

Presence of *Brucella abortus* Vaccinal Strain Rb51 in Vaginal Exudates of Aborted Cows

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Abstract: The present study was aimed to detect *B. abortus* DNA from aborted cows and differentiated its vaccinal and/or field origin. The PCR technique was used to identify and differentiate S19, RB51 and field strains. The research was performed in a dairy farm located in the state of Aguascalientes, Mexico, with reproductive problems and abortions. Cows were vaccinated when calves with RB51, normal dose and revaccinated with RB51 reduced dose 8 months before starting the study. The number of revaccinations of each cow, was not known since it is customary to revaccinate in one or more occasions. Samples of milk, blood serum and vaginal exudates were collected from 30 cows that had aborted between day 166 and 260 of gestation, all the abortions occurred 22-30 days before sampling; Card, rivanol and radial immunodiffusion tests were performed for Brucellosis diagnosis. For the bacteriological study samples of milk and vaginal swabs were used. The isolated strains were subjected to PCR using specific primers for RB51, S19 and field strains. From the 30 vaginal exudate samples bacteriological analyzed, we isolated smooth *B. abortus* biotype 1 was isolated from four cows. Thirteen out of the 30 cows vaginal exudates analyzed by PCR revealed the presence of DNA corresponding to RB51 vaccine, the 13 cows that were positive to RB51 through PCR had negative results in both the bacteriological and serological studies.

Key words: Aguascalientes, Brucellosis, RB51, cows, vaccinal strain, vaginal exudates

INTRODUCTION

In the state of Aguascalientes, Mexico, industrial producers represent 27% of the dairy-producing units, with 57% of the dairy cattle and generate 70% of the milk calculated in 426 million liters. In the state of Aguascalientes, Mexico, a Brucellosis prevalence study performed from 1966-2004, was reported that the 85.2% of herds had not positive animals; whereas the disease was distributed at different ranges of prevalence in the remaining herds (Alvarez, 2006).

Brucellosis is a chronic disease with importance on public and animal health, producing large economic losses due to the commercial restrictions imposed on the infected animals and their products. The most characteristic feature of Brucellosis is abortion during the last third of gestation; some cows during the first and second gestation are susceptible to abortion due to the infection, although they can give birth

normally in ulterior gestations despite being infected (Luna-Martinez and Mejia-Teran, 2002).

In Mexico, before 1998, *B. abortus* strain S19 vaccine was used to prevent Brucellosis in cattle, since 2005 its use became popular once again. The S19 vaccine, is a smooth, attenuated strain presenting a 702 pb deletion that affects genes *eryC* and *eryD* of the *ery* operon that participates in the catabolism of erythritol (Sangari and Aguero, 1994a, b). Since 1997, the RB51 strain of *B. abortus* has been approved as official vaccine for bovines in Mexico (Luna-Martinez and Mejia-Teran, 2002); this is a rough attenuated strain derived from strain 2308 of *B. abortus*, which is a smooth and virulent strain. RB51 vaccine was obtained through successive passes in a culture medium containing rifampicin and penicillin (Schurig *et al.*, 1991) its designation refers to a Rough strain (R) of *Brucella* (B) and 51 corresponds to the internal nomenclature of the laboratory used at the time it was derived. Strain RB51 presents an insertion

sequence of 842 pb known as insertion element IS711 that interrupts gene *wboA*, which encodes a glycosyltransferase, an essential enzyme for the biosynthesis of the lipopolysaccharide (Vemulapalli *et al.*, 1999). The deletion in the *ery* locus shown by strain S19 of *B. abortus*, as well as the mutation in gene *wboA* by element IS711 in strain RB51, are unique molecular characteristics that provide for a diagnostic method to identify these strains. Based on those genetic markers, (Sangari *et al.*, 1994b; Vemulapalli *et al.*, 1999) designed specific primers for a Polymerase Chain Reaction (PCR) capable of identifying and differentiating strains S19 and RB51, respectively, from other *Brucella* species and strains. In this study, we used both PCR assays to detect the presence of DNA from *B. abortus* vaccinal and field strains in vaginal exudates from cows that had aborted.

MATERIALS AND METHODS

Location: The present research was performed from June to December 2005, in a dairy farm located in the county of Pabellón de Arteaga, state of Aguascalientes, Mexico; the farm has an extension of 5,589 km² and is located between 101°50' and 102°53' W longitude and 20°30' 22' 28' N latitude, in central Mexico, limiting to the south and southeast with the state of Jalisco, to the north, east and west with the state of Zacatecas (Garcia, 1973). The dairy farm was chosen based on the disposition and cooperation of the owner.

Animals: The farm had 1,340 Holstein-breed cows and 533 heifers, under a dairy milk system characteristic of the region; animals are milked three times a day. Productive and reproductive records are kept for each animal and artificial insemination is used; replacements are usually acquired locally or from the state of Jalisco. Reproductive problems and abortions are frequent in this dairy farm. The sampled cows had been vaccinated as calves with a normal dose of RB51 with 1×10¹⁰ cfu and revaccinated with a reduced RB51 dose with 1×10⁹ cfu eight months before starting this study. The number of revaccinations for each cow was unknown, since it is a common practice in this geographical region to revaccinate one or more times with RB51 in reduced dose.

Samples: The collected samples were: milk, blood serum and vaginal exudate from 30 cows that had aborted in a 22-30 days period before the study. The time of gestation at the time of abortion ranged from 166-260 days.

Serological tests: Card, rivanol and radial immunodiffusion tests were performed in blood sera (Alton, 1988; Diaz *et al.*, 1981).

Table 1: Oligonucleotides sequence used for PCR

| Oligonucleotides | Sequence | Genes |
|------------------|----------------------------------|------------------|
| S19-1 | 5' TTGGCGGCAAGTCCGTCGGT 3' | <i>eryC-eryD</i> |
| S19-2 | 5' CCCAGAAGCGAGACGAAACG 3' | |
| RB51-1 | 5' TTAAGCGCTGATGCCATTTCCTTCAC 3' | <i>wboA</i> |
| RB51-2 | 5' GCCAACCAACCCAAATGCTCACAA 3' | |

Bacteriological study: The milk and vaginal exudate were subjected to bacteriological isolation, inoculating Brucella and Farrel agar plates, according to Alton *et al.* (1988) for the isolation, identification and differentiation between smooth and rough phenotypes. In addition, the isolated strains were subjected to PCR.

Bacterial strains: The *B. abortus* strains used for DNA extraction were 544, RB51 and S19. All strains were cultured in Brucella agar at 37°C from 24-72 h (Alton, 1988).

DNA extraction: DNA extraction from the vaginal exudates and from the *B. abortus* strains was performed through the phenol-chloroform technique described by Sambrook *et al.* (1989) 100 ng of the extracted DNA was used as template for the PCR.

PCR: To identify strain S19, the primers described by Sangari *et al.* (1994b) were used and, for strain RB51, those described by Vemulapalli *et al.* (1999) (Table 1).

The PCR, for the vaccinal, control and field strains was performed in a total volume of 50 µL, containing 25 µL of the commercial premix REDTaq ReadyMix that contains: 20 mM Tris-HCl, 100 mM KCl, 3 mM MgCl₂, 0.002% gelatin, 0.4 mM dNTPs and 0.06 units of Taq DNA polimerase/µL. Reaction cycles for the PCR to identify RB51 (PCR-1) were: Initial denaturation at 94 °C for 5 min, 30 cycles at 94°C for 30 s, 60°C during 30 s and 72°C for 90 min and a final extension at 72°C for 5 min. The reaction cycles to identify strain S19 (PCR-2) were: initial denaturation at 94°C for 5 min, 30 cycles at 94°C for 1 min, 59°C for 30 s and 72°C for 1 min and a final extension at 72°C for 2 min.

RESULTS

The standard bacteriological tests (Alton, 1988) allowed for the isolation of smooth biotype 1 *B. abortus* strains from 4 cows out of the 30 subjected to vaginal exudate analyses. Through PCR, these same isolates were identified as *B. abortus* field strains. From the exudate samples of the 30 analyzed cows, in 13 the PCR revealed the presence of DNA corresponding to strain RB51.

The 13 positive cows to RB51 through PCR, resulted negative to both the bacteriological and serological tests.

The four cows in which the smooth field strains were isolated resulted positive in the serological tests performed, i.e., Card test, rivanol in titers of 1:400 and the radial immunodiffusion test.

No isolations were obtained from the milk samples suggesting *Brucella* infection, nor were these samples positive to the PCR test.

DISCUSSION

The presence of Brucellosis in the state of Aguascalientes is a serious problem as confirmed by data obtained from 1996-2004, during which bovine Brucellosis depicted an individual prevalence of 5% and a herd prevalence of 14.9%, aside from a high variability in the eleven counties comprising the state (Alvarez, 2006). This prevalence places the state of Aguascalientes as a zone under control according to the official Mexican standard as it is above 3% (NOM-041-ZOO-1995). In the state of Aguascalientes, vaccination is performed since 1998 with RB51; however, in 54.3% of the dairy farms (2, 645 of the 4,871 herds) vaccination is applied without a previous serological diagnosis (Chávez, 2006). Between 1996 and 2004, the RB51 vaccine from Litton laboratories was applied in 73% (82,915) of the vaccinated cows in Aguascalientes, being higher the amount of cows immunized with the reduced dose which confirms the notion accepted by producers that this strain does not interfere with the diagnosis and monitoring programs of bovine Brucellosis (Chavez, 2006).

It is a common practice in the state of Aguascalientes to revaccinate repeatedly the cows with the cow dose and lately with the calf dose of RB51, which lacks scientific support (Moriyon *et al.*, 2004) and which could be responsible for the elimination of *Brucella* from the vaginal exudates (Leal-Hernandez *et al.*, 2005). Olsen *et al.* (2000) reported that vaccination with a single RB51 dose of 1×10^9 CFU of cows before their mating or artificial insemination protect against infection and abortion induced by the challenge with intra-conjunctival inoculation of 1×10^7 CFU of the virulent 2308 strain. In pregnant cows vaccinated with RB51 at a reduced dose (around 10^9), a residual low virulence is reported, but which was able to produce placentitis in cows inoculated intravenously with higher doses (Palmer *et al.*, 1996).

The use of the classical dose in adult cows is not recommended, but is rather to be used in extraordinary outbreak cases or when faced with increased abortions, since cows vaccinated with the normal S19 dose remain positive to the serological diagnostic tests; aside that it favors abortions and shedding of *Brucella* from the milk and vaginal exudate just like RB51 (Flores-Castro *et al.*, 1985, Leal-Hernandez *et al.*, 2005).

Some authors claim that the RB51 vaccine is safe at all ages and that there are no evidences of pathological effects on tissues, even when applying subcutaneously high doses to calves of 3 and 6 months of age (Cheville *et al.*, 1993).

In cows infected with *Brucella*, the field strains are eliminated in milk and vaginal exudates after parturition or abortion, during two or three months; after this period *Brucella* is shed sporadically, although these cows can remain as carriers for years (Herr *et al.*, 1990; Philippon *et al.*, 1979).

Although strain RB51 is a vaccinal rough and attenuated strain, it is characterized for being alive and therefore, can cause disease, as evidenced by reports of infection in humans (Ashford *et al.*, 2004) which is aggravated by the resistance presented by this strain to rifampicin (Marianelli *et al.*, 2004) which is one of the choice treatment against Brucellosis in Mexico.

Poester *et al.* (1998) have evidenced in different occasions that the vaccinal RB51 strain is not isolated from either vaginal secretions or from the milk from vaccinated animals and that this continues to be true for animals at 60 days of gestation. Their results indicate that vaccination with RB51 avoided 59.4% of abortions and 58.6% of infections in cows and 61% of infection in fetuses. The relative risk revealed that the non-vaccinated animals have a 2.462 times higher risk of aborting than those vaccinated with RB51 (Poester *et al.*, 2006).

On the other hand, Leal *et al.* (2005) and Uza *et al.* (2000) have demonstrated that the RB51 strain can be excreted in the milk and the vaginal exudate of vaccinated cows, although the frequency is not high. In addition Samartino *et al.* (2000) observed that only 1 in 57 revaccinated cows excreted RB51 in the milk, three days after parturition.

In an *in vitro* study, strain RB51 isolated from the milk, when assessed in its intracellular trafficking, retained its non-virulent characteristics (Arellano-Reynoso *et al.*, 2004). In 99 cows positive to serological tests, from a herd with natural *B. abortus* infection and which had been immunized with S19 and RB51, PCR assays identified 14 cows infected with *B. abortus*, 9 infected with the wild type and 5 with the wild type and RB51; strain S19 was not identified (Lavaroni *et al.*, 2004).

These values agree with our results, since PCR was more efficacious than the bacteriological study to demonstrate the presence of *Brucella abortus*, as it is a highly sensitive method and requires less amount of bacteria than a bacteriological study to confirm the infection; in addition, PCR does not need that bacteria be viable or culturable. This contrasts with the bacteriological study that requires special and specific growth conditions, therefore, this bacterium is considered a nuisance microorganism.

In this study we used PCR assays previously standardized (Sangari and Aguero, 1994a; Vemulapalli *et al.*, 1999) which are useful to differentiate *B. abortus* vaccinal strains from field strains. The PCR assays used in this work have been endorsed by the OIE (2004) for the differential diagnosis of Brucellosis, as they are capable of discriminating among strains based on the fact that the oligonucleotides, used to detect S19, flank the sequence of operon *ery* in which the 702 pb deletion is found and they amplify a 361 pb product; in contrast, it determines a 1063 pb product for the remainder *B. abortus* strains, because the latter possess a complete *ery* locus (Sangari and Aguero, 1994a). The oligonucleotides used to detect strain RB51 flank the gene *wboA*, in which the insertion IS711 is found. Hence, when dealing with strain RB51 the oligonucleotides amplify a 1298 pb product and when dealing with any other strain they detect a 456 pb product, since these do not possess the IS711 element in gene *wboA* (Vemulapalli *et al.*, 1999).

The fact that the cows in which the smooth field strain were positive to the Card, rivanol and radial immunodiffusion tests and that the cows in which RB51 was detected were negative to the serological tests is because the O chain, present in the smooth strains and absent in the rough ones, is the epitope to which the antibodies react in these tests this has been corroborated by other authors (Lavaroni *et al.*, 2004; Leal-Hernandez *et al.*, 2005).

CONCLUSION

The DNA of the RB51 vaccine strain was identified through PCR in 13 of the revaccinated cows sampled 22 to 30 days after aborting. These results suggest that the RB51 vaccine is eliminated throughout the vaginal exudates in aborted cows.

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