Use of a monovalent leptospiral vaccine to prevent renal colonization and urinary shedding in cattle exposed to Leptospira borgpetersenii serovar hardjo

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Objective—To determine whether a monovalent Leptospira borgpetersenii serovar hardjo (type hardjo-bovis) vaccine commercially available in Australia, New Zealand, Ireland, and the United Kingdom would protect cattle from renal colonization and urinary shedding when exposed to a US strain of Leptospira borgpetersenii serovar hardjo.

Animals—24 Hereford heifers that lacked detectable antibodies against serovar hardjo.

Procedure—Heifers received 2 doses, 4 weeks apart, of the commercial hardjo vaccine (n = 8) or a monovalent US reference hardjo vaccine (8) or were not vaccinated (controls; 8). Heifers were challenged 16 weeks later by intraperitoneal inoculation or conjunctival instillation. Serum antibody titers were measured weekly, and urine samples were examined for leptospires. Heifers were euthanatized 11 to 14 weeks after challenge, and kidney tissue was examined for evidence of colonization.

Results—All 8 heifers vaccinated with the reference vaccine were found to be shedding leptospires in their urine and had evidence of renal colonization. All 4 control heifers challenged by conjunctival instillation and 2 of 4 control heifers challenged by intraperitoneal inoculation shed leptospires in their urine, and all 8 had evidence of renal colonization. In contrast, leptospires were not detected in the urine or tissues of any of the 8 heifers that received the commercial hardjo vaccine. Heifers that received the commercial hardjo vaccine had significantly higher antibody titers than did heifers that received the reference vaccine.

Conclusions and Clinical Relevance—Results suggest that cattle that received 2 doses of the commercial hardjo vaccine were protected against renal colonization and urinary shedding when challenged with L. borgpetersenii serovar hardjo 203 four months after vaccination. (Am J Vet Res 2001;62:995–1000)

Leptospirosis is an important cause of reproductive failure and production losses in cattle throughout the world.1–12 The most common cause of leptospirosis among cattle in the United States and throughout much of the world is infection with leptospires belonging to serovar hardjo. Two serologically indistinguishable but genetically distinct types of serovar hardjo have been identified: Leptospira interrogans serovar hardjo (type hardjoprajitno) and L. borgpetersenii serovar hardjo (type hardjo-bovis). Serovar hardjo type hardjo-bovis is common in cattle populations throughout the world13–16; type hardjoprajitno is isolated primarily from cattle in the United Kingdom.17

Vaccination with whole-cell inactivated leptospiral vaccines containing serovars hardjo, canicola, pomona, grippotyphosa, and icterohaemorrhagiae is the primary means of controlling leptospirosis in cattle in the United States. However, experimental trials of vaccines available in the United States have found that these vaccines result in only a short duration of immunity and do not prevent infection, reproductive failure, or urinary shedding in cattle challenged with US strains of serovar hardjo.17,18 In some other countries, vaccines containing serovar hardjo alone or in combination with serovar pomona are available and have been shown to decrease the incidence of infection, the duration and intensity of urinary shedding, and the incidence of leptospirosis among humans in contact with the cattle.19,20

Differences in the efficacy of hardjo vaccines among studies are likely to be related to several factors, including differences in vaccine formulation, the challenge strain used, and the method of challenge. Most serovar hardjo vaccines are formulated with isolates of serovar hardjo type hardjoprajitno, and only 1 commercial vaccine contains serovar hardjo type hardjo-bovis. Other factors related to vaccine formulation that may influence vaccine efficacy include leptospiiral growth conditions (eg, culture medium, fermentation conditions), adjuvant content, and the number of serovars included in the vaccine.

The strain of serovar hardjo type hardjo-bovis that is used for challenge exposure of cattle may also have an important effect on the outcome of vaccine efficacy studies, as hardjo-bovis isolates from different parts of the world have substantial genetic variation21,22 and vary in regards to the immune responses induced in cattle and the patterns of tissue colonization.5 Therefore, the fact that a serovar hardjo vaccine is efficacious in other countries does not necessarily mean that it will protect cattle exposed to US strains of serovar hardjo.

The method of challenge may also play an impor-
tant role in the outcome of vaccine efficacy studies. In many studies of the efficacy of leptospiral vaccines, cattle have been challenged by parenteral injection of large numbers of the organism. However, however, cattle have been challenged by application of moderate numbers of the organism on the conjunctival mucous membrane. This conjunctival challenge method is thought to more closely mimic the natural exposure that may occur in the field and has been recommended for use by the World Health Organization Working Group on Leptospiral Vaccines.

A serovar hardjo type hardjo-bovis vaccine that is commercially available in Australia, New Zealand, Ireland, and the United Kingdom has been shown to have good efficacy in cattle. However, this commercial hardjo vaccine has not been evaluated for protection of cattle challenged by means of conjunctival instillation of serovar hardjo strains typical of those found in the United States. Therefore, the purpose of the study reported here was to determine whether this commercial hardjo vaccine would protect US cattle from renal colonization and urinary shedding when exposed to a US strain of *L. borgpetersenii* serovar hardjo. Along with unvaccinated cattle, cattle vaccinated with a reference vaccine representative of the serovar hardjo component of many US leptospiral vaccines were included as controls.

Materials and Methods

Cattle—Twenty-four 8- to 12-month-old Hereford heifers that lacked detectable serum antibodies against serovar hardjo, as determined by use of the microscopic agglutination test, were used. Heifers were housed in groups in outside pens during the initial part of the study. One week prior to challenge exposure, heifers were moved to individual pens in a biosafety level-2 containment facility approved by the Association for Assessment and Accreditation of Laboratory Animal Care.

Experimental design—Heifers were randomly assigned to 1 of 3 groups (n = 8/group). Group-1 heifers were vaccinated with the US reference vaccine, group-2 heifers were vaccinated with a commercial monovalent hardjo type hardjo-bovis vaccine, and group-3 heifers were maintained as unvaccinated controls. Group-1 and -2 heifers were vaccinated twice, 4 weeks apart. Both vaccines were given as a 2-ml dose in the lateral aspect of the neck; the reference vaccine was given (or at an equivalent time for control heifers).

All heifers were challenged with *L. borgpetersenii* serovar hardjo strain 93U that was originally isolated from a steer at the National Animal Disease Center. Leptospires were cultured in liquid Ellinghausen-McCullough-Johnson-Harris culture medium to a density of approximately $5 \times 10^6$ cells/ml. Bacterial cells were counted, using a Petroff-Hauser bacterial counting chamber, harvested by centrifugation, and resuspended in sterile PBS. Leptospirese were inactivated by addition of sterile 10% merthiolate solution to give a final concentration of 1:10,000. The mixture was incubated at 37°C for 24 hours, and the pH was adjusted to 7.4. The vaccine was checked for sterility by inoculation into leptospiral culture medium and thioglycolate broth and onto blood agar plates.

Aluminum hydroxide, equivalent to 2% Al$_2$O$_3$ gel, was added at the time of assembly to a final concentration of approximately 12% vol/vol; pH was adjusted to 7.4 after the addition of aluminum hydroxide. The final product contained 2.5 $X$ 10$^7$ leptospirese/ml. The commercial hardjo vaccine was obtained directly from the manufacturer and was shipped to the United States with cold packs.

Leptospiral culture—Four formulations of semi-solid leptospiral culture medium were used. One was Ellinghausen-McCullough-Johnson-Harris semi-solid leptospiral culture medium containing 100 µg of 5-fluorouracil/ml and 1% rabbit serum. The other 3 were formulations of Tween 80-Tween 40-lactalbumin hydrolysate semi-solid medium containing 0.4% rabbit serum and 0, 100, or 200 µg of 5-fluorouracil/ml. Medium was inoculated, incubated, and examined as described.

Serologic testing—Serum samples were tested for antibodies against serovar hardjo with a microscopic agglutination test, using hardjo-bovis (strain 93U) and hardjoprajitno antigens as antigens. An initial serum dilution of 1:12.5 and 2-fold dilutions were tested. Titer was recorded as the reciprocal of the highest dilution at which ≥ 50% of the leptospirese were agglutinated. For samples for which titers obtained with the 2 antigens were different, the higher titer was used.

Histologic examination and lesion scores—Samples of kidney were fixed in neutral-buffered 10% formalin, embedded in paraffin, and sectioned at 5 µm, using standard techniques. One section from each block was stained with hematoxylin and eosin, and another was stained with a modified dieterle silver stain. Kidney lesion scores were assigned as described on the basis of gross and microscopic lesions: 0 = no lesions; 1 = focal or multifocal 1- to 2-mm pale areas in the renal cortex, with lymphocytic interstitial nephritis; 2 = multiple 2- to 5-mm pale foci in the renal cortex, with lymphocytic interstitial nephritis; 3 = multiple 5- to 10-mm pale foci in the renal cortex and medulla, with extensive interstitial nephritis and tubular degeneration; 4 = pale foci (> 10 mm) in the renal cortex and medulla, with severe interstitial nephritis, tubular degeneration, and fibrosis.

Immunofluorescence assay—Urine and kidney tissue were collected 15 minutes after IV administration of 300 mg of furosemide. Urine samples were submitted for leptospiral culture and immunofluorescence testing.

Heifers were euthanatized by IV injection of an overdose of pentobarbital 11 to 14 weeks after challenge. A postmortem examination was performed, and kidneys were examined for evidence of gross lesions. Specimens of kidney tissue were collected and submitted for histologic examination, immunohistochemical and immunofluorescence testing, and leptospiral culture.

Vaccines—The reference vaccine was prepared according to USDA Animal and Plant Health Inspection Service (APHIS) guidelines with slight modifications. The vaccine was formulated, using a clone of *L. borgpetersenii* serovar hardjo strain 93U that was originally isolated from a steer at the National Animal Disease Center. Leptospirese were cultured in liquid Ellinghausen-McCullough-Johnson-Harris culture medium to a density of approximately $5 \times 10^6$ cells/ml. Bacterial cells were counted, using a Petroff-Hauser bacterial counting chamber, harvested by centrifugation, and resuspended in sterile PBS. Leptospirese were inactivated by addition of sterile 10% merthiolate solution to give a final concentration of 1:10,000. The mixture was incubated at 37°C for 24 hours, and the pH was adjusted to 7.4. The vaccine was checked for sterility by inoculation into leptospiral culture medium and thioglycolate broth and onto blood agar plates.

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were processed and stained with fluorescein-labeled rabbit anti-hardjo serum, as described. Leptospires were identified on the basis of typical shape and specific fluorescence when examined by incident-light fluorescence microscopy.

Immunohistochemical testing—Immunohistochemical testing was done as described to identify leptospires in kidney specimens collected at necropsy. Briefly, 5-µm-thick sections of formalin-fixed paraffin-embedded tissues were placed on pre-bond slides. Paraffin was removed, and sections were treated with trypsin to expose masked antigens. Tissue sections were incubated with the appropriate dilution of the primary antibody (polyclonal rabbit anti-hardjo serum) overnight at 4°C. Sections were then incubated with biotinylated goat anti-rabbit immunoglobulin followed by supersensitive streptavidin-alkaline phosphatase. New fuchsin was the enzyme substrate, and slides were counterstained with hematoxylin.

Statistical analyses—Analysis of variance followed by the least significant difference procedure was used to compare anti-hardjo titers among groups; titters were converted to logarithms for this analysis. Values of P < 0.05 were considered significant.

Results
All vaccinated animals developed an agglutinating antibody titer against serovar hardjo. However, mean peak postvaccination titer and mean titer at the time of challenge exposure were significantly higher among cattle that received the commercial hardjo vaccine than among cattle that received the reference vaccine (Fig 1, Table 1). For cattle that received the commercial hardjo vaccine, peak postvaccination titer ranged from 800 to 12,800, and titer at the time of challenge ranged from 100 to 800. For cattle that received the reference vaccine, peak postvaccination titer ranged from 100 to 400, and titer at the time of challenge ranged from 12.5 to 50. Control cattle had titers ≤ 12.5 up to the time of challenge.

Control cattle developed antibodies against serovar hardjo after challenge exposure, regardless of whether the challenge organism was given by the intraperitoneal or conjunctival route. Two control heifers, both chal-

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* Number of urine samples in which leptospires were detected/number tested (urine samples were collected beginning 14 days after challenge). 12gross and microscopic lesions were scored as follows: 0 = no lesions; 1 = focal or multifocal 1- to 2-mm pale areas in the renal cortex, with lymphocytic interstitial nephritis; 2 = multiple 2- to 5-mm pale foci in the renal cortex, with lymphocytic interstitial nephritis; 3 = multiple 5- to 10-mm pale foci in the renal cortex and medulla, with extensive interstitial nephritis and tubular degeneration; 4 = pale foci (≥ 10 mm) in the renal cortex and medulla, with severe interstitial nephritis, tubular degeneration, and fibrosis.
lenged by the intraperitoneal route, developed only modest antibody titers after challenge, with peak postchallenge titers of 50 and 200; in contrast, the other control heifers developed peak post-challenge titers of 800 to 3,200. Heifers vaccinated with the commercial hardjo or the reference vaccine had little increase in antibody titer after challenge by either route.

All 4 control heifers challenged by conjunctival instillation and 2 of 4 heifers challenged by intraperitoneal inoculation shed leptospires in their urine. All 8 control heifers, including the 2 control heifers challenged by intraperitoneal inoculation that did not shed leptospires in their urine, were demonstrated to have renal colonization by examination of kidney specimens at necropsy. Similarly, all 8 heifers vaccinated with the reference vaccine were found to be shedding leptospires in their urine; however, the percentage of urine samples positive for leptospires was lower for cattle given the reference vaccine (18%) than for control cattle (66%). Leptospires were not detected in the urine or tissues of any of the 8 heifers that received the commercial hardjo vaccine.

Kidney lesion scores ranged from 0 to 2.5 for cattle that received the commercial hardjo vaccine, from 0 to 3.5 for cattle that received the reference vaccine, and from 0 to 3.5 for control cattle.

**Discussion**

Results of the present study suggested that cattle that received 2 doses of the commercial hardjo vaccine were protected against renal colonization and urinary shedding when challenged with *L. borgpetersenii* serovar hardjo strain 203 by the conjunctival or intraperitoneal route 4 months after vaccination. None of the heifers in this group were shedding organisms in their urine or had leptospires in their kidneys at necropsy. In contrast, all 8 nonvaccinated control heifers became infected with serovar hardjo, and 6 shed leptospires in their urine. Further studies will be required to determine whether the commercial hardjo vaccine protects cattle against other strains of *L. borgpetersenii* serovar hardjo in the United States. In addition, because venereal transmission is thought to occur with naturally induced leptospirosis, further studies are required to determine whether the commercial hardjo vaccine provides protection when cattle are challenged by the venereal route. However, it seems likely that a vaccine that prevents renal colonization and urinary shedding will also prevent colonization of the reproductive tract.

The reference vaccine used in this study failed to prevent infection, renal colonization, and urinary shedding in cattle challenged with *L. borgpetersenii* serovar hardjo. However, the percentage of urine samples found to contain leptospires was lower for animals vaccinated with the reference vaccine than for the control animals, which may indicate that the vaccine had some effect in keeping the numbers of organisms low in these animals. Results for the reference vaccine in the present study are similar to results for a pentavalent vaccine licensed for use in the United States, an experimental pentavalent vaccine containing hardjo-bovis, and an experimental monovalent hardjo-bovis vaccine. The reference vaccine was prepared according to a protocol developed by the USDA:APHIS:National Veterinary Services Laboratory. Because of different seed stocks, culture conditions, antigen concentrations, and adjuvants used by various companies to formulate the hardjo component of their leptospiral vaccines, it is not possible to produce a vaccine that truly represents all of the serovar hardjo vaccines available in the United States. This reference vaccine was prepared with *L. borgpetersenii* serovar hardjo (type hardjo-bovis) rather than *L. interrogans* serovar hardjo (type hardjo-prajitno), which is used in many cattle leptospiral vaccines available in the United States, because *L. borgpetersenii* serovar hardjo is the organism that infects cattle in the United States. and a previous study has shown that a vaccine containing hardjo-bovis induces higher antibody titers in animals than 1 containing hardjo-prajitno. Titer differences induced by the commercial hardjo vaccine used in this study were significantly higher than titers induced by the reference vaccine and titers typically induced by pentavalent leptospiral vaccines available in the United States. In most cases, peak postvaccination titers in cattle given pentavalent vaccines in the United States are < 200, and titers are typically < 100 within 60 days after vaccination. Cattle vaccinated with the commercial hardjo vaccine developed antibody titers as high as 12,800 after vaccination, and titers remained > 100 for 4 months after vaccination. Because of the high and long-lasting antibody titers induced, use of this commercial hardjo vaccine may complicate serologic diagnosis of serovar hardjo infection in US cattle.

Leptospires were not detected in any of the urine samples from the 8 heifers vaccinated with the commercial hardjo vaccine. Six of these animals did have small foci of lymphoplasmacytic interstitial nephritis, which is characteristic of leptospirosis; however, in contrast to control cattle and cattle that received the reference vaccine, leptospires were not identified in the kidneys of the cattle given the commercial hardjo vaccine. Because of the lack of other evidence of infection, the cause of the renal lesions in cattle given the commercial hardjo vaccine is not clear. Perhaps a transient infection occurred, during which a few organisms reached the renal parenchyma and induced an inflammatory response. There was no evidence that leptospires colonized the renal tubules or were shed in the urine of these animals, making the risk of transmission of infection from these cattle negligible.

The commercial hardjo vaccine used in this study protected cattle against conjunctival challenge with a highly infectious US strain of serovar hardjo. This is consistent with studies of the efficacy of this vaccine (as a hardjo-pomona combination) in New Zealand but differed from results of studies of other hardjo vaccines conducted in the United States. The reason for the difference between the efficacy of the commercial hardjo vaccine evaluated here and the efficacy of other serovar hardjo vaccines tested is likely attributable to differences in formulation of the commercial hardjo vaccine, because a similar vaccination regimen, challenge model, and challenge strain were used in these studies.
Control animals challenged by either route became infected and shed serovar hardjo in their urine. However, only 2 of 4 animals challenged by the intraperitoneal route shed leptospires in their urine, whereas all 4 animals challenged by the conjunctival route did. It is not clear why 2 animals challenged by the intraperitoneal route did not shed organisms in their urine. Low numbers of organisms were detected in the kidneys of these animals at necropsy, indicating that they were infected. It is possible that they were shedding organisms in numbers less than the detection limit of our tests. However, the combination of leptospiroplasm and immunofluorescence is quite sensitive in detecting leptospires in the urine of unvaccinated animals, and multiple urine samples were collected. Both of these animals did develop an antibody response to serovar hardjo after challenge, but the response was muted in comparison to the response of the other 2 control animals challenged by the IP route.

In this study and others, vaccinated cattle failed to develop a marked anamnestic antibody response after challenge with serovar hardjo. In previous studies, it was postulated that the route of challenge (ie, conjunctival instillation and natural exposure) was such that too few organisms penetrated the mucosa to stimulate a secondary immune response. However, in the present study, vaccinated animals challenged by intraperitoneal inoculation also failed to mount a secondary immune response. This is in contrast to another report of cattle challenged by the intraperitoneal route after vaccination, but the reason for this difference is not clear. Clearly, whether a secondary immune response is or is not detected after challenge is not a good indicator of exposure or infection status in vaccinated animals.

References


