Bivalent vaccination against pneumonic pasteurellosis in domestic sheep and goats with modified-live in-frame lktA deletion mutants of Mannheimia haemolytica

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Abstract

A temperature-sensitive shuttle vector, pBB80C, was utilized to generate in-frame deletion mutants of the leukotoxin structural gene (lktA) of Mannheimia haemolytica serotypes 1, 2, 5, 6, 7, 8, 9, and 12. Culture supernatants from the mutants contained a truncated protein with an approximate molecular weight of 66 kDa which was reactive to anti-leukotoxin monoclonal antibody. No protein reactive to anti-LktA monoclonal antibody was detected at the molecular weight 100-105 kDa of native LktA. Sheep and goats vaccinated intramuscularly with a mixture of serotypes 5 and 6 mutants were resistant to virulent challenge with a mixture of the wild-type parent strains. These vaccinates responded serologically to both vaccine serotypes and exhibited markedly-reduced lung lesion volume and pulmonary infectious load compared to control animals. Control animals yielded a mixture of serotypes from lung lobes, but the proportion even within an individual animal varied widely from 95% serotype 5 to 95% serotype 6. Cultures recovered from liver were homogeneous, but two animals yielded serotype 5 and the other two yielded serotype 6 in pure culture.

1. Introduction

The bacterium Mannheimia haemolytica is the cause of significant economic losses to the cattle, sheep, and goat industries, and for significant death losses in wild populations of bighorn sheep in the United States [1–5]. This organism is part of the normal nasopharyngeal flora in cattle, sheep, and goats [1–3]. Under conditions of stress or respiratory viral-infection, M. haemolytica can become established in the lungs of these animals and cause fibrinous pneumonia [1].

Management and control of ruminant respiratory disease is variably achieved with a combination of appropriate husbandry to reduce stress, the use of therapeutic and metaphylactic antimicrobials, and immunization for common viral and bacterial agents [2,4,6]. While the value of management and husbandry for control of respiratory disease cannot be overemphasized, there are concerns regarding excessive agricultural use of antimicrobials and the development of antibiotic-resistance, antibiotic residues in food products, and animal welfare [3]. Effective alternate preventative measures are therefore needed.

The etiologic agent M. haemolytica possesses several virulence factors, including capsule, outer membrane proteins, adhesins, neuraminidase, endotoxin, and an exotoxic leukotoxin. Of these, several lines of evidence indicate that the leukotoxin is a primary virulence factor which plays a major role leading to lung pathology characteristic of the disease [7]. Previous work has shown that a mutant M. haemolytica which express no leukotoxin elicits dramatically reduced lung injury after experimental pulmonary instillation of the mutant compared to its wild-type parent strain [8]. The mutant strain was therefore effectively attenuated. It is believed, however, that an immune response to leukotoxin is important to disease resistance [1,3]. This experiment was designed to produce and evaluate attenuated strains of M. haemolytica which express and transport inactive but immunogenic leukotoxin protein for potential use as a vaccine in ruminant animals. The products were constructed to be non-revertible and to contain no foreign DNA or antibiotic-resistance genes. Because sheep, goats, and cattle are susceptible to multiple serotypes of M. haemolytica [9–11], an objective was to determine the immune response to multiple serotypes of live vaccine delivered simultaneously. While 8 serotypes of
M. haemolytica were modified with respect to LktA, only 2 were evaluated in small ruminants.

2. Materials and methods

2.1. Construction of leukotoxin mutants

M. haemolytica pneumatic lung isolates were selected from the NADC archives based on lack of plasmid and sensitivity to kanamycin selection. The selected strains were: NADC D632, ovine serotype 1; NADC D121, ovine serotype 2; NADC D110, ovine serotype 5; NADC D174, bovine serotype 6; NADC D102, ovine serotype 7; NADC D844, ovine serotype 8; NADC D122, ovine serotype 9; and NADC D712, ovine serotype 12.

Plasmid pBB80C lktA was utilized to introduce an in-frame genetic deletion into the leukotoxin structural gene of each strain (except serotype 2 NADC D121, described below), as previously described [12]. Briefly, electrotransformed colonies were recovered after 48 h incubation with kanamycin selection at 30 °C. Passage of transformants on blood agar plates with kanamycin at 37 °C selected for single-crossover mutants. Hemolytic putative single-crossover colonies were passed to Columbia broth without selection which was incubated overnight at 30 °C. The growth in broth was stuck for isolation on non-selective blood agar plates and incubated overnight at 37 °C. Non-hemolytic colonies were recovered from each strain (serotype) and were confirmed to be kanamycin-sensitive.

Because pBB80C lktA failed to generate mutant products in strain NADC D121 (serotype 2), a second replacement plasmid was constructed. Briefly, an EcoRV fragment of genomic DNA was ligated into pBB80C as previously described [12]. PCR primers with NgoMIV ends were used to amplify the replacement plasmid. Digestion of the ampiclon with NgoMIV and recircularization by ligation completed the second replacement plasmid. The product was sequenced to confirm the deletion was in-frame with lktA and designated pBB80C lktA2. As above, this plasmid was utilized to introduce an in-frame genetic deletion into NADC D121.

2.2. Characterization of the leukotoxin mutants

To define the chromosomal deletion, DNA was PCR amplified from whole cells as previously described [12]. Log-phase culture supernatants were concentrated and subjected to Western blot analysis as previously described [12].

2.3. Bacteria

M. haemolytica strains NADC D110 (serotype 5, ovine lung isolate), NADC D110 lktA, NADC D174 (serotype 6, bovine lung isolate), and NADC D174 lktA were grown separately in Columbia broth (Difco Laboratories, Detroit MI) approximately 3 h to late log-phase, about 2 × 10^9 CFU/ml. Growth was diluted in Earle's balanced salt solution (EBSS) 1:50 for the vaccine dose or 1:100 for the challenge dose. The two strains were mixed in equal volume and kept on ice (−2 h) prior to animal inoculation. The delivered CFU were calculated using plate counts of serial dilutions.

2.4. Animal vaccination

Four lambs (Columbia, approximately 80–100 days of age and approximately 25 kg) and six goats (Toggenburg, approximately 80–100 days of age and approximately 15 kg) were colostrum deprived and raised at the National Animal Disease Center, Ames, IA. Two lambs and three goats were randomly selected and vaccinated with 4 × 10^7 CFU each of M. haemolytica NADC D110 lktA and NADC D174 lktA (serotypes 5 and 6 respectively) in 1 ml Earle's balanced salt solution (EBSS). The suspension was delivered intramuscularly in the right mid-cervical region. After three weeks, the animals were re-vaccinated similarly. Ten days after the second vaccination all ten animals were challenged with 8.5 × 10^7 CFU each of the parent strains NADC D110 and NADC D174 mixed in a total volume of 5 ml EBSS instilled intratracheally at the tracheal bifurcation with a catheter. The inoculum was chased with 5 ml sterile EBSS. Five days after challenge all surviving animals were euthanized and necropsied. All animal work was conducted under local IACUC and IBC approved protocol and BSL-2N biocountainment.

2.5. Samples and data collection

Sera were collected the day of the first vaccination, 2 weeks later, the day of challenge exposure, and the day of necropsy. Rectal temperatures were recorded daily throughout the trial and twice daily from challenge exposure to necropsy. Clinical scores were subjectively assessed on the same schedule as rectal temperatures based on degree of depression and appetite. At necropsy, lung specimens from 1 to 3 g in weight were obtained from 3 to 4 lung lobes per animal, including areas containing abnormalities when possible, for bacterial enumeration. Swab specimens were obtained from trachea, kidney, and liver for bacterial isolation. Lung lesion volumes were estimated, by personnel blinded to treatment group, visually and by palpation for each lobe of the lung, including both consolidated areas and those which appeared merely atelectic. Total lung lesion scores were the sum of the 8 individual lobe's score where the estimated percent of each lobe's lung lesion volume was multiplied by an approximation of its contribution to air exchange as follows: right cranial lobe, 6%; right cranial half of the middle lobe, 5%; right caudal half of the middle lobe, 7%; right caudal lobe, 35%; accessory lobe, 4%; left cranial lobe, 4%; left middle lobe, 6%; and left caudal lobe, 33%.

2.6. Sample processing

Sera were tested for M. haemolytica antibody by an indirect haemagglutination assay (IHA) against serotypes 5 and 6 wherein glutaraldehyde-treated erythrocytes are sensitized with M. haemolytica culture supernatant and exposed to dilutions of sera [13]. Leukotoxin neutralization titters were assessed using BL-3 cells and MTT dye [14]. Lung specimens were weighed and EBSS was added to bring the tissue plus fluid volume to 10 times the weight. The specimens were ground to yield a homogenous suspension and ten-fold dilutions were made in EBSS. The dilutions (100 μl) were spread onto blood agar base plates containing 5% defibrinated bovine blood which were incubated overnight at 37 °C. Colonies exhibiting typical M. haemolytica morphology were enumerated and 20 representative colonies (where available) were serotyped using specific antiserum [15]. Swabs were rolled onto one third of fresh blood agar plates and then each side of a sterile loop was used to semi-quantitatively streak for isolation onto the remaining thirds consecutively.

2.7. Statistical analysis

Lung lesions, log(10) bacterial counts and log(2) serological titers were analyzed using independent samples t-test.

3. Results

3.1. Construction and evaluation of leukotoxin mutants

The selected M. haemolytica serotype 2 strain NADC D121, when subjected to replacement plasmid pBB80C lktA, appeared to
generate single-crossover integrant products at 37°C which consistently resolved to wild-type products when passed at 30°C. Sequencing of the serotype 2 leukotoxin operon and comparison with that of the replacement plasmid pBB80CΔlktA derived from serotype 1 revealed that homology of the upstream arm of the replacement plasmid was likely insufficient to allow homologous recombination. Mutant products were produced from serotypes 1, 5, 6, 7, 8, 9, and 12 using pBB80CΔlktA and serotype 2 using pBB80CΔlktA2.

PCR products from each mutant product were approximately 1 kb smaller than that from wild-type parent strains, indicating each contained chromosomal deletions consistent with the desired in-frame mutation. Western blot analysis revealed protein bands reactive with anti-leukotoxin monoclonal antibody at approximately 66 kDa and the absence of reactive protein at the 101 kDa size of the native leukotoxin products (Fig. 1).

3.2. Clinical response to vaccination

No local reaction was palpable or visible following first or second vaccination in any vaccinate. All animals remained alert and on-feed following both doses. The first dose of vaccine elicited a febrile response, particularly in the sheep which had a fever on day 2 and 3 and which peaked at 40.3°C on day 3. The second injection elicited no febrile response.

3.3. Antibody response to vaccination

Prior to vaccination, the animals had low IHA titers against both serotypes 5 and 6 of M. haemolytica (Table 1). After the first vaccination, vaccinates’ titer increased over 8-fold against both serotypes. No response was evident after the second dosage. Only a slight increase (not significant P > 0.05) in antibody titer occurred after challenge exposure of the vaccinated animals. The control animals’ titer increased slightly (not significant P > 0.05) prior to challenge exposure. Between the time of challenge exposure and necropsy, the one surviving control sheep increased its titer against both serotypes by about 32-fold.

Leukotoxin neutralization titers in the vaccinates increased variably. Both lambs and two goats seroconverted (increased at least 4-fold) after the first vaccination; one of which seroconverted again to the second dose. One goat remained leukotoxin-seronegative throughout the study. Control animals’ leukotoxin titers remained low through the challenge sampling. The one surviving control sheep seroconverted to leukotoxin following challenge exposure.

3.4. Clinical response to virulent challenge

Following challenge, none of the vaccinates had a fever at any time. They remained alert and eating all their food until euthanasia. The control animals had a fever the day after exposure averaging 40.7°C. All control goats and 1 control sheep died overnight between the 1st and 2nd day after exposure. The remaining control sheep remained febrile, anorexic, and depressed until necropsy.

3.5. Injection site reaction

Inspection of the vaccine injection site at necropsy revealed no detectable reaction in the muscle. Slight subcutaneous discoloration about 1 cm in diameter consistent with hemorrhage was detected in both sheep and two of the three goats.

3.6. Gross pathologic lung lesions

Lung lesion volume of vaccinates (corrected for ventilation capacity of each lobe) averaged 3.5% (Table 2). One sheep had tight adhesions of visceral to parietal pleura and to the pericardium ventrally on both right and left sides. One goat had moderately firm consolidation of 95% of its accessory lobe. The remaining lung lesions were soft, consistent with atelectasis. Lung lesion volume of the controls (corrected for ventilation capacity of each lobe) averaged 52% (Table 2). The four animals which died contained large amounts of fibrinous pleural effusion and fibrinous pleural adhesions. The lung lesions were firm or moderately firm and emphysematous and/or crepitous areas were evident. The sheep which survived until the time of necropsy contained about 100 cc pleural effusion and a large (about 250 cc) fibrinous mass occupying the pleural space over the right cranial and middle lobes. The lung lesions consisted primarily of firm fibrinous consolidation in this animal.

| Table 1 | IHA antibody titers against Mannheimia haemolytica serotypes 5 and 6 and leukotoxin neutralization titers before and after vaccination.a |
| Day 0 | Day 14 | Day 31 | Day 36 |
| Serotype 5 | Vaccine | 2.4 ± 0.4 | 6.0 ± 0.5b | 6.2 ± 0.5 | 6.8 ± 0.6 |
| Control | 1.2 ± 0.4 | 1.8 ± 0.5 | 2.6 ± 0.6 | 8b |
| Serotype 6 | Vaccine | 1.4 ± 0.3 | 6.2 ± 0.6b | 6.2 ± 0.5 | 6.8 ± 0.6 |
| Control | 0.8 ± 0.4 | 1.4 ± 0.4 | 1.4 ± 0.5 | 6b |
| Leukotoxin | Vaccine | 0.4 ± 0.2 | 3.0 ± 0.7b | 3.4 ± 0.9 | 3.6 ± 0.7 |
| Control | 0.6 ± 0.2 | 0.6 ± 0.2 | 1.0 ± 0.3 | 4.0b |

a Results geometric mean log(2) ± SEM. First dose of vaccine on day 0, 2nd dose on day 21. All animals intratracheally challenged with wild-type mixture of serotypes 5 and 6 on day 31 (n = 5 for each group).
b Different from prior titer, P < 0.01.
c One surviving sheep, 4 animals died approximately 36 h after challenge.

| Table 2 | Lung lesion scores and postmortem lung bacterial culture results 5 days after intratracheal challenge with Mannheimia haemolytica serotypes 5 and 6.3 |
| Mean percent lung lesionsb | Log(10) geometric mean M. haemolytica per gram lung |
| Vaccinates | 3.5 ± 1.4c | 1.1 ± 0.48c |
| Controlsd | 53 ± 11 | 7.8 ± 0.73 |

3 n = 5 for each group. Results expressed as mean ± SEM.
b Percentage involvement of each lobe estimated and multiplied by the lobes’ estimated contribution to overall air exchange.
c Significantly different from control values, P < 0.001.
d 4 animals died approximately 36 h after challenge and were posted on day 2 post-challenge.
3.7. Qualitative and quantitative bacterial culture

Of 19 lung specimens quantitatively cultured from vaccinates, 5 yielded *M. haemolytica*. One sheep and 1 goat yielded no *M. haemolytica* from their lung. The sheep with pleural adhesions yielded 2 × 10⁵ CFU/g from its right cranial lobe. The goat with accessory lobe involvement yielded 1.3 × 10⁶ CFU/g from that lobe and 1 × 10⁷ CFU/g from its right caudal half of the middle lobe, and moderate growth from its tracheal swab. The remaining goat yielded 2 × 10⁵ and 7 × 10⁴ CFU/g from its right cranial lobe and cranial half of the middle lobe respectively. All other lung specimens and tracheal swabs from vaccinates were culture negative as were swabs from liver and kidney.

Of 17 cultured lung specimens from control animals, all yielded *M. haemolytica* from as few as 2.5 × 10³ CFU/g to 4 × 10⁸ CFU/g. The geometric mean count for the four animals which died acutely was 2.5 × 10⁵ CFU/g; the surviving sheep had a mean count of 2.5 × 10⁵ CFU/g. Tracheal swabs from the four animals which died yielded heavy growth of *M. haemolytica*; the surviving sheep yielded light growth from its trachea. Liver swabs of all four, and kidney swabs of two of the animals which died yielded *M. haemolytica*. The surviving sheep was culture negative in both liver and kidney.

3.8. Serotyping of bacterial isolates

Serotyping of isolates from lung revealed that the few colonies recovered from vaccinates were of serotype 5, except for the actively infected accessory lobe of one goat which yielded equal amounts of both serotype 5 and 6. Control animals tended to yield a mixture of serotypes from each lobe, but the mixture varied widely from lobe to lobe in the animals which acutely died (e.g., 95% of serotype 5 in the right cranial lobe to only 5% of serotype 5 in the right caudal lobe). Isolates recovered from kidney and liver were homogenous with respect to serotype in any given animal, but two animals contained serotype 5 in these tissues and the other two serotype 6.

4. Discussion

Vaccination with a combination of two leukotoxin deletion mutants (modified-live vaccine combination) of *M. haemolytica* serotypes 5 and 6 protected against combined homologous challenge with the virulent wild-type parent strains, based on clinical signs, postmortem lesions, and results of bacterial culture. All vaccinates remained active, alert, afebrile, and on-feed while 80% of the control animals succumbed to pneumonic (and septicemic) pasteurellosis.

Reactions observed at the vaccine injection sites were minor and did not involve muscular tissue, consistent with findings using leukotoxin negative mutants of serotype 1 in cattle [12]. This contrasts greatly with the response to leukotoxin positive strains given intramuscularly to cattle which evidence large swellings and necrosis in the area, sometimes opening through the overlying skin (unpublished observations). It is likely that little or no local adverse reaction would occur had the dosage been subcutaneous or intradermal, an alternative that may also tend to reduce the observed febrile response to vaccination.

The failure to significantly stimulate further antibody (as measured by IHA) by the second dosage of vaccine may indicate two doses were unnecessary and that a single dose would have been sufficient. Lung challenge also failed to elicit significant increases in antibody titer in the vaccinated group. The control sheep which survived challenge seroconverted to both serotypes 5 and 6 (over 32-fold increase in IHA titer) between challenge and necropsy, indicating that sufficient time had elapsed for a measurable humoral immune response.

It is not clear what, if anything, the soft atelectic areas of the lungs of vaccinates mean with respect to infection. Most of the cultured areas were sterile, and those that contained *M. haemolytica* did so at very low concentration. One goat with a consolidated accessory lobe yielded considerable numbers of *M. haemolytica* from that lobe and also yielded positive culture from its trachea. This, we believe, is evidence of an active infection in that lobe. The remaining lobes were either sterile or nearly so, indicating other lobes may have been resistant to infection despite contamination of the trachea. The clinical condition of this affected animal was normal and afebrile, indicating the likelihood the infection was contained and that it would have resolved given more time, though the affected lobe would unlikely resume full function.

The acute deaths of 4 out of the 5 control animals might be due solely to the compromise of pulmonary tissues; air exchange certainly was impaired severely with an acute 50% reduction in lung volume. These animals also were suffering from septicemia, perhaps as an agonal event but more likely contributing to their clinical syndrome. Sheep are known to contract both pneumonia and septicemic disease by biotype *M. haemolytica* [11]. Our observation of rapid deaths of these 4 animals and their septicemia, it is not surprising that large numbers of *M. haemolytica* were recovered from tissues and tracheal swabs. The fifth animal which survived to necropsy, although not septicemic, yielded large numbers of *M. haemolytica* from all lung specimens as well as low numbers from its trachea. We consider the infection was active and not contained, though it may have eventually been overcome with substantial residual damage and fibrosis.

The variability of colonization of the two serotypes between the lung lobes and systemic tissues of individual control animals was an unexpected finding. Despite the fact the serotype 6 strain was of bovine origin, it evidenced virulence in this small ruminant model just as did the serotype 5 ovine strain. If one strain were to out-compete the other, a reasonable correlate with virulence, one would expect a fairly consistent predominance of that strain. The observed distribution is almost as if single infective micro-colonies spread to adjacent uninfected lobes and therefore colonized the lobe with predominantly one serotype. Because the samples taken were relatively small (1–3 g of lung), perhaps micro-colonies infected only a part of each lobe and the observed segregation of serotypes is on a smaller scale of partial lung lobes. Perhaps the same might be true for circulatory infection; a single micro-colony might have breached into the circulatory system and colonized. Again, serotypes 5 and 6 apparently did not differ greatly in their capacity to cause septicemia inasmuch as equal numbers of animals became septicemic with each serotype.

Nearly equivalent antibody responses were elicited against both serotypes 5 and 6, indicating that multivalent *M. haemolytica* vaccines can effectively induce a multivalent response. The practical significance of this is obvious: In both cattle and in small ruminants, multiple serotypes are involved in disease [9–11]. An effective multivalent acellular vaccine has been recently demonstrated in bighorn sheep, though repeated doses were necessary for the acellular vaccine to elicit progressively higher antibody titers to leukotoxin and outer membrane proteins [16]. Additional studies may show that a reduced number of doses are required for the acellular vaccine to elicit adequate resistance to challenge, and modification of the vaccine or delivery system may further reduce the requirement for repeated vaccination. However, the serologic evidence supports the possibility that a single dose of modified-live vaccine is nearly as effective as two doses for induction of immunity. In cattle, small
ruminants, and wild populations of bighorn sheep, it can be difficult and costly to physically handle the animals multiple times, so a single-dosage product is desirable. It may also be possible to vaccinate small ruminants with modified-live \textit{M. haemolytica} without any handling using a mucosal approach similar to that recently demonstrated in calves [12]. Additional studies are necessary to determine the safety of alternative routes of modified-live \textit{M. haemolytica} vaccine delivery and to demonstrate efficacy against all relevant serotypes of \textit{M. haemolytica} in small ruminants and cattle.

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References


