Bovine tuberculosis (bTB) remains a problem on many dairy farms in Mexico, as well as a public health risk. We previously found a high frequency of Mycobacterium bovis DNA in colostrum from dairy cows using a nested PCR to detect \textit{mpb70}. Since there are no reliable \textit{in vivo} tests to determine the effectiveness of booster \textit{Mycobacterium bovis} BCG vaccination against bTB, in this work we monitored \textit{M. bovis} DNA in colostrum by using this nested PCR. In order to decrease the risk of adverse reactions in animals likely containing viable \textit{M. bovis}, a single application of BCG and a subunit vaccine (EEP-1) formulated with \textit{M. bovis} culture filtrate proteins (CFP) and a copolymer as the adjuvant was performed in tuberculin skin test-negative cattle (TST\textsuperscript{−}), while TST reactor animals (TST\textsuperscript{+}) received EEP-1 only. Booster immunization using EEP-1 was applied to both groups, 2 months after primary vaccination to whole herds and 12 months later to lactating cows. Colostrum samples were collected from 6 farms where the cows were vaccinated over a 12-month period postvaccination and, for comparison, from one control farm where the cows were not vaccinated with comparable bTB prevalence. We observed an inverse relationship between the frequency of \textit{M. bovis} DNA detection and time postvaccination at the first (\textit{P} < 0.001) and second (\textit{P} < 0.0001) 6-month periods. Additionally, the concentration of gamma interferon (IFN-\gamma) was higher in \textit{mpb70} PCR-positive colostrum samples (\textit{P} = 0.0003). These results suggest that \textit{M. bovis} DNA frequency in colostrum could be a potentially useful biomarker for bTB vaccine efficacy on commercial dairy farms.

\textbf{Bovine tuberculosis (bTB)} is a chronic disease caused by Mycobacterium bovis, characterized by granulomatous lesions in bovines (1) and other mammals (2). In addition to causing livestock losses, bTB is a zoonosis transmitted via ingestion of unpasteurized dairy products or direct contact with infected animals (3–5). In Mexico, the national campaign for control of the disease (NOM-031-ZOO-1995) consists of the intradermal tuberculin test and the slaughter of reactors. Currently, 83.12% of the national territory is in an eradication phase. However, bTB prevalence on dairy farms continues to increase (6), and to avoid whole herd depopulation (7), alternative control strategies such as vaccination should be considered.

Vaccination with low doses of \textit{M. bovis} bacillus Calmette-Guérin (BCG) strain Pasteur 11732P2 has protected cattle against bTB in several trials (8, 9). The supporting evidence of BCG usefulness was the reduction in quantity and size of the lesions and the number of viable bacilli present (10). Booster BCG vaccination with subunits of \textit{M. bovis} proteins obtained from culture filtrates in combination with adjuvants further improved the protective efficacy of BCG (11). Moreover, culture filtrate proteins depleted of cell wall lipids are capable of evoking an antimycobacterial response without evidence of systemic toxicity or necrosis and with an immunotherapeutic effect in patients with active tuberculosis (12). However, there are neither reliable nor noninvasive tests to determine the effectiveness of vaccination against bTB on commercial dairy farms.

Aerosols from cattle with active bTB transmit \textit{M. bovis}. Pathogenesis of bTB starts with inhalation of mycobacteria by a susceptible host in close contact, which are taken up by various alveolar macrophages (M\textsubscript{Φ}), interstitial macrophages, dendritic cells (DC), and epithelial cells (13); then, mycobacteria are drained to the lymph nodes (14). \textit{M. bovis} induces the formation of lesions characterized by an inflammatory granulomatous exudate (1), which is composed of DC and M\textsubscript{Φ} with different phenotypes such as giant cell (Langhans), epithelial, and foamy, with B and T cells surrounding the lesions (15). The acquired T-cell responses develop after M\textsubscript{Φ} and DC present mycobacterial antigens (16, 17). Apoptotic vesicles carrying mycobacterial antigens as cargo within infected M\textsubscript{Φ} are engulfed by DC and can efficaciously stimulate CD4\textsuperscript{+}, CD8\textsuperscript{+}, and CD1-restricted T cells (18). M\textsubscript{Φ} instantaneously recognize molecular patterns via Toll-like receptor 2 (TLR2) among other receptors (19), and subsequent expression of their bactericidal capacity is enhanced by gamma interferon (IFN-\gamma) released by NK cells, WC1\textsuperscript{+} γδ, CD4\textsuperscript{+}, and CD8\textsuperscript{+} T cells (20). Granulomas may contain the infection and make the mycobacteria persistent in a nonreplicating state (21, 22). However, bacilli have pathogenic mechanisms to persist within M\textsubscript{Φ} (23, 24), triggering molecular signals in granulomas (25), which may exacerbate the inflammation and/or pathology during tuberculosis. The switch from a Th1 to a Th2 immune profile and tuberculosis progression have been associated with changes in hormonal profiles.
(26, 27) similar to those present during pregnancy (28, 29). Interaction between \( M. \text{bovis} \) and \( \Phi \text{p} \) results in a chronic inflammatory response to prevent mycobacterial growth but induces the development of granulomas, in which a balance of the immune response is needed to contain the infection. This balance might be affected by hormonal changes during the dry period and late pregnancy in dairy cattle.

To identify niches where \( M. \text{bovis} \) persists, we previously evaluated different clinical samples taken from dairy cows on a farm with 48% \( b\text{TB} \) prevalence. We used a nested PCR to amplify a 208-bp fragment of the \( \text{mpb70} \) gene specific to the \( \text{Mycobacterium tuberculosis} \) complex (30, 31). The results showed that 62% of the analyzed colostrum samples contained \( M. \text{bovis} \) DNA, while only 18% of the milk samples tested positive. In other samples, such as nasal swabs and bronchial alveolar lavage samples, the frequency was lower (30, 31). Currently, in order to validate an ancillary immunization strategy to reduce the prevalence of \( b\text{TB} \), we have vaccinated all cattle on 6 commercial dairy farms in the state of Jalisco, Mexico, using BCG and/or a subunit vaccine (called EEP-1) vaccinated all cattle on 6 commercial dairy farms in the state of Jalisco, Mexico, using BCG and/or a subunit vaccine (called EEP-1).

The purpose of the present study was to monitor the frequency of \( b\text{TB} \) in dairy cattle. To identify niches where \( M. \text{bovis} \) persists, we previously evaluated different clinical samples taken from dairy cows on a farm with 48% \( b\text{TB} \) prevalence. We used a nested PCR to amplify a 208-bp fragment of the \( \text{mpb70} \) gene specific to the \( \text{Mycobacterium tuberculosis} \) complex (30, 31). The results showed that 62% of the analyzed colostrum samples contained \( M. \text{bovis} \) DNA, while only 18% of the milk samples tested positive. In other samples, such as nasal swabs and bronchial alveolar lavage samples, the frequency was lower (30, 31). Currently, in order to validate an ancillary immunization strategy to reduce the prevalence of \( b\text{TB} \), we have vaccinated all cattle on 6 commercial dairy farms in the state of Jalisco, Mexico, using BCG and/or a subunit vaccine (called EEP-1) vaccinated all cattle on 6 commercial dairy farms in the state of Jalisco, Mexico, using BCG and/or a subunit vaccine (called EEP-1). The purpose of the present study was to monitor the frequency of \( M. \text{bovis} \) DNA in colostrum samples from vaccinated cattle in comparison with that in unvaccinated cattle using the previously validated nested PCR.

**MATERIALS AND METHODS**

**Study design and vaccination.** Dairy farms chosen for this study are located in a region with high \( b\text{TB} \) prevalence and within an area of around 20 square kilometers. These farms had a history of \( b\text{TB} \), with \( M. \text{bovis} \) infection confirmed by culture of specimens from tuberculin skin test (TST) reactors collected during postmortem inspection by personnel of the national campaign against \( b\text{TB} \). An immunization program was initiated in August 2010 with BCG plus EEP-1 (PRONABIVE) in all TST-confirmed animals, while TST-animals received only EEP-1 to avoid the risk of adverse reactions (32). A total of 1,007 doses of BCG and 1,206 doses of EEP-1 were applied on these 8 farms. Animals within 29 days prior to calving, 5 days postcalving, or visibly sick were excluded from vaccination. Subsequently, EEP-1 boosters were applied after primary vaccination to all animals at 6 to 8 weeks and to the lactating cattle at 10 to 12 months. In addition, the newborn calves were immunized before they were 8 days old with BCG plus EEP-1, and a booster with EEP-1 was applied 6 to 8 weeks later. Each vaccine was injected subcutaneously on the neck at different sites with a minimum distance of 10 cm between the sites. BCG was applied at 3 × 10⁶ CFU/ml, and subunit EEP-1 vaccine was applied at 400 µg/ml.

**Vaccines.** The live vaccine used in this study was prepared with \( M. \text{bovis} \) BCG strain Pasteur 11732 P2 (ATCC 35734). Bacteria were grown in Dorset Henley broth supplemented with 0.25% (wt/vol) glucose as previously described (33). Briefly, bacterial cultures were incubated at 37°C for 4 weeks and then harvested. The number of CFU was determined, and then vaccine was adjusted to contain 10⁶ bacteria per ml and lyophilized as described previously (8). The experimental subunit vaccine EEP-1 was prepared from \( M. \text{bovis} \) AN5 grown as a pellicle in Dorset Henley medium for 8 to 10 weeks as described previously (34) with some modifications. Briefly, culture supernatants were sterile filtered twice through a 0.2-µm-pore-size filter and tangentially size excluded using a 30-kDa cutoff filter (Pellicon membrane; Millipore, Inc.). The protein content of EEP-1 was adjusted to 400 µg/ml and mixed with a commercial adjuvant (Polygen; MVP Laboratories, Inc.) to a final concentration of 10% (vol/vol). Protein integrity was checked with SDS-PAGE and Western blotting as described previously (34), using an anti-\( \text{Mycobacterium tuberculosis} \) polyclonal antibody (ab905; Abcam, Cambridge, MA). EEP-1 mostly contains culture filtrate proteins of <30 kDa.

**Single intradermal tuberculin test (TST).** A tuberculin skin test was carried out by intradermal inoculation of 0.1 ml of bovine purified protein derivative (PPD) in the left caudal fold, prepared from \( M. \text{bovis} \) strain AN5 (0.1 mg/ml [5,000 IU]; PRONABIVE), for all animals older than 6 months, according to the existing policy in Mexico (NOM-031-ZOO-1995). For interpretation, any increase in thickness detected was considered a positive reaction. Prevalence was calculated by using the following formula: (number of TST reactors/number of total of animals tested) × 100. At the beginning of the study, the farms had the following numbers of animals: farm 1 (F1), 217; F2, 231; F3, 219; F4, 85; F5, 102; F6, 84. The mean prevalence for the 6 farms where the cows were vaccinated included in the study was 29.6% ± 9.3 (± standard error of the mean [SEM]), whereas for the control farm where the cows were not vaccinated, the prevalence was 20.9% (66 TST reactors).

**Colostrum sampling.** About once a week over a 12-month period postvaccination on the 6 farms where the cows were vaccinated, colostrum samples (50 ml) were collected (within 12 h after calving) as follows: farm 1 (F1), 12 samples; F2, 13; F3, 17; F4, 2; F5, 10; F6, 21. On the control farm where the cows were not vaccinated, colostrum samples were taken from almost every calving. The mammary gland was sanitized before milking, and samples were frozen at −20°C until processed. Truplicate samples were processed independently to obtain DNA and whey from colostrum. In total, 45 colostrum samples from TST+-animals vaccinated with EEP-1 and 30 of TST-animals vaccinated with BCG plus EEP-1 were analyzed by PCR. In order to make a comparison, 25 samples from TST+ and 12 from TST-animals from the unvaccinated control farm were also analyzed.

**DNA extraction.** DNA for the nested PCR was extracted from colostrum as previously described (30). Briefly, samples were centrifuged at 10,000 × g for 10 min, after which the pellets were washed, homogenized with 400 µl of Tris-EDTA (TE) buffer containing lysosome, and incubated for 1 h at 37°C. Next, 70 µl of 10% SDS was added, followed by 6 µl of proteinase K solution (10 mg/ml). The samples were then incubated for 10 min at 65°C. After incubation, 100 µl of 5 M NaCl was added to each sample followed by 80 µl of 5 M NaCl solution with 5% cetyl trimethyl ammonium bromide. Again, the mix was incubated for 10 min at 65°C. DNA was extracted with 1 volume of phenol-chloroform-isoamyl alcohol (24:24:1) and then precipitated with 0.6 volume of absolute isopropanol and washed with 1 ml of 70% ethanol. DNA was suspended in 25 µl of Milli-Q H₂O and quantified with a ND-1000 spectrophotometer (NanoDrop; Wilmington, DE). Finally, it was visualized by electrophoresis in 0.7% agarose gels stained with 0.005% ethidium bromide.

**PCR amplification.** PCRs were carried out as previously described (30) using 0.5 U of TaqGOLD polymerase (Applied Biosystems) and 500 ng of DNA in a Gene Amp PCR system 9700 (Applied Biosystems). The first PCR was run using specific primers (TB1F, 5’ GAA CAA TCC GGA GTT GAC AA 3’; TB1R, 5’ AGC AGC CTG CTA ATC TG 3’) for amplifying a 372-bp segment of the \( \text{mpb70} \) gene of the \( \text{Mycobacterium tuberculosis} \) complex (35). This was followed by a nested PCR run, using 1/10 of the product obtained from the previous reaction to amplify a 208-bp fragment with previously designed primers (M223F, 5’ GCT GAC GCG TGC ACT GTC GGC C 3’; M224R, 5’ CTT CCG GCC TGG TTT GGC C 3’) at the same temperature for annealing and extension (30). As a control of genomic DNA integrity, we carried out the amplification of the mitochondrial \( \text{cyt-b} \) gene using primers CYB1 and CYB2 (CYB1, 5’ CCA TCC AAC ATC TCA GCA TGA AA 3’; CYB2, 5’ GCC CCT CAG GAT ATT TGT CCT CA 3’) (36).

**IFN-γ assay in colostrum whey.** Colostrum samples from vaccinated animals with no further \textit{in vitro} stimulation were diluted with phosphate-buffered saline (PBS) (1:1; vol/vol) and centrifuged at 10,000 × g for 10 min. After centrifugation, whey was collected with a pipette tip, avoiding the pellet in the bottom and fat in the top of the 1.5-ml microtube. Whey samples were clarified by centrifugation at 10,000 × g for 3 min before triplicate enzyme-linked immunosorbent assay (ELISA) analyses were performed. The IFN-γ quantification was performed with a sandwich
TABLE 1 Comparison of changes in two consecutive TST in vaccinated and unvaccinated animals

<table>
<thead>
<tr>
<th>Cohort/group</th>
<th>Result [no. (%)] for animal group</th>
<th>Vaccinated</th>
<th>Unvaccinated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TST2</td>
<td>TST2*</td>
<td>TST2/3</td>
</tr>
<tr>
<td>TST1*</td>
<td>15 (30)</td>
<td>9 (21)</td>
<td>23 (56)</td>
</tr>
<tr>
<td>TST1*</td>
<td>22 (76)</td>
<td>9 (32)</td>
<td>2 (7)</td>
</tr>
</tbody>
</table>

* TST2*: animals changed from an initial TST-negative result to a TST-positive result.
  b TST2/3*: animals changed from an initial TST-negative result to a TST-positive result and maintained this positive reaction until the third test.
  c TST2/3*: animals changed from an initial TST-positive result to a TST-negative result.
  d Unchanged, animals did not show a change in their TST results after primary vaccination.

ELISA following the manufacturer’s specifications, and the optical density at 495 nm (OD495) was read (Bovigam; Prionics, Switzerland). The IFN-γ concentration was expressed in pg/ml, after extrapolation from standard recombinant bovine IFN-γ (Serotec, United Kingdom) curve (20, 100, 250, 500, 1,000, 5,000, and 10,000 pg readings. A total of 27 whey samples from TST* versus 48 samples from TST- animals, as well as 20 versus 55 samples from TST* animals, from farms where the animals were vaccinated were assayed and compared; in addition, 25 samples from TST* and 12 samples from TST* animals from the unvaccinated control farm were assayed.

Statistical analysis. Agreement among the bacterial elimination measured by nested PCR in colostrum and TST results from the second and third test was estimated using the kappa value (κ) using MedCalc statistical software version 12.0.3.0 for Windows and the following κ criteria: <0.20, poor; 0.21 to 0.40, weak; 0.41 to 0.60, moderate; 0.61 to 0.80, good; and 0.81 to 1.00, very good. PCR results were compared between those for the unvaccinated control farm (first and second period of 6 months) and those for the vaccinated farms (n = 37 for the first 6 months and 38 for the second period of 6 months) by Fisher’s exact test. The strength of association was quantified by calculating the relative risk (RR) using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA). PCR results obtained for vaccinated farms in the first (n = 37) versus the second (n = 38) period of 6 months were also compared. The IFN-γ concentration (mean ± SEM) in colostrum samples was compared in cohorts of tested samples (TST* versus TST-) and PCR (positives versus negatives) by the Mann-Whitney U test, using GraphPad Prism version 5.00 for Windows. Differences were considered statistically significant at a P of <0.05.

RESULTS

In order to assess the agreement between TST and PCR tests after vaccination, we previously surveyed TST result changes. BCG did not produce a major change in TST- animals in comparison with unvaccinated animals. A major change was observed in TST* animals (vaccinated only with EEP-1), which become TST- between the first (76%) and second TST (32%), but its change did not prevail after the third test in most of the animals (Table 1). In vaccinated cattle, we found no correlation between TST and PCR results, regardless of TST status (Table 2). However, in unvaccinated animals, we found variable correlation within a range similar to that previously reported (Table 2) (30).

Comparing vaccinated with unvaccinated cattle, we found significant differences. In the first period of 6 months we found 15% and 34% PCR-positive samples (P = 0.001) and 4% and 33% in the second period (P = 0.0001), respectively (Fig. 1). In addition, the risk of M. bovis DNA detection decreased from 2- to 7-fold between the first and second periods (Fig. 1). Taking into account the group of vaccinated animals alone, the risk decreased 0.76-fold (Fig. 1) from the first to the second period postvaccination (P = 0.02).

In order to know whether M. bovis DNA detection was associated with IFN-γ concentration in colostrum samples, we compared results obtained with samples from vaccinated and unvaccinated animals (Fig. 2). A significant difference in the IFN-γ concentrations found in colostrum samples from PCR- and PCR+ cohorts of vaccinated cows was found (P = 0.0009), with mean ± SEM values ranging from 373 ± 87 to 107 ± 52 pg/ml (Fig. 2); in comparison, no difference was found between TST- and TST+ results (P > 0.05), even though the values range from 216 ± 80 to 156 ± 57 pg/ml (Fig. 2). In addition, IFN-γ was not found in samples from the unvaccinated control farm (data not shown).

DISCUSSION

To determine the impact of field vaccination on bovine TB transmission in dairy cattle in Jalisco, Mexico, in places with a preva-

TABLE 2 Agreement of TST after vaccination with mpb70 PCR results in colostrum samples

<table>
<thead>
<tr>
<th>Group and cohort</th>
<th>TST, n</th>
<th>TST2, no. (%)</th>
<th>TST3, no. (%)</th>
<th>PCR, no. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TST* /EEP-1</td>
<td>45</td>
<td>13 (30)</td>
<td>14 (33)</td>
<td>5 (11)</td>
</tr>
<tr>
<td>TST* /BCG /EEP-1</td>
<td>30</td>
<td>7 (23)</td>
<td>14 (50)</td>
<td>9 (30)</td>
</tr>
<tr>
<td>TST* /EEP-1</td>
<td>25</td>
<td>8 (32)</td>
<td>7 (28)</td>
<td>15 (60)</td>
</tr>
<tr>
<td>TST* /BCG /EEP-1</td>
<td>12</td>
<td>8 (67)</td>
<td>6 (60)</td>
<td>10 (83)</td>
</tr>
</tbody>
</table>

* Animals included from farms where the animals were vaccinated in each cohort.
  b TST reactors at the second test.
  c Kappa’s coefficient between TST2 and PCR. 95% CI, 95% confidence interval.
  d TST reactors at the third test.
  e PCR-positive results in colostrum samples.

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ence equal to or higher than 10%, we have a vaccination strategy, namely, vaccination with BCG (10^6 CFU) administered to TST^− bovines and subunit boosting immunization administered to TST^+ animals. Differential vaccination was used in order to decrease the risk of adverse reactions in animals likely containing viable *M. bovis*. Furthermore, our vaccination strategy includes boosting with EEP-1 to all animals regardless of their TST status, 2 months post-primary vaccination, as well as 12 months post-primary vaccination of lactating cows. The purpose of the present study was to monitor the frequency of *M. bovis* DNA in colostrum samples from vaccinated cattle. Results shown in this report are for 12 months postvaccination.

The second and third TST performed 4 and 8 months after primary vaccination did not show an increase with respect to vaccination. Conversely, TST results changed from positive to negative from test 1 to test 2 in a significant number of animals, although this did not persist for all animals or for test 3. This could be because other reports tested animals free of bTB, vaccinated at birth, and sacrificed before 12 months old. In contrast, our study included cows that had already given birth and were older than 24 months and thus were likely to have been exposed or infected. Molecular typing of *M. bovis* suggests that the transmission occurs at regional, herd, and animal levels (37). Infected cattle with generalized bTB (73) but also those that were recently infected (74,
75) may promote transmission to susceptible animals within epidemiological units. Common risk factors that influence bTB susceptibility in cattle have been detected in case-control studies and on farms with a high incidence rate. Some of these factors include the purchase of cattle from infected stables and the herd size in the stable (38, 39). The immunosuppression caused by metabolic imbalance (40, 41), calving (48), and concurrent infections (42, 43) may be examples of nongenetic factors that explain multiple transmission or transmission following exposure to a particular infectious shedder cow (44).

We previously analyzed samples of nasal exudates from cattle with culture-confirmed bovine tuberculosis, some of them with gross lesions, using the same nested PCR. These previous studies showed that the frequency of M. bovis DNA detection was 26% in nasal exudates (31). Also, we found differences in detection frequency of M. bovis DNA in mammary fluids from lactating (milk) and recently calving cows (colostrum), 18% and 62%, respectively, and 0% in bronchoalveolar lavage specimens (30). Thus, considering that macrophages constitute more than 80% of the cells of the colostrum and bronchoalveolar lavage specimens, these previous results suggest that the antimycobacterial ability of macrophages could be significantly impaired in the mammary gland (45). In addition, we propose that the mammary gland microenvironment, including the hormonal profile during the peri-partum period and the O2 tension constraints and lipids present in colostrum, could affect antimycobacterial functions of macrophages, encouraging the replication of M. bovis (46, 47). Immunosuppression might occur during peripartum, diminishing the immune response as a consequence of hormonal changes. In persistently infected cows, this might promote reactivation (48). In some instances, we have obtained a mycobacterial PCR amplification product using samples from mammary glands (G. López-Rincón et al., unpublished data). Therefore, we hypothesized that the frequency of detection of M. bovis DNA in colostrum might be affected by the degree of immunity and enhanced after vaccination in either exposed (TST−) or infected (TST+) dairy cattle.

In this work we not only confirmed a high frequency of M. bovis DNA in colostrum specimens from bTB-infected animals but also found a time-dependent relationship between a vaccination strategy against bTB and the decrease in the frequency of detection of M. bovis in colostrum. This result suggests that immunization elicited by booster BCG vaccination with our subunit vaccine controlled the replication of M. bovis in macrophages, including those from the mammary gland. We think that this could be the consequence of possible immune activation that hinders further bacterial replication. Ingestion of maternal colostral leukocytes immediately after birth promotes development of the immune system in newborn calves (49). Since M. bovis depletion in colostrum was elicited by vaccination, our immunization strategy might help to control its transmission and enhance resistance in newborns fed fresh colostrum.

M. bovis displays different mechanisms of pathogenicity to persist within macrophages, including multiple mechanisms of inhibiting responses to IFN-γ (23, 24). The greater activation and subsequent expression of bactericidal capacity of the macrophage are induced by the IFN-γ derived from lymphocytes and NK cells in cattle (20). The secretion of IFN-γ is promoted by the interleukin-12 (IL-12) secreted by infected dendritic cells and macrophages (50), after recognition via TLR2 of lipids and lipoproteins of bacilli (19). In agreement with earlier reports showing high IFN-γ in colostrum mainly from CD8+ cells (51, 52), we also found higher IFN-γ concentrations associated with colostrum harboring M. bovis DNA in the vaccinated group but not in the unvaccinated control group. The concentration of IFN-γ was greater than the common basal level observed in nonstimulated peripheral blood samples from bovines infected with tuberculosis (11). Therefore, these results suggest that vaccination against bTB might induce the synthesis and release of IFN-γ in lymphocytes, where macrophages and/or mammary gland epithelial cells infected with M. bovis exist.

In addition, a lack of correlation between the nested PCR and TST results was noted after vaccination. The low sensitivity (ca. ~56%) of TST is a concern in controlling bTB, and the impact of the risk factor based on TST results might have been incorrectly extrapolated (53). However, if TST− cattle are infectious and there are susceptible animals in contact, the neglected animals constitute a real problem (54). Moreover, abattoir inspection has shown lower sensitivity than TST (55); thus, the bTB prevalence in cattle is higher than that estimated by the current testing-slaughtering policies in many countries, including Mexico. Pseudovirtual transmission in newborn calves from infected dams via the ingestion of M. bovis-contaminated colostrum and the infectivity of this fluid may be also underestimated.

In humans, breastfeeding has also been recognized as a route of transmission to the newborn of some important pathogens that may result in disease (56). Infants who consume breast milk may be exposed to a variety of environmental and medical contaminants, including intracellular pathogens (e.g., tuberculosis, hepatitis B virus, cytomegalovirus, Epstein-Barr virus, human immuno deficiency virus) (57). Isolation of Mycobacterium avium subsp. paratuberculosis from human breast milk of Crohn’s disease patients has been documented (58). In human beings, there is an apparent relationship between breastfeeding transmission of tuberculosis and maternal infection with M. tuberculosis. Mammary tuberculosis is a rare clinical entity, but tuberculous mastitis with granulomatous inflammation of benign or malignant origin might mimic breast cancer (59). Misdiagnosis of tuberculosis of the breast is common, as biopsy specimens are paucibacillary and negative. Ziehl-Neelsen staining and culture are frequently negative (60). Moreover, a higher risk of activation of the tuberculosis disease during the first 5 months postpartum has been noted (61). On the other hand, cattle in commercial dairies may be coinfeckted with various intracellular pathogens that can decrease the immune response to M. bovis. This allows the hematogenous or lymphatic dissemination of bacilli.

There are two well-known clinical forms of TB in humans: lung infection in adults and disseminated disease in young children (62). Since vaccination with BCG in newborns significantly reduced the disease in children (63), the reactivation of latent TB from a silent primary infection is a major concern around the world (64, 65). Thus, the emergence of new cases in cattle could be reduced if vaccination reduces the susceptibility to infection, for example by immunization (8, 9). However, a latency phase has not been well described in cattle (66) although it has been suggested (67). Moreover, our group has achieved detection of nonreplicating persistence-associated gene expression in naturally infected cattle (Beatriz Jiménez et al., unpublished data). Undetected persistent infections could be a source of transmission within and between herds (68, 69). The molecular monitoring of M. bovis has been achieved using a specific PCR, avoiding the problems of at-
tempting cultivation of this slow-growing group of mycobacteria with some nonreplicating states (70). Recent studies showed that *M. bovis* DNA detection correlates to the presence of viable bacilli (71, 72). Colostrum samples analyzed in this study were decontaminated and cultured, but no growth was observed (data not shown). Currently, we have modified the method of decontamination and achieved the isolation of *M. bovis* from colostrum samples from exposed and infected cows. Nevertheless, we need to conduct real-time PCR tests, assessing the expression of genes known to be required for active replication, as well as those required for entering a nonreplicating state.

In conclusion, the booster BCG vaccination scheme implemented on dairy farms with BCG and/or subunit vaccines was associated with a decreased frequency of *M. bovis* DNA detection in colostrum samples from both exposed and infected cattle. Therefore, *M. bovis* DNA frequency detected by nested PCR could be useful as an indicator of vaccination effectiveness against BTB.

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