Efficacy of a flexible schedule for administration of a *Leptospira borgpetersenii* serovar Hardjo bacterin to beef calves

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Objective—To determine whether a flexible vaccination regimen provides protection against challenge exposure with a virulent *Leptospira borgpetersenii* serovar Hardjo isolate.

Animals—Fifty-five 4-week-old calves seronegative for antibodies against *L borgpetersenii* serovar Hardjo.

Procedures—Calves were assigned to 3 groups and administered 2 doses of adjuvant (control calves; n = 11), 1 dose of serovar Hardjo bacterin and 1 dose of adjuvant (22), or 2 doses of the serovar Hardjo bacterin (22); there was a 16-week interval between dose administrations. Three weeks after the second dose, all calves were challenge exposed by use of conjunctival instillation of a heterologous strain of *L borgpetersenii* serovar Hardjo for 3 consecutive days. Urine samples for leptospiral culture were collected for 5 weeks after challenge exposure; at that time, all calves were euthanized and kidney samples collected for leptospiral culture.

Results—Antibody titers increased in both leptospiral-vaccinated groups of calves. A significant increase in antibody titers against *L borgpetersenii* serovar Hardjo was detected after administration of the second dose of *L borgpetersenii* serovar Hardjo bacterin and challenge exposure. In 10 of 11 adjuvant-treated control calves, serovar Hardjo was isolated from both urine and kidney samples. *Leptospira borgpetersenii* serovar Hardjo was not isolated from the urine or kidney samples obtained from any of the 21 remaining calves that received 1 dose of bacterin or the 20 remaining calves that received 2 doses of bacterin.

Conclusions and Clinical Relevance—Protection in young calves was induced by vaccination with 1 or 2 doses of a serovar Hardjo bacterin. (*Am J Vet Res* 2014;75:507–512)

Leptospirosis, which is caused by *Leptospira* spirochetes, is found throughout the world and remains one of the most important infectious diseases affecting cattle reproduction in US dairy and cow-calf operations despite routine use of leptospiral vaccines.[1] Pathogenic leptospiral species can cause disease in a multitude of hosts and are recognized as important zoonotic organisms.[2,3] Transmission is via infection of the renal tubules and excretion of infectious leptospires in the urine.[4] Initially, all pathogenic leptospires were classified as *Leptospira interrogans*, and serovar determination was based on the antigenic diversity of surface antigens. More recently, genetic sequencing has led to the reclassification of the genus. Pathogenic leptospires that most commonly affect cattle in the United States currently are allocated into 7 serovars (*L interrogans* serovars Hardjo, Pomona, Icterohemorrhagiae, Canicola, and Bratislava; *Leptospira borgpetersenii* serovar Hardjo; and *Leptospira kirschneri* serovar Grippotyphosa).[5]

Abbreviation

MAT Microscopic agglutination test

All infections attributable to pathogenic *Leptospira* spp are typically classified by 2 methods: host specificity and method of transmission. Reservoir hosts (maintenance hosts) exist for most pathogenic leptospires. The disease is maintained within that animal species, and spread of infection is primarily through direct transmission.[6] Infections in maintenance hosts are generally characterized by mild disease, prolonged (sometimes lifetime) shedding of leptospires, nonprotective immune responses, and few clinical signs. In contrast, infections in incidental hosts typically result in more severe disease and clinical signs, strong immune responses that are protective against subsequent exposures, and a short period of leptospire shedding (usually ≤ 3 months).[6]

Many serovars of *Leptospira* spp can cause disease in cattle throughout the world. In addition to the aforementioned 7 serovars, serovars Hebdomadis, Kremastos, Tarassovi, Autumnalis, Australis, Sejroe, and Bataviae have been isolated from outbreaks in cattle.[7] Only 2 species of *Leptospira* organisms (*L borgpetersе-*

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nii serovar Hardjo and *L interrogans* serovar Hardjo) are considered to have cattle as their maintenance host.[7] *Leptospira borgpetersenii* serovar Hardjo is the predominant serovar in cattle and has been isolated wherever cattle are raised.[1] Outbreaks of severe clinical disease with accompanying abortions typically are associated with leptospiral infections of cattle, but these events most often occur with infections in incidental hosts.[6]

Although maintenance host infections in cattle can cause severe disease (usually the first time an animal is exposed), they typically result in more subtle clinical signs. Disease is generally mild and often inapparent. Chronic infections with long-term shedding have been identified (some cattle may be infected and shed throughout their entire lifetime).[8] Spontaneous clearance of infection does not provide substantive protection against reinfection.[8] Infection with *L borgpetersenii* serovar Hardjo rarely causes abortions and is more often associated with conception failure and early embryonic death loss (or both).[1,8] Investigators in 1 study[9] found that cows seronegative for *L interrogans* serovar Hardjo were 8 times as likely to become pregnant as were seropositive cows. In addition, human health implications are important because of the likelihood for the exposure of workers to cattle urine.[3]

Much confusion exists regarding vaccination as a means of control for *L borgpetersenii* serovar Hardjo. The efficacy of conventional serovar Hardjo vaccines may be suboptimal. As mentioned previously, infections attributable to *L borgpetersenii* serovar Hardjo are common in herds despite routine vaccination. Investigators in several studies[10–14] found that use of conventional leptospiral vaccines did not provide protection against colonization of the renal or genital tract or transplacental infection after challenge exposure with *L borgpetersenii* serovar Hardjo, even though the vaccines decreased the frequency of infection and the duration of shedding of the organisms in urine. Cattle were not protected even though vaccination stimulated the development of antibodies against *L borgpetersenii* serovar Hardjo. In subsequent studies,[15–18] it has been proposed that cell-mediated immunity may play a role in protective immunity against *L borgpetersenii* serovar Hardjo in cattle. A vaccine containing *L borgpetersenii* serovar Hardjo has provided cattle with protection against infection, tissue colonization, shedding, and transplacental infection.[18,19] Each of these studies[15–19] was performed by administration of 2 doses of vaccine, with a 4-week interval between vaccine administrations. The study reported here was performed to determine whether 1 dose of vaccine would provide protection for an extended period and whether delayed administration of a second dose would function as a booster vaccination.

Materials and Methods

Animals—Fifty-five colostrum-deprived female beef calves were purchased and used in a study with 2 phases (vaccination phase and challenge-exposure phase). Calves were purchased when they were between 1 and 3 days of age; calves were purchased and enrolled in the study over a 9-day period. Calves were housed initially at a facility in Oakland, Neb. Calves had negative results when tested at a diagnostic laboratory[a] for persistent infection with bovine viral diarrhea virus and were seronegative (MAT antibody titers ≤ 12.5 for serovar Hardjo and ≤ 50 for all other serovars) for preexisting antibodies against *L borgpetersenii* serovar Hardjo and *L interrogans* serovars Hardjo, Australis, Autumnalis, Canicola, Grippotyphosa, Hebdomonadis, Icterohemorrhagiae, Pomona, and Sejroe. Calves were identified by placing a unique numeric tag in each ear and were housed individually in calf huts. Calves were fed medicated milk replacer that contained oxytetracycline and neomycin and were weaned onto a medicated calf starter feed that contained decoquinate. Animal protocol and management procedures for the vaccination phase were approved by an institutional animal care and use committee at Midwest Veterinary Services Inc, a contract research facility in Oakland, Neb, and animal protocol and management procedures for the challenge-exposure phase were approved by an institutional animal care and use committee at the Department of Veterinary Medical Research and Development, Zoetis, Kalamazoo, Mich.

Procedures—Calves were allowed to acclimatize to the facility for 7 days before the study was initiated. Calves were allocated into 3 groups (11, 22, and 22 calves in groups 1 through 3, respectively) by use of a generalized randomized block design. Blocking was based on order of enrollment (5 calves/block; each block comprised 1 calf of group 1, 2 calves of group 2, and 2 calves of group 3). Procedures and results of laboratory tests were recorded such that researchers were not aware of treatment group assignments.

On day 0, all calves (range, 20 to 29 days of age) received a 5-way modified-live viral vaccine.[b] In addition, each of the calves received 1 dose (2 mL) of an assigned product, SC, in the left side of the neck. Calves in group 1 (control group) received an adjuvant, whereas calves in groups 2 and 3 received a dose of an *L borgpetersenii* serovar Hardjo bacterin.[c] On day 14, all calves were administered a dose of a pour-on endectocide.[d]

When calves were 3 to 4 months old, they were transported to Richland, Mich. Three calves were removed from the study (1 calf of group 2 because of injury and 2 calves of group 3 because 1 was a bull calf and the other died after excessive consumption of wood chip bedding). Calves were housed in groups (commingled) in 3 biosafety level 2 rooms; calves within the same block were housed in the same room. Blocks were assigned to rooms such that variability of order of enrollment (age of calves) was minimized within each room. Calves were allowed a 7-day period to acclimatize after arrival at the facility in Michigan.

All remaining 52 calves received a second product, which was administered on the same day. The interval between administration of the first and second doses ranged from 111 to 159 days (mean, 136 days) for all calves in the 3 groups. Calves received the second assigned product (dose, 2 mL), SC, in the right side of the neck. Calves in groups 1 and 2 received a dose of the adjuvant, and calves in group 3 received a dose of the *L borgpetersenii* serovar Hardjo bacterin.[c]

Challenge exposure—Beginning on day 22 after the second administration, a challenge-exposure dose

of 1 mL (0.5 mL instilled into each conjunctival sac) that contained approximately 2.8×10^6 organisms/mL was administered to each calf for 3 consecutive days. The challenge-exposure inoculum was second-passage organisms isolated from urine of cattle experimentally infected with *L borgpetersenii* serovar Hardjo strain 203 and prepared in broth as described elsewhere.[19,20] The challenge-exposure strain of *L borgpetersenii* serovar Hardjo was considered to be heterologous to the vaccinal strain because the challenge-exposure strain was an isolate from the United States and the vaccinal strain was an isolate from Australia.

Blood sample collection and serologic evaluation—Blood samples were collected for serum harvest from all calves before the first SC administration (day 0), on days 14 and 62 or 63, before the second SC administration, on the first day of challenge exposure, and 14 and 35 days after the first day of challenge exposure. Serum samples obtained before product administration on day 0 and before conjunctival administration on the first day of challenge exposure were assayed with an MAT to detect antibodies against serovars Australis, Autumnalis, Canicola, Grippotyphosa, Hebdomonadis, Icterohemorrhagiae, Pomona, and Sejroe. Serum obtained on all days was assayed with an MAT to detect antibodies against *L interrogans* serovar Hardjo and *L borgpetersenii* serovar Hardjo. The MAT was a modification of a standard procedure (National Veterinary Services Laboratory protocol BTYP-PR04001.02).[21] Briefly, a culture of *L interrogans* serovar Hardjo and *L borgpetersenii* serovar *Hardjo* (strain NU) was prepared in Ellinghausen-McCullough-Johnson-Harris growth medium. Six to 14 days later, the antigen was standardized (concentration range, 1×10^8 cells/mL to 5×10^8 cells/mL). Serum samples were diluted 1:6.25 in PBS solution and placed in the first well of a flat-bottom microtiter plate. Serial 2-fold dilutions were created, followed by the addition of an equal volume of the standardized *Leptospira* antigen (final dilution of serum in the first well, 1:12.5). Positive and negative control samples were included in each assay. Plates were covered, gently shaken for 5 to 10 seconds, and incubated at 28° to 30°C for 2 to 3 hours. Each well was examined by use of dark-field microscopy, and agglutination was scored as follows: − = no agglutination, ± = trace agglutination, 1+ = approximately 2% to 4% agglutinated, 2+ = approximately 45% to 75% agglutinated, 3+ = approximately 75% to 95% agglutinated, and 4+ = 100% agglutinated. Results were expressed as the highest dilution of serum with a reaction > 2+.

Urine sample collection and culture of *Leptospira* spp—Urine samples were collected from each calf before the first day of challenge exposure and on days 14, 21, 28, and 35 after the first day of challenge exposure. A diuretic (2 mg of furosemide/kg) was administered IV to each calf prior to urine collection. Samples of urine were collected by midstream catch into a sterile tube. Samples were maintained at ambient temperature and cultured for *Leptospira* spp within 3 hours after collection, as previously described.[13] Samples with positive results collected on day 28 after the first day of challenge exposure were subjected to PCR assay to confirm the recovered organisms were *L interrogans* serovar Hardjo or *L borgpetersenii* serovar Hardjo.[21,e,f] All serologic evaluations, leptospiral culture, and PCR assays were performed by personnel at the same laboratory.[e]

Collection of kidney samples—One calf of group 2 was euthanized 32 days after the first day of challenge exposure because of an inability to stand. The remaining 51 calves were euthanized 36 days after the first day of challenge exposure. Euthanasia was performed with a captive bolt followed by exsanguination. Routine postmortem examinations were performed, at which time both kidneys were aseptically removed with the renal capsule intact. The right kidney was examined for gross lesions, and any lesions were recorded. The right kidney was then transported to a diagnostic laboratory[a] within 2 hours after collection and used for culture of *Leptospira* spp.[13]

The renal capsule was removed from the left kidney, and the kidney was examined for gross lesions. Tissue sections (which targeted the area of gross lesions, if present) were collected and preserved in neutral-buffered 10% formalin; however, a tissue section was collected from each left kidney, regardless of whether gross lesions were present.

Kidney tissue collected at necropsy was evaluated grossly for lesions (yes or no). Tissues were histologically evaluated for evidence of nephritis by personnel at the diagnostic laboratory.[a] Tissues were microscopically scored for nephritis on a scale of 0 (no interstitial infiltrates) to 5 (> 5% of the parenchyma involved).

Statistical analysis—Data were analyzed with a mixed-model or categorical procedure.[22,23] Calf was the experimental unit, and $P \leq 0.05$ was used to assess significant differences.

Serum antibody titers were analyzed with a linear mixed model with repeated measures that included the fixed effects of treatment, day of study, and the treatment × day of study interaction in addition to the random effects of pen, block within pen, and calf. A significant effect for treatment or the treatment × day of study interaction was required before pairwise tests were conducted to detect differences among treatments. Back-transformed least squares means, SE, ranges, and 95% confidence intervals were reported for each time point. For statistical analyses, titers < 12.5 were analyzed as 6.25.

The primary variable was isolation of *L borgpetersenii* serovar Hardjo from urine and kidney samples. Calves were considered to be positive for *L borgpetersenii* serovar Hardjo if at least 1 urine or kidney sample had positive results. The status of each calf with regard to isolation of *L borgpetersenii* serovar Hardjo was summarized, and frequency tables were created for each treatment group.

Results

Calves—All injections of the adjuvant and bacterin were tolerated well by the calves. No adverse effects were attributed to products after the first or second administrations. Necropsy of the calf of group 2 that was euthanized 32 days after the first day of challenge exposure because of difficulty standing revealed chronic

Table 1—Group geometric mean (range) serum MAT titers* against *Leptospira borgpetersenii* serovar Hardjo in calves administered 2 doses† of adjuvant (control calves; group 1), calves administered 1 dose of *L borgpetersenii* serovar Hardjo bacterin and 1 dose of adjuvant (group 2), and calves administered 2 doses of *L borgpetersenii* serovar Hardjo bacterin (group 3).

Group	First dose	14 days after first dose	62 or 63 days after first dose	Second dose‡	First day of challenge exposure§	14 days after first day of challenge exposure	35 days after first day of challenge exposure
1 (n = 11)	6 (6–6)	6 (6–6)[a]	8 (6–50)[a]	6 (6–6)[a]	6 (6–6)[a]	1,704 (200–12,800)[a]	1,029 (200–3,200)[a]
2 (n = 22)	6 (6–6)	33 (6–200)[b]	129 (6–400)[b]	51 (6–400) ‖[b]	38 (6–800) ‖[b]	74 (25–800) ‖[b]	27 (6–200)¶[b]
3 (n = 22)	6 (6–6)	29 (6–400)[b]	137 (6–1,600)[b]	38 (6–400) ¶[b]	1,841 (6–6,400)¶[c]	2,350 (100–25,600)¶[a,c]	1,173 (50–6,400)¶[a,c]

*Values for the first dose are descriptive geometric means; all other values are back-transformed geometric least squares means. Titers < 12.5 were analyzed as 6.25. †All doses were 2 mL and were administered SC in the neck. ‡Interval between the first and second dose was 111 to 159 days (mean, 136 days). §All calves were challenge exposed by use of conjunctival instillation of a heterologous strain of *L borgpetersenii* serovar Hardjo for 3 consecutive days beginning 22 days after the second dose. ‖Represents results for 21 calves. ¶Represents results for 20 calves.

[a–c]Within a column, means with different superscript letters differ significantly ($P = 0.01$).

Table 2—Results for isolation of *L borgpetersenii* serovar Hardjo from urine or kidney samples obtained from 3 groups of calves after administration of an adjuvant or *L borgpetersenii* serovar Hardjo bacterin and challenge exposure* with an *L borgpetersenii* serovar Hardjo isolate.

Group	First day of challenge exposure	14 days after first day of challenge exposure	21 days after first day of challenge exposure	28 days after first day of challenge exposure	35 days after first day of challenge exposure
1	0/11	8/11	10/11	10/11	10/11
2	0/21	0/21	0/21	0/21	0/21
3	0/20	0/20	0/20	0/20	0/20

Values reported are the number of calves with positive culture results/number of calves from which samples were obtained for culture.

*All calves were challenge exposed by use of conjunctival instillation of a heterologous strain of *L borgpetersenii* serovar Hardjo for 3 consecutive days beginning 22 days after the second dose.

See Table 1 for remainder of key.

pneumonia and malabsorptive diarrhea unrelated to the challenge exposure. Data for this calf were included in the study analyses because all study samples were collected, except for those at 35 days after the first day of challenge exposure. Kidney samples were collected from this calf at the time of necropsy.

Serologic evaluation—All calves were seronegative for *L borgpetersenii* serovar Hardjo and *L interrogans* serovar Hardjo and serovars Australis, Autumnalis, Canicola, Grippotyphosa, Hebdomonadis, Icterohemorrhagiae, Pomona, and Sejroe at the time of enrollment (day 0). All calves in the control group remained seronegative for *L interrogans* serovar Hardjo and *L borgpetersenii* serovar Hardjo before challenge exposure, except for 1 calf that had an MAT titer of 50 on day 62 (**Table 1**). The MAT titer for that calf decreased to < 12.5 before challenge exposure. There were significant ($P = 0.01$) increases in MAT antibody titers for both leptospiral-vaccinated groups (groups 2 and 3) within 14 days after the initial vaccination. A significant ($P < 0.001$) increase in MAT antibody titer against *L interrogans* serovar Hardjo and *L borgpetersenii* serovar Hardjo was detected after administration of the second dose of *L borgpetersenii* serovar Hardjo bacterin for group 3, compared with the antibody titer after administration of the adjuvant for group 2. This difference between the 2 leptospiral-vaccinated groups was maintained after challenge exposure.

Culture of urine and kidney samples—Urine collected from each calf in all treatment groups on the day before challenge exposure yielded negative results for *Leptospira* organisms. Leptospires were detected by microbial culture in the urine and kidney samples collected after challenge exposure in 10 of 11 calves in the control group, whereas culture of urine and kidney samples from calves vaccinated once or twice with the leptospiral bacterin yielded negative results (**Table 2**). All calves that had leptospires in urine had leptospires in kidney tissues obtained during necropsy. Results of PCR assay performed on 10 positive urine samples collected from the control calves 28 days after the first day of challenge exposure had positive results for *L borgpetersenii* serovar Hardjo.

Gross renal lesions and histologic scores—Gross renal lesions were observed in the kidneys of 6 of 11 calves in group 1, 9 of 21 calves in group 2 (1 dose of leptospiral bacterin), and 13 of 20 calves in group 3 (2 doses of leptospiral bacterin). Histologic evaluation performed at the veterinary diagnostic laboratory indicated that scores of 3 (moderate interstitial nephritis) or higher were detected in kidney tissues obtained from 6 of 11, 7 of 21, and 6 of 20 calves in groups 1, 2, and 3, respectively.

Discussion

The objective of the study reported here was to determine the ability of a leptospiral bacterin to induce a protective immune response against challenge exposure with virulent *L borgpetersenii* serovar Hardjo in calves administered a single dose or 2 doses of bacterin with a prolonged interval between vaccinations. All calves were seronegative (MAT titer ≤ 12.5) at the start of the study for all serovars of *Leptospira* spp tested. Efficacy for use of this vaccine after administration of 2 doses

approximately 4 weeks apart has been reported elsewhere.[17–19] For various reasons, not all animals are vaccinated in accordance with the product label, and the present study was conducted to evaluate the potential impact that variation in the vaccination regimen might have on efficacy.

Microbial culture of urine and kidney samples obtained from all calves in groups 2 (1 dose of leptospiral bacterin) and 3 (2 doses of leptospiral bacterin) had negative results for *Leptospira* organisms throughout the period after challenge exposure. The ability of the challenge-exposure organism to colonize susceptible calves was evident in the control group, in which 10 of 11 calves were culture positive for both urine and kidney samples. Microscopic examination of sections of renal tissues revealed that vaccinates had a score of ≤ 2 (scale of 0 to 5) for interstitial nephritis (14/21 calves and 13/20 calves receiving 1 or 2 doses of leptospiral bacterin, respectively), whereas 6 of 11 calves in the control group had a score of ≥ 3, which was indicative of more severe nephritis in the control calves. However, the relevance of this result is not clear because histologic examination is not quantitative and nephritis can result from causes other than leptospirosis and thus is not definitive for *L borgpetersenii* serovar Hardjo infection.

All calves in both groups that received the leptospiral bacterin were protected and had negative results for leptospires in urine and kidney samples following challenge exposure with *L borgpetersenii* serovar Hardjo. In contrast, 10 of 11 control calves became infected following challenge exposure, harbored the challenge-exposure organism in their kidneys, and shed leptospires in their urine.

The significant increase in antibody titers against *L borgpetersenii* serovar Hardjo detected after administration of the second dose of leptospiral bacterin to the calves of group 3 indicated the ability of the bacterin to stimulate an anamnestic response 4 to 5 months after the initial vaccination. This finding supports observations from an earlier study[g] in which young calves were administered 2 doses of the same *L borgpetersenii* serovar Hardjo bacterin at a 4-month interval; those calves were protected at 3 weeks after administration of the booster vaccination when challenge exposed by use of conjunctival instillation of a heterologous strain of *L borgpetersenii* serovar Hardjo for 3 consecutive days. In contrast to results for the control group in the present study, there was relatively little change in the antibody titers in calves of group 3 (2 doses of leptospiral bacterin) after challenge exposure. These observations are consistent with previous reports[13,21] following use of this bacterin and other *L borgpetersenii* serovar Hardjo bacterins. The reason for this lack of an anamnestic antibody response following challenge exposure is unknown. Although the antibody titer in the calves receiving a single dose of bacterin remained relatively low throughout the entire period prior to administration of the bacterin, a similar lack of a secondary antibody response was observed in this group after challenge exposure.

Prevention of infections with serovar Hardjo requires that vaccination begin at a young age. Whereas prebreeding vaccination of heifers works well for incidental host serovars, vaccination at that point in the production cycle may be too late to prevent infections attributable to serovar Hardjo. Heifers can be exposed soon after birth and can remain infected for extended periods. Therefore, control of infections caused by serovar Hardjo should begin with the vaccination of young calves well before the time when they are commingled with older cattle. Because bulls can carry serovar Hardjo and transmit these organisms during breeding, they should be included in a vaccination program that is started when the animals are young.

Results of the present study indicated that administration of a single dose of *L borgpetersenii* serovar Hardjo bacterin to young calves provides protective efficacy for 4 to 5 months and that administration of a second dose of the bacterin 4 to 5 months after the initial dose will elicit a substantial anamnestic response. The flexibility of the dosing regimen used in this study likely will facilitate the design of efficacious vaccination programs that better fit modern production methods for both beef and dairy herds.

a. Veterinary Diagnostic Center, University of Nebraska, Lincoln, Neb.
b. Bovi-Shield GOLD 5, Zoetis, Madison, NJ.
c. Spirovac, Zoetis, Madison, NJ.
d. Dectomax pour-on solution, Zoetis, Madison, NJ.
e. Zoetis Veterinary Medical Research and Development, Kalamazoo, Mich.
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