Journal of Animal and Veterinary Advances 8 (11): 2171-2176, 2009
ISSN: 1680-5593
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Field Evaluation of the *Mycobacterium bovis*-BCG Vaccine Against Tuberculosis in Holstein Dairy Cows

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Abstract: An epidemiological field study was performed on a commercial dairy herd with a 40% prevalence of tuberculosis (on their 1st year of age and 45 days after first partum), in order to evaluate in two stages the *Mycobacterium bovis*-Bacillus Calmette-Guerin (Mb-BCG) vaccine. For this study, a total of 130 calves (2 weeks or younger), which tested negative to tuberculosis where arranged into two groups: Group 1 included 65 vaccinated calves (with a single dose 1×10^{-6}) and group 2 included 65 non vaccinated animals. During the first 3 days after birth both groups were given pasteurized colostrums and up to weaning time (60 days of age) were fed with pasteurized milk and concentrate. Later from the second to their 24 months of age both groups were gathered in the same pen being exposed in a natural way to animals with tuberculosis. For this study, it was considered that an animal was infected with tuberculosis when a positive reaction was the result to the next series of tests: Tuberculin, IFNγ PPD-B and IFNγ ESAT6-CFP10. The results for the first year showed that the frequency of tuberculosis between both groups was different $p = 0.02$ (24.5 vs. 8.9%). Also the non-vaccinated calves had a 2.7 higher risk of infection than the vaccinated ones (IC95%: 1.05-7.17). The frequency of the non-vaccinated group during the 2nd year was of 57.1% (24/42), while the vaccinated one was of 36.1% (13/36). This frequency between both groups had no statistical significance perhaps due to the sample size on which the study ended. In addition, the vaccine efficacy by PCR test was determined by nasal secretion. The difference in the elimination rates between both groups were not statistically different with a $p = 0.06$ (16.6% from the non-vaccinated against 7.7% vaccinated). The results in this study show a protective efficacy at 1 year of age; unfortunately, it was not possible to effectively measure the real efficacy at the 2nd year because of the limited sample size. Future studies should include a higher number of experimental units anticipating factors that compromise the sample size such as: sales or elimination to slaughter by other reasons than tuberculosis.

Key words: Vaccination, *Bovine tuberculosis*, *Mycobacterium bovis*, BCG, calves

INTRODUCTION

Bovine tuberculosis caused by *Mycobacterium bovis* is one of the most important diseases of the bovines considered to be of a very high risk for public health in many underdeveloped countries, where non pasteurized milk is consumed. Some of these countries have been able to eradicate or at least diminish significantly this illness by using eradication programs based upon the tuberculin test and by eliminating infected animals (Buddle *et al*., 2001). Due to the high incidence of tuberculosis in certain regions of Mexico, it has been very difficult to eradicate this problem using the tuberculin test and elimination programs because of economic reasons and this is why vaccination programs are considered effective for the reduction of tuberculosis cases. The model to evaluate the Mb-BCG vaccine under experimental studies is well described by Buddle *et al*. (2003, 2008) and Wedlock *et al*. (2007). With this model, animals testing negative to tuberculosis are vaccinated and challenged with a pathogenic strain in order to detect tissue damage at post-mortem inspection. It has been determined by the

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results of these studies that vaccines do protect even though, some researchers suggest that this should be evaluated under natural infectious conditions (Buddle *et al*., 2001; Vordermeier *et al*., 2006). In a previous evaluation of the Mb-BCG vaccine done by the researchers under field conditions with calves of up to 1 year of age, the results, where that the vaccinated group was protected against infection when compared to the non vaccinated group (9.2 vs. 22.7%). Nevertheless, it is necessary to evaluate the vaccine in female cows due to their great value in the milk industry and because of the length of time that they are part of the herd. It is clear that in production herds where the Mb-BCG vaccine has been evaluated, animals should not be sent for slaughter once the study period is over, for which it is necessary to find other options to determine the protective efficacy of the vaccine. This study evaluates the Mb-BCG vaccine under field conditions in up to 45 days old calves using a series of tuberculin tests, gamma interferon bovis (IFNγ bovis) and IFNγ ESAT6-CFP10. In addition to determine the efficacy protection, the PCR test is used to detect the presence of *Mycobacterium bovis* in nasal secretion.

MATERIALS AND METHODS

Study site: The study was performed in Tijuana Baja California, Mexico at a dairy farm in the Carrizo Valley, which is located at 32°43'19" (latitude North) and 117°.043'19" (longitude West) with an average annual rainfall of 192.2 mm. The valley has approximately 3,800 cows in production, distributed among 10 commercial dairy farms, which share the same general handling conditions, milking gets done twice daily with an average production of 28 L day^{-1}, an average of 120 open days and 2.5 services per conception. The dairy farm tested has 600 Holstein Freisian cows with a tuberculosis prevalence of >40% (determined by tuberculin testing) and with a history of bacteriological isolation of *M. bovis* in the herd (IICV-UABC tuberculosis laboratory).

Study design: To evaluate, the vaccine we designed an observational epidemiology field study, which included 130 calves (2 weeks or younger) who tested negative to a double comparative tuberculin test (using bovine PPD and avian PPD). They also tested negative to BOVIGAM, which measures IFNγ bovis and IFNγ avium (Wood and Rothel, 1994). Positive reactors to any of these tests were not included in the study. Each group was identified with a plastic ear tag and divided as follows: the non vaccinated group of calves (n = 65) and the vaccinated one (n = 65) inoculated with one dose of Mb-BCG (1×10^6) of Tokyo strain. Both groups were given pasteurized colostrums the first 3 days of age and then were fed with pasteurized milk, plus a concentrated diet until weaned (60 days of age). At age of 2-24 months, both groups where put in the same corral and exposed to tuberculosis under natural conditions through contact, while allocating them with sick animals. In this study, there were two ways of transmission; direct (by sharing common water tanks with sick cows) and indirect (through aerosols) by being kept in corrals separated from sick animals only by a fence. The animals were kept under these conditions until their first partum (approximately at 24 months of age). Inclusion criteria: a selection of 130 Holstein Friesian calves from under 2 weeks of age took place after testing negative to a double comparative test and to IFNγ bovis and IFNγ avium tests (Wood and Rothel, 1994). Any positive reactor to any of the tests was not included in the study.

Sample size: Each sample size per group was of 65 calves, the sample was obtained using the statistical package Win Episcope 2.0. Assuming the following values: a 25% illness rate of the vaccinated animals a minimum relative risk of 2 to be detected (we expected twice the number of sick cases within the non vaccinated group); an 85% of test power and a significance level of 90%. The sample size based on this data was of 53 animals per group and 12 more were added to each group at the beginning anticipating a mortality rate of 20% for the study period.

Tuberculin tests: A double comparative tuberculin test was applied on both groups before they reached 2 weeks of age and in two more occasions, at 12 months and 45 days after their first partum. Before applying the tuberculin the inoculation area was shaved (upper third of the neck). The upper application site was approximately 10 cm below the crest, while the bottom site was approximately 13 cm underneath the other. Prior to inoculation a skin fold was lifted in the center of the shaved area and measured with a vernier to record the thickness. We proceeded to inoculate subcutaneously 0.1 mL of avian PPD into the upper shaved area, while 0.1 mL of bovine PPD was applied into the inferior area. The test was read 72±6 h after application by measuring with the vernier the thickness of the reactions. Any animal that had a reaction with a difference ≥4 mm between the initial and final readings was considered to be positive to the tuberculin test (Buddle *et al*., 2001).

Blood collection for the gamma Interferon (IFNγ) assays: During the length of the study three blood samples were

obtained from all animals: the first sample before 2 weeks of age, the second at 12 months and the last was on day 45 after birth. About 6 mL of blood were obtained in vacationer tubes with lithium heparin from the caudal or jugular vein. These samples were stored at 25°C and sent to the laboratory for processing within 18 h after their collection.

Antigens for IFNγ: To detect the presence of IFNγ the following type of antigens were used: PPD-B, PPD-A (CSL, Melbourn, Australia), the early secretion antigen 6 (ESAT6) and also a purified filtered culture 10 (CFP10) (supplied by Ray Walters, Agricultural Department of the United States of America, Ames, Iowa). As a negative control (Nil) a phosphate buffered saline solution (PBS, pH 7.2) was used.

IFNγ test: To measure the *in vitro* response of the T lymphocytes, the IFNγ test was used. Tests were performed according to the protocol described by Wood and Rothel (1994). The IFNγ test was divided in 2 phases; phase 1 sensitization was obtained by placing four 1.5 mL blood samples with heparin in sterile culture wells, each sample was added with: 100 μ of buffered saline solution PBS 7.2; 100 μ of Stimulogen PPD bovis; 100 μ of Stimulogen PPD avium and 50 μ of ESAT6-CFP10. After 24 h, 400 μ of the sensitized plasma was placed in sterile vials and frozen at -20°C until analysis. Each sample was identified with the ear tag number and date of collection. Phase 2, the ELISA test was used to determine *in vitro* production of IFNγ in the following antigens: PPD-B, PPD-A and ESAT6-CFP10, this was done to each of the plasma samples according with the procedure recommended in the Bovigam test kit (CSL, Australia). A brief procedure description is as follows: 50 μ of a green dilution was placed in each well adding 50 μ of plasma, the plate then was incubated at a temperature of 22±5°C for 1 h. Once incubated the plate was washed with a buffered solution 1:20 and 100 μ of diluted conjugate in a 1:100 blue dilution was added and then incubated once more at laboratory temperature (22±5°C) for an hour. Done this, we washed it again 6 more times with a buffered solution and at the end 100 μ of substrate was added. The reaction was stopped using a finalization enzyme. The plates were read with an ELISA reader at 450 nanometers and a positive and negative control were used in each test. A positive result was the one in which the PPD-B Optic Density (OD) minus the Nil was ≥0.1 and the PPD-B (OD) minus the PPD-A (OD) was ≥0.1. A negative result was the one in which the PPD-B (OD) minus the Nil was <0.1 or the PPD-B (OD) minus the PPD-A (OD) was <0.1. For the ESAT6-CFP10 a positive result was one in which ESAT6-CFP10 (OD) minus Nil (OD) was ≥0.1.

***M. bovis*-BCG vaccine production:** The BCG (Tokyo strain) vaccine was cultured for 12-15 days (mid-log fase) in Dubos broth (Difco, Labs.) supplemented with Middlebrook OADC enriched medium. After being centrifuged, 5 mL of the supernatant was eliminated and replaced by 5 mL of Dubos broth with 10% glycerol. The vials were hemogenized by agitation (Buddle *et al*., 1995). The total content of the suspension was stored in sterile vials in amounts of 50 μ and frozen at -80°C until their use. The UFC was determined by standard count in double dilutions in a Middlebrook medium (Buddle *et al*., 1995).

Vaccination: The female calves in the Mb-BCG vaccinated group were inoculated subcutaneously on the humerus-scapula joint region with 1 mL (one dose 1×10^6 UFC) of the Mb-BCG vaccine (Tokyo strain), while the control group was inoculated with 1 mL of saline solution. Since birth and until their 2nd month of age the calves were set in consecutive individual pens according to their date of birth. They were randomly assigned to a group; the first calf was chosen by the flip of a coin and assigned to the vaccinated group, the next calf was automatically assigned to the non vaccinated group and so on until having a number of 65 animals in each group.

Nasal secretion collection for the detection of *M. bovis* using a Chain Reaction Polymerase (PCR) test: To determine the amount of sick animals with the presence of *M. bovis* in their nasal secretion, 10 mL samples of nasal flush were collected from this animals using sterile saline solution at their 45th day after first partum. All samples were identified and stored in 15 mL. sterile Falcon centrifuge vials, which were sent to the laboratory to be frozen at -20°C until their analysis with PCR.

Chain Reaction Polymerase (PCR) test: In order to inactivate the mycobacterium present in the samples, 1 mL of each sample was heated to a temperature of 95°C for an hour. The already inactivated samples were centrifuged at 13,792× g for 5 min using an Eppendorf centrifuge. The supernatant was discarded and the pellet with the genetic material was used to purify the DNA. The DNA extraction from the nasal inactivated samples was obtained by using the Qlamp mini kit (Qlagen, Inc.) according to the instructions by the manufacturer. The oligonucleotide (Invitrogen, Inc.) used were: IS41, 5' CCT GCG AGC GTA GGC GTC GG 3'; IS43, 5' TCA GCC GCG TCC ACG CCG CCA 3'; complimentary to a sequence flanking the repetitive insertion element IS6110 specific of the tuberculosis complex (Liébana *et al*., 1995). Briefly the reaction consisted of 100 ng of DNA from each sample,

1× Taq buffer (Invitrogen, Inc.), 1.5 mM $MgCI_2$, 0.2 mM deoxynucleotide triphosphate, 2.5 U Taq polymerase (Invitrogen, Inc.) and 25 pM from each oligonucleotide in a total volume of 50 μL. The DNA was amplified with an Express Hybaid thermocycler, using one cycle at 94°C for 10 min, 30 cycles for denaturing at 94°C for 2 min, alignment at 68°C for 2 min, an extension of 72°C for 2 min and finally 1 cycle at 72°C for 10 min. The expected amplified DNA band was of 317 bp (Liébana *et al*., 1995). The AN5 strain was used as a positive control, while for the negative control, water and DNA samples from nasal exudate collected from cows free of tuberculosis were used. PCR products were tested by electrophoresis in 1.5% agarose gels and stained with 5 μg of ethyl bromide and observed under an ultraviolet lamp (Bio Rad, Inc.)

Statistical analysis: In order to estimate the rate of cases per group, the number of positive tuberculosis cases was determined and then divided by the analyzed number of animals from each group (Martin *et al*., 1987). Three tests were used to determine the positive ones to tuberculosis: positive reaction to tuberculin testing, positive reactions to IFNγ-PPD-B and also to IFNγ-ESAT6-CFP10. Animals testing positive to only one or two of this, tests were considered as negative. The proportion of tuberculosis between the vaccinated and the non vaccinated groups was analyzed by Pearsons χ^2 using the program.

The risk of infection between groups was estimated by the analysis of relative risk (Martin *et al*., 1987). The difference between the proportions of animals with *M. bovis* in nasal secretion was analyzed by Fishers exact test in the Epi-info 6 program (CDC, Atlanta, USA).

RESULTS

Frequency of tuberculosis in non-vaccinated and vaccinated calves at 1 year of age: During the 1st year of the experiment, 12 calves from the non vaccinated group and 9 from the vaccinated one died; showing a frequency of tuberculosis of a 24.5% (13/53) in the non vaccinated group, while the vaccinated one showed an IFNγ 8.9% (5/56). The frequency of tuberculosis between both groups was statistically significant (p = 0.02). While, estimating the relative risk, we observed that in the non vaccinated group the probability is 2.7 higher than in the vaccinated one (Table 1).

Frequency of Tuberculosis in non vaccinated calves and vaccinated at 45 days after their first partum: This study began with 130 calves divided into two groups (65 vaccinated and 65 non vaccinated). During the 2 years period of the study, 23 animals from the non vaccinated group and 29 from the vaccinated one were eliminated due to different reasons; infectious deaths (diarrhea), injuries, loose of their ear tag and sales. Within the non vaccinated, the tuberculosis frequency was of 57.1% (24/42), while the vaccinated group had a frequency of 36.1% (13/30). The frequency of tuberculosis between this two groups had no statistical significance (p = 0.064). While estimating the relative risk, we observed that there is a 1.5 higher probability that non vaccinated calves become infected while being compared to the vaccinated ones (Table 2).

Presence of *M. bovis* in Nasal exudates: Using PCR, exudates from positive animals of both groups, where tested (n = 37). In 16.6% (4/24) of animals diagnosed as infected in the non vaccinated group *M. bovis* was detected in their nasal secretion, while 7.7% (1/13) of the animals in the vaccinated group that tested positive the *M. bovis* was present. In the positive animals from the non vaccinated group, the presence of *M. bovis* in the nasal exudate was higher when compared to the vaccinated number, however no statistical difference was found (Table 3).

Table 1: Frequency of Tuberculosis between non-vaccinated and vaccinated animals with Mb-BCG at 12 months of age using as diagnostic tests in series: Tuberculin, IFNγ-B and IFNγ-ESAT6-CFP10

Groups	Positive*	Negative	n	Freq. (%)**	RR (CI 95%)
Non vaccinated	13	40	53	24.5	2.74 (1.05-7.17)
Vaccinated	5	51	56	8.9	
Total	18	101	109		

*An animal that came positive to the next three series of tests was considered with Tuberculosis: Tuberculin, IFNγ-B and IFNγ-ESAT6-CFP10; **The frequencies are statistically different (p = 0.02); RR = Relative Risk; CI = Confidence Interval at 95%; Confidence

Table 2: Frequency of tuberculosis among non-vaccinated calves and vaccinated with Mb-BCG 45 days after their first born using for diagnosis a series of tests: Tuberculin, IFNγ-B and INFγ-ESAT6-CFP10

Groups	Positive*	Negative	n	Freq. (%)**	RR (CI 95%)
Non vaccinated	24	18	42	57.1	1.58 (0.91-2.74)
Vaccinated	13	23	36	36.1	
Total	37	41	78	47.4	

*An animal that came positive to the next three series of tests was considered with tuberculosis: Tuberculin, IFNγ-B and IFNγ-ESAT6-CFP10; **Frequencies are not statistically different (p = 0.064). RR = Relative Risk; CI = Confidence Interval at 95%; Confidence

Table 3: Protective efficacy of the *Mycobacterium bovis*-BCG vaccine in vaccinated and non vaccinated female Holstein calves based upon the presence of *M bovis* in nasal exudate

Groups	Animals with TB*	PCR^+	PCR^-	Freq. (%)	p-value
Non vaccinated	24	4	20	16.6	0.063
Vaccinated	13	1	12	7.7	
Total	37	5	32		

*An animal that came positive to the next three series of tests was considered with Tuberculosis: Tuberculin, IFNγ-B and IFNγ-ESAT6-CFP10

DISCUSSION

The objective of the study was the evaluation of the efficacy of the *M. bovis* BCG vaccine under natural exposure conditions in calves during 2 stages: the 1st stage evaluated the presence of tuberculosis up to their 1st year of age and during the 2nd stage, the presence of tuberculosis was evaluated 45 days after birth in a high incidence herd. We also analyzed nasal secretion from all sick animals through a polymer chain reaction. After researching references of the last 28 years, the investigation is the first to evaluate the efficacy of Mb-BCG vaccine in calves up to 45 days after their first partum under natural exposure conditions in which at least three aspects were considered giving the importance to other experimental studies in making a good evaluation of the BCG vaccine and this are: the dosage of the vaccine in use (most appropriate 1×10^6), not having any previous exposure to the *Mycobacterium prior* to inoculation and Age at vaccination (before the 2nd week of age) (Buddle *et al*., 2003). The results of the evaluation as to the 1st year of age shows the effectiveness of the vaccine due to a difference present ($p<0.05$) between both groups (8% vaccinated vs. 24% non vaccinated). In a previous study and under the same conditions, we evaluated de Mb-BCG vaccine but in male animals, showing a very similar behaviour with a 9% of tuberculosis cases within the vaccinated calves against a 22% of the non vaccinated. The results of these studies concur with the ones reported by Morris *et al*. (1994), who declares that there is no relation for infection regarding the sex of the animal. By evaluating, the vaccine during the second phase (45 days post partum) the frequency of tuberculosis on those not vaccinated animals was of 57.1%, while in the vaccinated group was only 36.1%. The frequency of tuberculosis between both groups was not significant ($p = 0.064$) and a possible explanation for this behaviour is that the size of the sample was insufficient in order to detect a statistical difference. It is important in order to evaluate the vaccine under field conditions to do this kind of testing in commercial milking herds with tuberculosis; also some important decisions that are out of control from the researcher (selling, switching or to be slaughter before the study ends) limits the reach of the project as shown in this case.

The model for evaluating the Mb-BCG vaccine under experimental studies is well established (Buddle *et al*., 2003, 2008; Wedlock *et al*., 2007). In this, the animal gets vaccinated and challenged with pathogenic strains to be later sent for follow up after slaughter for a postmortem detection of tuberculosis lesions. When a Mb-BCG vaccine is evaluated under field conditions in productive cows, it is clear that when the study is completed its impossible to have a postmortem evaluation and for this reason an excellent option to measure the protective efficacy of a vaccine under this circumstances is to determine the amount of animals in which *Mycobacterium bovis* was isolated from their nasal secretion. In present study, the *Mycobacterium* found in the nasal secretions from non vaccinated infected animals was higher (16%) than the vaccinated group (7%) and although, it could not establish a statistically significant difference, it can be explained because only 73% of the animals (78/106) included in the size of the sample ended the study period. It is important to mention that on future studies when the protective efficacy of the Mb-BCG vaccine shall be evaluated under field conditions to consider trying it for a longer period and used on a larger number of animals due to the elimination of some experimental units before the end of the study.

CONCLUSION

The use of the Mb-BCG vaccine applied to calves before 2 weeks age and evaluated until their 1st year was effective against bovine tuberculosis, because the vaccinated heifers showed a lower number of cases (8.9%) in comparison with not vaccinated animals (24.5%). One of the major limitations in present study when we intent to evaluate under field conditions the 2 years old heifers (45 days post partum) was that a large number of animals were out of study before we end it, principally for out of researchers control situation. Although was not possible to detect a statistical difference between vaccinated and not vaccinated groups at two years old, it was due at the sample size, but it was evident that vaccinated animals shown a less tuberculosis cases in comparison with not vaccinated. The model of vaccination used to evaluate the vaccine under natural infections conditions in a high prevalence dairy herd, considering the additional diagnostic tests is viable. However, it will be improved increasing the study time and the sample size in both groups.

ACKNOWLEDGEMENTS

This research is part of the requisites to obtain the grade of Doctor en Ciencias Agropecuarias of the present first author (Facultad de Medicina Veterinaria y Zootecnia, Universidad Autónoma de Yucatán). This research was partially supported by CONACYTSAGARP

AC02-2003-03. We thank Raquel Pio Garcia, Carloman Herrera for excellent technical assistance and M.V.Z. Javier Jimenez Serrano for his interest in this study and logistic support.

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