sensitive, accurate, and faster methods to assist in the control of this zoonosis.

Polymerase chain reaction (PCR) has been successfully applied to detect members of the *M. tuberculosis* complex and is especially useful for the direct detection of *M. bovis* in bovine tissue samples.[13,14,16,24,33–37] A study evaluating different methodologies for the detection of *M. bovis* in spiked milk found that immunomagnetic separation, followed by nested PCR in a single tube, is a suitable method for routine diagnosis.[1] Another study reported the development of a real-time PCR protocol, which, besides being faster, allows the quantification of the initial bacterial load in tissue samples.[30] Other workers have applied multiplex PCR for the simultaneous detection of *Brucella abortus* and *M. bovis* in bovine milk and nasal secretions.[28]

The primers most commonly used in *M. bovis* PCR-based assays target IS*6110*, an insertion sequence that usually has more than 5 copies in the genome of *M. tuberculosis* and less than 5 copies (most isolates have only 1 copy) in *M. bovis*.[2,6,7,8,9,19,23,27,32] The detection limit reported by using different PCR-based methods varies between 1 and 100 bacilli. Clarridge et al.[2] reported on the use of an IS*6110*-targeted PCR system for diagnosis of human tuberculosis on more than 5,000 samples; in comparison with *M. tuberculosis* culture, they obtained a relative sensitivity of 94% for smear-positive specimens and 62% for smear-negative specimens and a 99% relative specificity. In contrast, in an assessment of reliability and reproducibility among laboratories that routinely used IS*6110* PCR, Noordhoek et al. found that sensitivity and specificity for detecting the vaccine strain of *M. bovis* was highly variable among the laboratories.[20,21] Their report emphasized the need for better interlaboratory standardization of reagents and methodology. Other authors have found similar difficulties in standardizing PCR in Latin American laboratories.[29] This study developed a touch-down cycling procedure for PCR targeting IS*6110*, IS*1081*, and *hsp65* to enhance the detection level of *M. bovis* and to eliminate spurious electrophoretic bands sometimes observed in bovine samples such as milk and nasal swabs.

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Table 1. Primers used for the amplification of fragments of targeted genes.

Name	Sequence	Target	Amplicon size (bp)	Reference
INS1	cgtgagggcatcgaggtggc	IS6110	245	11
INS2	gcgtaggcgtcggtgacaaa			
IS2	cctgcgagcgtaggcgtcgg	IS6110	123	7
IS1	ctcgtccagcgccgcttcgg			
1081-l	tcgcgtgatccttcgaaacg	IS1081	237	3
1081-9	gccgttgcgctgattggacc			
TB11	accaacgatggtgtgtccat	Hsp65	440	30
TB12	cttgtcgaaccgcataccct			

Materials and methods

Nasal swabs and milk samples from *M. bovis*–positive cattle

Nasal swabs were collected from purified protein derivative (PPD)–positive Friesian cows using 50-cm-long homemade sterile swabs made of aluminum wire and gauze. The swabs were expunged into 10 ml of sterile phosphate-buffered saline in 50-ml centrifuge tubes and the tubes were centrifuged at 4,000 × *g* for 20 min. Ten milliliters of raw milk samples were collected from each cow of the same herd.

Bacterial strains and culture media

Mycobacterium bovis BCG Pasteur, *M. bovis* AN5, and a local wild type isolate of *M. bovis* 534 were cultivated in M7H11 solid media[a] supplemented with albumin dextrose complex (0.4% albumin, 0.5% glucose) and 0.4% sodium pyruvate at 37 C for 1 mo.

The DNA extraction from nasal swabs and cultured bacteria

The pellets from nasal swabs or from 1 ml of liquid cultured *M. bovis* were resuspended in 400 μl of 1× Tris–ethylenediamine tetraacetic acid buffer. Bacteria were inactivated by heating at 80 C for 30 min. Then 50 μl of 10 mg/ml lysozyme[b] was added and incubated overnight. One hundred microliters of 10% sodium dodecyl sulfate (SDS) and 10 μl of 10 mg/ml proteinase K[c] (PK) were added and incubated for 10 min at 65 C. One hundred microliters of 5 M NaCl and 100 μl of *N*-cetyl-*N,N,N*,trimethlyl ammonium bromide/NaCl were added. The suspension was mixed thoroughly until it formed a white, milky suspension. It was then incubated for 10 min at 65 C, treated with chloroform–isoamylic alcohol (24:1, vol/vol), and centrifuged for 10 min at 15,000 × *g*. The supernatant was transferred to a new tube and 0.6 volumes of isopropanol were added followed by overnight incubation at −20 C. The tube was centrifuged at 15,000 × *g* for 15 min, the supernatant discarded, and 2 washes were performed in 70% ethanol. The DNA pellet was dried, resuspended in 20 μl of water, and 2 μl was used for PCR.

The DNA extraction from milk

One milliliter of milk sample was incubated with 20 μl of PK (10 mg/ml) and 100 μl of 10% SDS for 40 min at 65 C. The treated milk was boiled for 10 min, treated twice with phenol:chloroform:isoamylic alcohol (24:24:1, vol/vol) and once with chloroform:isoamylic alcohol (24:1). Then 100 μl of 5 M NaCl was added, after which DNA was precipitated with 2 volumes of 100% ethanol at −20 C overnight. The sample was centrifuged for 15 min at 15,000 × *g*, after which the supernatant was discarded and the pellet washed with 70% ethanol. The remaining ethanol was evaporated by drying, and the pellet was resuspended in 40 μl of water. An additional 1:40 dilution in water was made and 2 μl was used for PCR.

Polymerase chain reaction

Table 1 shows the primers used.

The IS6110 amplification using INS1 and INS2 primers. Conventional amplification was performed as described previously[11] with the following modifications: an initial denaturing step at 96 C for 3 min and 30 or 38 cycles of 96 C for 1 min, 65 C for 1 min, and 72 C for 2 min. At the end, an 8-min final extension period at 72 C was performed. Touch-down amplification was performed with an initial step of 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 72 C for 1 min for extension. 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 IS6110 amplification using IS1 and IS2 primers. Conventional amplification was performed as previously described[7] with the following modifications with an initial step of 96 C for 3 min and 30 or 38 cycles of 96 C for 45 sec, 70 C for 30 sec, and 72 C for 30 sec. At the end, a 5-min extension period at 72 C was performed. Touch-down amplification was performed with an initial step of 96 C for 3 min; followed by 8 cycles of 96 C for 45 sec, annealing temperatures starting at 78 C for 30 sec (decreasing 1 C/cycle), and 72 C for 30 sec for extension. This step was followed by 30 cycles of 96 C for 45 sec, 70 C for 30 sec, 72 C for 30 sec, and finally 72 C for 5 min.

The IS1081 amplification using 1081-l and 1081-9 primers. Conventional amplification was performed as described previously[3] with an initial step of 96 C for 3 min and 30 or 38 cycles of 96 C for 1 min, 65 C for 1 min, 72 C for 2 min. At the end, an extension at 72 C for 8 min was performed. Touch-down amplification was performed with an initial step of 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 1 C/cycle), and 72 C for 1 min for extension.

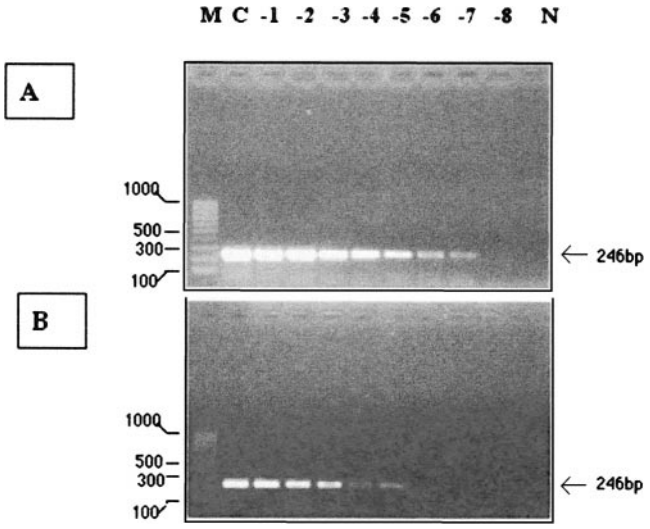

Figure 1. Sensitivity profile of DNA detection in water. Agarose gel electrophoresis of PCR products from 10-fold dilutions of *Mycobacterium bovis* DNA in water. Amplifications were performed using INS1 and INS2 primers. Lanes M, molecular weight markers; C, positive control; N, negative control (reagents only); 10^{-1} through 10^{-8} 10-fold dilutions. **A**, touch-down cycling, **B**, conventional cycling.

This step was followed by 30 cycles of 96 C for 1 min, 65 C for 1 min, 72 C for 2 min, and finally 72 C for 8 min.

The hsp65 amplification. Conventional amplification was performed as described previously,[31] with an initial step of 96 C for 3 min and 45 cycles of 96 C for 1 min, 60 C for 1 min, and 72 C for 1 min. At the end, an extension at 72 C for 8 min was performed. Touch-down amplification was performed with an initial step of 96 C for 3 min followed by 10 cycles of 96 C for 1 min, annealing temperatures starting at 70 C for 1 min (decreasing at 1 C/cycle), 72 C 1 min for extension. This step was followed by 35 cycles of 96 C for 1 min, 60 C for 1 min, 72 C for 1 min, and finally 72 C for 8 min.

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 (for INS1/INS2 and 1081-l/1081-9) or 1.5 mM (for IS1/IS2 and TB11/TB12) $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate, 1 μM of each primer, 2 μl template, and 1.25 U of *Taq* DNA polymerase,[b] in a final volume of 50 μl. The mix was covered with 1 volume of mineral oil. The sizes of amplified products are given in Table 1.

Results

Determination of the PCR sensitivity using DNA in water. As a first step, the detection levels of conventional and touch-down protocols were compared by performing 10-fold dilutions of *M. bovis* BCG (1.8×10^9 colony forming units [CFU] per milliliter, as determined by plating) in water. The dilutions were tested by PCR for the presence of *M. bovis* DNA. The sensitivities of conventional and touch-down amplifications using primers INS1 and INS2[11] targeting IS*6110* were compared. The detection limits of touch-down and conventional PCR were 9 CFU (equivalent to about 45 fg of DNA[34]) and 900 CFU, respectively (Fig. 1). Thus, the touch-down protocol had a detection threshold 2 logs higher than the conventional protocol under these experimental condition.

Comparison of touch-down and conventional amplification in milk. When serial 10-fold dilutions of DNA from *M. bovis*–spiked raw milk was tested by touch-down and conventional PCR using the INS1/INS2 primer set, the detection limits were 3 CFU and 300 CFU, respectively (Fig. 2). However, when the conventional protocol was extended to 38 cycles (to use the same number of cycles as the touch-down protocol), the same analytical sensitivity as touch-down was obtained, but the bands were fainter. When another pair of primers (IS1 and IS2) targeting IS*6110* was used, the enhancing effect of touch-down was again observed (Fig. 3).

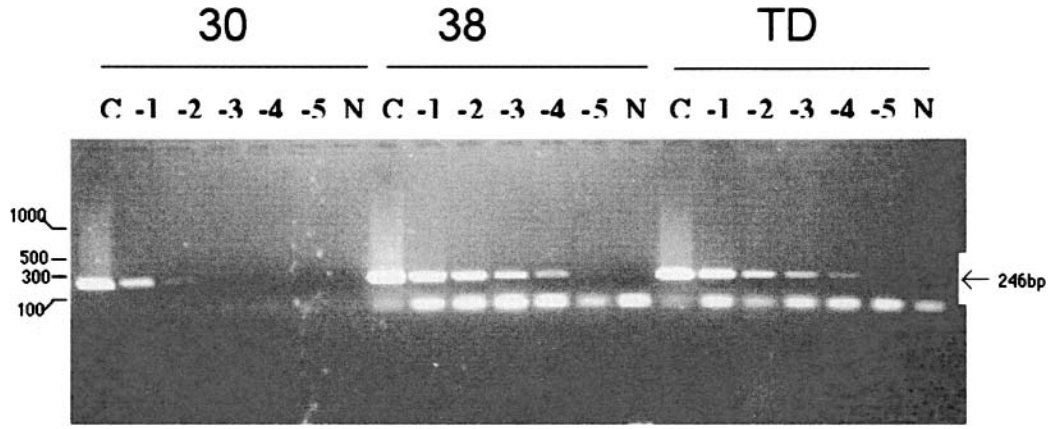

Figure 2. Sensitivity profile milk spiked with *Mycobacterium bovis.* Agarose gel electrophoresis of PCR products from 10-fold dilutions of *M. bovis* in raw milk. Amplifications were performed using INS1 and INS2 primers. Lanes C, positive control; 10^{-1} to 10^{-5} 10-fold dilutions; N, negative control (reagents only). 38, 30, and TD represent conventional PCR with 30 or 38 cycles and touch-down, respectively.

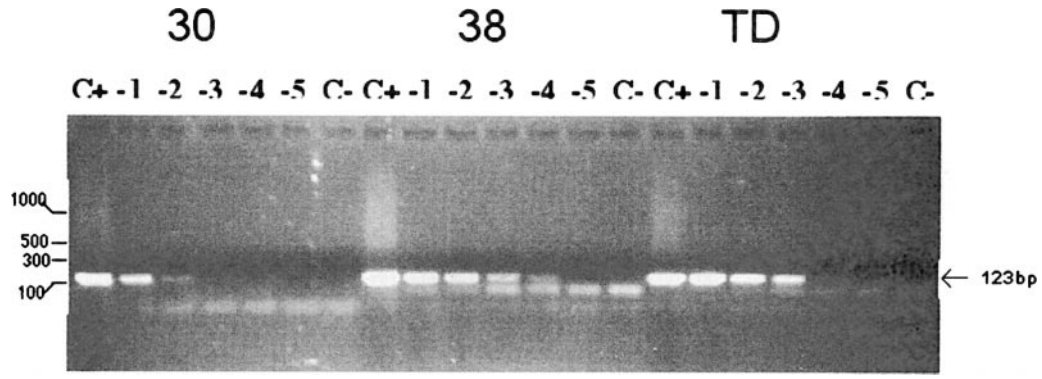

Figure 3. Sensitivity profile in milk spiked with *Mycobacterium bovis* using IS1 and IS2 primers. Agarose gel electrophoresis of PCR products from 10-fold dilutions of *M. bovis* in raw milk. Lanes C, positive control; 10^{-1} through 10^{-5} 10-fold dilutions; N, negative control (reagents only). 38, 30, and TD represent conventional PCR with 30 or 38 cycles and touch-down, respectively.

Effect of touch-down on other PCR targets. Primers targeting *hsp65* and IS*1081* were tested using the same dilutions of *M. bovis* isolate in raw milk. Touch-down increased the analytical sensitivity achieved with IS*1081* primers (Fig. 4a) by 1 log (30 CFU vs. 300 CFU). However, it had no effect on the *hsp65* (Fig. 4b) amplification levels.

The IS6110 touch-down PCR in nasal swabs and milk from M. bovis–infected cattle. Eighteen nasal swab samples were collected from a *M. bovis* PPD-positive herd. When INS1 and INS2 primers were used, 17 samples were positive by touch-down PCR, 17- by 38-cycle PCR, and 2- by 30-cycles PCR. Two samples were positive by standard culture in Stonebrink solid media. Despite the fact that the same number of samples was positive by touch-down and 38-cycle PCR, a larger size, spurious band was observed in all the samples when the 38-cycle PCR was performed (Fig. 5). Some of these samples were confirmed as PCR positive by another pair of primers

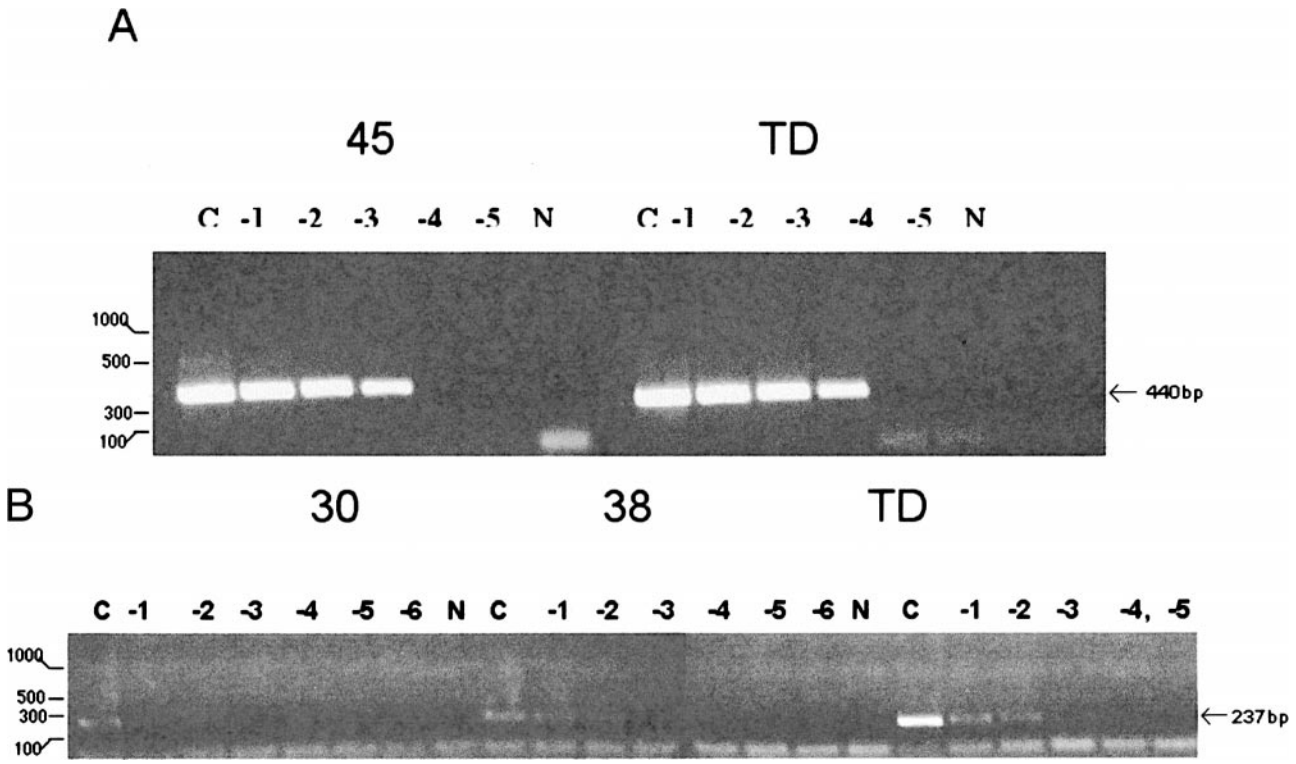

Figure 4. Sensitivity profile in milk spiked with *Mycobacterium bovis* using TB11 and TB12 (**A**) and IS*1081*-1 and IS*1081*-9 (**B**) primers. Agarose gel electrophoresis of PCR products from 10-fold dilutions of *M. bovis* in raw milk. Lanes C, positive control, 10^{-1} to 10^{-5} (10^{-1} to 10^{-6} in **B**) 10-fold dilutions; N, negative control (reagents only). 30, 38, 45, and TD represent conventional PCR with 30, 38, or 45 cycles and touch-down, respectively.

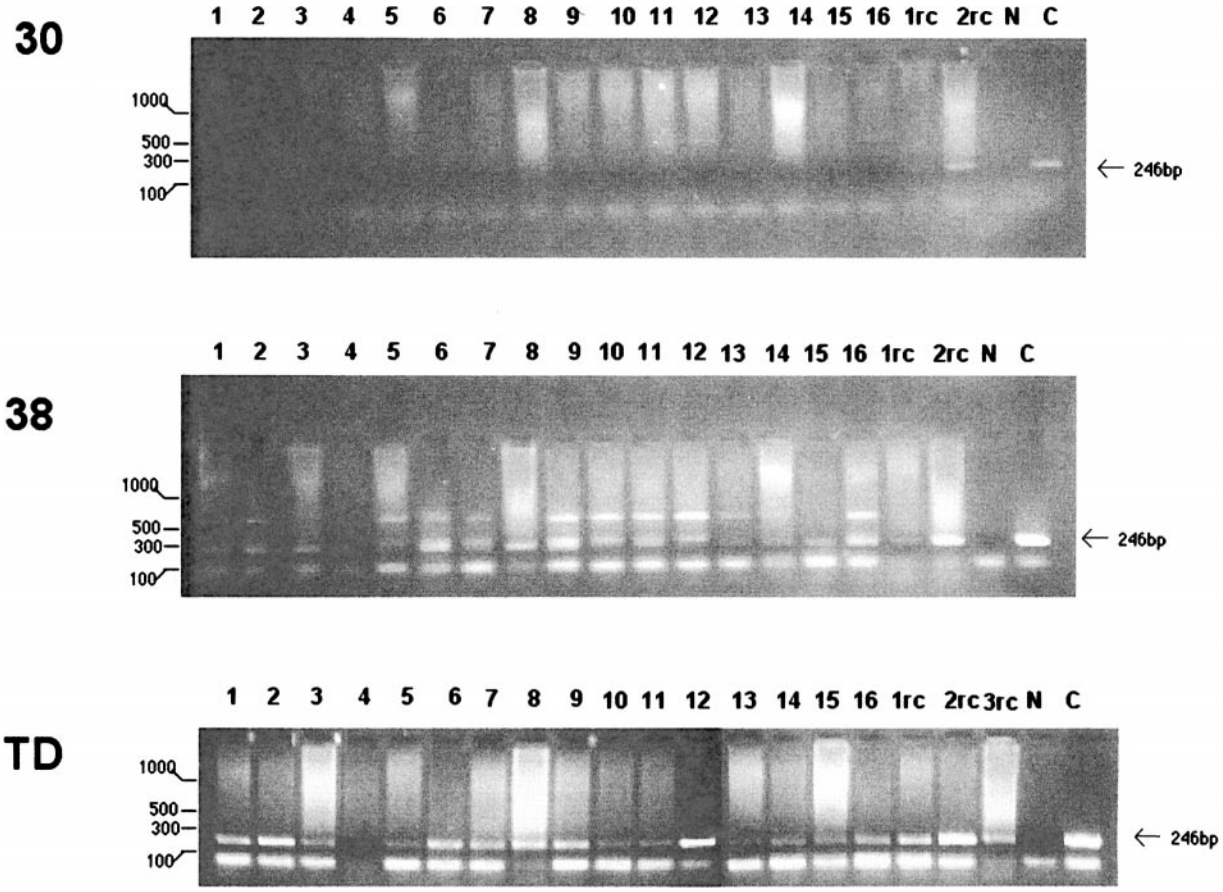

Figure 5. *Mycobacterium bovis* detection by PCR in nasal swabs from a PPD-positive herd using INS1 and INS2 primers. Agarose gel electrophoresis of PCR products from nasal swabs. Numbers at the top of lanes correspond to the identification of animals, N, negative control (reagents only), C+, positive control. 38, 30, and TD represent conventional PCR with 30 or 38 cycles and touch-down, respectively.

(BW8/BW9)[34] targeting IS*1081* (data not shown). Nasal samples from a PPD-negative herd were also tested, and all the 19 samples were negative (data not shown) by the methods described. Fifty-three milk samples from the same positive herd mentioned above were used to detect *M. bovis* by PCR and standard culture. Fifteen were positive by touch-down using INS1 and INS2 primers, 12 by 38-cycle conventional PCR with INS1 and INS2 primers, 8- by 30-cycle conventional PCR with IS1 and IS2 primers, 0 by conventional 30-cycle PCR with INS1 and INS2 primers, and only 1 by culture. In total, 23 samples were positive by at least 1 of the IS*6110*-targeted PCRs. Seven of these samples were positive on amplification with the TB11 and TB12 primers targeting the *hsp65* gene. As described for nasal swabs, milk samples from a PPD-negative herd were also tested, and all the 34 samples were found negative.

Discussion

Polymerase chain reaction offers a powerful diagnostic alternative to culture methods for detection of *M. bovis* in animal samples. It is fast, sensitive, and specific and allows a herd-based screening for bovine tuberculosis. Vitale et al.[33] studied the performance of PCR compared with necropsy examination and found 100% relative sensitivity and specificity for milk and lymph node samples. Wards et al.[34] described an amplification method that detects as few as 200 organisms in tissue samples. Cornejo et al.[4] used PCR to efficiently detect *M. bovis* in milk samples. Miller et al.[18] showed that PCR is reliable for the identification of *M. bovis* in paraffin-embedded tissues from samples in which *M. bovis* could not be cultured.

Despite the initial enthusiasm for PCR, it brings new problems to diagnostics. These include: 1) a high risk of laboratory contamination leading to false positives, 2) the possible presence of substances in clinical samples that inhibit the activity of *Taq* polymerase, and 3) the lack of standardization of in-house PCR protocols leading to poor reproducibility of results. In bovine tuberculosis, these problems are further complicated by the scarcity of *M. bovis* bacteria in bovine secretions. The protocol developed in this study is highly sensitive and fast. This single-tube (nonnested)

PCR protocol can detect a very low number of bacteria in bovine samples.

Several targets have been used to detect *M. bovis* by PCR: IS*1081* that has 5 or 6 copies in *M. bovis*,[34,35] *mpb70* gene,[34] *hsp65* gene,[28] and IS*6110*.[19,22,30,36,37] Despite the fact that IS*6110* is generally present in a single copy in *M. bovis*, the data of this study indicate that it is highly efficient as a target for PCR amplification. This high level of amplification was achieved using the INS1/INS2 primers that amplify a 245-bp amplicon and the IS1/IS2 primers that amplify a 123-bp sequence inside of the same target (INS2 and IS2 annealing sites overlap). Several authors used INS1/INS2 primers to detect *M. tuberculosis*[13,14,25] and *M. bovis*.[22,36,37] In addition, the IS1/IS2 primers designed by Eisenach et al.[7] have also been used widely.[18,30] However, the use of touch-down cycling has not been reported previously for *M. bovis*. In spiked milk, a higher detection limit was reached with touch-down cycling when compared with 30-cycle PCR. Although the detection level was similar between conventional 38-cycle PCR and touch-down cycling, spurious bands affecting specificity were absent in the latter procedure. In the case of milk samples from PPD-positive cases, the authors observed that touch-down PCR with INS1/INS2 primers allowed the highest detection of *M. bovis* DNA. However, in 7 samples, *M. bovis* DNA was detected by touch-down using IS1/IS2 (4 cases) or by 38-cycle PCR (3 cases) but not by touch-down using INS1/INS2. Although there is no obvious explanation for this discrepancy, it is possible that the number of bacteria or the amount of DNA in milk is so low and so close to the amplification threshold that amplification may randomly occur with either primer pair. In other cases, the DNA might be degraded, thus making PCRs that amplify shorter fragments (IS1/IS2) more sensitive than those that amplify longer fragments (INS1/INS2). In many cases the samples amplified by IS*6110* and *hsp65* primers did not match. This is probably because TB11 and TB12 primers amplify *hsp65* genes from all mycobacteria. Species other than *M. tuberculosis* complex may contaminate milk samples. The cases where a sample was positive by IS*6110* primers and not by *hsp65* primers may be explained by the fact that the sensitivity of *hsp65* primers is lower than that of IS*6110* primers. Further optimization is needed to reduce this variability. Nevertheless, for herd-based diagnosis, the observed variability is within acceptable limits.

Polymerase chain reaction sensitivity increased by 2 logs using the touch-down program as compared with conventional PCR. For bovine samples such as milk or nasal swabs, PCR is more sensitive than culture methods. Higher sensitivity of PCR compared with culture of milk samples has been reported by other authors.[4,33] Lower sensitivity of culture could be attributed to the use of drastic preculture decontamination procedures that may kill *M. bovis* or to the fact that bacilli in milk might have already been killed by mammary macrophages. Although negative samples were also tested and yielded 100% specificity, this study was not aimed at determining the sensitivity and specificity of touch-down PCR; the goal was to improve amplification sensitivity using touch-down PCR. It will be necessary to test more samples from different locations before the sensitivity and specificity can be adequately defined.

Other authors have applied PCR to detect *M. bovis* in milk samples from Argentine cattle[22,28] but obtained negative results. The same samples were not available for comparative testing using the touch-down protocol described in this study, but it is possible that use of a less sensitive protocol than touch-down PCR might explain why no positive results were obtained.

The enhancing effect of touch-down cycling seems to be specific for certain target and primer combinations. For example, amplification was enhanced with the IS*6110* primers INS1 and INS2 but not with *hsp65* or certain IS*1081* primers (data not shown). Other workers[10] have also reported that touch-down protocols increase both the specificity and sensitivity of PCR. The enhancing effect of touch-down PCR is believed to be because of its design in which the annealing temperature starts above the melting temperature and ends either at or below the melting temperature. The first steps at higher temperature favor specific binding and avoid mispriming, which can lead to spurious amplification. In the last steps, when the annealing temperature decreases, the template copy number is higher, thus increasing the yield of amplicons.[15,26] In the protocol described in this study, touch-down starts at 8 C or 10 C above the melting temperature. It is likely that a touch-down PCR would also be beneficial when the melting temperature of the 2 primers is different.

Sources and manufacturers

a. Difco, Detroit, MI.
b. Sigma Aldrich, St. Louis, MO.
c. Promega, Madison, WI.

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