Immunogenicity of recombinant *Mannheimia haemolytica* serotype 1 outer membrane protein PlpE and augmentation of a commercial vaccine

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Abstract

*Mannheimia haemolytica* is the major cause of severe bacterial pneumonia associated with shipping fever in cattle. The gene for *M. haemolytica* outer membrane protein (OMP) PlpE was cloned into the expression vector pRSETA. The cloned gene was then expressed in BL21(DE3)pLysS and the recombinant PlpE (rPlpE) was purified and used in immunological and vaccination studies. Vaccination of cattle with commercial *M. haemolytica* vaccines stimulated no significant (*P > 0.05) antibody responses to rPlpE. Recombinant PlpE in a commercial proprietary adjuvant was highly immunogenic when injected subcutaneously into cattle. Vaccination of cattle with 100 μg of rPlpE to a commercial *M. haemolytica* vaccine, Prespone®, significantly enhanced (*P < 0.05) protection afforded by the vaccine against experimental challenge. Addition of rPlpE to commercial *M. haemolytica* vaccines could greatly enhance vaccine efficacy.

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1. Introduction

Bovine respiratory disease arises from the interaction of numerous contributing factors including physical stresses associated with weaning, shipment, inclement weather, and overcrowding coupled with viral and bacterial infections [1]. The end result in severe cases is colonization of the lungs with pathogenic bacteria resulting in severe pneumonia. *Pasteurella multocida*, *Haemophilus somnus* and *Mannheimia* (formerly *Pasteurella*) *haemolytica* are associated with bovine pneumonia. However, *Mannheimia haemolytica* serotype 1 (S1) is by far the most important and commonly isolated bacterial pathogen in development of the often-fatal fibrinous pleuropneumonia in beef cattle known as pneumonia aerobicus or shipping fever [2,3]. Despite the availability of numerous commercial vaccines for *M. haemolytica*, pneumonic pasteurellosis continues to be a major bovine health problem. Perino and Hunsaker [4] reviewed published field studies on commercial *M. haemolytica* vaccines and found that efficacy could be established in only 50% of the trials. Therefore, it is imperative that *M. haemolytica* vaccines be improved.

Immunity against *M. haemolytica* is thought to be primarily through production of serum antibodies that neutralize the secreted leukotoxin (LKT) and antibodies against surface antigens [5]. The specific surface antigens that are important in stimulating host immunity to *M. haemolytica* are not known; however, several studies point towards the importance of outer membrane proteins (OMPs). Pandher et al. [6] demonstrated 21 surface-exposed immunogenic outer membrane proteins in *M. haemolytica* S1 using protease treatment and Western blotting. High antibody responses to outer membranes, as measured by ELISA, and to several specific OMPs, as measured by quantitative Western Blotting, consistently correlated with resistance to challenge with virulent *M. haemolytica* S1 [7,8]. Vaccination of cattle with OMP-enriched cellular fractions, from *M. haemolytica* S1 significantly enhanced resistance of cattle against experimental challenge [9] in the absence of antibodies to LKT. One of the *M. haemolytica* OMPs to which high antibody responses correlated with resistance against experimental challenge is a major 45 kDa OMP [6,8]. Therefore, studies were undertaken to clone and characterize the gene for the major 45 kDa *M. haemolytica* S1 outer membrane lipoprotein (designated PlpE), which genetically has 32–35%...
similarity to an immunogenic lipoprotein, OmlA, demonstrated in *Actinobacillus pleuropneumoniae* serotypes 1 and 5 [11]. Affinity-purified, anti-PlpE antibodies recognized an OMP in all serotypes of *M. haemolytica* except in serotype 11, which has since been reclassified as a member of *Mannheimia glucosida* [10]. In addition, PlpE is surface-exposed and immunogenic in cattle. In complement-mediated killing assays, a significant reduction in killing of *M. haemolytica* was demonstrated when bovine immune serum depleted of anti-PlpE antibodies was used as the source of antibody. This suggests that antibodies against PlpE may contribute to host defense against the bacterium.

The purposes of these studies was to determine the immunogenicity of outer membrane lipoprotein PlpE from *M. haemolytica* S1, determine if commercial vaccines stimulate antibodies to it, and examine if addition of recombinant PlpE (rPlpE) to a commercial *M. haemolytica* vaccine will augment vaccine-induced immunity.

### 2. Materials and methods

#### 2.1. Bacterial culture

*M. haemolytica* S1 Oklahoma Strain was used for serology antigen preparation and for challenge of animals [12]. Frozen stock cultures were plated on brain–heart infusion (BHI) blood agar and grown at 37 °C in a 5% CO₂ environment for 18 h. An isolated colony from each was propagated in 10 ml BHI broth with rotary shaking at 120 oscillations/min for 18 h at 37 °C. One hundred microfilters of suspension was added to 11 of BHI broth and grown overnight. The bacteria were sedimeted by centrifugation of the suspension was added to 1 l of BHI broth and grown to the *OD*₆₀₀ = 1.350 OD₆₀₀. LKT was prepared from mannheimia glucosida S1 Oklahoma Strain was used for serology antigen preparation and for challenge of animals [12]. Antibodies to formalin-killed *M. haemolytica* whole bacterial cells (WC), to LKT, and to rPlpE were determined by centrifugation at 12,000 rpm for 30 min at 4 °C, and the supernatant containing the recombinant protein was passed through a 0.45 µm filter (Nalge Nunc, Rochester, NY) and loaded onto an affinity column packed with ProBond nickel-chelating resin that selectively binds recombinant proteins with 6 histidine residues (His-Tag) at either the N- or carboxy-terminus. In this instance, the His-Tag was at the N-terminus. The recombinant protein bound to the resin was then eluted with either a low pH buffer or by competition with imidazole. Because rPlpE is an insoluble protein, all of the purification steps were done in the presence of 6 M urea, which was removed by step-wise dialysis prior to injection of the protein. The purity of each preparation was determined by SDS-PAGE followed by Coomassie stain and Western blot with murine anti-rPlpE polyclonal ascites fluid (Fig. 1) that was produced against rPlpE by the Hybridoma Center at Oklahoma State University.

To produce polyclonal ascites fluid, multiple intraperitoneal 50–100 µg injections of rPlpE were given to Balb/c mice following the previously published protocol with the exception that RIBI adjuvant system (Corixa Corporation, Seattle, WA) was used [13]. To stimulate ascites the peritoneum was injected with 5 × 10⁷ Swiss Webster Sarcoma 180 ascites cells (ATCC, No. TIB-66, Manassas, VA).

#### 2.2. Cloning and purification of PlpE

The truncated form of plpE lacking the sequence encoding the putative signal peptide was amplified from pB4522 [10] with the help of a forward primer starting 5′-end and priming into the open reading frame of plpE and a reverse primer which is complementary to the 3′-end of the gene [10]. The amplimer was cut with BamHI and HinflII and ligated into an expression vector, pRSETA (Invitrogen, Carlsbad, CA), cut with the same restriction enzymes. Competent *E. coli* DH5α (genotype F-, Φ80lacZΔM15, 8 lacZYA-argF, U169, deoR, recA1, hsdR17(rk−,mk−), gal-, phoA, mpro44, lambda-, thi-1, gyrA96, relA1, phoA, Invitrogen, Carlsbad, CA) cells were transformed with the ligation mixture and transformants were plated on Luria–Bertani (LB) agar plates with 50 µg/ml of ampicillin. Transformants were screened and appropriate subclones were identified. Plasmid DNA isolated from such subclones was submitted to the Oklahoma State University Core Facility where the nucleotide sequence was determined by the ABI Model 3700 (Biosciences) automated DNA sequencing system. Once the nucleotide sequence of a representative subclone was compared to that deposited in the GenBank (AF059036), the recombinant plasmid was introduced into BL21(DE3)ΔλlysS (Novagen, Madison, WI) by transformation to express and purify rPlpE.

The expression of rPlpE was done according to the protocol recommended by the manufacturer of the vector and the expression host (Invitrogen, CA and Novagen, WI, respectively). Briefly, single colonies of BL21(DE3)ΔλlysS harboring the truncated_plpE in pRSETA, were inoculated into appropriate volumes of LB broth with 50 µg/ml ampicillin and 34 µg/ml chloramphenicol. The culture was incubated at 37 °C until *OD*₆₀₀ = 0.5 was attained at which time the synthesis of the recombinant protein was induced by adding IPTG (1 mM final concentration) and the induction was continued for at least 3 h. In order to purify rPlpE, the culture was harvested and lysed by sonication. The cellular debris was then removed by centrifugation at 12,000 rpm for 30 min at 4 °C, and the supernatant containing the recombinant protein was passed through a 0.45 µm filter (Nalge Nunc, Rochester, NY) and loaded onto an affinity column packed with ProBond nickel-chelating resin that selectively binds recombinant proteins with 6 histidine residues (His-Tag) at either the N- or carboxy-terminus. In this instance, the His-Tag was at the N-terminus. The recombinant protein bound to the resin was then eluted with either a low pH buffer or by competition with imidazole. Because rPlpE is an insoluble protein, all of the purification steps were done in the presence of 6 M urea, which was removed by step-wise dialysis prior to injection of the protein. The purity of each preparation was determined by SDS-PAGE followed by Coomassie stain and Western blot with murine anti-rPlpE polyclonal ascites fluid (Fig. 1) that was produced against rPlpE by the Hybridoma Center at Oklahoma State University. To produce polyclonal ascites fluid, multiple intraperitoneal 50–100 µg injections of rPlpE were given to Balb/c mice following the previously published protocol with the exception that RIBI adjuvant system (Corixa Corporation, Seattle, WA) was used [13]. To stimulate ascites the peritoneum was injected with 5 × 10⁷ Swiss Webster Sarcoma 180 ascites cells (ATCC, No. TIB-66, Manassas, VA).

#### 2.3. Serology

Antibodies to formalin-killed *M. haemolytica* whole bacterial cells (WC), to LKT, and to rPlpE were determined by enzyme-linked immunosorbent assays (ELISAs) [7,14]. For WC preparation, *M. haemolytica* S1 were prepared from a washed 24 h culture by suspending cells in 0.4% formalinized saline at a concentration determined spectrophotometrically to be 1.350 OD₆₀₀. LKT was prepared from
supernatant from a 3-h culture of *M. haemolytica* S1 grown in RPMI-1640 medium at 37 °C in a shaking incubator. The LKT was partially purified by precipitation with 40–60% ammonium sulfate as previously described [15]. The precipitate was resuspended in 3 M guanidine containing 59 mM NaHPO₄ and 100 mM NaCl. By SDS-PAGE of the LKT preparation, one intensely staining band was identified at 105 kDa and confirmed to be LKT on a western blot using an anti-LKT monoclonal antibody [14]. Leukotoxic activity was 10⁵ LKT U/ml [14]. The 2-keto-3-deoxyoctonate concentration was 7.5 μg per mg of protein [16].

Wells of 96-well microtiter plates were coated with WC at an optical density reading equivalent to 10⁵ CFU of a 24 h culture, with LKT at 50 ng per well, or with rPlpE at 50 ng per well. Sera were diluted in PBS-Tween 20 containing 1% BSA. ELISA for detection of serum antibodies to rPlpE was done in the first immunogenicity study using serum dilutions ranging from 1:400 to 1:819,200. Otherwise, sera were tested against various antigens at dilutions of 1:800 for WC, 1:1600 for LKT, and 1:1600 for rPlpE, which were in the linear range of established dilution curves. The extent of antibody binding was detected using a 1:400 dilution of horseradish peroxidase-conjugated, affinity purified rabbit anti-bovine IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Antibody responses are expressed as nanogram of immunoglobulin binding based on a set of IgG standards on each plate.

### 2.4. Animals

A total of 82 normal healthy beef calves (Hereford or Angus/Hereford cross) of mixed sex were used. The calves were weaned at around 6–8 months of age. All calves were vaccinated with 7-way Clostridial vaccine and leptospiral vaccine, and treated with anthelmintic 30 days prior to the study. The calves received free choice native grass hay supplemented with grain ration throughout the study. All animal studies followed protocols approved by the University Institutional Animal Care and Use Committee (Protocol #112).

#### 2.5. Anti-rPlpE responses with *M. haemolytica* vaccines

To determine if vaccination of calves with commercial or experimental *M. haemolytica* vaccines stimulate anti-rPlpE antibodies, two studies were done. The first experiment was a retrospective study using sera from 18 calves from previous vaccine studies [9,17]. Serum antibodies to rPlpE were determined using samples from the day of vaccination (day 0) and from day 14. On day 0, three calves each were vaccinated subcutaneously with one of the following commercial vaccines: *P. haemolytica* Toxoid, BR-SV-BVD-IBR-P-Vaccine (Pyramid™&Prespone®, Fort Dodge Laboratories), *P. haemolytica*-multocida Bacterin-Toxoid (Pulmo-guard™ PH-M, Boehringer Ingelheim), *P. haemolytica*-multocida-Salmonella typhimurium Bacterin-Toxoid (Poly-Bac B® 1, Texas Vet Labs) [17]. Sera was used from three calves that had each been vaccinated with 2 mg of an *M. haemolytica* Serotype 1 (Oklahoma strain), Sarcosyl: insoluble, outer membrane preparation in Freund’s incomplete adjuvant or from three calves that were subcutaneously vaccinated with 10⁵ CFU of live *M. haemolytica* Serotype 1 (Oklahoma strain) [9]. In addition, sera were analyzed from three nonvaccinated calves that had previously spontaneously seroconverted to *M. haemolytica* Serotype 1 based on increased antibody responses to WC and LKT in paired serum samples and were designated as Natural Exposure. Anti-WC and anti-LKT antibody responses have previously been reported for these sera [9,17,18].

The second vaccine experiment was a prospective study designed to follow the anti-rPlpE antibodies for 42 days after a single dose of a commercial *M. haemolytica* vaccine or rPlpE. Thirty calves were divided equally among six groups and vaccinated subcutaneously once each on day 0 with Prespone®. *P. haemolytica* Bacterin-Toxoid (One Shot™, Pfizer), an avirulent *M. haemolytica* culture (Once PMH™, Intervet), Pulmo-guard™ PH-M, or 100 μg of rPlpE in commercial adjuvant (Pfizer). Five unvaccinated calves served as controls. Sera were obtained on days 0, 7, 14, 21, 28, and 42, and antibodies to WC, LKT and rPlpE were determined.

#### 2.6. Recombinant PlpE Immunogenicity studies

For the rPlpE immunogenicity experiments, sera from all calves were screened for anti-*M. haemolytica* antibodies as measured by the ELISA to WC. All calves used in that experiment had measured serum antibody concentrations of <0.4 ng of IgG, a concentration that we have found previously to be considered as normal background for this assay with animals being susceptible to challenge with *M. haemolytica*. 

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**Fig. 1.** Coomassie stain of varying concentrations of purified recombinant PlpE used in the 4th Vaccination Trial (Dec 11, 2001).
To determine if rPlpE was immunogenic, one calf each was vaccinated once with either 10, 50, or 100 μg of rPlpE in a commercial proprietary adjuvant (Pfizer, Inc., Lincoln, NE). One calf remained as a nonvaccinated control. Sera were obtained on days 0 and 21 days after vaccination and evaluated for end-point antibody titers against rPlpE using two-fold serial dilutions. Twenty-four days after the initial vaccination, each calf and a nonvaccinated calf were transthoracically challenged with 5.0 × 10⁹ CFU of live M. haemolytica from an overnight culture, as previously described [12]. Four days later, calves were humanely killed, and lung lesion scores determined on a 20-point scale as previously described [12,19].

In a second cattle experiment, six calves were vaccinated with 100 μg of rPlpE in commercial adjuvant on day 0 and six calves remained as nonvaccinated controls. On day 21, all calves were challenged transthoracically with 1 × 10⁹ CFU of virulent M. haemolytica. Calves were humanely killed on day 25, and lung lesion scores determined. Antibody responses against rPlpE and M. haemolytica WC were determined on days 0, 7, 14 and 21 after vaccination.

In a third cattle experiment, Presponse® was obtained from the manufacturer, and 18 weanling beef steers were equally allocated among the following vaccine groups: Group 1—Presponse®, Group 2—Presponse® + 100 μg rPlpE, and Group 3—nonvaccinated. Calves were vaccinated on day 0 with 2 ml of Presponse (manufacturer’s recommended dosage) or 2 ml of Presponse mixed with 0.5 ml of rPlpE (100 μg). Antibody responses to M. haemolytica WC, rPlpE or to LKT were determined by ELISA on days 0, 7, 15, and 23. On day 24, calves in Groups 1, 2, and 3 were challenged transthoracically with 3.0 × 10⁹ CFU of M. haemolytica. Four days later, calves were humanely killed, and lung lesion scores determined.

2.7. Statistical analysis

Mean rectal temperatures, antibody responses and lesion scores among the various groups were compared by Student’s t-tests [20]. Mean rectal temperatures and antibody responses within groups were compared by paired t-tests. Differences were considered significant when P < 0.05. Linear regression analyses were done to determine if there was a significant correlation between antibody response and lesion score [20].

3. Results

3.1. M. haemolytica vaccines

In the first vaccine experiment, vaccination of calves with commercial vaccines, M. haemolytica outer membranes, and live M. haemolytica resulted in a nonsignificant increase (P > 0.05) in antibodies to rPlpE (Fig. 2). In contrast, sera from those calves that had previously had a natural exposure to M. haemolytica, as indicated by spontaneous seroconversion, had a significant increase (P < 0.05) in anti-rPlpE antibodies. All vaccine-induced responses and natural exposure were substantially less than the antibodies produced in a calf vaccinated with 100 μg of rPlpE in commercial adjuvant. There were no significant differences (P > 0.05) among the antibody responses to rPlpE on day 14 for any of the commercial vaccines, live M. haemolytica vaccinated, or natural exposure groups. Antibody responses to M.

![Fig. 2. Anti-rPlpE antibody responses of cattle that spontaneously seroconverted to M. haemolytica (natural infection) or were vaccinated with commercial vaccines, M. haemolytica outer membranes (OMPs), or live M. haemolytica. The bar labeled rPlpE represents one calf vaccinated with 100 μg of rPlpE in a commercial adjuvant and is included as a positive reference point.](image-url)
haemolytica LKT and WC significantly increased ($P < 0.05$) for Pulmo-guard™, and the live bacteria-vaccinated and natural exposure calves, whereas vaccination with outer membranes stimulated a significant ($P < 0.05$) antibody response to WC and vaccination with Poly-Bac and Presponse® failed to stimulate significant ($P > 0.05$) antibody responses to either *M. haemolytica* antigen. Those data were previously published [9,17].

In the second vaccine experiment, vaccination of calves with one of four commercial *M. haemolytica* vaccines resulted in nonsignificant increases ($P > 0.05$) in antibodies to rPlpE (Fig. 3). Vaccination of calves with 100 μg of rPlpE in commercial adjuvant stimulated a significant increase ($P < 0.05$) in antibody responses to rPlpE by day 7. That response continued to increase until it peaked on day 21 after vaccination. Vaccination with each commercial vaccine and with rPlpE resulted in significant increases ($P < 0.05$) in antibodies to *M. haemolytica* WC by day 7 (One Shot and Presponse®) and by day 14 (Once PMH, Pulmo-guard™, and rPlpE) (Fig. 3). Those responses remained significantly increased ($P < 0.05$) through day 14 (Once PMH and Presponse®) and day 42 (One Shot, Pulmo-guard™, and rPlpE). Peak antibody responses for One Shot—vaccinated calves were significantly greater ($P < 0.05$) than peak responses for Once PMH, Presponse® or rPlpE vaccinates. Although antibody responses to LKT increased after vaccination with each commercial vaccine, only the responses initiated by Pulmo-guard™ and One
fig. 4. Anti-rPlpE antibody response of six cattle vaccinated with 100 μg of rPlpE on day 0. Anti-M. haemolytica LKT (A), anti-M. haemolytica whole cells (B), and anti-rPlpE (C).

Shot were significantly increased (P < 0.05) beginning on day 7 through day 28. Anti-LKT antibodies did not increase for the rPlpE vaccinates. Peak anti-LKT antibody responses for Pulmo-guard™-vaccinated calves were significantly greater (P < 0.05) than peak responses for Once PMH, Presponse® or rPlpE vaccinates, whereas peak anti-LKT antibody responses for One Shot—vaccinated calves were significantly greater (P < 0.05) than peak responses for Presponse® vaccinates.

3.2. Recombinant PlpE immunogenicity

In the first immunogenicity experiment that determined end-point anti-rPlpE titers in response to various doses of rPlpE, serum from the nonvaccinated calf had an end-point antibody titer of 1:400 against rPlpE. Sera from the 10, 50, and 100 μg vaccinates had titers of 1:12,800, 1:25,600, and 1:25,600, respectively. Transthoracic challenge of those calves with virulent M. haemolytica resulted in clinical signs of lethargy, anorexia and dyspnea that lasted <24 h for the vaccinates and for 4 days for the nonvaccinated calf. Evaluations of lungs post-mortem indicated a lesion score of 15.5 (20 maximum severity) for the nonvaccinated control calf. Lesion scores for the 10, 50, and 100 μg-vaccinates were 4.5, 3.0, and 3.5, respectively.

In the second immunogenicity experiment, vaccination with rPlpE on day 0 stimulated a significant increase (P < 0.05) in antibodies to rPlpE and to M. haemolytica WC on day 7 (Fig. 4). Those responses continued to increase to a maximum on day 20 and declined insignificantly (P > 0.05) on day 25, whereas antibodies to rPlpE and to WC failed to increase for the nonvaccinated calves. Anti-LKT antibodies did not significantly increase (P > 0.05) for either the rPlpE-vaccinated or control groups. Transthoracic challenge of calves with virulent M. haemolytica resulted in clinical signs of lethargy, anorexia and dyspnea that lasted <24 h for the vaccinates and for two–three days for the nonvaccinated calves. All calves survived the challenge, and mean lesion scores (±standard deviation) after challenge were 7.0 ± 3.8 for nonvaccinates and 4.1 ± 3.0 for the rPlpE vaccinates, a 41.4% reduction in lesion scores. Those differences were not statistically significant (P = 0.07). Vaccination and challenge conditions were similar for the control calves and for the 100 μg-vaccinates between immunogenicity experiments one and two. Therefore, the lesion score data from experiment one was combined with the lesion score data from
experiment two. This resulted in the mean lesion score for nonvaccinated controls was 8.2 ± 4.7 and mean lesion score for rPlpE vaccinates was 3.9 ± 2.6, a 52.1% reduction in lesion scores, which were significantly different (P < 0.05).

3.3. Augmentation of commercial vaccine with rPlpE

Because vaccination with commercial *M. haemolytica* vaccines stimulated low antibody responses to rPlpE, we investigated the augmentation of a commercial vaccine with rPlpE. Vaccination with Presponse® stimulated a nonsignificant increase (P > 0.05) in anti-rPlpE antibodies on day 15. Those responses were not significantly different (P > 0.05) than were antibody responses of the nonvaccinated control calves on days 7, 15, and 23 (Fig. 5). Presponse®-rPlpE vaccination stimulated a significant increase (P < 0.05) in anti-rPlpE antibodies on days 7, 15 and 23, and those responses were significantly higher (P < 0.05) than responses for the Presponse®-vaccinated or nonvaccinated control calves. Anti-WC and anti-LKT responses were significantly increased (P < 0.05) on days 7 and 15 for the Presponse® and Presponse®-rPlpE vaccinates. Those responses were not significantly different (P > 0.05) between those groups, whereas they were significantly greater (P < 0.05) than were anti-WC and anti-LKT antibody values for the nonvaccinated control group.

Rectal temperatures were taken on the day of challenge (day 24) and for the next 3 days. Rectal temperatures remained essentially normal for all calves except for the nonvaccinated Control group. Clinical signs of depression and dyspnea subsided within 12 h for the vaccinates. In the control group, rectal temperatures significantly increased (P < 0.05) on days 25 and 26, declining insignificantly (P > 0.05) on day 27. All control calves demonstrated depression, anorexia and dyspnea for 1.5–4 days after challenge. On days 26 and 27, mean rectal temperatures for the nonvaccinated Control group were significantly greater (P < 0.05) than for either the Presponse® or Presponse®-rPlpE groups. At necropsy, mean lung lesion scores were 7.9 ± 3.6 for nonvaccinated controls, 3.0 ± 1.3 for Presponse® vaccines (62.0% reduction in lesion score), and 1.1 ± 0.9 for Presponse®-rPlpE vaccines.
(86.1% reduction in lesion scores). Differences between the Presponse® and Control and Presponse®/rPlpE and Control lesion scores were significant (P < 0.05). In addition, mean lesion score for the Presponse®/rPlpE group was significantly lower (P < 0.05) than for the Presponse® group. There was a significant correlation (r = −0.598, P < 0.001) between high serum antibody responses to rPlpE at day 23 and low lesion scores.

4. Discussion

Pandher et al. [10] previously found that M. haemolytica and recombinant E. coli expressing rPlpE were capable of absorbing anti-rPlpE antibodies from bovine immune serum, indicating that rPlpE is surface exposed. In complement-mediated killing assays, they found a significant reduction in killing of M. haemolytica when bovine immune serum was depleted of anti-rPlpE antibodies and concluded that antibodies against rPlpE may contribute to host defense against M. haemolytica. In the current studies, we demonstrated that rPlpE is highly immunogenic for cattle and that vaccination with rPlpE reduced lung lesions after experimental challenge with the bacterium. Therefore, our in vivo studies corroborated previous in vitro studies and indicated that anti-rPlpE antibodies can contribute to host defense against M. haemolytica infection.

In the current study, natural exposure or vaccination of cattle with commercial M. haemolytica vaccines, live M. haemolytica or outer membranes stimulated low antibody responses to rPlpE. For the vaccines, the rise in antibodies to rPlpE as measured on various days was not significant, and even those vaccines that stimulated high antibodies to M. haemolytica WC and LKT still stimulated low anti-rPlpE response. Commercial vaccine-induced anti-rPlpE antibody responses were substantially lower than those stimulated by vaccination with 100 µg of rPlpE in a commercial adjuvant. This was an expected finding, because commercial vaccines vary greatly in their composition in that some are composed of culture supernatants and bacterial cell components, others contain whole bacterial cells, and one is a live mutant. A somewhat surprising finding was that calves previously vaccinated with M. haemolytica outer membranes in Freund’s incomplete adjuvant had low antibody responses to rPlpE on day 14 [9]. Therefore, although PlpE is a major outer membrane protein, its concentrations in commercial and experimental vaccines are most likely low and variable. In addition, the adjuvant used may play an important role in stimulating antibodies to PlpE.

Because commercial vaccines stimulated low antibodies to rPlpE, we added 100 µg rPlpE to the commercial vaccine Presponse® to augment the antibody responses to rPlpE. Results of that study demonstrated that Presponse®/rPlpE stimulated greater protection against challenge than did Presponse® alone. Conlon et al. [21] previously demonstrated that addition of recombinant LKT enhanced the efficacy of a culture supernatant vaccine and decreased clinical signs and pneumonic lesions. Therefore, addition of one or more recombinant proteins to a M. haemolytica vaccine could be used by animal health companies to provide better products for protection of cattle against shipping fever.

In the present study, anti-LKT and anti-WC antibody responses were consistently, though nonsignificantly, lower in cattle vaccinated with Presponse®/rPlpE compared to those vaccinated with Presponse® alone. The cause of this observation is not known; however, no additional adjuvant was added to the Presponse®/rPlpE vaccine. Therefore, the somewhat lower antibody responses in the Presponse®/rPlpE group could have been related to less than optimal adjuvant concentrations for the additional protein. Despite that finding, the Presponse®/rPlpE group still had lower lesion scores even though their anti-LKT and anti-WC antibodies were lower underscoring the potential importance of anti-rPlpE antibodies in enhancing resistance against M. haemolytica infection.

In a recent survey, researchers found that M. haemolytica isolates from bovine respiratory disease from upper Midwestern United States were 60% S1, 26% S6 and 7% S2 with the remaining isolates from A9, A11 and untypeable strains [22]. In another study, 60% of M. haemolytica isolates from cattle in a Texas feedyard were S1, whereas 40% were serotypes S2, S6, or S5 [2]. Therefore, although serotype 1 is the most common isolate from shipping fever, other serotypes play a role in the disease. Currently available M. haemolytica vaccines contain serotype 1 exclusively and therefore may or may not provide efficacious immunity against other serotypes. Cross serotype protection as stimulated by outer membrane vaccines or bacterins is limited. Others and we have shown that antibodies against M. haemolytica serotype 1 LKT will cross neutralize the toxin prepared from other serotypes [23,24]. Therefore, commercial vaccines that stimulate anti-LKT antibodies should provide some cross protection against other serotypes. However, Conlon et al. [21] demonstrated that vaccination with recombinant LKT alone failed to stimulate protection against experimental M. haemolytica challenge, and Purdy et al. [25] found that vaccination of goats with LKT-impregnated agar beads stimulated incomplete immunity. Shewen and Wilkie [5] demonstrated that immunity to M. haemolytica was directed against both surface antigens and LKT. The actual surface antigen of importance in stimulating protection is not known; however, studies indicate that it is most likely outer membrane proteins and not capsular polysaccharide or lipopolysaccharide. Pandher et al. [10] demonstrated the presence of a PlpE—like protein in outer membranes of all M. haemolytica serotypes except serotype 11, which has since been reclassified as a member of Mannheimia glucosida. There was some variation in molecular masses among the various proteins. With the findings of this study, demonstrating immunogenicity of rPlpE and augmentation of a commercial vaccine that stimulates anti-LKT antibodies, the previous demonstration of a PlpE—like protein in...
most serotypes and that anti-LKT antibodies can neutralize LKT from other serotypes, the addition of rPlpE to a commercial vaccine that stimulates anti-LKT antibodies could enhance cross serotype protection in shipping fever.

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