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RESEARCH ARTICLE

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Vaccination programs, parity, and calving season as factors affecting the risk of fetal losses and mummified fetuses in Holstein cows

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Abstract

Aim of study: To investigate vaccination programs, parity, and calving season as factors affecting the risk of abortion and mummified fetuses in Holstein cows.

Area of study: Hot zone of Northeast Mexico.

Material and methods: Multiple logistic regression models were used to examine the relationship between peripartum disorders, parity, previous occurrence of abortion, season of calving, vaccination program, incidence of abortion, and mummified fetuses in Holstein cows.

Main results: For 7014 pregnancies (2886 cows), the percentage of cows aborting and having mummified fetuses was 17.7% and 1.1%, respectively. As the number of brucellosis vaccinations increased, the incidence of abortion increased (10.4% for a single vaccination and 38.0% for 6 accumulated vaccinations). Abortion for cows having 1-2 previous abortions (56%) and >2 abortions (77%) was fivefold and sevenfold greater ($p<0.01$), respectively, than that for cows without previous abortion. Other important risk factors for abortion were number of calvings (19.8% for nulliparous and primiparous *vs*. 13.8% for >3 parturitions; OR=1.7, $p<0.01$), leptospirosis vaccine application <55 days postpartum (dpp; OR=1.3, $p<0.05$), viral vaccine application >37 dpp (OR=1.3, $p<0.01$), brucellosis vaccine application >20 dpp (OR=1.6, $p<0.01$), and no application of clostridial vaccine (OR=3.7, $p<0.01$). Significant risk factors for mummified fetuses were application of ≥3 brucellosis vaccinations (OR=3.3, $p<0.01$), no application of 10-way clostridial vaccine (OR=2.3, $p<0.01$), >2 previous abortions (OR=18.4, $p<0.01$), and calving in autumn (OR=0.4, compared to winter, $p<0.05$).

Research highlights: Risk of abortion and mummified fetuses in Holstein cows has been found to be related to vaccination programs.

Additional key words: bovine abortion; clostridial vaccination; *Brucella abortus* RB51 vaccine; repeated abortion; Leptospira vaccine

Abbreviations used: dpc (days post-calving); dpp (days postpartum) OR (odds ratio); CI (confidence intervals).

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Introduction

Reproductive efficiency plays a crucial part in the profitability of intensive dairy operations, as it affects milk yield and the productive lifetime of dairy cows which affects the net returns in dairy herds (De Vries, 2006). Components of reduced reproductive performance of high-milk yielding cows in intensive dairy operations include delayed resumption of ovulation after parturition (Crowe *et al.*, 2014; Santos *et al.*, 2016), lower expression of estrus signs (Madureira *et al.*, 2015), uterine health (Krause *et al.*, 2014; Moore *et al.*, 2014), lower pregnancy rates to first and subsequent inseminations (Flores *et al.*, 2019), increased incidence of embryonic death (Diskin *et al.*, 2016) and abortion (Mellado *et al.*, 2016, 2019). The latter is very costly because it leads to significant economic losses to dairy producers. After all, rebreeding of aborted cows result in long calving intervals, loss of calf returns, culling of cows, and the replacement cost if the cow is culled (Lee & Kim, 2007).

An adequate vaccination program may avoid the infectious causes of abortion; however, non-infectious causes of abortion in bovines are more difficult to prevent in intensive dairy herds (Grimard *et al.*, 2006). Non-infectious causes of abortion include high milk production (Grimard *et al.*, 2006; Mellado *et al.*, 2019), heat stress (García-Ispierto *et al.*, 2006; Mellado *et al.*, 2016), parity (Labèrnia *et al.*, 1996; López-Gatius *et al.*, 2009), previous postpartum disorders (López-Gatius, 2012) and twin pregnancies (López-Gatius *et al.*, 2004; Mellado *et al.*, 2019).

Fetal mummification does not account for a substantial loss in earnings in intensive dairy farms; however, this gestational disorder is a cause of failure to yield a calf per year per cow and imposes economic loss by extending the inter-calving period, the occurrence of fetal loss, and treatment to remove the mummified fetus, which frequently includes a cesarean section (Dutt *et al.*, 2018). Therefore, it is essential to know the causes of mummified fetuses to help rule out particular situations, increasing this reproductive disorder's risk. Various risk factors have been described for bovine abortion/fetal loss (Mellado *et al.*, 2016, 2019); however, risk factors associated with vaccination programs in adult dairy cows are unknown. Therefore, the objective of this investigation was to discern whether vaccination programs under field conditions and in a hot environment, parity, previous abortion, time from calving to vaccination, and calving season would influence the risk for abortion or mummified fetuses. The hypothesis was that adult cows repeatedly vaccinated against brucellosis, the time from calving to vaccination against abortifacient organisms, and cows with previous abortions would increase the risk of future abortion. Also, we hypothesized that season and time from calving to application of vaccinations would increase the occurrence of mummified fetuses. These hypotheses are proposed because it is plausible to expect that repeated cases of abortion in cows (Keshavarzi *et al.*, 2017), greater parity (Norman *et al.*, 2012), late gestation during warm months (Norman *et al.*, 2012), and repeated inoculation against *Brucella abortus* (Sanz *et al.*, 2010) will increase the risk of the premature expulsion of the fetus.

Material and methods

Animals and herd management

The experimental procedures and animal care conditions were approved by the Ethics Committee of the Research Department of the Autonomous Agrarian University Antonio Narro (protocol 42520-3001-2258). Holstein cows from a single large commercial dairy farm (3000 milking animals) located in a hot-arid environment of northern Mexico (25° N, 103° W, elevation 1140 m, mean annual rainfall 230 mm, mean annual temperature 23.7 ºC) were included in this retrospective study. The herd's annual daily milk yield was 34 kg/day, and the mean annual culling rate was 34%. Due to the low fertility of this herd because of the high ambient temperature, abortion was not a cause for culling, and therefore, cows often presented extended lactations (>500 days). Thus, two abortions never occurred in the same lactation.

A total of 7014 pregnancies from 2886 cows were included in this retrospective investigation from 2016 to 2019. Cows were housed in open-dirt pens equipped with a fixed metal framework shades in the center of the pens and additional shades covering the feed alleys. Lactating cows were fed total mixed rations twice per day, and ≈5% of feed refusals were removed immediately before each morning feeding.

Calves were open bucket fed and had access to free-choice water and pelleted calf starter. Bodyweight was registered with a mechanic scale at birth before colostrum ingestion and at weaning. Female calves were vaccinated subcutaneously with a standard label dose (10×10^9 CFU) of brucellosis vaccine strain RB51 (MSD Salud Animal Mexico, Mexico City, Mexico) at ≈4 months of age (this vaccination was considered for getting the sum of the total number of vaccinations against *B. abortus* applied to cows). All animals included in this investigation were revaccinated against brucellosis every year before the first breeding post-calving. It has been suggested that booster RB51 vaccination is required between 4 and 5 years of age to maintain high levels of protection after calfhood vaccination (Olsen & Stoffregen, 2005), although no improvement in the immunological response resulting from RB51-revaccination has been demonstrated in adult cattle (Dorneles *et al.*, 2014). The practice of annual revaccination against *B. abortus* in northern Mexico arises from a widespread dogma among veterinarians involved in dairy practice who have come to believe that repeated vaccinations against *B. abortus* strengthen the immunity of cows against this bacteria.

Some cows were vaccinated with a 10-way clostridial vaccine (toxoids of *Clostridium perfringens* types A, B, C and D, *C. septicum*, *C. sordelli, C. novyi, C. haemolyticum, C. tetani,* and inactivated cells of *C. chauvoei*; MSD Salud Animal México, Mexico City) previously to the first breeding and previously to the first service after calving. No particular criteria existed for applying this vaccine; inventory availability dictated the use of this vaccine.

All animals were vaccinated annually against infectious bovine rhinotracheitis, bovine respiratory syncytial virus, bovine viral diarrhea types 1 and 2, para-influenza 3, and leptospirosis caused by five *Leptospira* serovars (CattleMaster Gold FP5®, Zoetis, Mexico DF, Mexico). Cows were also annually vaccinated against leptospirosis

(5-serovars; LEPTAVOID-H®; Merck Sharp & Dohme Corp., Mexico DF) 30 days postpartum (dpp). All cows were periodically tested for brucellosis, and seropositive reactors to the card tests were culled. The herd prevalence of brucellosis was 1.5%, and none of the seropositive animals were included in the investigation.

Estrus was detected by direct observation and with the aid of tail chalk that was applied daily. After a voluntary waiting period of 50 dpp, cows in estrus were artificially inseminated following the a.m./p.m. guideline. Controlled breeding programs (Ovsynch) were used in all repeat-breeding animals. Commercial frozen-thawed semen from 69 high genetic merit bulls from the USA was used. Pregnancy diagnoses were performed at 45±3 days from their last recorded AI by the herd veterinary. Second palpation was carried out between 105 and 145 d following insemination.

Loss of pregnancy between 42-48 (pregnancy diagnosis by palpation per rectum) and 260 days of gestation was considered an abortion (Mee, 2020). Most of these cows displayed expulsed recognizable lifeless fetuses, had the presence of extraembryonic membranes and vaginal discharges, or resulted negative to the second palpation carried out between 105 and 145 d following insemination. Also, abortion was considered when cows returned to service after being confirmed pregnant. The abortion date was the day when distinctive signs of abortion were observed. In cows returning to service after being declared pregnant, abortion date was estimated as 12 d before the day of palpation when uterine horns were thick-walled and distended. Puerperal metritis was defined as cows presenting abnormally enlarged uterus with a fetid watery red-brown uterine discharge, related with signs of systemic illness and fever, within 21 days after parturition (Sheldon *et al.*, 2006). Fetuses and placentas were not submitted to the laboratory for an abortion investigation.

Mummies were detected with transrectal palpation and ultrasonographic examination. They were defined as a compact, firm fetal structure dehydrated, shriveled, and shrunken in appearance without fetal fluid and no history of systemic disease (Lefebvre *et al.*, 2009). Most diagnoses of mummies were made when cows were examined for going considerably over-term or because cows showed no udder development near the time they were expected to calve. Cases of abortion and mummified fetuses were not combined because, although fetal death is common for both reproductive problems, these are different conditions; *e.g.*, luteolysis and the opening of the cervix occur in one case but not in the other (Kumar & Saxena, 2018).

Statistical analyses

The response variable for the cow-level risk factors was binomial, with cows classified as presenting abortion or mummified fetuses or having normal parturition. A screening process to detect explanatory candidate variables ($p \leq 0.15$) was performed using univariate logistic regression analyses using SAS (SAS Inst. Inc., Cary, NC, USA) to subsequently build a multivariate model. The preliminary models contained the following potentially explanatory variables: number of brucellosis vaccinations (1, 2-3, >3) per cow, parity (1, 2-4, >4), days from delivery to leptospirosis vaccination (≤55 and >55 days), the interval from calving to various virus vaccination (≤37 and >37 days), days from delivery to brucellosis vaccination (≤20 and >20 days), application of 10-way clostridial vaccine (yes *vs.* no), occurrence of puerperal metritis (yes *vs.* no), number of previous abortions (0, 1-2, >2), season of delivery, age at first calving (≤700 and >700 days), birth weight (≤37 and >37 kg), weaning weight (≤77 and >77 kg), occurrence of ketosis (yes *vs.* no), dystocic parturition (yes *vs.* no) and season of calving (winter months being January–March; spring, April–June; summer, July–September; and fall, October–December). Also, two-way interactions were included in the model. The cut-off values for the time of postpartum application of various vaccinations were based on the mean interval between calving and vaccination (values above and below the mean).

Because gestations of the same cow cannot be considered independent events, for cows with various pregnancies, the sampling unit (pregnancy) was nested within cows to account for the cluster effect. Analysis of putative risk factors was performed using PROC GLIMMIX of SAS. The multivariate models were assessed using a binary distribution for the occurrence of abortion and mummified fetuses. The main confounders identified were the number of calvings per cow and the previous number of abortions; therefore, this set of baseline variables was included in the multivariate model for controlling these confounders. Additional covariates that caused effect modification of explanatory variables were added to the model based on significant interactions. All covariates included in the model were significant at $p<0.05$. Cow within gestation number was a random effect, and other main effects and their interactions were considered fixed.

Multivariate mixed logistic regression models using the GLIMMIX procedure produced odds ratios (OR) as estimates of the strength of association between the potential risk factors and the reproductive disorders studied. The Cochran-Armitage trend test (PROC FREQ of SAS) was used to test the null hypothesis that abortion is independent of days of gestation. The cumulative probability of pregnant cows aborting to defined times of pregnancy was determined using Kaplan-Meier survival curves produced with the Statgraphics Centurion XV statistical software (Statpoint Technologies Inc., Warrenton, VA, USA). Finally, the association between the number of *B. abortus* vaccinations received per cow and the abortion rate was assessed using the CurveExpert Professional 2.5.6

software (Hyams Development, Madison, AL, USA). For all statistical analyses, values with $p<0.05$ were regarded as statistically significant.

Results

Occurrence of abortion

The proportion of pregnant cows presenting abortion from 2016 to 2019 was 17.7 (1242/7014). The median time from calving to abortion was 129 days with two well-defined ($p<0.01$) peaks at around 86 and 168 days of gestation (Fig. 1). The risk factors adjusted for parity and previous abortion for current abortion are listed in Table 1. Throughout their productive life, cows with ≥2 brucellosis vaccinations had 7.4 times more risk of abortion as had cows with one vaccination against *B. abortus* (adjusted for the effects of parity, previous abortion, annual clostridial vaccination, and days to postpartum leptospirosis vaccination; $p<0.01$). The impact of repeated vaccination against brucellosis on the occurrence of abortions was more pronounced for cows receiving five or six vaccinations (Fig. 2). Furthermore, the interval from breeding to abortion depended on the number of *B. abortus* vaccinations applied. Cumulative *B. abortus* vaccinations were associated with a decreased interval from breeding to abortion (Fig. 3).

There was an interaction between the number of brucellosis vaccinations applied to cows and the pre-breeding vaccination of cows for leptospirosis ($p<0.01$) for the incidence of abortions. The proportion of abortions of unknown cause was twice as much in cows receiving the leptospirosis vaccine >55 d post-calving in cows with two or more *B. abortus* vaccine applications compared with that observed in cows receiving the annual leptospirosis booster vaccine >55 days post-calving (dpc) but with single brucellosis vaccination.

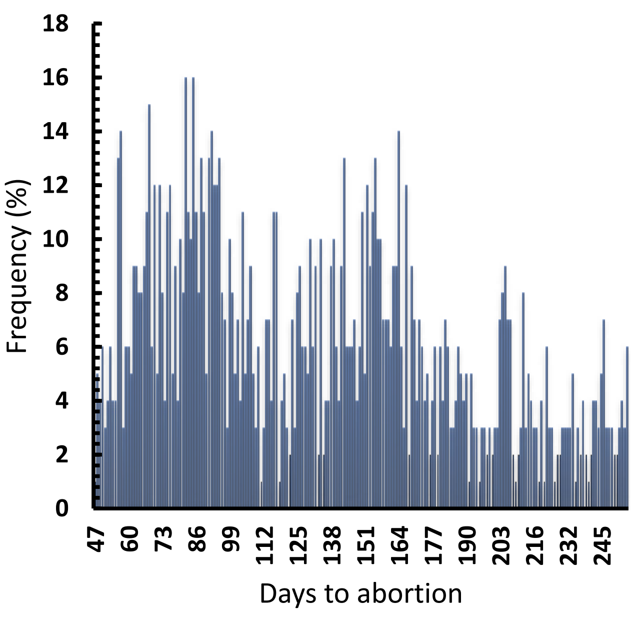

Figure 1. Frequency of abortions of unknown cause in Holstein cows in a hot environment relative to days of gestation when abortion occurred.

Also, there was a significant interaction detected between the number of brucellosis vaccinations per cow and the application of the clostridial vaccine. A remarkable low (0.7%; $p<0.01$) incidence of abortion was observed in cows with >3 application of brucellosis vaccine and given the multicomponent clostridial vaccine compared with cows getting a single *B. abortus* RB51 vaccine and receiving the clostridial vaccine (7.7%). The interaction between the number of brucellosis vaccinations per cow and parity was significant ($p<0.05$). Despite the interdependence between these variables (the number of vaccinations against *B. abortus* increased linearly with parity), the interaction occurred because abortion rate followed a cubic trend relative to parity.

Comparisons among the different parity groups showed that nulliparous and primiparous cows were 1.6 more likely ($p<0.01$) to have an abortion than multiparous cows (Table 1). Cows vaccinated against leptospirosis <55 dpp were 3.4 more likely to have an abortion when compared with cows immunized against this disease >55 dpp (Table 1). There was a significant interaction between dpc vaccination against leptospirosis and the occurrence of previous abortion. Abortions were 56.5% for cows with ≥2 abortions receiving the leptospirosis vaccination <55 dpc compared with 82.8% for cows with ≥2 abortions receiving the leptospirosis booster vaccine >55 dpc.

Cows vaccinated <37 dpp against various viral diseases-causing abortion had a lower ($p<0.01$) risk of abortion relative to cows vaccinated >37 dpc (Table 1). There was a significant interaction of viral vaccine applied at different times post-calving with previous abortion: cows with two previous abortions that received the viral vaccination <37 dpp had a reduced risk of abortion (OR=0.35; $p<0.01$) compared with cows with two previous abortions and receiving the viral vaccination >37 dpp.

Cows vaccinated >20 dpc against brucellosis had 1.8 times more risk of abortion than cows vaccinated <20 dpp ($p<0.01$). Cows no receiving clostridial vaccines (adjusted for parity and number of previous abortions) had nearly 4 times the risk of abortion as had cows receiving this vaccine (Table 1). There was a significant interaction of the application of the clostridial vaccine with the occurrence of previous abortion: cows that received the clostridial vaccine with two previous vaccinations against brucellosis had a reduced ($p<0.01$) risk of abortion (OR= 0.37) than cows receiving the clostridial vaccine with one or ≥3 previous vaccinations against brucellosis.

Cows not experiencing puerperal metritis had a reduced risk of abortion as had cows free of this postpartum disease (Table 1). Previous abortions had the strongest association with subsequent abortions (adjusted for days to

Table 1. Final multivariate logistic regression model for factors associated with the incidence of abortions of unknown cause in high-yielding Holstein cows in a hot arid environment

Variables	Abortions, n (%)	Odds ratio (OR)	95% CI OR	*p*
Brucellosis vaccinations per cow[1,2,3,4]				<0.0001
>3	559/2556 (21.9)	7.4	5.7 – 9.8	
2-3	430/2028 (21.2)	4.3	3.3 – 5.6	
≤2	253/2430 (10.4)	Reference		
Parity				<0.0001
Nulliparous and primiparous	905/4571 (19.8)	1.6	1.2 – 2.1	
Two to three parturitions	169/1214 (13.9)	1.0	0.8 – 1.3	
>3 parturitions	168/1229 (13.7)	Reference		
Days postpartum leptospirosis vaccination[5,6]				0.0272
<55	1163/6450 (18.0)	3.4	2.4 – 4.7	
>55	84/585 (14.4)	Reference		
Days postpartum viral vaccine[7,8]				<0.0001
<37	754/4644 (16.2)	0.6	0. 5 – 0.7	
>37	488/2370 (20.6)	Reference		
Days postpartum to *B. abortus* vaccine				<0.0001
>20	815/3985 (20.4)	1.8	1.5 – 2.1	
<20	427/3029 (14.1)	Reference		
Application of 10-way clostridial vaccine[9,10]				<0.0001
No	1103/5041 (21.9)	3.7	3.1 – 4.5	
Yes	139/1973 (7.1)	Reference		
Puerperal metritis				0.0035
No	1130/6517 (17.3)	0.72	0.6 – 0.9	
Yes	112/497 (22.5)	Reference		
Number of previous abortions				<0.0001
>2	218/283 (77.0)	26.1	19.6 – 34.8	
1-2	323/577 (56.0)	9.9	8.2 – 11.9	
0	701/6154 (11.4)	Reference		

[1]*Brucella abortus* strain RB51 vaccine, including the vaccine applied at four months of age.
Significant (p<0.05) interactions: [2]number of brucellosis vaccinations per cow × postpartum leptospirosis vaccination; [3]number of brucellosis vaccinations per cow × application of the clostridial vaccine; [4]number of brucellosis vaccinations per cow × parity.
Significant (p<0.05) interactions: [5]days post-calving vaccination against leptospirosis × occurrence of previous abortion; [6]days postpartum for leptospirosis vaccination × occurrence of previous abortion.
[7]CattleMaster Gold FP5®, Zoetis, Mexico DF, Mexico: bovine rhinotracheitis, bovine viral diarrhea Types 1 and 2, parainfluenza3 virus, bovine respiratory syncytial virus, and leptospirosis caused by five *Leptospira* serovars.
[8]Significant (p<0.05) interactions of time of viral vaccination post-calving × occurrence of previous abortion.
[9]Toxoids of *Clostridium perfringens* types A, B, C and D, *C. septicum, C. sordelli, C. novyi, C. haemolyticum, C. tetani*, and inactivated cells of *C. chauvoei.*
[10]Significant (p<0.05) interactions of application of clostridial vaccine × occurrence of previous abortion.

leptospirosis vaccination post-calving, number of brucellosis vaccinations applied during the productive life, parity, and dpp to brucellosis vaccination), which indicates that some cows are predisposed to recurrent abortion. The interaction between previous abortion and dpp of leptospirosis vaccination was significant (p<0.01). The occurrence of previous abortion by-number of anti-brucellosis vaccinations was significant (p<0.05) for abortion, indicating that cows with more than one previous abortion had a higher abortion rate than cows with just one abortion.

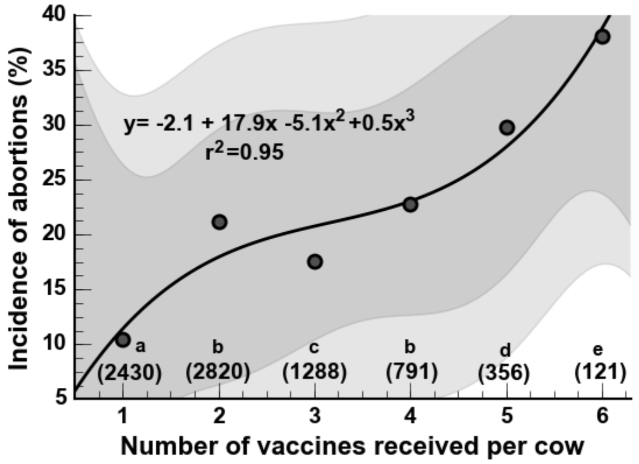

Figure 2. Association between the number of vaccinations against *Brucella abortus* strain RB51 received by cow and the incidence of abortion of unknown cause in Holstein cows in a hot arid environment. Dark bands are 95% confidence intervals for estimated values. Light bands are 95% confidence intervals for actual values. Values in brackets are number of observations. Number of vaccinations with different letter differ (p<0.05).

Occurrence of mummified fetuses

The incidence of mummified fetuses was 1.1% (77/7014). The risk factors for mummified fetuses (adjusted for parity and number of previous abortions) are listed in Table 2. The odds of mummified fetuses in cows with >2 *B. abortus* S19 vaccine were three times higher (p<0.01) than cows receiving a single vaccination. In addition, there was an interaction between number of brucellosis vaccinations per cow and the annual boosting leptospirosis vaccination (p<0.05).

Cows no receiving clostridial vaccines were four times more likely (p<0.01) to present mummified fetuses than cows immunized against these bacteria (Table 2). Cows no receiving bovine leptospirosis vaccines postpartum had about half the risk (p<0.01) of presenting mummified fetuses than cows receiving this vaccine. As in the case of abortion, the higher the number of previous abortions, the higher (p<0.01) the risk of mummified fetuses. Calving in autumn decreased (p<0.05) the risk of mummified fetuses (Table 2). Cows with a single vaccination against brucellosis had half the chance of presenting mummified fetuses than cows repeatedly vaccinated against this disease.

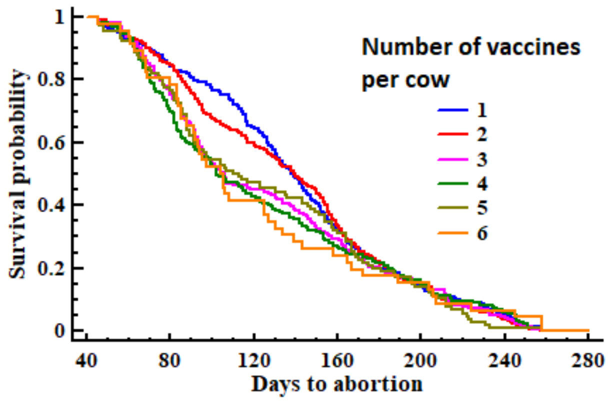

Figure 3. Kaplan-Meier survival analysis for cows receiving one to six *Brucella abortus* RB51 vaccinations. Cows with multiple *B. abortus* vaccinations had shorter conception to abortion intervals than cows receiving one or two vaccinations (p<0.0001; Wilcoxon test).

Discussion

Occurrence of abortion

Abortion in the present study was defined as the loss of a fetus that occurs from the moment of pregnancy diagnosis by palpation per rectum until 260 days of gestation (the point at which the fetus can survive outside the uterus). We caution about this definition because there are numerous abortion definitions used internationally, including early ultrasonographic pregnancy diagnosis (≈ 30 days).

This study showed that despite the sound vaccination program for reproductive diseases and protection of the developing fetus, abortions of unknown cause in this farm were two to three-fold higher than the abortion/fetal loss rates of 1.3-5.0% reported in North-American (Forar *et al.*, 1995; Jousan *et al.*, 2005; Norman *et al.*, 2012), 1.7-10.3% found in European (Mee, 1992; Andreu-Vázquez *et al.*, 2012; Barański *et al.*, 2012), 6-7% in New Zealand and Australian (Norton *et al.*, 1989; McDougall *et al.*, 2005) and 6.9% observed in Asian (Lee & Kim, 2007) intensive dairy herds. Nonetheless, abortions observed in the present investigation are close to those observed in Holstein herds, where the etiology of abortions was not investigated in the temperate zone of Mexico (24-29%; Albuja *et al.*, 2019; Mellado *et al.*, 2019).

Abortions had bimodal days of gestation distribution. The first abortion peak around 86 days of pregnancy agrees with previous studies, where the occurrence of abortions of unknown etiology peaks during the first three months of pregnancy (Albuja *et al.*, 2019). However, twinning has been a significant contributor to pregnancy loss during this period (López-Gatius *et al.*, 2004). The second peak of abortions occurred in the second trimester of gestation, which agrees with Garcia-Ispierto & López-Gatius (2019), who observed the highest occurrence of abortion between 135 and 154 days of pregnancy, being unicornual twins a significant cause of fetal losses during this period. In the present study, abortions were likely due to many bacterial, viral, protozoan, and fungal infections because bovine abortion during mid-to-late-gestation is primarily due to infectious causes (Anderson, 2007; Reichel *et al.*, 2018).

For a given pregnancy, abortions were higher among cows who previously had this reproductive disorder than those who had not. These results align with Markusfeld-Nir (1997) observations, where the proportion of aborted cows

Table 2. Final multivariate logistic regression model for factors associated with the occurrences of mummified fetuses in high-yielding Holstein cows in a hot arid environment.

Variables	Mummified fetuses, n (%)	Odds ratio (OR)	95% CI OR	*p*
Number of brucellosis vaccinations per cow[1]				<0.001
>3	36/2556 (1.4)	3.0	1.2–7.0	
2-3	30/2028 (1.5)	3.3	1.7–6.6	
1	11/2430 (0.5)	Reference		
Application of 10-way clostridial vaccine[2]				0.0005
No	70/5041 (1.4)	4.0	1.8–8.6	
Yes	7/1973 (0.4)	Reference		
Leptospirosis vaccination[3]				0.0284
No	8/1456 (0.6)	0.4	0.2–0.9	
Yes	69/497 (1.2)	Reference		
Number of previous abortions				<0.0001
>2	27/283 (9.5)	20.2	12.0–34.2	
1-2	15/577 (2.6)	3.7	1.9–7.1	
0	35/6154 (0.6)	Reference		
Season of delivery				0.0425
Spring *vs.* winter	1.51 *vs.* 1.44	1.1	0.5–2.1	
Summer *vs.* winter	1.44 *vs.* 1.44	1.0	0.5–1.9	
Autumn *vs.* winter	0.62 *vs.* 1.44	0.4	0.2–0.8	

[1] *Brucella abortus* strain RB51 vaccine, including the vaccine applied at four months of age. [2] Toxoids of *Clostridium perfringens* types A, B, C and D, *C. septicum, C. sordelli, C. novyi, C. haemolyticum, C. tetani*, and inactivated cells of *C. chauvoei.* [3] Number of brucellosis vaccinations per cow × annual boosting leptospirosis vaccination interaction detected ($p<0.05$).

with a previous abortion was three times higher than that of the whole population. Likewise, Gehrke & Zbylut (2011) reported that abortions in cows presenting repeated abortion were two-fold higher than that registered in cows of the entire population.

Abortion happens due to many causes, although infections are the most common origin of this reproductive disorder (Wolf-Jäckel *et al.*, 2020). Some infectious abortions by one single pathogen only occur once in the cow's life due to acquired immunity after their first abortion (Megid *et al.*, 2010). Yet, *Neospora caninum* is now recognized as the most common cause of repeated abortions in cattle. In the present study, few cows were culled because of abortion; thus, the permanence of aborting cows in the herd, possibly due to *N. caninum*, increased the abortion risk in subsequent pregnancies. The explanation for this response could be the endemic infections of *N. caninum* in cattle populations of Mexico (García-Vázquez *et al.*, 2005; Medina-Esparza *et al.*, 2018), as chronic seropositive cows to this protozoa are reported to have recurrent abortions (Corbellini *et al.*, 2006; Pabón *et al.*, 2007). Given that *N. caninum* does not induce abortion below day 90 of pregnancy (Wilson *et al.*, 2016; Melendez *et al.*, 2020), abortions before 90 days of pregnancy could have resulted from advanced age, chronic endometritis, luteal phase deficiency, uterine anomalies, unicorneal twining or abnormal immunologic response.

Annual revaccination of adult non-pregnant cattle with *B. abortus* RB51 markedly increased abortions in cows, suggesting that this vaccine should not be applied annually. Even though retrospective analysis has methodological limitations, these data were abundant, complete, balanced, and accurate on both the risk and outcome factor to answer the study question; therefore, it is considered that these data provide valid information about causal effects of factors used in this study using observational evidence.

Fluegel Dougherty *et al.* (2013) found that vaccination of adult pregnant beef cattle with *B. abortus* RB51 resulted in 5.3% pregnancy losses. Likewise, field reports have shown the brucellosis vaccine strain RB51 in the abortion of some cows (Yazdi *et al.*, 2009; Sanz *et al.*, 2010). Thus, it could be that the attenuated strain *B. abortus* RB51 could be harbored in non-pregnant cows, and once pregnancy is established, *B. abortus* can cause fetal infection and abortion, as it has been reported by Van Metre *et al.* (1999) and Yazdi *et al.* (2009). Thus, another explanation for the harmful effects of repeated RB51 immunization is that the recurrent use of the RB51 vaccine could increase the risk of reversion of this attenuated strain RB51 vaccine to wild-type virulence.

Hitherto no extensive study has proven the vaccine's responsibility against *B. abortus* strain RB51. This investigation shows strong evidence that annual revaccination against this disease is not safe for non-pregnant Holstein cows in early lactation. This study also indicates that the accumulation of *B. abortu*s vaccinations markedly increases the risk of abortion and triggered abortion earlier in pregnancy. These results suggest that this live *B. abortus* strain RB51vaccine applied repeatedly can cause abortion. These results would be similar to the live vaccine strain 1B of *Chlamydia abortus* used to prevent abortion in small ruminants, which may enter circulation and cause abortion in some vaccinated animals (Longbottom *et al.*, 2018). It could be that the reaction of cows to repeated vaccinations against *B. abortus* is not due to the agent but to an immune-mediated response by susceptible cows to adverse reactions to vaccines.

Additionally, revaccination with *B. abortus* RB51 after 20 dpp increased the risk of fetal loss in cows, compared to cows vaccinated <20 dpc. This greater incidence of *B. abortus* RB51-induced abortion in cows revaccinated close or during breeding indicates that vaccination of adult non-pregnant cattle with RB51 does not seem advisable if more than 20 days have elapsed between calving and vaccination. This response is unknown, but perhaps the depression of the immune system in high-yielding cows during the transition period (Esposito *et al.*, 2014) could have contributed to the higher *B. abortus* RB51-induced abortion (Uzal *et al.*, 2000; Fluegel Dougherty *et al.*, 2013). Further analyses of the association of abortions and cows vaccinated after 20 dpp are required to clarify this relationship.

In the present study, the time of postpartum immunization against viruses linked to abortion was an important factor affecting this reproductive disorder. Possibly, impairment of the immune response in the immediate postpartum period of dairy cows (Mann *et al.*, 2018), which coincided with revaccination (>37 dpp), is cause-and-effect related. Unfortunately, few data are available from controlled clinical trials that have assessed the efficacy of this vaccine for protection against abortion in adult cows. Fetal immunity against viral infection is not absolute in vaccinated animals (Van Campen *et al.*, 2000). Other reports have demonstrated insufficient fetal protection of inactivated viral vaccines (Grooms, 2004; Rodning *et al.*, 2010). Thus, these results suggest that the appropriate window to vaccinate cows postpartum and pre-breeding against viral diseases linked to abortion is <37 dpc. This is so because the onset of immunity from inactivated virus vaccines may be delayed four to six weeks from the time of initial vaccination, which may result in vaccination failure (Newcomer *et al.*, 2017).

An interesting finding of this investigation was that, for a given gravidity, abortions were lower among cows that were revaccinated against different *Clostridium* species compared with cows that did not receive this vaccine. This finding has to be seen in light of some limitations of the present study, such as whether vaccination or not vaccination against clostridial infections was not controlled at all. Most commercial clostridial vaccines recommend annual revaccination in cattle, but this recommendation is largely ignored (Uzal, 2012). Results of the present investigation suggest that the antibody responses elicited by the 10-way clostridial vaccine somehow reduced the incidence of abortion. Antibody titers against clostridial antigens have been considered the primary connection of vaccine-induced protection against these infectious diseases.

Given that antibody levels after applying clostridial vaccines remain above the minimal level required for protection only for one year, annual booster vaccinations are required to protect against clostridial diseases. In the present study, the protection of this vaccine apparently extended to the safety of the growing fetus while in the uterus. However, causality between clostridial diseases and abortion in ruminants has not been established (Anderson, 2007; Borel *et al.*, 2014; Vidal *et al.*, 2017). Thus, probably cross-reactivity (immune response against abortifacient organisms not specifically targeted by the vaccine antigen) and cross-protection (defense against non-vaccine microorganism varieties) could have happened as it is the case of protection against *M. leprae* by *M. tuberculosis,* or protection against *N. gonorrhoeae* by *M. meningitis*, just to mention a few examples (Vojtek *et al.*, 2019).

Cow parity was associated with the occurrence of abortions in this investigation. The incidence of abortion was greatest in primiparous cows, contrary to observations of Thurmond *et al.* (1990) and Lee & Kim (2007), who found that fetal losses were higher in cows with three or more parturitions. Other studies have reported no effect of parity on pregnancy losses (Labèrnia *et al.*, 1996; Moore *et al.*, 2005), whereas observations of Jousan *et al.* (2005) and Gehrke & Zbylut (2011) show that the highest risk of pregnancy/fetal loss occurs in first or second-lactation cows. The contrasting results among studies on this matter seem to be due to the different definitions of pregnancy loss, the methods of pregnancy diagnosis (manual, laboratory, ultrasound), the criteria used for time of fetus mortality, and the signs used to diagnose abortion (late-term abortions are the easiest to notice, but abortions can happen at any stage of pregnancy). It is worth noting that despite the higher abortion rate in nulliparous/primiparous heifers, the abortion rate increased with the number of *B. abortus* vaccinations, which implies that the older the cows, the higher the abortion rate. This apparent contradiction may be due to uncontrolled interferences exerted by other factors included in the model, or that heifers are more susceptible to infections leading to abortion compared with primiparous and multiparous cows (Alfieri & Alfieri, 2017).

Cows that received the pentavalent leptospira vaccine >37 dpp had an increased risk of fetal loss than cows vaccinated <37 dpc. However, given the observational nature of this study, the occurrence of this association does not necessarily imply causation. Therefore, limitations exist in the present observational research in assessing causal associations. For dairy cows, energy balance during the postpartum period directly influences fertility and susceptibility to infectious disease (Mulligan & Doherty, 2008). Therefore, it is believed that the loss of body condition score peripartum (>37 dpp; Chebel *et al.*, 2018) was a predisposing factor associated with a lesser response of cows to the leptospira vaccine, which apparently resulted in a greater occurrence of abortion.

The occurrence of puerperal metritis was a risk factor associated with abortion. However, this association is not clear, as cows with metritis treated with antimicrobial therapy present a high recovery rate from this disease (Ordell *et al.*, 2016). Additionally, uterine infections in postpartum dairy cows may interrupt successful reproduction at several crucial stages, although these detrimental effects do not proceed beyond the early embryo development (Gilbert, 2012). Puerperal metritis could be mediated by a diminished ovarian function altered by bacterial products or inflammatory agents acting directly on the ovary or indirectly on the hypothalamus or pituitary gland (Sheldon *et al.*, 2006; Cheong *et al.*, 2017). This hampered ovarian function can cause smaller dominant follicles and corpus luteum, with the consequent lower blood estradiol and progesterone concentrations (Williams *et al.*, 2007) which could ultimately affect early fetus survival.

Mummified fetuses

So far, no specific causes for the mummification of bovine fetuses have been recognized (Drost, 2007), basically because of tissue degeneration and autolysis. However, in the present study, it was found that the risk of the occurrence of mummified fetuses increased with increasing the number of *B. abortus* vaccinations applied to adult animals. This response is intriguing. Given that fetal mummification occurs after the development of the placenta and fetal ossification (after 70 days of pregnancy), it could be that attenuated *B. abortus* bacteria reactivated after the third month of pregnancy and infected maternal and fetal tissues, as has been the case of previous studies in cattle (Palmer *et al.*, 1996; Fluegel Dougherty *et al.*, 2013).

It was found a markedly increased risk of mummified fetuses in those cows receiving the bovine clostridial vaccine. However, this association may not be as straightforward as it may appear, considering that this was not a randomized controlled trial. However, several studies assessing agreement between similar hypotheses tested using randomized controlled trials compared to observational designs have found that agreement between the two is high (Boyko, 2013). The exact manner of the harmful effect of this multi-way clostridial vaccine is unknown. It could be that the inactivated toxins (toxoid) used to immunize cows against a wide variety of *Clostridium* species could reach the uterus causing fetal demise and subsequent fetal mummification without causing further contamination and irritation of the uterus. Although *Clostridium* species are not considered abortifacients, reports in foals (Ortega *et al.*, 2007), mink (Hammer *et al.*, 2017), and sheep (Brozos *et al.*, 2012) have implicated these microorganisms in omphalitis and abortion. Of interest, the time of vaccination post-calving against leptospirosis, various viral diseases, and brucellosis also had higher risks of developing subsequent mummified fetuses.

Conclusions

Repeated postpartum *B. abortus* RB51 vaccination in adult Holstein cows was not associated with a reduced abortion and mummification rate. Yet, it was associated with a raised risk of these reproductive disorders, emphasizing the importance of modifying vaccination protocols of cows. These findings are of great practical significance because they contradict the view that revaccination of cows increases immunity against this disease. The reasons for the repeated *B. abortus* RB51 vaccination associated with increased risk for abortion warrant further research. The number of previous abortions was the most crucial risk factor for abortion. Further research to understand the cause of these repeated abortions is highly needed.

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