



## Immunogenicity and protective efficacy of the recombinant *Pasteurella* lipoprotein E and outer membrane protein H from *Pasteurella multocida* A:3 in mice

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### ABSTRACT

*Pasteurella multocida* serotype A:3 is a Gram-negative bacterial pathogen, one of the causative agents of shipping fever of cattle. In this study, outer membrane protein H (*ompH*) and *Pasteurella* lipoprotein E (*plpE*) genes were cloned and *plpEC-ompH* fusion was constructed and expressed in *Escherichia coli*. Recombinant PlpE, OmpH and PlpEC-OmpH fusion proteins were purified and formulated with oil-based and oil-based CpG ODN adjuvants. Antibody responses in mice vaccinated with recombinant PlpE and PlpEC-OmpH proteins formulated with both adjuvants were significantly ( $p < 0.05$ ) increased. However, a significant ( $p < 0.05$ ) increment in serum IFN- $\gamma$  level was only observed upon immunization with oil-based CpG formulations. Protectivity of the vaccines were evaluated via intraperitoneal challenge of mice with 10  $LD_{50}$  of *P. multocida* A:3. The recombinant proteins PlpE and PlpEC-OmpH fusion conferred 100% protection when formulated with oil-based CpG ODN while the protectivity was found to be 80% and 60%, respectively when only oil-based adjuvant was used in respective formulations. These findings indicated that the recombinant PlpE or PlpEC-OmpH fusion proteins formulated with oil-based CpG ODN adjuvant are possible acellular vaccine candidates against shipping fever.

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### 1. Introduction

Bovine respiratory diseases (BRDs) are one of the most serious problems in the cattle industry causing high mortality and economic loss. BRDs are associated with stressful conditions, such as commingling and shipment of cattle, and adverse climatic conditions coupled with bacterial and viral infections. *Pasteurella multocida* is a facultative intracellular bacterial pathogen associated with the clinical syndromes of the BRD complex including neonatal calf (enzootic) pneumonia and beef cattle pneumonia (shipping fever). Infections by *P. multocida* serogroup A are responsible for severe bronchopneumonia in young dairy calves, and the majority of samples from affected cattle have been found to be positive for *P. multocida* serotype A:3 (Dabo et al., 2008b).

Currently available *P. multocida* cattle vaccines are predominantly traditional bacterins and a live streptomycin-dependent (STR<sup>D</sup>) mutant (Dabo et al., 2008b). These vaccines have some limitations such as being serotype-specific (Catt et al., 1985) or conferring partial protection (Mathy et al., 2002). Moreover, Dowling et al. (2004) reported that intratracheal vaccination of calves with

formalin-killed *P. multocida* did not result in protection against experimental pulmonary challenge. Inactivated vaccines are generally ineffective at inducing a potent cell-mediated immune response which is important in the elimination of intracellular pathogens (Singh and O'Hagan, 2002). Commercial vaccines against BRD are monovalent or divalent, limiting their efficacy. Recent studies have focused on multivalent vaccines containing antigenic proteins to simplify the vaccination schedule and increase the protection range (Cho et al., 2008) as well as genetic fusions to deliver diverse antigens to the immune system (Ayalew et al., 2008). On the other hand, a proper adjuvant plays an important role in the success of the vaccine formulations containing recombinant proteins (Buchman et al., 2010).

Recent studies have shown that several *P. multocida* outer membrane proteins (OMPs) contributed to the pathogenesis and possess immunogenic and bactericidal properties (Basagoudanavar et al., 2006; Lee et al., 2007; Tan et al., 2010). OmpH is an antigenic, surface-exposed and conserved OMP porin that is detected in 100% of bovine isolates investigated and has been mooted as a potential vaccine candidate (Dabo et al., 2008b). *Pasteurella* lipoprotein E (PlpE) is another immunogenic OMP of *P. multocida* and mice or chickens immunized with recombinant PlpE were protected against challenge with other *P. multocida* serotypes (Wu et al., 2007). To date, protection conferred by recombinant PlpE and

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OmpH proteins from a bovine isolate of *P. multocida* A:3 has not been investigated.

The aim of the present study was to investigate the efficacy of the recombinant PlpE, OmpH and PlpEC-OmpH proteins from *P. multocida* A:3, one of the causative agents of shipping fever, in a mouse model. The recombinant proteins were formulated with oil-based or oil-based CpG ODN adjuvants and inoculated into BALB/c mice. Protectivity of the formulations was evaluated by survival of the mice upon challenge with a lethal dose of the pathogen. Our data indicated that rPlpE or rPlpEC-OmpH formulated with an oil-based CpG ODN conferred 100% survival against 10  $LD_{50}$  of *P. multocida* in mice.

## 2. Materials and methods

### 2.1. Bacterial strains and plasmids

*Escherichia coli* DH5 $\alpha$  (ATCC) and *E. coli* BL21 (Novagen, Germany) were the bacterial hosts for cloning and expression of the genes from *P. multocida* P-1062 (ATCC 15743, serotype A:3). The cloning vector pGEMT Easy (Promega, USA) and expression vector pET28a(+) (Novagen, Germany) were used.

### 2.2. Construction of recombinant plasmids

*plpE* and *ompH* genes were amplified via PCR using chromosomal DNA of *P. multocida* P-1062. The *plpE* gene was amplified with PlpEF: ggatccatgtgtagcgggtggtgg (*Bam*HI) and PlpER: agatcttctgtcctgtgactt (*Bgl*II) primers. Primers for the C-terminal fragment were PlpECF: ggatccatgcccttcagcagattacaa (*Bam*HI) and PlpER. The *ompH* gene was amplified with OmpHF: agatctatggcaacagtttcaaa (*Bgl*II) and OmpHR: agatctttagaagtgtacgcgta (*Bgl*II) primers. PCR products were ligated to pGEMT Easy vector and introduced into *E. coli* DH5 $\alpha$ . The pGEMT-plpEC and pGEMT-ompH plasmids were cut with *Bgl*II and the *ompH* fragment was ligated to pGEMT-plpEC to obtain *plpEC-ompH* fusion. Recombinant plasmids were verified with restriction enzyme digestion and nucleotide sequence analysis. The genes were subsequently cloned in pET28a to express His-tagged proteins (pET28-plpE, pET28-ompH and pET28-plpEC-ompH).

### 2.3. Purification of recombinant proteins and preparation of vaccine formulations

*E. coli* BL21 cells carrying pET28-plpEC-ompH, pET28-plpE and pET28-ompH were grown in Luria Broth (LB; Merck, Germany) supplemented with 30  $\mu$ g of kanamycin/ml. Expression was induced at OD<sub>600</sub> of 0.6 by adding isopropyl- $\beta$ -D-galactopyranoside (IPTG; Sigma, Germany) to 1 mM final concentration and incubated at 37 °C for 5 h in a shaker incubator at 200 rpm. Cells were harvested by centrifugation and resuspended in LEW buffer (8 M urea, 300 mM NaCl, 50 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 8.0). Following the sonication using a CP70T Ultrasonic Processor (Cole-Parmer, USA) for 6  $\times$  10 s at 60% amplitude, cellular debris was removed by centrifugation. The supernatants containing the recombinant proteins were purified on Protino Ni-TED 2000 packed columns (Macherey-Nagel, Germany) according to the supplier's recommendations. Eluted proteins were dialyzed against DB buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 500 mM NaCl, 4 M urea, pH 8.0) and sterilized through a 0.2  $\mu$ m membrane filter. The purity of proteins was determined by SDS-PAGE. The recombinant proteins were formulated with oil based (Montanide ISA 206 VG, Seppic, France) or oil based-CpG ODN 1555 adjuvant. The sequence of a phosphorothioate modified, 15-mer CpG 1555 is reported elsewhere (Gursel et al., 2001). Ten microgram of CpG ODN was used per dose.

### 2.4. Immunization and challenge experiments

Female BALB/c mice weighing 16–18 g were used in animal experiments. 100  $\mu$ g of recombinant PlpE, OmpH and PlpEC-OmpH proteins formulated with the oil-based or oil-based CpG ODN adjuvant were intraperitoneally (IP) inoculated into mice at days 0 and 21 and the mice were challenged at day 31. Mice in the two parallel control groups received either oil-based or oil-based CpG ODN adjuvant only. The mice were tail bled a day prior to both booster vaccination and challenge (days 20 and 30, respectively). The sera were maintained at –20 °C until further use. Animals were challenged IP with 10  $LD_{50}$  of *P. multocida* A:3 (55 CFU) in 500  $\mu$ l of saline solution. Survivors were recorded daily for seven days. Animal experiments were performed under the approval of the Ethics Committee on Animal Experimentation, Middle East Technical University, Turkey.

### 2.5. Western blot analysis

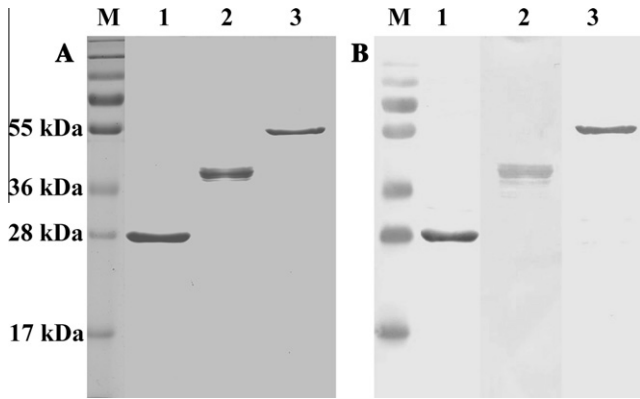
Recombinant proteins were run on 12% SDS-polyacrylamide gel and transferred to nitrocellulose membranes by a modified method of Towbin et al. (1979) using a semi-dry blotting system (Cleaver Scientific Ltd, UK). Each lane for rPlpE, rOmpH and PlpEC-OmpH was separated cutting the nitrocellulose membrane. Western blot analysis was carried out using the respective primary antibodies raised against rPlpE, rOmpH or PlpEC-OmpH fusion proteins at day 30 post primary vaccination at a dilution of 1:200. Rabbit anti-mouse IgG conjugated to alkaline phosphatase (Sigma, Germany) was used as the secondary antibody at a dilution of 1:10,000. AP Conjugate Substrate Kit (Bio-Rad, USA) was used for color detection. PageRuler™ Plus Prestained Protein Ladder (Fermentas, Thermo Scientific, USA) was used as the size marker.

### 2.6. Determination of antibody response to vaccine formulations

Specific antibody response was determined measuring the IgG titers via ELISA using sera collected from vaccinated mice. Purified recombinant PlpE, OmpH and PlpEC-OmpH proteins were used as coating antigens at concentrations of 1  $\mu$ g/well for determination of anti-PlpE, anti-OmpH and anti-PlpEC-OmpH antibody levels, respectively. Two-fold serial dilutions of the murine sera ranging from 1:100 to 1:12800 were used in triplicates as primary antibody and rabbit anti-mouse IgG conjugated to alkaline phosphatase (Sigma, Germany) was used as secondary antibody at a dilution of 1:2000. PNPP substrate (*p*-Nitrophenyl Phosphate Disodium Salt, Thermo Scientific, USA) was used as colorimetric reagent. Plates were read at 405 nm to determine optical density on a microtiter plate reader.

### 2.7. ELISA for detection of serum IFN- $\gamma$ titers

Mouse IFN- $\gamma$  Minikit (Pierce, Thermo Scientific, USA) was used for the detection of serum IFN- $\gamma$  levels of vaccinated mice. The protocol was applied according to the manufacturer's recommendations. Briefly, 96-well plates were coated with Coating Antibody and blocked with Blocking Buffer. Serum samples were added at 1:4 dilutions in Assay Buffer and incubated at RT overnight. After washing the plate, Detection Antibody was added to each well and incubated 1 h at RT. The plate was washed and Streptavidin-HRP (Pierce, Thermo Scientific, USA) was added at a dilution of 1:10,000. The plate was incubated at RT for 30 min and washed. TMB substrate (Pierce, Thermo Scientific, USA) was added to each well and incubated at RT for 30 min. The reaction was stopped with 0.18 M sulphuric acid and the absorbance read at 450 nm on a microtiter plate reader. The ng values were extrapolated from a ng vs absorbance curve for a set of standards on the plates.



**Fig. 1.** SDS–polyacrylamide gel (A) and Western blot (B) analyses of the rPlpE protein (lane 1), rOmpH protein (lane 2) and PlpEC-OmpH fusion protein (lane 3). M: protein molecular weight marker. Western blot analysis was performed cutting nitrocellulose membrane for each lane and using anti-rPlpE, anti-rOmpH and anti-PlpEC-OmpH sera collected at day 30 as primary antibodies for lanes 1, 2 and 3, respectively.

**2.8. Statistical analyses**

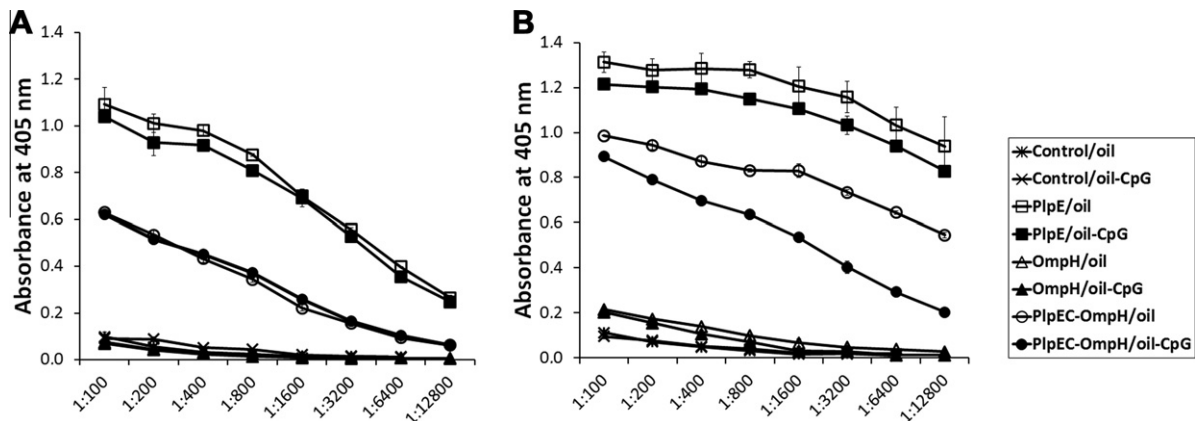
An analysis of variance (ANOVA) and the Tukey’s test were used for mean comparison of antibody response between groups.

Survival data were compared using the chi-square test (two-sided). Statistical analyses for immune responses and survival were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, USA). The significance level (*p*) for all analyses was set at 0.05. Standard deviations were calculated using Microsoft Office Excel 2010 program.

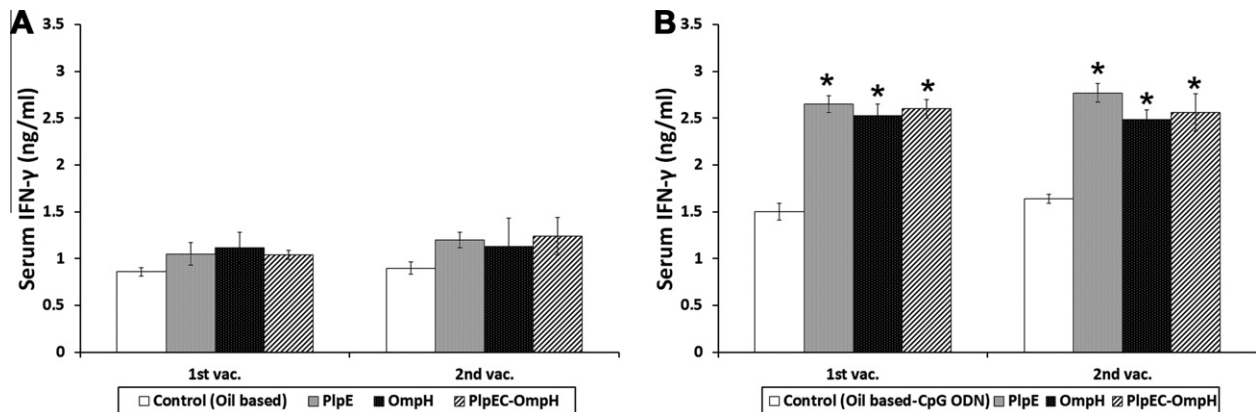
**3. Results**

**3.1. Cloning of *plpE* and *ompH* genes from *P. multocida* A:3 and purification of the recombinant proteins**

*plpE* gene (936 bp) without signal sequence, 459 bp C-terminal (*plpEC*) fragment and 969 bp *ompH* gene were cloned via PCR from the genomic DNA of *P. multocida* A:3 and the sequences were verified. In this study, the C-terminal of PlpE was used to obtain chimeric protein with OmpH since it was determined to be more immunogenic as compared to an N-terminal fragment (Okay et al., unpublished data). *plpE*, *ompH* and *plpEC-ompH* were cloned in pET28a for His-tagged protein expression. The purity of the recombinant proteins was visualized on SDS–polyacrylamide gel by Coomassie blue staining and antigenicity was shown by Western blot analysis (Fig. 1). Purified recombinant OmpH and PlpEC-OmpH proteins had bands with expected molecular masses of 37 and 54 kDa, respectively on SDS–polyacrylamide gels. rPlpE protein



**Fig. 2.** Serum IgG titers in mice vaccinated with rPlpE, rOmpH and PlpEC-OmpH formulated with oil-based and oil-based CpG ODN adjuvant measured by ELISA coating the plates with rPlpE, rOmpH and PlpEC-OmpH fusion protein, respectively. 1:100 to 1:12800 dilutions of the sera collected after first vaccination, at day 20 (A) and booster vaccination, at day 30 (B) were used and averages of triplicate samples were represented with standard deviations.



**Fig. 3.** Serum IFN-γ responses in mice vaccinated with rPlpE, rOmpH and PlpEC-OmpH formulated with oil-based (A) or oil-based CpG ODN (B) adjuvant measured by ELISA using 1:4 dilution of the sera collected after first vaccination (at day 20) and booster vaccination (at day 30). The ng values were extrapolated from a ng vs absorbance curve for a set of standards on the plates. \*indicates statistical difference (*p* < 0.05) between control and vaccinated groups.

**Table 1**

Protection conferred in BALB/c mice vaccinated IP with 100 µg of rPlpE, rOmpH and rPlpEC-OmpH proteins formulated with oil-based or oil-based CpG ODN adjuvants, respectively upon IP challenge with 10 LD<sub>50</sub> of live *P. multocida* A:3.

Vaccine formulation (protein/adjuvant)	No. of mice survived/challenged	Protection (%)
rPlpE/oil-based	4/5	80*
rPlpE/oil-based CpG	5/5	100*
rOmpH/oil-based	2/5	40
rOmpH/oil-based CpG	2/5	40
PlpEC-OmpH/oil-based	3/5	60*
PlpEC-OmpH/oil-based CpG	5/5	100*
Control (oil-based adjuvant)	0/5	0
Control (oil based CpG adjuvant)	0/5	0

\* Statistically significant as compared to control group,  $p < 0.05$ .

was, on the other hand, 28 kDa in both IPTG-induced culture lysate (data not shown) and the eluate, while its calculated mass was 36.5 kDa, indicating its processing in *E. coli*. Western blot analysis using specific antibodies showed that these antibodies reacted with purified recombinant protein antigens.

### 3.2. Immune responses against PlpE, OmpH and PlpEC-OmpH proteins and their protective efficacies

BALB/c mice were inoculated IP twice with 100 µg of rPlpE, rOmpH and PlpEC-OmpH proteins formulated with oil-based and oil-based CpG ODN adjuvants. The sera were collected prior to both booster inoculation and challenge (days 20 and 30, respectively) and used for the measurement of IgG titers and IFN-γ levels. As shown in Fig. 2, serum IgG levels in mice vaccinated with rPlpE and rPlpEC-OmpH significantly ( $p < 0.05$ ) increased after first and second immunizations. On the other hand, the increment in IgG level upon injection with rOmpH formulated with oil-based or oil-based CpG ODN was only significant after second vaccination and at lower dilutions.

Serum IFN-γ levels in mice IP vaccinated with rPlpE, rOmpH and PlpEC-OmpH formulated with oil-based and oil-based CpG ODN adjuvants were determined by ELISA (Fig. 3). Oil-based CpG ODN adjuvanted formulations significantly ( $p < 0.05$ ) increased serum IFN-γ titers after first and second vaccinations while the increment was not statistically significant with oil-based adjuvant alone.

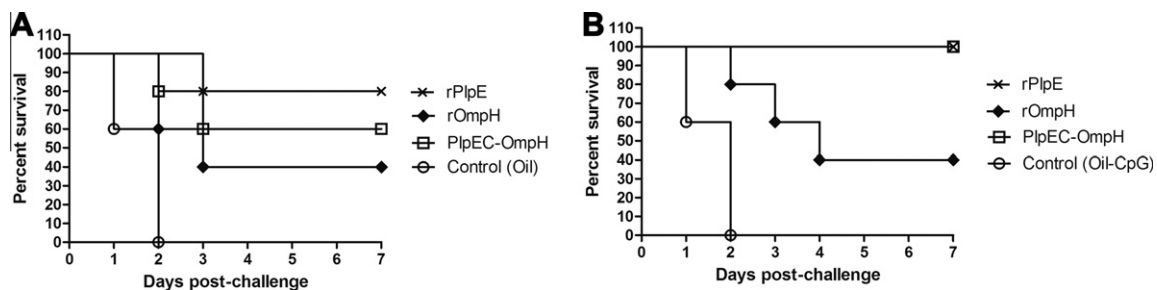
The protective efficacy of rPlpE, rOmpH and PlpEC-OmpH proteins formulated with oil-based or oil-based CpG ODN adjuvants was investigated after IP challenge of the immunized mice with 10 LD<sub>50</sub> of live *P. multocida* A:3 (Table 1, Fig. 4). Vaccine formulations composed of rPlpE with oil-based or oil-based CpG ODN conferred 80% and 100% protection, respectively. Protectivity of rPlpEC-OmpH fusion proteins formulated with oil-based or oil-based CpG ODN was 60% and 100%, respectively. However,

formulations containing rOmpH provided 40% protection, not statistically significant.

## 4. Discussion

Outer membrane protein H (OmpH) and *Pasteurella* lipoprotein E (PlpE) are of interest in recombinant vaccine studies against different serotypes of *P. multocida*. Wu et al. (2007) reported that rPlpE from *P. multocida* serotype A:1 causing fowl cholera conferred 63–100% protection in mice and chickens against challenge with serotypes A:1, A:3 and A:4. Luo et al. (1997) vaccinated chickens with native and recombinant OmpH from *P. multocida* serotype A:1 and reported 100% and 18% protection, respectively. However, Sthitmatee et al. (2008) showed that both native and recombinant OmpH from serotype A:1 and its identical protein, Cp39 from serotype A:3, conferred 60–100% protection in chickens against challenge with serotypes A:1 and A:3. Tan et al. (2010) immunized mice IP and subcutaneously with rOmpH from *P. multocida* serotype B:2 causing haemorrhagic septicaemia and obtained 80% and 100% protection, respectively. Moreover, rOmpH from *P. multocida* isolated from a case of atrophic rhinitis provided 70% protection in mice (Lee et al., 2007). Dabo et al. (2008a) vaccinated mice with rOmpA from a bovine isolate of *P. multocida* serotype A:3 but no protection was obtained. Till now, there has been no report on protectivity of rPlpE or rOmpH proteins isolated from *P. multocida* A:3 causing shipping fever. In the present study, we showed that rPlpE, rOmpH and PlpEC-OmpH fusion proteins provided 80–100%, 40% and 60–100% homologous protection, respectively depending on the adjuvant used in the formulation. A cross-protection experiment was not performed in this study since the only *P. multocida* serotype involved in shipping fever is A:3.

Selection of appropriate adjuvants that boost antigen immunogenicity is one of the critical steps in vaccine development (Rojomontejo et al., 2011). CpG ODNs have been utilized as vaccine adjuvants in studies on animals because they induce Th1-type cell mediated immunity, as evidenced by increased IFN-γ through binding to TLR9, which is involved in the recognition of pathogen-associated molecular patterns (Klinman et al., 2004). Oil-based adjuvants (emulsions) have been used in vaccine formulations since 1945 (Aucouturier et al., 2006). Montanide ISA 206 is a safe water-in-oil-in-water emulsion commonly used in vaccine studies against animal pathogens including *P. multocida* (Basagoudanavar et al., 2006; Dupuis et al., 2006). It has been shown that CpG ODN in combination with Montanide ISA 206 synergistically induced higher immune responses to VP1 antigen of FMDV in mice and cattle as compared to an either CpG ODN or Montanide only formulation (Ren et al., 2011). Consistently, our study showed that Montanide plus CpG ODN adjuvanted formulations induced both IgG and serum IFN-γ in vaccinated mice whilst Montanide only formulations did not increase serum IFN-γ. The control group mice were inoculated with oil-based CpG ODN adjuvant alone. The high-



**Fig. 4.** Kaplan Meier survival curves of mice ( $n = 5$ ) immunized with rPlpE, rOmpH and PlpEC-OmpH fusion protein formulated with (A) oil-based or (B) oil-based CpG ODN adjuvants upon IP challenge with 10 LD<sub>50</sub> of live *P. multocida* A:3.



er IFN- $\gamma$  levels in mice received vaccine formulations as compared to that in control group showed that the increase in IFN- $\gamma$  titers was antigen-specific. Still, the use of splenocyte cultures stimulated with the antigen for the detection of IFN- $\gamma$  levels would provide convincing evidence.

Our study constitutes the first recombinant vaccination strategy utilizing PlpE and OmpH against *P. multocida* A:3, demonstrating that rPlpE or PlpEC-OmpH fusion proteins conferred 100% survival in mice when injected together with Montanide modified-CpG ODN, and our further work will involve the trials of vaccination in cattle using these formulations.

### Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could have inappropriately influenced or biased the content of this paper.

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