Effect of intranasal exposure to leukotoxin-deficient *Mannheimia haemolytica* at the time of arrival at the feedyard on subsequent isolation of *M haemolytica* from nasal secretions of calves

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Objective—To determine the effect of intranasal exposure to live leukotoxin (LktA)-deficient *Mannheimia haemolytica* (MH) at the time of feedyard arrival on nasopharyngeal colonization by wild-type MH in calves.

Animals—200 calves.

Procedure—Calves from Arkansas (AR calves; n = 100; mean body weight, 205 kg) were purchased from an order buyer barn. Calves from New Mexico (NM calves; n = 100; mean body weight, 188 kg) were obtained from a single ranch. Calves were transported to a feedyard, where half of each group was exposed intranasally with LktA-deficient MH at the time of arrival. Calves were observed daily for respiratory tract disease (RTD), and nasal swab specimens were collected periodically to determine nasopharyngeal colonization status with MH. Serum samples were assayed for antibodies to MH.

Results—15 AR calves had nasopharyngeal colonization by wild-type MH at the order buyer barn, whereas none of the NM calves had nasopharyngeal colonization. Intranasal exposure to LktA-deficient MH elicited an increase in serum antibody titers against MH in NM calves, but titers were less in NM calves treated for RTD. Exposure of NM calves to LktA-deficient MH offered protection from nasopharyngeal colonization by wild-type MH.

Conclusions and Clinical Relevance—Exposure of calves to LktA-deficient MH elicited an increase in serum antibody titers against MH and decreased colonization of the nasopharynx by wild-type MH. Earlier exposure would likely allow an immune response to develop before transportation and offer protection from nasopharyngeal colonization and pneumonia caused by wild-type MH. (*Am J Vet Res* 2003;64:580–585)

Mannheimia haemolytica (MH) inhabits the tonsils and nasal passages of healthy cattle as a minor component of the normal bacterial flora.[1-5] After transport or during viral-induced illnesses, MH serotype A1 can undergo a rapid selective growth in the nasopharynx. This selective and substantial population increase is a likely prerequisite for the onset of pneumonic pasteurellosis.[6,7] An experimental parenterally administered MH bacterin inhibited colonization of the nasopharynx by MH.[4] In a previous experiment,[8] calves were vaccinated intranasally with a modified-live MH 3 days before transport to a feedyard, followed by prophylactic administration of antimicrobials at the time of arrival. The medication inhibited colonization by the vaccine strain of MH, and serum antibody titers against MH were decreased.[8] The study was designed to determine the effect of intranasal exposure of calves (at the time of arrival at the feedyard) to a live **leukotoxin (LktA)**-deficient MH on colonization of the nasopharynx by wild-type MH.

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Materials and Methods

Experimental design—Calves from Arkansas (AR calves; n = 100; body weight range, 175 to 241 kg; mean body weight, 205 kg; 54 bulls and 46 steers) were accumulated at a local order buyer barn on day –1. Calves were given sequential ear tag numbers as they were processed. Fifty calves (alternate sequentially numbered pairs) were vaccinated with a modified-live virus vaccine[a] that contained infectious bovine rhinotracheitis virus, bovine viral diarrhea virus types I and II, parainfluenza 3 virus, and bovine respiratory syncytial virus. All calves were transported by truck to the feedlot at the Clayton Livestock Research Center on day -1 and arrived on day 0. At the time of arrival, 50 odd-numbered calves (25 virus-vaccinated and 25 nonvaccinated) were exposed intranasally with live MH serotype 1 (exposed calves). The MH used for exposure had an approximate 1-kilobase deletion in the *lktA* gene encoding for leukotoxin (LktA-deficient MH).[9] This MH expresses and transports an inactive but immunogenic product, approximately 66 kDa molecular weight, from the partially-deleted *lktA* gene. The culture (3.5×10^7 colony-forming units/mL) was given intranasally (2 mL/nostril) with an automatic injector-drencher.[b] The nozzle of the injector-drencher was constricted to enhance projection of the effluent stream.

Calves from New Mexico (NM calves; n = 100; body weight range, 146 to 234 kg; mean body weight, 188 kg) originated from a single ranch. On day -1 at the ranch, calves were given sequential ear tag numbers as they were processed. Alternate sequentially numbered pairs were vaccinated with the modified-live virus vaccine. All calves were transported by truck to the feedlot at the Clayton Livestock Research Center on day 0 and arrived on day 0. At the time of arrival, 50 odd-numbered calves (25 virus-vaccinated, 25 nonvaccinated)

were exposed intranasally with the LktA-deficient MH by the same procedure that was used for AR calves.

At the time of arrival, all calves received SC vaccination with a clostridial antigen[c] and treatment for parasites.[d] Calves were weighed, branded, horn tipped, and received an injection[e] containing vitamins A and D_3. Bull calves were castrated. Calves were randomly assigned to 16 pens (12 to 13 calves/pen), within the constraints determined for each pen. Two pens contained exposed AR calves, 2 pens contained nonexposed AR calves, 2 pens contained exposed NM calves, 2 pens contained nonexposed NM calves, 4 pens contained exposed AR and NM calves (mixed pens), and 4 pens contained nonexposed AR and NM calves (mixed pens). All pens contained a balanced number of virus-vaccinates and nonvaccinates. Calves vaccinated with the modified-live virus vaccine were vaccinated with a modified-live bovine respiratory syncytial virus vaccine[f] on day 12.

Feed was provided for ad libitum consumption, and a continuous supply of water was available. Calves were monitored daily for clinical signs of **respiratory tract disease (RTD)**. Clinical signs included nasal or ocular discharge, labored breathing, lethargy, and emaciated body condition. Calves that had clinical signs of RTD were taken to a processing facility, and those with a rectal temperature > 39.7°C received medical treatment. Calves were given tilmicosin[g] (10 mg/kg, SC) for the first treatment and florfenicol[h] (40 mg/kg, SC) and flunixin megulmine[i] (10 mg/kg, IM) for the second treatment, if necessary. A third treatment, enrofloxacin[j] (10 mg/kg, SC), was given if necessary. Additional treatments consisted of repeating the first 3 treatments. After each treatment, calves were returned to their pens.

All calves were weighed and rectal temperatures were recorded on days 0, 1, 6, 12, 19, and 33. Calves that had rectal temperatures > 39.7°C but had not been removed because of clinical signs of RTD were not treated. At the feedyard, pens of calves entered the chute for processing in an established sequence to control the spread of MH and viral exposure during processing. First to be processed were nonexposed NM calves from the nonmixed pens, then all nonexposed calves from the mixed pens, and then nonexposed AR calves from the nonmixed pens. The crowding pen, working alley, and squeeze chute were then disinfected with a spray.[k] After disinfection, exposed calves from nonmixed and mixed pens were processed in the same order as nonexposed calves, after which the handling equipment was again disinfected.

Nasal swab specimen and blood sample collection and bacteriologic culture—Nasal swab specimens were collected from all calves at the order buyer barn or ranch (day -1) and at the feedyard on days 1, 6, 12, 19, and 33. Blood samples were collected on the same days (except for day 1) to obtain serum samples. Nasal swab specimens were obtained by inserting 1 cotton-tipped swab into each ventral nasal meatus. Each pair of swabs was placed in a dry tube, sealed, and stored on dry ice for transport to the National Animal Disease Center, where they were stored at –70°C. For analysis, nasal swab specimens were applied to blood agar base[l] plates containing 5% bovine blood, using a constant procedure to form 3 zones of decreasing growth.[10] Zone 3 was an approximate 160-fold (mean, 161.5 ± 44.3-fold) dilution of zone 1. Cultures were incubated overnight at 37°C, and MH colonies were identified and serotyped.[11,12] The LktA-deficient MH was nonhemolytic, in contrast to the hemolytic wild-type. Serum antibody titers were determined by use of an indirect hemagglutination procedure.[13] A calf was considered to have nasopharyngeal colonization by MH if the nasal swab specimen had positive bacteriologic culture results for MH.

Statistical analyses—The effect of LktA-deficient MH exposure on isolation of wild-type MH and on the proportion of calves treated was evaluated by χ^2 and logistic regression analyses. The proportion of calves treated anytime, on day 2 or later, and on day 6 or later was evaluated. Potential confounding variables analyzed in the logistic model included source of calves, pen identification number, vaccine virus administration, and whether they were in a mixed pen. Results from the most parsimonious model are presented for the 3 dependent variables. A value of $P < 0.05$ was considered significant.

Results

Effects of exposure on RTD—No deaths occurred as a result of RTD. Most calves (73.5%; 147/200) were treated on at least 1 day. Sixty-two NM calves and 85 AR calves were treated on at least 1 day with antimicrobials (**Table 1**). All but 24 treatment days occurred on day 6 of feed or earlier. More AR calves than NM calves were treated on day 6 or earlier (**Fig 1**). Calves that were exposed to LktA-deficient MH were significantly less likely to be treated after day 6 on feed, compared with nonexposed calves (37.5% [9/24] vs 62.5% [15/24], respectively). This effect was most notable in NM calves. Exposure had no effect on the proportion of calves treated on day 6 of feed or earlier. Findings on logistic regression models revealed that the exposure to LktA-deficient MH decreased the probability of treatment on day 6 or later, but not on day 2 or later (odds ratio, 0.43). The model included the covariates of source (New Mexico or Arkansas), whether calves were from a mixed pen, and whether calves had received the virus vaccine.

Colonization by MH—Colonization of the nasopharynx by wild-type MH was influenced by origin, exposure to LktA-deficient MH, and whether calves were kept in a mixed pen. Fifteen AR calves had colonization by wild-type MH at the order buyer barn, whereas none of the NM calves had colonization by wild-type MH at the ranch (**Table 2 and 3**; Fig 1). On the first day after arrival (day 1), 43 to 83% of the AR calves from each mixed pen and 50 to 77% of the AR calves from each nonmixed pen had nasopharyngeal colonization by wild-type MH. The infection spread to NM calves from mixed pens and peaked at day 12. Wild-type MH was recovered from nonexposed NM calves from mixed pens by day 6,

Table 1—Calves treated for respiratory tract disease with antimicrobials

			Calves	
Source	Exposed*	Mixed†	Total No.	Total No. treated
Arkansas	Yes	Yes	24	21
Arkansas	Yes	No	26	24
Arkansas	No	Yes	26	22
Arkansas	No	No	24	18
New Mexico	Yes	Yes	26	19
New Mexico	Yes	No	24	18
New Mexico	No	Yes	24	17
New Mexico	No	No	26	8

*Exposed intranasally to live *Mannheimia haemolytica* serotype 1 with a deletion in the *lktA* gene encoding for leukotoxin at the time of arrival at a feedyard in New Mexico. †Held in same pen with calves from the other source.

but was not recovered from exposed NM calves from mixed pens until day 12. Wild-type MH was not recovered from nonexposed NM calves from nonmixed pens until day 12. Isolation rates seemed to peak around day 33, but no data are available beyond day 33. One day after intranasal exposure to LktA-deficient MH, wild-type MH was isolated from 51 AR calves (22 exposed and 29 nonexposed), whereas the LktA-deficient MH was isolated from 23 of 50 exposed AR calves. The LktA-deficient MH was isolated from 26 of 50 exposed NM calves on day 1 and from 13 of 50 exposed NM calves on day 6. None of the nonexposed calves had nasopharyngeal colonization by LktA-deficient MH during the trial. Given that NM calves were exposed at the feedyard and AR calves seemed to bring infection to the feedyard, NM calves could be viewed as the study population for determination of the effect of LktA-deficient MH. Isolation of wild-type MH on day 6 or later was significantly lower in all exposed calves, compared with nonexposed calves (30.9% [29/94] vs 69.2% [65/94], respectively). No wild-type MH was isolated from exposed NM calves from nonmixed pens. Wild-type MH was isolated from only 42% (21/50) of exposed NM calves from mixed pens. On the basis of the logistic regression model, the odds of recovering wild-type MH were 5-fold less for exposed calves than nonexposed.

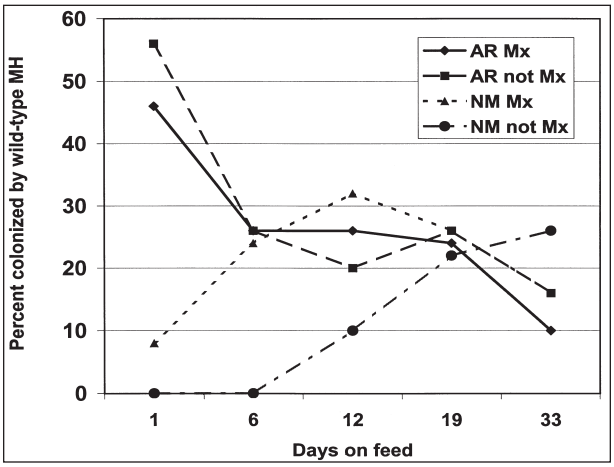

Figure 1—Isolation rate of wild-type *Mannheimia haemolytica* (MH) by source and pen (mixed or nonmixed). Calves were randomly assigned to 16 pens, with 12 to 13 calves in each. Four pens contained only calves from Arkansas (AR calves), and 4 contained only calves from New Mexico (NM calves). Four pens contained exposed AR calves and exposed NM calves (mixed pens), and 4 contained nonexposed AR calves and nonexposed NM calves (mixed pens). Mx = Mixed pen.

Table 2—Effects at the time of feedyard arrival of intranasal exposure of calves from Arkansas with LktA-deficient *Mannheimia haemolytica* (MH) and of placement in pens with calves from New Mexico (mixed pens) on the recovery of wild-type and LktA-deficient MH from nasal swab specimens

				No. of calves with nasopharyngeal colonization	
Exposed	Mixed pen	Day of study	No. of calves	Wild-type MH	LktA-deficient MH
Yes	Yes	–1	24	5	0
Yes	No	–1	26	3	0
No	Yes	–1	26	3	0
No	No	–1	24	4	0
Yes	Yes	1	24	8	11*
Yes	No	1	26	14	12*
No	Yes	1	26	15	0
No	No	1	24	14	0
Yes	Yes	6	24	6	3
Yes	No	6	26	4	0
No	Yes	6	26	7	0
No	No	6	24	8	0
Yes	Yes	12	24	6	2
Yes	No	12	26	3	0
No	Yes	12	26	7	0
No	No	12	24	7	0
Yes	Yes	19	24	6	2
Yes	No	19	26	4	0
No	Yes	19	26	6	0
No	No	19	23	9	0
Yes	Yes	33	24	3	0
Yes	No	33	26	1	0
No	Yes	33	26	2	0
No	No	33	23	7	0

*Significantly ($P < 0.05$) greater than number of nonexposed calves.

Effects of antimicrobial treatment on MH isolation—The first treatments for RTD were administered on day 1. On day 6, MH was isolated from 19 of 81 treat-

Table 3—Effects at the time of feedyard arrival of intranasal exposure of calves from New Mexico with LktA-deficient MH and of placement in pens with calves from Arkansas (mixed pens) on the recovery of wild-type and LktA-deficient MH from nasal swab specimens

				No. of calves with nasopharyngeal colonization	
Exposed	Mixed pen	Day of study	No. of calves	Wild-type MH	LktA-deficient MH
Yes	Yes	–1	26	0	0
Yes	No	–1	24	0	0
No	Yes	–1	24	0	0
No	No	–1	26	0	0
Yes	Yes	1	26	0	16*
Yes	No	1	24	0	10*
No	Yes	1	24	4	0
No	No	1	26	0	0
Yes	Yes	6	26	5	7*
Yes	No	6	24	0	6*
No	Yes	6	24	7†	0
No	No	6	26	0	0
Yes	Yes	12	26	5	3
Yes	No	12	24	0	3
No	Yes	12	24	11‡	0
No	No	12	26	5	0
Yes	Yes	19	26	5	0
Yes	No	19	24	0	1
No	Yes	19	24	8‡	0
No	No	19	26	11§	0
Yes	Yes	33	26	2	0
Yes	No	33	24	0	1
No	Yes	33	24	6‡	0
No	No	33	26	13§¶	0

*Significantly ($P < 0.05$) greater than number of nonexposed calves. †Significantly ($P < 0.05$) greater than number of calves from nonmixed pens. ‡Significantly ($P < 0.05$) greater than number of exposed calves from nonmixed pens. §Significantly ($P < 0.05$) greater than number of exposed calves. ¶Significantly ($P < 0.05$) greater than number of exposed calves from mixed pens.

Table 4—Isolation of all MH (deletion mutant and wild-type) from nasal swab specimens of calves treated and untreated for respiratory tract disease within 6 days prior to specimen collection

Source	Day of study	No. of calves*	
		Treated	Not treated
Arkansas	6	19/81	8/19
Arkansas	12	0/21	25/79†
Arkansas	19	0/13	25/87
Arkansas	33	0/0	13/100
New Mexico	6	4/41	20/59†
New Mexico	12	0/5	25/95
New Mexico	19	0/18	23/82†
New Mexico	33	0/0	22/100

*Total number of calves from which MH was isolated over number of calves that were treated or not treated. †MH isolated from significantly ($P < 0.05$) more untreated calves than treated calves.

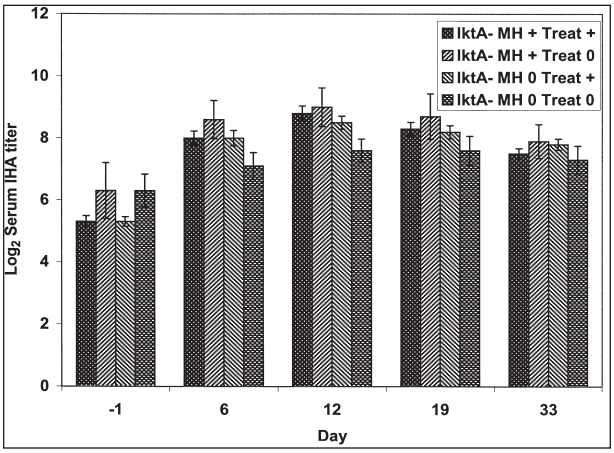

Figure 2—Geometric mean (± SE) serum antibody titers of AR calves against MH as determined by means of an indirect hemagglutination (IHA) inhibition assay.IktA-MH + = Calf exposed intranasally at the time of feedyard arrival to live MH serotype 1 that had a deletion in the *lktA* gene encoding for leukotoxin. IktA-MH 0 = Calf not exposed. Treat + = Calf treated for respiratory tract disease during days 1 to 5 of study. Treat 0 = Calf not treated during days 1 to 5.

ed calves and from 8 of 19 calves that were not treated during days 1 to 5. On days 12, 19, and 33, MH was not isolated from 34 nasal swab specimens of calves that were treated within 7 days before sample collection, but MH was isolated from 66 of 266 nasal swab specimens of untreated calves (**Table 4**). Treatment for RTD during days 1 to 5 significantly decreased the number of NM calves from which MH was isolated on day 6.

Serotypes of MH isolates—Two serotypes of wild-type MH, A1 and A2, were isolated from bacteriologic cultures of nasal swab specimens. At the order buyer barn, 11 of 26 isolates from AR calves were serotype A2, whereas no MH was isolated from NM calves at the ranch. In the feedyard, serotype A1 comprised 123 of 134 (93%) of all wild-type MH isolates from AR calves and 81 of 93 (87%) of the wild-type isolates from NM calves. Most of the wild-type serotype A1 MH colonies tested were resistant to kanamycin.[m]

Serum antibody titers against MH—Serum antibody titers against MH were determined by an indirect hemagglutination procedure. Serum antibody titers of AR calves increased by day 6, but serum antibody titers

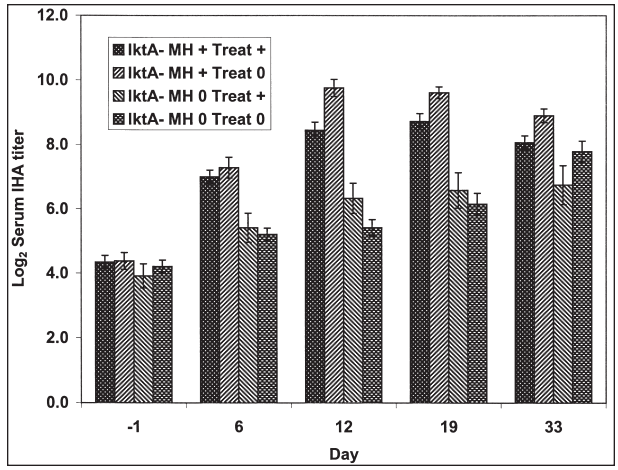

Figure 3—Geometric mean (± SE) serum antibody titers of NM calves against MH as determined by means of an IHA inhibition assay. *See* Figure 2 for remainder of key.

against MH between exposed and nonexposed calves were not significantly different (**Fig 2**). Treatment for RTD during days 1 to 5 did not inhibit the development of serum antibody titers against MH by day 6. However, serum antibody titers against MH of nonexposed AR calves that were not treated were significantly lower than those of the other groups on days 6 and 12.

Serum antibody titers against MH of NM calves increased by day 6, and serum antibody titers of exposed NM calves were significantly greater than those of nonexposed calves through day 19 (**Fig 3**). From days 12 to 33, exposed NM calves that were treated for RTD during days 1 to 5 had significantly lower serum antibody titers against MH than those of untreated calves. Serum antibody titers of nonexposed NM calves that were not treated were significantly lower than those of the other groups on day 12 but were significantly greater than nonexposed treated calves by day 33. Nonexposed NM calves from mixed pens developed greater serum antibody titers against MH than those of nonexposed NM calves from nonmixed pens through day 19. By day 33, serum antibody titers of nonexposed NM calves were no longer significantly different between calves from mixed and nonmixed pens.

Discussion

Acute RTD, in the form of an acute pneumonic pasteurellosis, usually develops in transported calves within the first week of arrival at the feedyard and was found in the calves of our study. *Mannheimia haemolytica* inhabits the tonsils and nasal passages of healthy cattle as a minor component of the normal bacterial flora.[1-5] After transport, MH serotype A1 can undergo rapid selective growth in the nasopharynx,[6,7] which was also found in the calves of our study. In our study, 2 groups of calves (1 from Arkansas and 1 from New Mexico) were brought to a common feedyard, where half of each group was placed in pens with calves from the other source and half of each group was not. At least 15 AR calves had colonization of the nasopharynx by MH serotype A1 before transport, and 51 AR calves had colonization on day 1 after transport. However, NM calves appeared to be free of MH colonization, because

none had positive bacteriologic culture results for wild-type MH before transport or on day 1. This presented an opportunity to study the effect of exposure to LktA-deficient MH on wild-type MH colonization of calves with and without preexisting MH infections.

Spread of wild-type MH infection was influenced by preexisting MH infections, exposure to LktA-deficient MH, placement of uninfected calves in pens with calves that had colonization of the nasopharynx by MH, and treatment for RTD. Because many of the AR calves already had colonization of the nasopharynx by wild-type MH, results of the LktA-deficient MH exposure were more evident in the NM calves. Exposure to LktA-deficient MH at the time of arrival inhibited colonization by wild-type MH in NM calves from nonmixed pens, whereas some nonexposed calves had colonization of the nasopharynx by MH within 12 days, even though they had not been placed in pens with AR calves.

Tilmicosin and florfenicol are used to treat RTD. Both antimicrobials inhibit colonization of the nasopharynx by MH for several days after administration.[8,10,14] In our study, treatment did not significantly decrease the number of AR calves with detectable colonization on day 6. However, most treated calves were shedding less MH organisms than untreated calves. With the procedure used to streak the agar plates to create 3 zones of a decreasing number of colonies, zone 3 is an approximate 160-fold dilution of zone 1. Of the 19 treated calves from which MH was isolated on day 6, bacteriologic culture results of nasal swab specimens revealed that 4 calves had only 1 detectable MH colony in zone 1, 6 calves had multiple MH colonies in zone 1 only, 7 calves had MH colonies in zone 2, and 2 calves had MH colonies in zone 3. Of the 8 untreated calves from which MH was isolated on day 6, bacteriologic culture results of nasal swab specimens revealed that 1 calf had MH colonies only in zone 1, 2 calves had MH colonies in zone 2, and 5 calves had MH colonies in zone 3. The number of untreated calves with MH colonies in zone 3 was significantly greater than that of treated calves.

In the AR calves, serum antibody titers against MH increased by day 6 with no significant differences in geometric mean titer between exposed and nonexposed calves. Serum antibody titers against MH of untreated nonexposed calves were less than those of the other groups. Serum antibody titers against MH of exposed untreated calves were greater than those of nonexposed untreated calves, supporting the probability that exposure elicited an increase in serum antibody titer. In the NM calves, exposure elicited significantly higher serum antibody titers against MH, compared with nonexposed NM calves.

Because a dramatic selective increase in the MH population of the nasopharynx is a prerequisite for the development of pneumonic pasteurellosis, inhibition of colonization of the nasopharynx of calves by MH is a desirable goal to seek in order to control the disease. One method of inhibition was the prophylactic use of tilmicosin or florfenicol before transport or at the time of arrival at the feedyard.[8,14] Another was by vaccination with an experimental MH serotype A1 bacterin made by growing the bacteria in the presence of bovine lung lavage and spleen cells.[4] The MH organisms grown in bovine cellular material may produce some of the same antigens that they would produce in vivo. Exposure to live attenuated MH intranasally would allow the bacteria to produce the products necessary for survival in the host and the host to produce antibodies against these products. In our study, calves were exposed to live attenuated MH that colonized the nasopharynx. Even though the exposure was not given early enough to allow immune responses to develop before contact with wild-type MH, an antibody response to the attenuated MH and some protection against colonization by wild-type MH were observed.

One drawback to using live bacteria as a vaccine for RTD at the time of arrival at the feedyard is that antimicrobial treatment for RTD would inhibit its growth in the host and result in a decrease in the antibody response.[8,14] In our study, the first treatments for RTD were given 1 day after arrival at the feedyard, which was 1 day after exposure. Treatment caused the development of serum antibody titers to be decreased in exposed and nonexposed NM calves. Presumably, the decrease in the development of serum antibody titers against MH was caused by suppression of natural MH infection by the treatment.

In our study, intranasal exposure of calves to LktA-deficient MH at the time of arrival at the feedyard increased serum antibody titers to MH and decreased the amount of colonization of the nasopharynx by wild-type MH. Therefore, it would seem that exposing calves to LktA-deficient MH at an earlier time to allow an immune response to develop before calves are stressed by transport to a feedyard could offer protection from colonization and from pneumonia caused by wild-type MH.

[a]Titanium 5, Agri Laboratories Ltd, St Joseph, Mo.
[b]Allflex, NJ Phillips Pty Ltd, Somersby, NSW 2250, Australia.
[c]Electroid 7, Schering-Plough Animal Health Corp, Omaha, Neb.
[d]Cydectin, Fort Dodge Animal Health, Fort Dodge, Iowa.
[e]Vita-Jec A and D, RXV Products, Grapevine, Tex.
[f]Titanium BRSV, Agri Laboratories Ltd, St Joseph, Mo.
[g]Micotil, Elanco Animal Health, Indianapolis, Ind.
[h]Nuflor, Schering-Plough Corp, Kenilworth, NJ.
[i]Banamine, Schering-Plough Corp, Kenilworth, NJ.
[j]Baytril, Bayer Corp, Shawnee Mission, Kan.
[k]Lysol IC, Racket and Coleman Inc, Montvale, NJ.
[l]Bacto blood agar base, Difco Laboratories Inc, Detroit, Mich.
[m]Kanamycin A, Sigma Chemical Co, St Louis, Mo.

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