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LKTA and PlpE small fragments fusion protein protect against *Mannheimia haemolytica* challenge

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ABSTRACT

Bovine respiratory disease (BRD) complex is a major cause of economic losses for the cattle backgrounding and feedlot industries. *Mannheimia haemolytica* is considered the most important pathogen associated with this disease. Vaccines against *M. haemolytica* have been prepared and used for many decades, but traditional bacterins have failed to demonstrate effective protection and their use has often exacerbated disease in vaccinated animals. Thus, the BRD complex continues to exert a strong adverse effect on the health and wellbeing of stocker and feeder cattle. Therefore, generation of recombinant proteins has been helpful in formulating enhanced vaccines against *M. haemolytica*, which could confer better protection against BRD. In the present study, we formulated a vaccine preparation enriched with recombinant small fragments of leukotoxin A (LKTA) and outer-membrane lipoprotein (PlpE) proteins, and demonstrated its ability to generate high antibody titers in rabbits and sheep, which protected against *M. haemolytica* bacterial challenge in mice.

1. Introduction

Bovine respiratory disease (BRD) complex is a major cause of economic losses for the cattle backgrounding and feedlot industries (Gagea et al., 2006; Snowder et al., 2006). BRD research has provided significant understanding of the disease over the past 30 years (Fulton, 2009). Modern research tools have been used to generate advances in products including vaccines as well as technological, biological, and pharmacological developments. The bacterial component of the BRD complex continues to have a major adverse effect on health and wellbeing of stocker and feeder cattle (McVey, 2009; Griffin, 2010).

BRD involves complex interactions among viral and bacterial pathogens that can lead to intense pulmonary inflammation (fibrinous pleuropneumonia) (Czuprynski, 2009). Among bacterial pathogens associated with the disease, two Gram negative bacteria are the more relevant: *Histophilus somni*, which causes respiratory disease, septicemia, thrombotic meningoencephalitis, myocarditis, arthritis, and abortion (Corbeil, 2007; O'Toole et al., 2009; Sandal and Inzana, 2010), and *Mannheimia haemolytica*, which is considered the most important (Confer, 2009). Pathogens involved in the BRD complex have developed intricate mechanisms to thwart both the innate and adaptive immune responses of their hosts. These immune evasion strategies are likely to contribute to the failure of currently available vaccines to provide complete protection to cattle against these pathogens (Srikumaran et al., 2007).

M. haemolytica is an opportunist bacterium, gaining access to the lungs, when host defenses are compromised by stress or infection with respiratory viruses or mycoplasma. Although, several serotypes act as commensals, A1 and A6 are the most common isolates from pneumonic lungs (Rice et al., 2007; Confer, 2009).

Among potential virulence factors of *M. haemolytica*, leukotoxin A (LKTA), an exotoxin that is cytolytic for macrophages, neutrophils and all other leukocyte subsets (Berggren et al., 1981; Shewen and Wilkie, 1982), is critical in the induction of pneumonia (Jeyaseelan et al., 2002; Rice et al., 2007; Czuprynski, 2009) and antibodies

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against it are needed to generate immunity against BRD (Shewen and Wilkie, 1982). Protein LKTA (GenBank M20730) has 953 amino acids and a molecular mass of 104 kDa (Fig. 1). It is a member of the repeats-in-toxin (RTX) family of multidomain exotoxins (Lo, 1990) and contains several highly conserved glycine-rich repeats of nine amino acids near the C-terminal end of its structure (Lo, 1990; Coote, 1992).

Another immunogenically relevant protein is a 45-kDa outer-membrane lipoprotein, PlpE (Pandher et al., 1999; Ayalew et al., 2004; GenBank ABB20693.1; Fig. 2). It has been demonstrated that when PlpE is added to the vaccine formulation, the vaccinated cattle have significantly greater resistance against experimental challenge (Confer et al., 2003, 2006, 2009a,b).

Recently, Confer et al. (2009 a,b) showed in a relevant work series that the PlpE–LKTA chimeric protein, in combination with a bacterin of *M. haemolytica*, stimulated significant protection against a severe transthoracic challenge with the bacterium (Ayalew et al., 2008; Confer et al., 2009a,b). In this case, they included the epitope involved in neutralization of LKTA (localized at 146-amino acid region of the C-terminus of native LKTA, Fig. 1) in the chimeric protein (Lainson et al., 1996; Rajeev et al., 2001). To complement these investigations, we show in the present work that a recombinant fragment of LKTA (it includes five haemolysin binding domains also) that constitutes a neutralization epitope, even its first 46 amino acids (one third), mixed with a fragment of PlpE, also protected against the bacterial challenge. In addition, we used aluminum hydroxide [$Al(OH)_3$] as the adjuvant in vaccine preparation because it is a well-documented adjuvant for stimulating immunity (Exley et al., 2010) by potentiating the immune response (Seubert et al., 2008). It was used instead of the cholera toxin or Freund's incomplete adjuvant, which were used as adjuvants in other studies.

Vaccination aims to mimic development of naturally acquired immunity by inoculation of immunogenic components of the pathogen in question (Meeusen et al., 2007) and has been shown to be the single most efficient means of preventing bacterial, viral and parasitic infections (Potter et al., 2008). Vaccines have been used for many decades, but traditional bacterins have failed to demonstrate effective protection and their use has often exacerbated disease in vaccinated animals (Rice et al., 2007). Modern-day vaccines preferentially use culture supernatants containing LKTA and other soluble antigens. Nevertheless, these vaccines have 50–70% efficacy in preventing *M. haemolytica* pneumonia (Rice et al., 2007) and can cause severe adverse effects. Therefore, more effective control of *M. haemolytica* pneumonia requires a combination of more precise diagnosis, efficacious vaccines, therapeutic intervention and improved management practices. Therefore, generation of recombinant proteins is helpful in formulating recombinant vaccines against *M. haemolytica*, which could confer better protection against BRD.

2. Material and methods

2.1. Bacterial strains and culture conditions

Escherichia coli TOP10 or M15 (Invitrogen) was used as the host for cloning and propagation of plasmids, which were cultured in Luria–Bertani (LB) broth supplemented with thymine (50 mg/ml) and ampicillin (100 mg/ml), chloramphenicol (25 mg/ml), or kanamycin (50 mg/ml), as necessary. *M. haemolytica A1* (ATCC: 43270) was used to obtain bacterial DNA for the PCR.

2.2. Extraction and quantification of genomic DNA and recombinant methods

Bacterial genomic DNA was obtained by Illustra bacteria genomic Prep Mini Spin kit (GE Healthcare). DNA integrity was routinely evaluated by electrophoresis in agarose gels stained with Safe DNA gel stain (Invitrogen) and quality evaluated by A_{260}/A_{280} absorbance. All DNA cloning and ligation were carried out using standard recombinant DNA techniques (Ausubel et al., 2001; Sambrook et al., 2001).

2.3. Oligonucleotide design and PCR

LKTA is a soluble secreted and highly toxic protein. As attempts to study immunogenicity of its carboxyl-terminal region (that includes the RTX domain) have not been successful (Lainson et al., 1996), we decided to produce a fusion protein with a fragment of LKTA from amino acid 573 to 845 to obtain antibodies against it (Fig. 1). This 273-amino acid polypeptide includes five repeated hemolysin-type calcium-binding domains, which are highly conserved sequence regions among different serotypes. For the PlpE construct, we decided to eliminate first 18 amino acids, which encodes for a signaling peptide, and our construct therefore included remaining 338 amino acids (Fig. 2).

Oligonucleotide sequences of the primers used for PCR were as follows: for *lktA* gene, the sense primer 5′-GAAAAGGCCTGATG GTGCAGCAAGTTCTAC-3′ and the antisense primer 5′-GGCACAAGC TTACGAAATCAGCCTCTCGG-3′ were used to amplify an 846-bp fragment that encoded for 273-amino acid fragment of the LKTA. For *plpE* gene, the sense primer 5′-AATAGGCCTGCGGAGGAAGCGG-TAGC-3′ and the antisense primer 5′-ATAAGCTTATTTTTTCTCGCTA ACCATTA-3′ were used to amplify a 1014-bp fragment that encoded for 338 amino acids of PlpE. Primers were designed with enzyme restriction sites introduced into each. *Stu*I site was introduced in both forward primers and *Hind*III in both reverse primers in order to achieve directional cloning in the expression vector.

PCR was carried out in a Perkin-Elmer GeneAmp PCR System 2400 Thermocycler (Perkin-Elmer, Foster City, California). For all

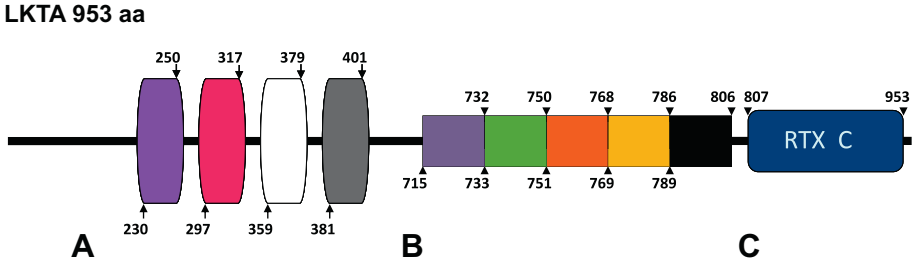

Fig. 1. LKTA protein domain structure, which is 953 amino acids long. (A) Possible transmembrane domains, (B) five repeated hemolysin-type calcium-binding regions, and (C) RTX domain at the carboxyl-terminal region. Numbers indicate amino acid position. Sequence was obtained from UniProt sequence (accession number Q9EV32) and analyses were performed based on the software by Hau et al. (2007).

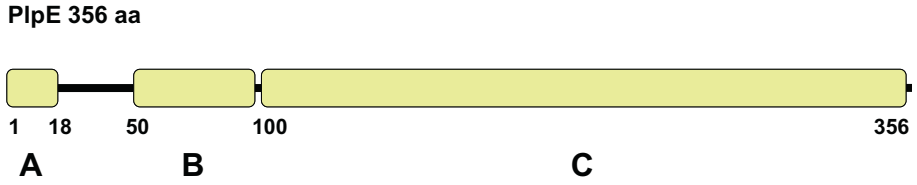

Fig. 2. PlpE protein domain structure, which is 356 amino acids long. (A) Signaling peptide, (B) hexapeptide repeats rich in glutamine, and (C) bacterial lipoprotein domain. Numbers indicate amino acid position. Protein sequence was obtained from GeneBank (accession number AAC82640.1) and analyses were performed based on the software by Hau et al. (2007).

PCR experiments, Platinum PCR SuperMix High Fidelity (Invitrogen) was used. PCR products were visualized in 1% agarose gels stained with Safe DNA gel stain (Invitrogen). Optimal annealing temperature was initially established by testing a temperature gradient. All PCR reactions were performed after a single denaturation step at 94 °C for 5 min and involved 30 cycles of denaturation at 94 °C for 1 min, annealing at 63 °C for *lktA* primers or 60 °C for *plpE* primers for 1 min, and extension at 72 °C for 1 min followed by a final extension for 5 min at 72 °C.

2.4. Construction of lktA-pQE 30 Xa and plpE-pQE 30 Xa and molecular cloning

To clone the PCR products, fragments were purified from agarose gels by GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare) and directly cloned in pCR® 2.1 TOPO vector (Invitrogen). TOP10 bacteria were transformed by the $CaCl_2$ method and positive clones were selected by means of the β-galactosidase reaction. Plasmid DNA was obtained from *E. coli* by plasmid Prep Mini Spin Kit (GE Healthcare). For each insert, at least three clones were sequenced in order to verify the sequence reported for both genes (GenBank M20730 for LKTA and GenBank AF059036 for PlpE). Only clones that precisely matched the reported sequence were used for subcloning and expression of the fusion protein. Sequences were obtained in a capillary ABI Prism 310 sequencer (Applied Biosystems). The sequences obtained were compiled with the Chromas v2.31 and DNASTAR Inc. software packages and compared with databases using the Blast program.

For fusion protein, pQE-30 Xa vector (Qiagen) was used. It introduces a six-histidine tag and a site for factor Xa cleavage. Plasmid DNA with PCR inserts, previously cloned, were digested and religated at the *Stu*I and *Hind*III sites located at the ends of the *lktA* and *plpE* sequences. Therefore, we generated two constructs that were able to express fusion proteins, designated as ΔLKTA-pQE 30 Xa and ΔPlpE-pQE 30 Xa that should theoretically express a 30.9- and 38.4-kDa derivative, respectively. Constructs were transformed in *E. coli* M15 (Invitrogen) for fusion protein expression.

2.5. Fusion protein expression by IPTG and purification

For expression of fusion proteins, we used isopropyl β-D-1-thiogalactopyranoside (IPTG). From overnight bacterial cultures, 1 ml was used to inoculate 50 ml of LB medium and let it grew for 2 h. Then, 1 mM of IPTG was added and the culture was allowed to grow for three more hours. Finally, crude total extracts were obtained and examined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

To purify the fusion proteins, QIAexpress Assay System (Qiagen) was used. It is based on the affinity of histidine tag, included in the fusion protein, for nickel-nitriloacetic acid (Ni-NTA) resin. Acid or basic buffers were then used to elute fusion protein from the column, in 500 μl fractions and by the action of factor Xa, and isolate it from the total extracts. Total proteins were quantified by the Lowry method (Lowry et al., 1951). A small aliquot of the extract (5 μg of total protein) was examined by SDS–PAGE to confirm the presence of proteins corresponding to the truncated LKTA and PlpE fusion proteins.

2.6. Production of anti-LKTA and anti-PlpE antibodies in rabbits

To prepare the immunogen, purified proteins in the elution buffer (8 M urea, 0.01 M Tris–HCl, 0.1 M NaH_2PO_4) were mixed (1/8 v/v) with 0.25% $Al(OH)_3$ in sterile phosphate buffer saline (PBS), pH 7.2, as adjuvant. Once prepared, the immunogen was inoculated subcutaneously into New Zealand white rabbits weighing around 2,000 g. Two rabbits were inoculated subcutaneously with each fusion protein (90 μg) on days 0, 14 and 21. Serum was collected on day 0 (before the first inoculation and used as pre-immune serum control), and after every inoculation (on day 14 and 21). Finally, total serum was obtained by complete exsanguination from the rabbit on day 28. Crude serum was inactivated by heat and stored at −20 °C until analysis by Western blotting.

2.7. Protein electrophoresis and Western blot analysis

Western blots were used to detect the production of antibodies against LKTA and PlpE in rabbits immunized with the purified fusion proteins. Crude extracts of total protein were obtained in lysis buffer (PBS pH 7.2, SDS 4%). Homogenates were then centrifuged at 14,000*g* for 15 min at 4 °C. Supernatants were collected and protein content was determined using the Lowry method. Samples were placed in PBS pH 7.2, with SDS 4%, denatured by boiling and separated by electrophoresis in 12% acrylamide gels. Thereafter, proteins were transferred onto nitrocellulose sheets (Millipore, Bedford, MA). Membranes were blocked overnight at 4 °C with 80 g/L non-fat milk in tween 0.1% tris saline buffer (TBST) and then incubated with crude rabbit serum (1:1000) for 1 h at room temperature (RT). After that, blots were washed thoroughly in TBST and incubated for 1 h with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (1:5000, Millipore, Bedford, MA). Immunoreactive proteins were revealed with ECL (ECL Western blotting Analysis System, GE Healthcare, United Kingdom) and detected by ChemiDoc (Bio-Rad Laboratories, Hercules, California, USA).

2.8. Vaccination and production of polyclonal antibodies in rabbits and sheep

Twenty-five New Zealand white rabbits weighing around 2,000*g* were used and divided into five groups (Table 1). The vaccines (each 500 μl) were inoculated subcutaneously two times on days 0 and 14. Group A (positive control) was inoculated with a commercial bacterin (®Biobac 11 vías) composed of *Clostridium chauvoei*, *Clostridium septicum*, *Clostridium novyi*, *Clostridium sordellii*, *Clostridium perfringens* type C, *Clostridium perfringens* type D, *Pasteurella multocida* type A, *Pasteurella multocida* type D, *Mannheimia haemolytica* serotype A1, *Histophilus somni* and $Al(OH)_3$ as adjuvant. Group B was vaccinated with the recombinant preparation composed of

Table 1
Rabbit and sheep groups.

Groups	Rabbits immunogen	Sheep immunogen	Vaccination days	Sampling: blood (sera)
A	®Biobac 11 vías	®Biobac 11 vías	0, 14	0, 14, 21, 28, 35, 42
B	Recombinant (30 μg)	Recombinant (50 μg)	0, 14	0, 14, 21, 28, 35, 42
C	Recombinant (60 μg)	Recombinant (100 μg)	0, 14	0, 14, 21, 28, 35, 42
D	®Biobac 7 vías	®Biobac 7 vías	0, 14	0, 14, 21, 28, 35, 42
E	None (PBS + $Al(OH)_3$)	None (PBS + $Al(OH)_3$)	0, 14	0, 14, 21, 28, 35, 42

30 μg recombinant LKTA [rLKTA], 30 μg recombinant PlpE [rPlpE] and complemented with the commercial vaccine ®Biobac 7 vías (*Clostridium chauvoei*, *Clostridium septicum*, *Clostridium novyi*, *Clostridium sordellii*, *Clostridium perfringens* type C, *Clostridium perfringens* type D and $Al(OH)_3$). In group C, the rabbits were inoculated with recombinant preparation composed of 60 μg rLKTA, 60 μg rPlpE and ®Biobac 7 vías; while the vaccine used in group D (negative control) was ®Biobac 7 vías; and PBS and adjuvant for group E (negative control).

For the second experiment, 10 healthy sheep weighing around 30 kg, female and a hair hybrid race of Pelibuey, Katahdin and Blackbelly were used in groups of two and housed in adequate corrals and de-wormed orally with Closantil. The animals were divided into five groups and vaccinated in the lateral neck region on days 0 and 14 with 2.5 ml of each vaccine. The groups were as follows: group A (positive control) was inoculated with a commercial bacterin (®Biobac 11 vías); group B with recombinant preparation composed of 50 μg rLKTA, 50 μg rPlpE and ®Biobac 7 vías; group C with recombinant preparation composed of 100 μg rLKTA, 100 μg rPlpE and ®Biobac 7 vías; group D (negative control) with ®Biobac 7 vías and group E (negative control) with PBS and $Al(OH)_3$.

For antibody determinations, serum samples were obtained on days 0, 14, 21, 28, 35 and 42. Serum samples were assayed for anti rLKTA, rPlpE and against *M. haemolytica* whole bacterial cells crude protein extract (WC) by enzyme-linked immunosorbent assay (ELISA). Protein extracts (WC) were prepared as previously mentioned and total amount of protein of each line for assay was 2 μg.

2.9. ELISA

The 96-well polystyrene microplates were coated with *M. haemolytica* WC in lysis buffer and recombinant proteins separately. The plates were coated with 100 ng/well of each protein (LktA or PlpE) diluted in carbonate buffer (0.1 M sodium carbonate, 0.1 M bicarbonate sodium, pH 9.6) and incubated for 16–18 h at 4 °C. The supernatant was removed and plates were washed four times in buffer (1.25 M NaCl, 250 mM Tris–HCl pH 7.9, 1% Tween-20) blocked with 2% non-fat milk in 0.1% Tween–PBS and incubated at 37 °C for 1 h. They were then washed and samples were applied. The serum dilutions were 1:800 for WC, rLKTA and rPlpE, and incubated for 1 h at 37 °C.

Microplates were washed four times and secondary HRP-conjugated anti-rabbit IgG antibody (1:1000, Millipore, Bedford, MA) was added and incubated at 37 °C for 1 h. O-phenylendiamine (OPD, Sigma) was added as substrate for the peroxidase enzyme and left to incubate for 5 min at room temperature. Afterwards, microplates were read at 415 nm, and the results were expressed as absorbance. To determine anti-rLKTA and rPlpE for sheep IgG antibodies, previous steps were repeated. The serum dilutions were 1:800 and the secondary antibody was HRP-conjugated rabbit anti-sheep IgG (H + L) used at a dilution of 1:1000 (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD). Results were expressed as absorbance.

2.10. Bacterial challenge in BALB/c mice

Seventy-five male BALB/c mice, weighing 26–32 g, were used in this study and distributed into experimental groups. Animals were kept under controlled temperature, humidity and light conditions.

The BALB/c mice were divided into five groups and injected intraperitoneally twice on days 0 and 14 with 250 μl of each vaccine. Group A (positive control) was immunized with ®Biobac 11 vías, group B with a recombinant preparation composed of 10 μg rLKTA + 10 μg rPlpE + ®Biobac 7 vías, group C with a recombinant preparation composed of 20 μg rLKTA + 20 μg rPlpE + ®Biobac 7 vías and the negative control groups of D and E with ®Biobac 7 vías and PBS + $Al(OH)_3$, respectively. Because the final objective was to formulate and probe a combined vaccine, we did not include groups for testing both recombinant proteins separately.

All the mice were challenged on day 28 after first immunization with approximately 1.6×10^7 CFU of virulent *M. haemolytica* intraperitoneally, and monitored for 10 days for their survival rates. To verify effect of the bacteria, necropsies were performed when the animals died.

2.11. Animals care and use

The present study complied with the Institution's Guidelines and the Mexican Official Regulation (NOM-062-ZOO-1999;2008) regarding technical specifications for production, care, and use of laboratory animals. The protocol was also approved by the local Animal Ethics Committee. Every effort was made to minimize animal suffering and to reduce the number of animals used.

2.12. Statistical analysis

To determine protection against *M. haemolytica*, statistical analysis was performed by Fisher's exact test on percentage survival data. ANOVA test were performed to analyze the ELISA results.

3. Results

3.1. Induction of the expression of ΔLKTA-pQE 30 Xa and ΔPlpE-pQE 30 Xa

Fragments from PCR of each gene were initially cloned into pCR® 2.1 TOPO (Invitrogen). Afterwards, the digested and gel-purified *Stu*I–*Hind*III restriction fragments were subcloned into pQE-30 Xa expression vector (Qiagen). Expression of the fusion proteins was performed in M15 *E. coli* (Invitrogen) transformed by heat shock. The LKTA fragment was clearly induced by IPTG at approximately 35 kDa (Fig. 3A), and PlpE fusion protein at 50 kDa (Fig. 4A). Because the clones used were previously sequenced, therefore, increase in electrophoretic mobility could be due to a combined effect of terminal 18 amino acids deletion plus addition of histidine tag and factor Xa recognition site on this particular fragment.

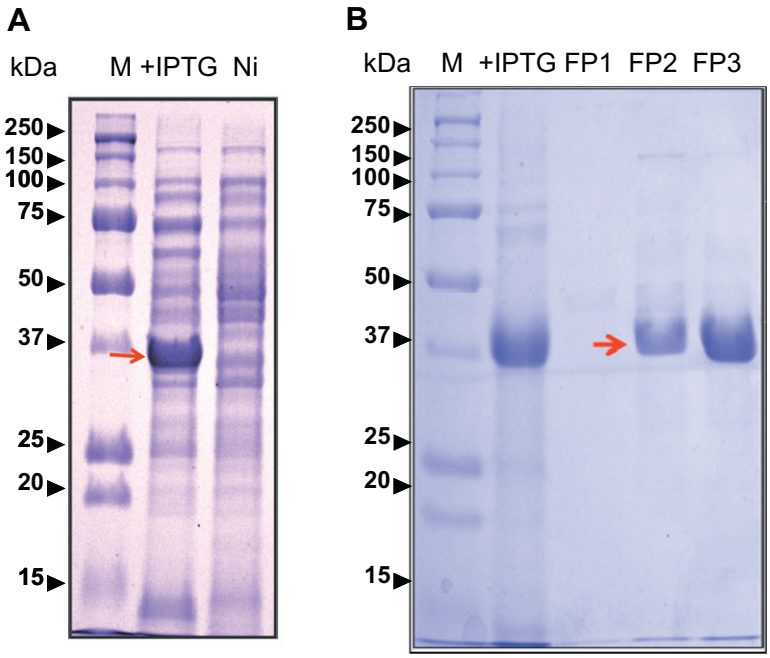

Fig. 3. Expression and purification of ΔlktA-pQE 30 Xa. (A) Induction of the expression of fusion proteins and (B) purification after elution from the column.

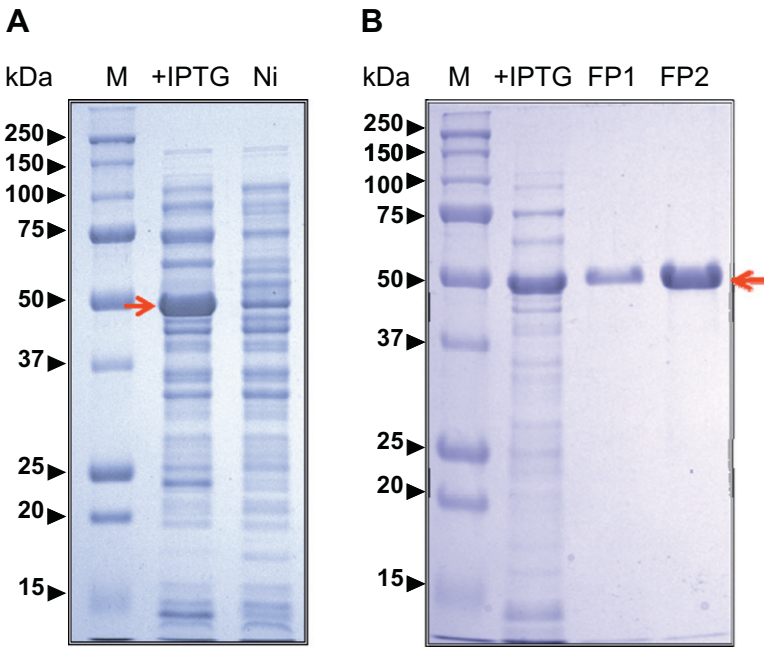

Fig. 4. Expression and purification of ΔplpE-pQE 30 Xa. (A) Induction of the expression of fusion proteins and (B) purification after elution from the column.

3.2. Purification of fusion proteins and production of antibodies

Fusion proteins were purified from the crude extracts using QIAexpress Assay System (Qiagen), as described in Section 2. Purified proteins were analyzed by SDS–PAGE (Figs. 3B and 4B).

Three New Zealand rabbits were immunized subcutaneously three times on days 0, 14 and 21, with 30 μg of each protein (for a total of 90 μg). Before the first immunization, we obtained pre-immune serum as internal control. Ten days after the last administration, the rabbits were sacrificed by exsanguination to obtain the final serum. All serum samples were tested by Western blot against total protein extracts with or without induced proteins. Results of the Western blots are presented in Fig. 5A for LKTA and Fig. 5B for PlpE.

3.3. Analysis of antibody response by ELISA

Antibodies against *M. haemolytica*, rLKTA and rPlpE were evaluated by ELISA in two experimental models (rabbits and sheep) (Fig. 6). Vaccination of rabbits with commercial bacterin (®Biobac 11 vías) and recombinant vaccines stimulated detectable anti-LKTA and anti-PlpE antibodies (Fig. 6). On day 21, antibody production increased significantly. Antibody response was significantly greater for group C (rLKTA 60 μg) than for groups A and B, while

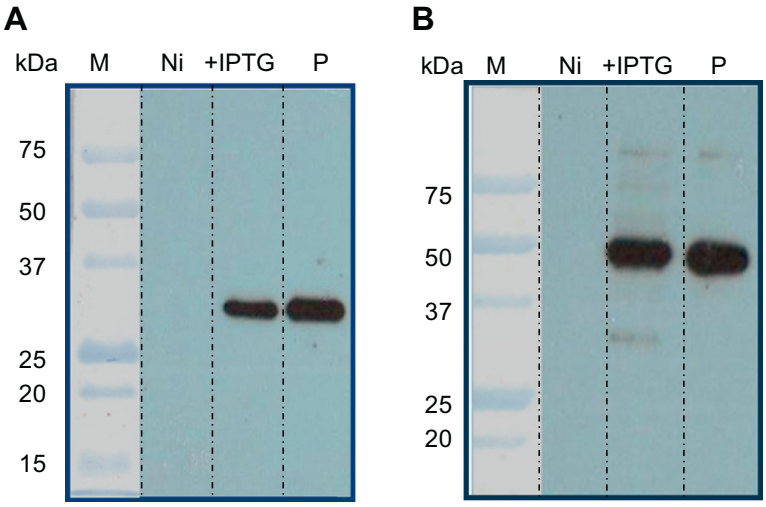

Fig. 5. Western blot analysis of rabbit serum samples. (A) Serum with antibodies against LKTA and (B) serum with antibodies against PlpE.

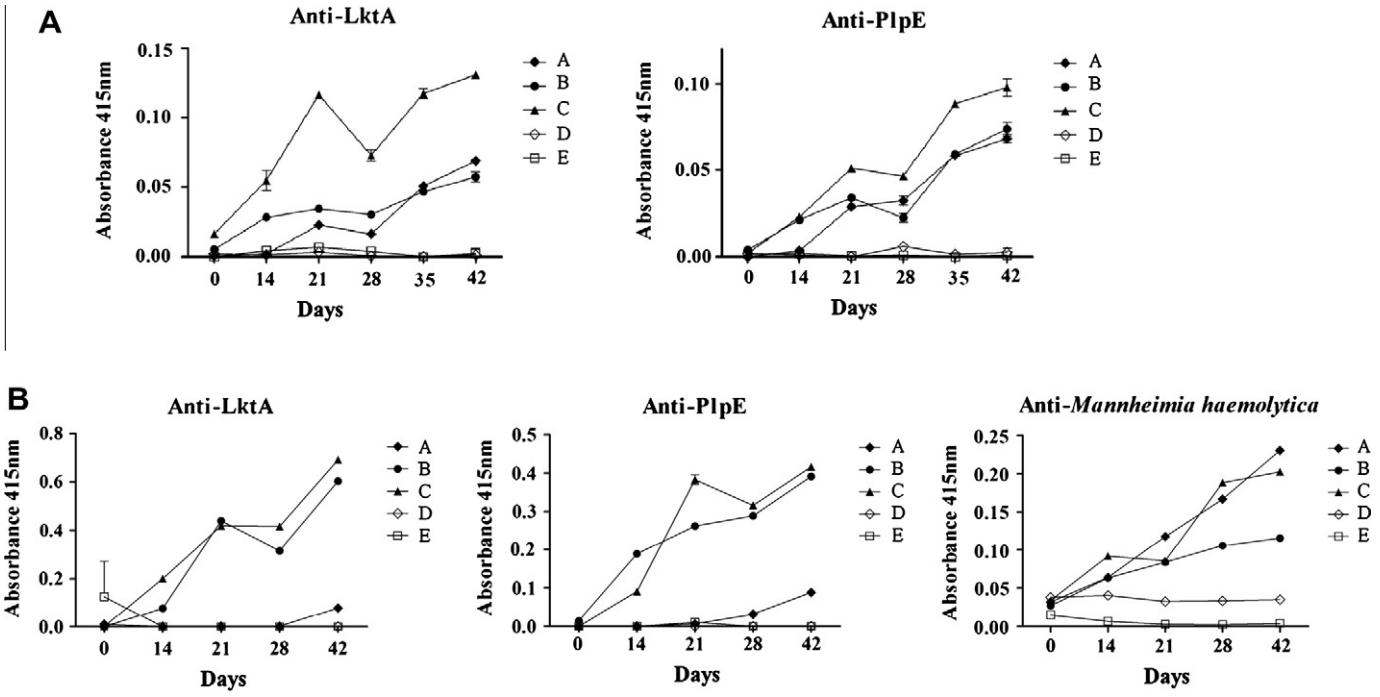

Fig. 6. Results from ELISA analysis. (A) *Rabbits:* Group A (positive control) inoculated with a commercial bacterin (®Biobac 11 vías) composition as stated in the Section 2. Group B was vaccinated with the recombinant preparation composed of 30 μg recombinant LKTA (rLKTA), 30 μg recombinant PlpE (rPlpE) and complemented with the commercial vaccine ®Biobac 7 vías. Group C corresponds to recombinant preparation composed of 60 μg rLKTA, 60 μg rPlpE and ®Biobac 7 vías, while the vaccine used in group D (negative control) was ®Biobac 7 vías, and PBS and adjuvant for group E (negative control). (B) *Sheep:* Group A (positive control) immunized with recombinant preparation alone; group B with recombinant preparation composed of 50 μg rLKTA, 50 μg rPlpE and ®Biobac 7 vías; group C were inoculated with recombinant preparation composed of 100 μg rLKTA, 100 μg rPlpE and ®Biobac 7 vías; group D (negative control) with ®Biobac 7 vías and group E (negative control) with PBS and $Al(OH)_3$.

groups D and E (negative controls) did not demonstrate any antibody production.

For the second experiment (Fig. 6 B), serum samples from sheep were analyzed for rLktA and rPlpE and WC *M. haemolytica*. Anti-rLKTA and anti-rPlpE increased by day 21 only in groups B and C (recombinant vaccines), with the responses higher in group C (rLKTA + rPlpE, 100 μg) than in group B (rLKTA + rPlpE, 50 μg). Anti-WC *M. haemolytica* increased by day 21 in the groups treated with commercial and recombinant vaccines.

Immunization of rabbits and sheep with vaccines complemented with recombinant proteins strongly stimulated antibody production against LKTA and PlpE (Fig. 6), demonstrating that the recombinant proteins used in these experiments are clearly immunogenic and dose-dependent, and can be used in the formulation of vaccines against BRD.

3.4. Bacterial challenge

Protection against bacterial challenge with pathogenic *M. haemolytica* was analyzed by Fisher's exact test and the results are summarized in Table 2. Groups A, B and C were protected in the challenge. Group A (®Biobac 11 vías) was protected with an efficacy of 93.3% survival, group B immunized with a lower dose of recombinant vaccine (rLKTA + rPlpE 10 μg each) demonstrated

Table 2
Result of challenge test in BALB/c mice.

Groups	Mice immunogen	Vaccination days	% Survival
A	®Biobac 11 vías	0, 14	93.3 (1/14)
B	Recombinant (10 μg)	0, 14	86.7 (2/13)
C	Recombinant (20 μg)	0, 14	100 (0/15)
D	®Biobac 7 vías	0, 14	0 (15/0)
E	None (PBS + $Al(OH)_3$)	0, 14	0 (15/0)

86.7% survival, whereas group C immunized with a higher dose of 20 μg rLKTA + rPlpE were completely protected (100% survival).

The negative controls (groups D and E) were not protected (0% survival) against the infection with 1.6×10^7 CFU of virulent *M. haemolytica*. After the challenge, mice were monitored for 10 days. Those belonging to groups D and E developed gross lesions including pulmonary congestion, edema and hemorrhages. Protection elicited by commercial (®Biobac 11 vías) and recombinant vaccines (containing rLKTA + rPlpE) were significantly greater ($p < 0.0001$) than that of the negative controls (®Biobac 7 vías and adjuvant).

4. Discussion

Attempts have been made to document that prevention of BRD by proper vaccination and management prior to exposure to infectious agents can minimize disease and serve as economic incentives for certified health programs (Griffin, 2010). Therefore, it is important to use vaccines as preventive measures. Several commercial vaccines were reported before the 1990s (Rice et al., 2007), but most of them caused secondary adverse reactions (Ellis and Yong, 1997), hence the relevance of generating recombinant vaccines, wherein presence of more than one antigen may contribute to its efficacy. In agreement with this, generation and adequate use of vaccine preparations that protect against BRD pathogens are still of interest. As demonstrated in the present work, a recombinant vaccine that included protein fragments of two antigens of *M. haemolytica*, LKTA and PlpE, induced high antibody titers and was able to protect against bacterial challenge.

Confer et al. (2009b) reported that after intranasal vaccination of calves with *M. haemolytica* PlpE-LKTA chimeric protein, alongwith cholera toxin as adjuvant, nasal antibodies to PlpE and LKTA antigens were stimulated, but serum antibody responses were limited. In other study, subcutaneous vaccination with PlpE-LKTA chimeric protein and *M. haemolytica* bacterin alongwith Freunds incomplete adjuvant, stimulated significant protection in cattle against a severe transthoracic challenge with the bacterium (Confer et al., 2009a). In this study, subcutaneous administration of LKTA and PlpE recombinant proteins alone, without any combination of *M. haemolytica* bacterin, induce high levels of antibodies, as demonstrated by ELISA quantifications (Fig. 6), rabbits and sheep. Further, instead of Freund's incomplete adjuvant or cholera toxin, as reported by Confer et al. (2009 a,b); $Al(OH)_3$ was used as the adjuvant. The aluminum adjuvants are reported to ensure potency and efficacy of those antigens that are sparingly available (Exley et al., 2010), thus, enhance the immune response (Seubert et al., 2008). Although, it is necessary to perform immunogenic studies in beef cattle, the results clearly demonstrate (Fig. 6A and B) that rLktA and rPlpE fragments are highly immunogenic in sheep than in rabbits, yielding higher response in sheep than the response generated with the commercial bacterin ®Biobac 11 vías. Therefore, the fusion proteins may be suitable to be included in a formulation of a recombinant vaccine that can be administered in combination to elicit protection against BRD.

The results also demonstrated that in the same formulation of commercial vaccine for other bacterial diseases (®Biobac 7 vías), subcutaneous administration of only recombinant fragments of either the LKTA or PlpE proteins alone without addition of *M. haemolytica* bacterin and $Al(OH)_3$ as adjuvant, enabled generation of high antibody titers in rabbits and sheep, with protection against *M. haemolytica* bacterial challenge in mice.

Underlying reason for the viral/bacterial synergism and the manner in which the cattle respond to virulence strategies of the bacterial pathogens is not completely understood (Czuprynski, 2009). Additionally, given that LKTA-mediated infiltration and destruction of leukocytes impair bacterial clearance and contribute to the development of fibrinous pneumonia (Rice et al., 2007), it is useful to obtain antibodies, such as the ones generated in this study, which identify LKTA and can be used to study these processes at a cellular level.

Recently, a wide immunoproteomic analysis was performed to characterize the outer membrane proteome of *M. haemolytica* (Ayalew et al., 2010). The authors proposed 55 proteins as immunoreactive candidate proteins, and concluded that further research was needed for complete knowledge of the immunogenicity of the outer membrane proteins of *M. haemolytica*. Even so, given that once an immunogenic protein has been identified by the immune system, it reacts against the same immunogen at the second encounter, and that the use of large amount of immunogens could result in an exacerbated immune response. It is, thus, of practical interest to develop a recombinant vaccine based on a couple of proteins, which could elicit an immunological response and protect against infection.

Finally, and because BRD are multifactorial, it is reasonable to propose that a vaccine composed of combined antigenic surface proteins from different pathogens, for instance from *M. haemolytica* and *Histophilus somni*, would be helpful in preventing infection and reducing the incidence of BRD.

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References

Ausubel, F., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., et al., 2001. Current Protocols in Molecular Biology, first ed. John Wiley and Sons, New York.

Ayalew, S., Confer, A.W., Blackwood, E.R., 2004. Characterization of immunodominant and potentially protective epitopes of *Mannheimia haemolytica* serotype 1 outer membrane lipoprotein PlpE. Infection and Immunity 72 (12), 7265–7274.

Ayalew, S., Confer, A.W., Payton, M.E., Garrels, K.D., Shrestha, B., Ingram, K.R., Montelongo, M.A., Taylor, J.D., 2008. *Mannheimia haemolytica* chimeric protein vaccine composed of the major surface-exposed epitope of outer membrane lipoprotein PlpE and the neutralizing epitope of leukotoxin. Vaccine 26 (38), 4955–4961.

Ayalew, S., Confer, A.W., Hartson, S.D., Shrestha, B., 2010. Immunoproteomic analyses of outer membrane proteins of *Mannheimia haemolytica* and identification of potential vaccine candidates. Proteomics 10 (11), 2151–2164.

Berggren, K.A., Baluyut, C.S., Simonson, R.R., Bemrick, W.J., Maheswaran, S.K., 1981. Cytotoxic effects of *Pasteurella haemolytica* on bovine neutrophils. American Journal of Veterinary Research 42 (8), 1383–1388.

Confer, A.W., Ayalew, S., Panciera, R.J., Montelongo, M., Whitworth, L.C., Hammer, J.D., 2003. Immunogenicity of recombinant *Mannheimia haemolytica* serotype 1

outer membrane protein PlpE and augmentation of a commercial vaccine. Vaccine 21 (21–22), 2821–2829.
Confer, A.W., Ayalew, S., Panciera, R.J., Montelongo, M., Wray, J.H., 2006. Recombinant *Mannheimia haemolytica* serotype 1 outer membrane protein PlpE enhances commercial *M. haemolytica* vaccine-induced resistance against serotype 6 challenge. Vaccine 24 (13), 2248–2255.
Confer, A.W., 2009. Update on bacterial pathogenesis in BRD. Animal Health Research Reviews 10 (2), 145–148.
Confer, A.W., Ayalew, S., Montelongo, M., Step, D.L., Wray, J.H., Hansen, R.D., Panciera, R.J., 2009a. Immunity of cattle following vaccination with a *Mannheimia haemolytica* chimeric PlpE-LKT (SAC89) protein. Vaccine 27 (11), 1771–1776.
Confer, A.W., Ayalew, S., Step, D.L., Trojan, B., Montelongo, M., 2009b. Intranasal vaccination of young Holstein calves with *Mannheimia haemolytica* chimeric protein PlpE-LKT (SAC89) and cholera toxin. Veterinary Immunology and Immunopathology 132 (2–4), 232–236.
Coote, J.G., 1992. Structural and functional relationships among the RTX toxin determinants of gram-negative bacteria. FEMS Microbiology Reviews 8 (2), 137–161.
Corbeil, L.B., 2007. Histophilus somni host-parasite relationships. Animal Health Research Reviews 8 (2), 151–160.
Czuprynski, C.J., 2009. Host response to bovine respiratory pathogens. Animal Health Research Reviews 10 (2), 141–143.
Ellis, J.A., Yong, C., 1997. Systemic adverse reactions in young Simmental calves following administration of a combination vaccine. The Canadian Veterinary Journal 38, 45–47.
Exley, C., Siesjo, P., Eriksson, H., 2010. The immunobiology of aluminium adjuvants: How do they really work? Trends in Immunology 31 (3), 103–109.
Fulton, R.W., 2009. Bovine respiratory disease research (1983–2009). Animal Health Research Reviews 10 (2), 131–139.
Gagea, M.I., Bateman, K.G., van Dreumel, T., McEwen, B.J., Carman, S., Archambault, M., Shanahan, R.A., Caswell, J.L., 2006. Diseases and pathogens associated with mortality in Ontario beef feedlots. Journal of Veterinary Diagnostic Investigations 18 (1), 18–28.
Griffin, D., 2010. Bovine pasteurellosis and other bacterial infections of the respiratory tract. Veterinary Clinical North America Food Animal Practice 26 (1), 57–71.
Hau, J., Muller, M., Pagni, M., 2007. HitKeeper, a generic software package for hit list management. Source Code for Biology and Medicine 2, 2.
Jeyaseelan, S., Sreevatsan, S., Maheswaran, S.K., 2002. Role of *Mannheimia haemolytica* leukotoxin in the pathogenesis of bovine pneumonic pasteurellosis. Animal Health Research Reviews 3 (2), 69–82.
Lainson, F.A., Murray, J., Davies, R.C., Donachie, W., 1996. Characterization of epitopes involved in the neutralization of *Pasteurella haemolytica* serotype A1 leukotoxin. Microbiology 142 (Pt. 9), 2499–2507.
Lo, R.Y., 1990. Molecular characterization of cytotoxins produced by *Haemophilus*, *Actinobacillus*, *Pasteurella*. Canadian Journal of Veterinary Research 54 (Suppl.), S33–5.
Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry 193 (1), 265–275.
McVey, D.S., 2009. BRD research needs in the next 10–20 years. Animal Health Research Reviews 10 (2), 165–167.
Meeusen, E.N., Walker, J., Peters, A., Pastoret, P.P., Jungersen, G., 2007. Current status of veterinary vaccines. Clinical Microbiology Reviews 20 (3), 489–510.
Norma Oficial Mexicana (NOM-062-ZOO-1999). Especificaciones técnicas para la producción, cuidado y uso de los animales de laboratorio. Modificated: november 26, 2008. Available from <www.senasica.gob.mx/default.asp?doc=743>.
O'Toole, D., Allen, T., Hunter, R., Corbeil, L.B., 2009. Diagnostic exercise: Myocarditis due to *Histophilus somni* in feedlot and backgrounded cattle. Veterinary Pathology 46 (5), 1015–1017.
Pandher, K., Murphy, G.L., Confer, A.W., 1999. Identification of immunogenic, surface-exposed outer membrane proteins of *Pasteurella haemolytica* serotype 1. Veterinary Microbiology 65 (3), 215–226.
Potter, A., Gerdts, V., Littel-van den Hurk, S.D., 2008. Veterinary vaccines: Alternatives to antibiotics? Animal Health Research Reviews 9 (2), 187–199.
Rajeev, S., Kania, S.A., Nair, R.V., McPherson, J.T., Moore, R.N., Bemis, D.A., 2001. Bordetella bronchiseptica fimbrial protein-enhanced immunogenicity of a *Mannheimia haemolytica* leukotoxin fragment. Vaccine 19 (32), 4842–4850.
Rice, J.A., Carrasco-Medina, L., Hodgins, D.C., Shewen, P.E., 2007. *Mannheimia haemolytica* and bovine respiratory disease. Animal Health Research Reviews 8 (2), 117–128.
Sambrook, J., Fritsch, E.F., Maniatis, T., 2001. Molecular Cloning: A Laboratory Manual, third ed. Cold Spring Harbor Laboratory Press, New York.
Sandal, I., Inzana, T.J., 2010. A genomic window into the virulence of *Histophilus somni*. Trends in Microbiology 18 (2), 90–99.
Seubert, A., Monaci, E., Pizza, M., O'Hagan, D.T., Wack, A., 2008. The adjuvants aluminum hydroxide and MF59 induce monocyte and granulocyte chemoattractants and enhance monocyte differentiation toward dendritic cells. Journal of Immunology 180 (8), 5402–5412.
Shewen, P.E., Wilkie, B.N., 1982. Cytotoxin of *Pasteurella haemolytica* acting on bovine leukocytes. Infection and Immunity 35 (1), 91–94 (PMCID: 351000).
Snowder, G.D., Van Vleck, L.D., Cundiff, L.V., Bennett, G.L., 2006. Bovine respiratory disease in feedlot cattle: Environmental, genetic, and economic factors. Journal of Animal Science 84 (8), 1999–2008.
Srikumaran, S., Kelling, C.L., Ambagala, A., 2007. Immune evasion by pathogens of bovine respiratory disease complex. Animal Health Research Reviews 8 (2), 215–229.