Proteomic Analysis and Immunogenicity of *Mannheimia haemolytica* Vesicles

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*Mannheimia haemolytica*, a major causative agent in bovine respiratory disease, inflicts extensive losses each year on cattle producers. Commercially available vaccines are only partially efficacious. Immunity to *M. haemolytica* requires antibodies to secreted toxins and outer membrane proteins (OMPs) of the bacterium. Gram-negative bacteria produce membrane blebs or vesicles, the membrane components of which are primarily derived from OMPs. Accordingly, vesicles have been used as immunogens with various degrees of success. This study characterized components of *M. haemolytica* vesicles and determined their immunogenicity in mice and cattle. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of vesicles from this bacterium identified 226 proteins, of which 58 (25.6%) were OMPs and periplasmic and one (0.44%) was extracellular.

Vesicles were used to vaccinate dairy calves and BALB/c mice. Analyses of sera from calves and mice by enzyme-linked immunosorbent assay (ELISA) showed that circulating antibodies against *M. haemolytica* whole cells and leukotoxin were significantly higher on days 21 and 28 (*P* < 0.05) than on day 0. For control calves and mice, there were no significant differences in serum anti-whole-cell and leukotoxin antibody levels from days 0 and 21 or 28, respectively. Lesion scores of lungs from vaccinated calves (15.95%) were significantly (*P* < 0.05) lower than those from nonvaccinated calves (42.65%). Sera from mice on day 28 and calves on day 21 showed 100% serum bactericidal activity. Sera from vesicle-vaccinated mice neutralized leukotoxin.

*M. haemolytica* is the major causative agent of severe, often fatal, respiratory disease in cattle (1). Vaccination of cattle with commercial *M. haemolytica* vaccines is only partially efficacious, and antimicrobial treatment of *M. haemolytica* cases is costly and impractical (2).

Immunity to *M. haemolytica* is based on the immune response to leukotoxin (LKT) and outer membrane proteins (OMPs) (3). An immunoproteomic study of *M. haemolytica* OMPs conducted in our laboratory identified 57 OMPs that may have the potential to be developed into vaccines (4). The immunogenicity of recombinant forms of several *M. haemolytica* OMPs, including PlpE, OmpA, Plf, OmpP2, serotype 1-specific antigen (SSA-1), and OmpD15, has been studied (5–10). Vaccination of calves with recombinant PlpE partially protects cattle against challenge with virulent *M. haemolytica* and significantly enhances the efficacy of commercial vaccines (7–9). Chimeric vaccines comprising one or more copies of the immunodominant epitope of PlpE and the neutralizing epitope of LKT (11) stimulated antibodies with potent complement-mediated cell killing and LKT-neutralizing activities, whereas cattle vaccinated with chimeric vaccines in combination with *M. haemolytica* bacterins had 71% fewer lung lesions than did control cattle (7).

Commercial animal health companies market culture supernatants or bacterin-toxoid combination vaccines (12). Supplementing commercial vaccines with recombinant OMPs substantially enhanced their efficacy (8, 9). However, with the low profit margin on bovine vaccines, commercialization of recombinant-protein-based or recombinant-protein-augmented vaccines has not come to fruition.

Inexpensive, efficacious, and alternative approaches to bovine bacterial vaccines are needed as substitutes for traditional bacteria and recombinant proteins. One such approach from other bacterial studies is bacterial vesicle vaccines. Growing, Gram-negative bacteria produce closed outer membrane blebs that detach as vesicles, which contain OMPs, lipopolysaccharide (LPS), periplasmic proteins, peptidoglycans, and secretory components such as toxins (13, 14). Because they contain a full complement of surface antigens, secretory proteins, and toxins, use of membrane vesicles as a nonliving, acellular vaccine has been studied with several bacteria (15–18). In addition, vesicles can serve as their own adjuvants, which can further decrease production costs (19). To our knowledge, outer membrane vesicles have not previously been demonstrated in *M. haemolytica*. We, therefore, undertook to identify proteins in *M. haemolytica* vesicles (MHVs) and to determine the immunogenicity of MHVs.

**MATERIALS AND METHODS**

**Bacterial strain and growth conditions.** *Mannheimia haemolytica* serotype S1, strain 89010807N, originally isolated from a case of calf pneumonia, was used for this study (20). Growth conditions of the bacterium have been described previously (4).

**Preparation of *M. haemolytica* vesicles.** Membrane vesicles were extracted and purified as previously described with slight modifications (21, 22). An overnight starter culture was used to seed larger volumes of brain heart infusion (BHI) broth in 1- to 2-liter Erlenmeyer flasks. The culture was incubated in a 37°C shaker incubator until the optical density at 600 nm (OD$_{600}$) was 1.0. The cells were removed by centrifugation at 10,000 × g, and the supernatant was filtered through an 0.2-μm filter. The
supernatant was concentrated by a centrifugal filter device with a molecular mass cutoff of 100,000 Da (EMD Millipore, Billerica, MA). Debris was removed by centrifugation at 20,000 × g, 4°C, and pellets containing vesicles were collected by centrifugation at 150,000 × g, 4°C, for 3 h. Pellets were resuspended in Dulbecco’s phosphate-buffered saline (DPBS), and the concentration of vesicles was determined by the bicinchoninic acid (BCA) assay (Thermo Scientific, Rockford, IL).

The shapes and appearances of negatively stained MHV preparations were determined with a JEOL JEM-2100 scanning transmission electron microscope (JEOL, Tokyo, Japan) in scanning electron microscope mode.

### Identification of proteins with LC-MS/MS

Proteins from MHV’s were cleared using a two-dimensional (2-D) cleanup kit (Bio-Rad, Hercules, CA), dissolved in Tris-buffered urea, reduced and alkylated using Tris(2-carboxyethyl)phosphine and iodoacetamide (11), and then diluted 4-fold and digested with trypsin. Peptides were separated by nano-liquid chromatography (nano-LC) on C18 columns and eluted directly into an LTQ-OrbitrapXL mass spectrometer for one full-range Fourier transform mass spectroscopy (FT-MS) scan and six concurrent data-dependent tandem mass spectrometry (MS/MS) scans. The LC-MS/MS data were searched via Mascot v2.2.04 (Matrix Science) and X! Tandem dem mass spectrometry (MS/MS) scans. The viability of control and LKT-treated target cells was determined by enzyme-linked immunosorbent assay (ELISA) using 2-fold serial dilutions of murine sera ranging from 1:400 to 1:819,200, whereas bovine serum antibodies were determined by a single-dilution ELISA (5, 9, 26). Sera collected from vaccinated or control mice and calves were used as primary antibodies. Affinity-purified, horseradish peroxidase-conjugated goat anti-mouse or goat anti-bovine IgG(H + L) (Kirkegaard and Perry Labs, Gaithersburg, MD) was used as secondary antibody, and o-phenylenediamine (Amresco, Solon, OH) was used as the substrate as described previously (3). Plates were read at 490 nm on a Vmax kinetic microtiter plate reader (Molecular Devices, Sunnyvale, CA). Statistically defined endpoint titers were calculated by the method of Frey et al. (27).

### Complement-mediated, serum bactericidal assay

Complement-mediated killing assays were performed as previously described (11). Briefly, decapsulated *M. haemolytica* cells were incubated with heat-inactivated sera in the presence or absence of complement source for 30 min at 37°C. Aliquots were plated on BHI blood agar plates at the beginning ($T_0$) and after 30 min ($T_{30}$) of incubation. All plates were incubated at 37°C overnight. Percent killing was calculated as $\frac{(T_0 - T_{30})}{T_0} \times 100$. Bovine hyperimmune serum from a calf vaccinated with rPpE (26) and naive bovine serum were used as positive and negative controls, respectively (8). The assay was repeated at least three times.

### Leukotoxin neutralization assay

A colorimetric microtitration assay for quantifying LKT cytotoxicity to the BL3.1 cell line was done as previously described (11). The viability of control and LKT-treated target cells with and without sera was determined using the methylthiazole tetrazolium (MTT) assay (Sigma, St. Louis, MO). Mouse sera from three mice each from the two 50-µg MHV, Pulmo-Guard PHM-1, and control groups were used in the assay. Percent neutralization was calculated using the formula $1 - \left( \frac{OD_{untreated} - OD_{untreated control}}{OD_{test sample wells containing toxin})/OD_{untreated control cells - OD}_{complete-toxicity wells} \right) \times 100$.

### Statistical analyses

Antibody responses, cumulative clinical and *M. haemolytica* reisolation scores, and percent lung lesions between vaccine-vaccinated and control calves were compared by the Student t test (23). Antibody responses among mouse groups were compared by analysis of variance (ANOVA).

### RESULTS

**Electron microscopy**. Electron microscopic examination of *M. haemolytica* demonstrated numerous membrane vesicles that were oval to round and 10 to 20 nm in diameter (Fig. 1).

**Proteomic analysis of *M. haemolytica* vesicles**. A total of 226 proteins (see Table S1 in the supplemental material) were identified in vesicle preparations of *M. haemolytica* grown in BHI broth to an A$_{600}$ of 1.0. The subcellular locales of identified proteins were predicted by PSORTb (Fig. 2). A total of 104 (46%) proteins were identified in vesicle preparations of *M. haemolytica* grown in BHI broth to an A$_{600}$ of 1.0. The subcellular locales of identified proteins were predicted by PSORTb (Fig. 2). A total of 104 (46%) proteins were

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**TABLE 1** Vaccine formulations for immunization of mice

| Group | Antigen dose | FIA | No. of mice |
|-------|--------------|-----|-------------|
| 1     | 10 µg        | Yes | 20          |
|       | 25 µg        |     | 20          |
|       | 50 µg        |     | 20          |
| 2     | 10 µg        | No  | 20          |
|       | 25 µg        |     | 20          |
|       | 50 µg        |     | 20          |
| 3 (Pulmo-Guard PHM-1) | 1/10 cattle dose | No | 20 |
| 4 (Control) | 0 | Yes | 30 |

*Vaccination was performed on days 0 and 14. Bleeding was performed on days 14 and 28 (10 mice).*

**TABLE 2** Protocol for experiment with calves

| Group | Antigen dose | FIA | No. of calves | Vaccination days | Bleed times |
|-------|--------------|-----|---------------|------------------|-------------|
| Vesicle 1 | 150 µg | Yes | 4 | 0 and 14 | Days 0, 7, 14, 21, and 28 |
| Control | PBS | Yes | 4 | 0 and 14 | Days 0, 7, 14, 21, and 28 |

*Total 8*
characterized as being of cytoplasmic origin, 58 (25.6%) were from either the periplasmic or the outer membrane, one (0.4%) was extracellular, 5 (2.2%) were from the cytoplasmic membrane, and 58 (25.6%) were of unknown locale. Analysis of 58 unknown proteins by LipoP, an algorithm that predicts whether the proteins form signal peptides or lipoproteins with signal peptides, showed that of the 58 proteins, 16 (27.5%) were predicted to be cytoplasmic, 22 (38%) were lipoproteins with signal peptides, and 20 (34.5%) were proteins with signal peptides. Overall, 100 (44.2%) of the proteins identified in vesicle preparations of this bacterium are proteins that are identified in other bacterial vesicle preparations.

Functional analysis of proteins of MHVs shows that 31.8% (72 proteins) of the MHV proteins are involved in transport, cell structure, virulence, etc., and therefore may contribute to immunity against *M. haemolytica*.

**Serologic responses of mice.** Anti-whole-cell and LKT antibodies in sera from mice vaccinated with MHV or Pulmo-Guard PHM-1 were determined. Statistical analyses showed that there was a significant increase \( (P < 0.05) \) in antibodies to both ligands between days 0 and 28 in sera from mice vaccinated with MHV and Pulmo-Guard PHM-1 (Fig. 3A). Antibody responses to both ligands in sera from nonvaccinated mice remained low. Analysis of the data demonstrated a significant increase of anti-LKT and anti-whole-cell antibodies with increasing MHV doses \( (P < 0.05) \) (Table 3).

**Calf immunization and challenge.** Calves vaccinated with MHV showed a significant increase in anti-whole-cell and anti-LKT antibody titers between days 0 and 21 \( (P < 0.05) \) and a significantly higher antibody response than that of controls \( (P < 0.05) \) (Fig. 3B). Following challenge, calves from each group developed mild to moderate clinical signs of respiratory disease, in-

![FIG 1](image1.jpg) **FIG 1** Electron micrographs of *M. haemolytica* cells (A) magnified \( \times 6,000 \) and vesicles (B) with a magnification of \( \times 50,000 \).

![FIG 2](image2.jpg) **FIG 2** Chart showing subcellular locations of proteins identified in *M. haemolytica* vesicles as determined by PSORTb. Of particular interest are the 58 (25.66%) proteins that constitute periplasmic and outer membrane proteins and the single extracellular protein, which happens to be leukotoxin.

![FIG 3](image3.jpg) **FIG 3** (A) Mean ELISA showing anti-whole-cell (Anti-WC) and antileukotoxin (Anti-LKT) antibody levels in day 28 sera collected from 8 groups of mice \( (n = 10/\text{group}) \) vaccinated with MHV or Pulmo-Guard PHM-1 (PMG) and nonvaccinates (CNTRL). (B) Circulating anti-whole-cell and anti-LKT antibodies in day 0 and 21 sera collected from 4 calves vaccinated with MHV and 4 nonvaccinates (Neg. Cntrl).

**TABLE 3** Leukotoxin neutralization by 1:10 dilution of sera from mice vaccinated with 50 \( \mug \) of MHV with or without FIA, a 1/10 cattle dose of Pulmo-Guard PHM-1, or PBS with FIA.

| Target with or without LKT | Sera from vaccinates | Mean \( OD_{690} \pm SD^a \) | % neutralization$^b$ |
|---------------------------|---------------------|---------------------------|----------------------|
| BL3 cells only            | None                | 1.2 \( \pm \) 0.28        | 0                    |
| BL3 cells + LKT           | None                | 0.24 \( \pm \) 0.08       | 0                    |
| BL3 cells + LKT + MHV     | MHV + FIA           | 0.87 \( \pm \) 0.17       | 65.6                 |
| BL3 cells + LKT           | MHV alone           | 0.67 \( \pm \) 0.10       | 44.8                 |
| BL3 cells + LKT + PHM-1   | Pulmo-Guard PHM-1   | 0.71 \( \pm \) 0.11       | 49.0                 |
| BL3 cells + LKT + PBS     | FIA                 | 0.27 \( \pm \) 0.05       | 3.1                  |

$^a$ OD values followed by different capital letters were significantly different \( (P > 0.05) \).

$^b$ % neutralization = \( 1 - \frac{\text{OD of untreated control cells} - \text{OD of test sample wells containing toxin}}{\text{OD of untreated control cells} - \text{OD of complete-toxicity wells}} \) \( \times 100 \).
cluding dyspnea, fever, and depression. The mean cumulative clinical score for control calves (9.5 ± 2.1) was significantly higher (P < 0.05) than that for the vaccinated calves (5.3 ± 2.8 (Table 4). Lung lesions were 62.9% smaller for MHV-vaccinated calves (15.9% ± 9.8%) than for control calves (42.7% ± 18.0%) (P < 0.05).

**Serum bactericidal assay.** Sera from nonvaccinated mice on days 0 and 28 or sera from MHV-vaccinated mice on day 0 had no bactericidal activity in the presence or absence of complement (Fig. 4 and 5). Complement-mediated killing by day 28 sera from mice vaccinated with MHV was 100% in the presence of complement and almost nonexistent in the absence of complement. Even though Pulmo-Guard PHM-1 induced the strongest immune response as measured by ELISA, sera from those mice did not exhibit any bactericidal activity.

Due to natural seroconversion, the bactericidal activity of sera from calves was more complicated. Undiluted sera from calves that were vaccinated and nonvaccinated with MHV exhibited 100% killing activity in the presence of a source of complement. However, when both sets of calf sera were diluted 100-fold, bactericidal activity of nonvaccinates on days 0 and 21 was reduced to background level, whereas MHV-vaccinated calves remained at or close to 100% killing in the presence of complement.

**DISCUSSION**

Comparison of proteins identified in our earlier immunoproteomic analysis of *M. haemolytica* OMPs (4) to the current findings shows that generally more proteins (226) were identified in MHV preparations than in the former study, in which 132 proteins were characterized. Prediction with PSORTb of subcellular locales of both sets of proteins followed the same trend. Accordingly, the MHV and immunoproteomic OMP study identified 28 and 16 proteins as OMPs, 30 and 8 as periplasmic proteins, 104 and 55 as cytoplasmic proteins, 5 and 7 as cytoplasmic membrane proteins, 1 and 1 as extracellular proteins, and 58 and 45 as unknown proteins, respectively. The apparent difference is because the immunoproteomic study identified only proteins that reacted with immune sera obtained from cattle either naturally exposed to *M. haemolytica* or vaccinated with components of the bacterium, as opposed to all proteins in the MHV study. Close scrutiny of OMPs identified in both studies shows that only 11 of the 16 OMPs identified in the immunoproteomic analysis were found in vesicles of *M. haemolytica*, even though 28 OMPs were identified in MHV. Possible explanations for the differences could be due to methods of extraction of MHVs and OMPs and databases used in the two studies. The database used in our earlier studies was an incomplete *M. haemolytica* genome sequence, whereas the MHV findings were compared to current, more complete genomic data.

MHVs or blebs are constantly shed by Gram-negative bacteria and are believed to be a mechanism for secretion and transfer of macromolecules to animals (28, 29). Moreover, bacterial vesicles have been successfully used as vaccines or components of vaccines in pathogenic bacteria, including *Vibrio cholerae* (30), the oral

**FIG 4** Mean complement-mediated bacterial killing of sera from mice vaccinated with *M. haemolytica* MHV and Pulmo-Guard PHM-1 (PMG) and nonvaccinated control mice (Neg. Cntrl). Serum from a calf vaccinated with rPlpE, a highly immunogenic outer membrane protein from *M. haemolytica*, was used as a positive control. C+ and C− designate the presence and absence, respectively, of complement in the assays.

**FIG 5** Mean bactericidal activity of sera from Holstein calves that were vaccinated with *M. haemolytica* MHV and nonvaccinated control calves (Neg. Cntrl). C+ and C− designate the presence and absence, respectively, of complement in each assay. The positive control is serum from a calf vaccinated with the recombinant form of the highly immunogenic *M. haemolytica* outer membrane protein PlpE. Note that there is no killing activity in the absence of complement in all cases.

**TABLE 4** Clinical, microbiologic, and pathological data from *M. haemolytica* vesicle-vaccinated and control calves after challenge with live *M. haemolytica*

| Group    | Adjuvant | Vaccination days | Challenge day | Mean cumulative clinical score ± SD (% reduction compared to control value) | Mean cumulative *M. haemolytica* isolation score ± SD (% reduction compared to control value) | Mean % pneumonia ± SD (% reduction compared to control value) |
|----------|----------|------------------|---------------|--------------------------------------------------------------------------------|---------------------------------------------------------------------------------|---------------------------------------------------------------|
| Vesicle vaccinated | FIA      | 0, 14            | 21            | 5.3 ± 2.8* (44.2)                                                              | 1.4 ± 0.9* (60)                                                                | 15.9 ± 9.8* (62.8)                                           |
| Control  | FIA      | 0, 14            | 21            | 9.5 ± 2.1                                                                       | 3.5 ± 1.7                                                                      | 42.7 ± 18.0                                                  |

*P < 0.05 compared to control values.

*P = 0.0515 compared to control values.
neutralizing function. The cause of the lack of functional bacteri-
late complement-mediated killing; however, it did have an LKT-
antibodies. Surprisingly, serum from those mice failed to stimu-
with the bacterin-toxoid developed high anti-whole-cell and LKT
properties have been demonstrated using meningococcal and E.
coli bacterial vesicles (19, 34). Assets of the vesicles responsible for
adjuvant property include lipopolysaccharide, a strong B-cell
stimulant, and other OMPs, such as OmpA, that can function as
pathogen-associated molecular patterns for pattern recognition
by the innate and adaptive arms of the immune system (16, 35).
Several studies have proposed that bacterial vesicles can be used as
substitutes for chemical adjuvants with viral vaccines, conjugated
to other bacterial immunogens, or for intranasal delivery (36, 37).
Bacterial vesicle vaccines, however, are not uniformly efficacious
in that meningococcal vesicles conjugated with capsular polysac-
charide are weak immunogens in infants and neonatal mice (38).

In this study, we evaluated MHV vaccine efficacy using an M.
haemolytica challenge model in young dairy calves. M. haemolytica
vaccines have traditionally been tested using one to two doses of
vaccine with intratracheal, intrabronchial, or transthoracic chal-
lenge approximately 14 days after the last vaccination (12, 39).
Clinical signs are usually evaluated and cattle are euthanized and
necropsied 4 to 6 days after challenge, with lung lesions quantified
and compared to lesions in nonvaccinates. The assessment of clin-
ical signs and lesions 4 to 6 days after challenge has been com-
monly used in assessment of M. haemolytica vaccine (7, 39–42). As
early as 1977, it was reported that clinical signs peaked at 9 to 18 h
after challenge, and lesions of acute pneumonia were readily ac-
cessible at day 3 with evidence of early healing by day 7 (43, 44). In
the current study, clinical scores peaked on day 24 after challenge
and remained steady in control calves (data not shown). There-
fore, given the literature, our previous experience with M. haemo-
lytica challenge, and the clinical findings in this experiment, day 5
was selected for necropsy and evaluation. In our cattle vaccine
experiment, we were restricted by the number of cattle available,
and because of their young ages, we opted to include adjuvant in
our vaccine preparations. Future studies are needed to determine
if adjuvant is required for MHV vaccine in cattle and, if so, to
examine numerous adjuvants for their efficacy in augmenting
MHV vaccine-induced immunity.

In the current study, we used a commercial M. haemolytica
bacterin-toxoid as a positive control for antibody production in
mice. The 1/10 cattle dose is the USDA-recommended dose for
testing a cattle vaccine in mice (3). A previous cattle study using
that vaccine as part of a bovine respiratory disease control pro-
gram demonstrated efficacy in a feedlot (45). Mice vaccinated
with the bacterin-toxoid developed high anti-whole-cell and LKT
antibodies. Surprisingly, serum from those mice failed to stimu-
late complement-mediated killing; however, it did have an LKT-
neutralizing function. The cause of the lack of functional bacteri-
cidal antibodies is not known. Others have shown differences
between immune responses and vaccine efficacy among different
mouse strains (46). In other Gram-negative bacteria, development
of blocking antibodies against OMPs and of serum resis-
tance has been demonstrated (47). Blocking antibodies directed
against an N. meningitidis lipoprotein reduced meningococcal
killing (48).

In conclusion, MHV vaccines stimulated a protective immune
response in calves. Interestingly, some of the proteins identified by
the proteomic analysis of MHV are OMPs such as PlpE (8, 9, 26);
PlpE (6); and OmpD15, OmpP2, SSA-1 (6), etc., that we have
shown to be highly immunogenic and, in the case of PlpE, protec-
tive. Further characterization of MHV as an alternative M. haemo-
lytica vaccine is warranted.

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