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**Original Article**

# Immunization with recombinant PlpE of ovine *Mannheimia haemolytica* isolate provides protection against lethal challenge in mice

**Yektaseresht, A.[1*]; Hemati, Z.[2]; Sabet Sarvestani, F.[1]; Hosseini, A.[1] and Vir Singh, S.[3]**

[1]Department of Pathobiology, School of Veterinary Medicine, Shiraz University, Shiraz, Iran; [2]Department of Pathobiology, Faculty of Veterinary Medicine, University of Shahrekord, Shahrekord, Iran; [3]Department of Biotechnology, Institute of Applied Sciences and Humanities, GLA University, Ajhai, Mathura, Uttar Pradesh, India

[*]***Correspondence:*** *A. Yektaseresht, Department of Pathobiology, School of Veterinary Medicine, Shiraz University, Shiraz, Iran. E-mail address: azadeh_neisi@shirazu.ac.ir*

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## Abstract

**Background:** *Mannheimia haemolytica* primarily causes pneumonia leading to heavy morbidity and mortality in domestic livestock world-wide. Recently, outer membrane lipoproteins have emerged as targets for inducing protective immunity against the *Pasteurella* infection. **Aims:** This study aimed to evaluate recombinant outer membrane lipoprotein E (PlpE) from the ovine *M. haemolytica* isolate, as a potential vaccine candidate. **Methods:** Recombinant PlpE was constructed using pET26 (b) expression vector in *Escherichia coli*. Expressed recombinant PlpE was purified and injected subcutaneously to mice. The protection index of the vaccine was evaluated by challenge of mice intraperitoneally. **Results:** Anti-PlpE antibody responses in the immunized mice was significantly increased in comparison with the control group which in turn, provided effective protection when challenged with strain of virulent *M. haemolytica*. **Conclusion:** Recombinant PlpE from ovine *M. haemolytica* isolate had the potential to be used as a vaccine candidate against *M. haemolytica* infection in sheep flocks.

**Key words:** *Mannheimia haemolytica*, PlpE, Recombinant vaccine, Sheep

## Introduction

As a commensal microorganism, *Mannheimia haemolytica* is part of natural flora of the upper respiratory tract of domestic ruminants. Sudden fluctuations in weather often lead to outbreak of disease in sheep causing pneumonia and heavy mortality and morbidity (Quinn *et al*., 2011). Of the four major domestic livestock species, sheep are most susceptible to *Mannheimia* infections. Non-judicious, indiscriminate, and frequent use of antimicrobials leads to the development of resistance against antibiotics (Guzman-Brambila *et al*., 2012). Effective vaccinations are the priority to save livestock (Ozbey *et al*., 2006). Currently, there are some licensed live-attenuated/killed vaccines incorporating whole cells or components of bacilli (Purdy *et al*., 1996; Aubry *et al*., 2001; Rajeev *et al*., 2003). The development of resistant strains necessitate the work on newer vaccines. Recombinant proteins have been considered safe and effective candidates. Recent focus has been on virulence factors e.g., capsule lipopolysaccharide, adhesins, outer membrane proteins, and leukotoxin (Lkt) to improve vaccine quality. Surface antigens and Lkt, (exotoxin of *M. haemolytica* secreted by all serotypes) are important in stimulating immunity (Shewenet *et al*., 1985). Low quantities of Lkt induce both neutrophils and macrophages, resulting in the release of cytokines (TNFα, IL-1) by mast cells (Maheswaran *et al*., 1992; Hsuan *et al*., 1999), whereas, higher concentrations in the host's system lead to apoptosis and subsequent necrosis of cells by creating hydrophilic pores on their membranes (Clinkenbeard *et al*., 1989). Outer membrane proteins (OMPs) are good candidates for developing novel vaccines (Gatto *et al*., 2002; Carpenter *et al*., 2007). OMP antigens have the ability to elicit higher antibody responses (Confer *et al*., 1995; Pandher *et al*., 1998). Immunization of cattle with OMPs from the *M. haemolytica* S1 strain enhances protection against challenges with *M. haemolytica* (Morton *et al*., 1995). Several *M. haemolytica* OMPs have been reported to possess immunogenic properties. Outer membrane lipoprotein E (PlpE) (45 kDa) is one of the OMPs which has immunogenic properties and is present in all the serotypes of *M. haemolytica* (Pandher *et al*., 1999). In view of the heavy economic losses to the livestock industry by *M. haemolytica*, work on the identification of appropriate antigens, as 'vaccine candidates', is in focus. The present study proposes to

evaluate the immunogenic potential of the recombinant PlpE antigen from ovine *M. haemolytica* lung isolates against *M. haemolytica* infection as a vaccine candidate in sheep.

## Materials and Methods

### Bacteria

*M. haemolytica* was recovered from pneumonic lungs of sheep from a slaughter house in Shiraz, Iran. The surface of suspected lungs was sterilized and sampled with sterile swabs. Colonies on blood agar were tested for hemolysis and morphology of colonies. Bacilli were identified on the basis of metabolic activity with different sugar moeties (Quinn *et al*., 2011). Bacilli were cultured in brain heart infusion broth (BHI, Merck, Germany) for 48 h at 37°C.

### Preparation of recombinant protein

*PlpE* gene was amplified from the genomic DNA of *M. haemolytica* using the following pair of oligonucleotide primers: CTC TAA TTA GAA TTC CGG AGG AAG CGG TAG CGG and GCC GGC CCT CGA GTT TTT TCT CGC TAA CCA TTA T. Amplified DNA fragment was cloned into the pET26b (+) vector to obtain His-tagged protein (pET26b-plpE) (Novagen, Madison, WI, USA). The nucleotide sequence of PlpE was deposited in the NCBI GenBank (accession No.: KY795962). Recombinant plasmid encoding PlpE protein was expressed in *E. coli* BL21 (DE3). A colony of the recombinant clone was cultured in LB medium (Merck, Germany) (50 µg/ml kanamycin) overnight at 37°C. The culture was diluted (1:100) and cultured until its optical density at 600 nm reached 0.6. Protein expression was induced with a final concentration of 1 mM isopropyl-*D*-1-thiogalactopyranoside (IPTG, Cinagen, Iran). The culture was incubated at 37°C for 16 h in a shaker incubator. Cells were harvested by centrifugation (9000 g, 10 min, 4°C) and resuspended in an equilibration buffer (200 mM NaCl, 50 mM Tris-HCl, pH = 8.0). Cell suspension was lysed on ice using sonication at 30% amplitude for 20 cycles (20 s pulse on, 20 s pulse off). His6-tagged PlpE was purified using nickel affinity column (Qiagen, Germany). Crude lysate was equilibrated with 1 ml of pre-washed Ni-NTA (Qiagen, Germany) resin for 8 h at 4°C. The suspension was centrifuged (2000 g, 10 min, 4°C) and the pellet was washed three times with wash buffer. The captured rPlpE proteins were eluted by increasing the imidazole concentration to 250 mM in the elution buffer (Yektaseresht *et al*., 2019). Collected fractions were dialyzed against PBS overnight at 4°C. The purified recombinant protein was evaluated by SDS-PAGE and western blotting. Recombinant PlpE was electrophoresed through SDS-PAGE using 4% stacking and 12% separating acrylamide gels, and transferred to nitrocellulose membrane using a semi-dry system. The membrane was blocked with 3% skimmed milk in PBS and incubated overnight. Following three rounds of washing with PBS-tween 20 (PBST buffer), membrane was incubated with 1:10000 dilution of mouse anti histidine monoclonal antibody (Sigma Aldrich, USA) for 1 h at room temperature. After washing with PBST, membrane was developed using substrate (0.5 mg/ml Diaminobenzidine, DAB, 0.005% $H_2O_2$) (Sigma Aldrich, USA).

### Immunization and challenge

Twenty BALB/c female mice (20 g, 6-8 weeks old) were purchased from Razi Institute (Tehran, Iran). In the first round of immunization, 10 mice were subcutaneously injected with 100 µg of rPlpE Freund's complete adjuvant (1:1), (Sigma Aldrich, USA). From the second to the fifth immunization, Freund's complete adjuvant was replaced with Freund's incomplete adjuvant. An interval of two weeks was kept between successive vaccinations. The control group (10 mice) was injected with PBS and the adjuvant. Serum samples were collected before each immunization and seven days after the last injection. Five days after the last injection, mice were challenged intraperitoneally with 500 µL ($3.6 \times 10^{10}$/ml) of *M. haemolytica* culture and observed for seven days. All animal experiments were performed as per the established guidelines and following the norms of the Ethical Committee of Shiraz University, Iran.

### ELISA

Anti-rPlpE antibody levels were assessed using ELISA. ELISA plates (Greiner Bio-One, Austria) were coated (100 µL/well) with 0.1 µg/ml purified rPlpE in 50 mM Carbonate/bicarbonate buffer (pH = 9.6) overnight. Free spaces were blocked with 3% skimmed milk. After washing with PBS-0.05% Tween 20 (PBST), mice serum samples were diluted 50 times with PBS-1% bovine serum albumin and incubated at 37°C for 1 h. Plates were washed with PBST, HRP-conjugated goat anti-mouse IgG (Sigma-Aldrich, USA) diluted 1:6000 for 1 h at 37°C. After washing, O-Phenylene diamine dihydrochloride (OPD) (Sigma Aldrich, USA) was used as the substrate, and the absorbance of each well was determined at 450 nm.

### Western blot analysis

Specificity of mouse polyclonal antibodies against rPlpE was evaluated by western blot analysis. Crude cell lysate of *M. haemolytica* was resolved on 12% SDS-PAGE (polyacrylamide gel electrophoresis) and transferred onto a nitrocellulose membrane. After blocking overnight at RT with 3% skimmed milk in PBS, membrane was washed with PBST and incubated with rPlpE-specific mouse polyclonal antibody at the dilution of 1:50 for 1 h at 37°C. After washing, horseradish peroxidase-conjugated goat anti-mouse IgG (Sigma-Aldrich, USA) diluted at 1:10000 was added and incubated for 1 h at 37°C. Finally, membrane was washed and placed into a substrate solution ($H_2O_2$/Diaminobenzidine, DAB) (Sigma Aldrich, USA).

### Statistical analysis

Statistical analysis was carried out using SPSS

version 16. All data was represented as mean±standard deviation (SD). Differences in the groups were analyzed by one-factor analysis of variance (ANOVA) and Tukey's post-hoc test in SPSS. P-values <0.05 were considered as statistically significant.

## Results

### Expression and purification of rPlpE

The expression of His6-tagged PlpE was induced with IPTG in *E. coli* BL21. SDS-PAGE analysis showed rPlpE with a molecular weight of 48 kDa (Fig. 1A). Recombinant PlpE was purified and a protein of 48 kDa was observed (Fig. 1A). In western blot analysis, anti-His monoclonal antibody reacted specifically with a ~48 kDa a protein in *E. coli* BL21, corresponding to rPlpE (Fig. 1B).

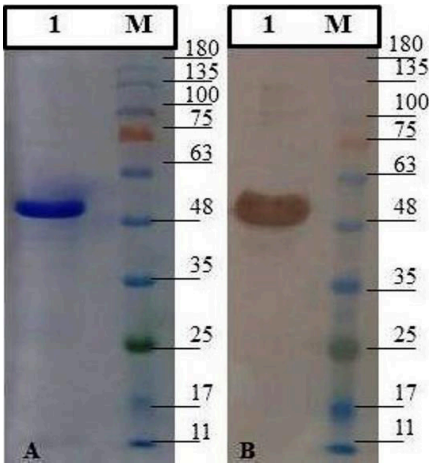


**Fig. 1:** Characterization and confirmation of purified recombinant PlpE protein using SDS-PAGE and Western blotting. (**A**) Characterization of purified recombinant PlpE protein expressed in *E. coli* strain BL21 using 12% SDS-PAGE gel electrophoresis, and (**B**) Western blotting results of purified rPlpE protein using anti-His antibodies. Lane M: Protein molecular weight marker (Cinnagen [SL7012] prestained protein ladder). Lane 1: Purified r PlpE protein

### Immune responses elicited by the immunization with rPlpE

Serum samples from mice were collected to evaluate their immune responses using ELISA. Anti rPlpE antibody titers against recombinant PlpE were detected in the immunized mice. Anti-rPlpE antibodies were seen on 7, 14, 21, and 28 days post immunization, and anti rPlpE antibody levels increased on day 28 post vaccination. Non-immunized mice showed low antibody titers (P<0.05) (Fig. 2).

### Specificity of anti-rPlpE antibodies against PlpE from *M. haemolytica*

Specificity of anti-rPlpE antibodies were evaluated using western blot analysis. As shown in Fig. 3, lane 1, antibodies raised against rPlpE reacted with PlpE with the whole cells of *M. haemolytica* but not with the control serum in lane 2 (Figs. 3A and B). Our results showed that the anti-rPlpE antibodies produced were able to specifically detect PlpE expressed by *M. haemolytica.*

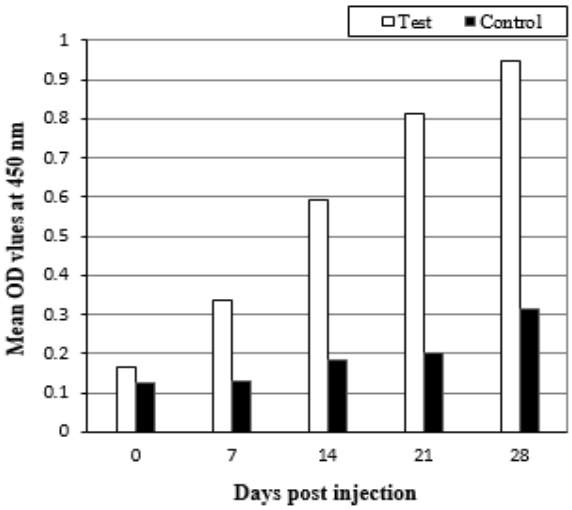


**Fig. 2:** Antibody titers were determined in sera of mice immunized with recombinant PlpE protein. Mice were immunized with rPlpE protein, or PBS as a control, and sera were collected at the indicated time points post-immunization to detect rPlpE specific antibodies by ELISA. Means of duplicate samples were represented with standard deviations

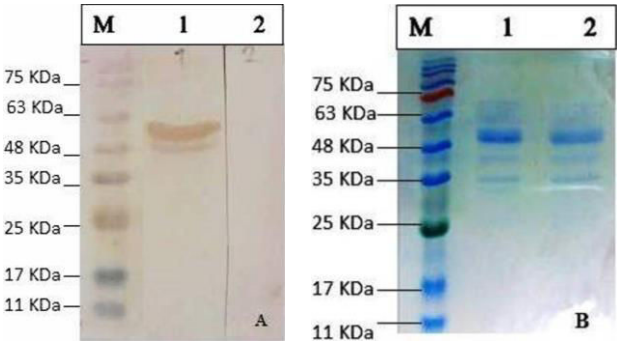


**Fig. 3:** Western blot (**A**) and SDS-polyacrylamide gel (**B**) analysis using polyclonal antibodies against the recombinant PlpE protein and control serum as primary antibodies. M: Protein molecular weight marker (Cinnagen [SL7012] prestained protein ladder). Lanes 1 and 2: The culture supernatant of *Mannheimia haemolytica*

### Protective effect of immunization with rPlpE antigens to the challenge of lethal *M. haemolytica*

Immunized mice were challenged intraperitoneally with live *M. hemolytica* culture. Results indicated that rPlpE could significantly protect mice against the challenge of infection. The protective index of rPlpE as 'vaccine candidate' was 90% in the immunized group. Nine out of 10 (90%) immunized mice survived while 10 out of the 10 (100%) of mice in the control group died. The spleen of the dead mice infected with *M. haemolytica* were removed and cultured on the blood agar plates. The presence of *M. haemolytica* in the tissues were confirmed biochemically (Quinn *et al*.,

2011).

## Discussion

Three (LPS, OMPs and Lkt) immuno-dominant proteins of *M. haemolytica* are the best known stimulators of immunity against pasteurellosis (Lee *et al*., 2000). High neutralizing antibody responses to Lkt protein in the vaccinated cattle have been correlated with resistance to experimental challenge with live *M. haemolytica* (Colon *et al*., 1991). Outer membrane proteins (antigens) of *M. haemolytica* are important in protecting domestic livestock against *M. haemolytica* infection. Therefore, recombinant outer membrane lipoprotein (PlpE) was developed and evaluated and shown to be a potent immunogen that induced effective immunity against *M. haemolytica* (Confer *et al*., 2003, 2006, 2009). Confer *et al*. (2003) reported that commercial *M. haemolytica* vaccine with recombinant *M. haemolytica* S1 (Oklahoma Strain) PlpE conferred protection against challenge with the homologous serotype in cattle. Confer *et al*. (2006) vaccinated cattle with rPlpE from the *M. haemolytica* serotype A:1 and reported protection against challenge with serotype A:6. Ayalew *et al*. (2008) studied immunogenicity of chimeric PlpE-Lkt protein in mice. Confer *et al*. (2009) vaccinated cattle with chimeric PlpE-Lkt protein and *M. haemolytica* bacterin and reported that they could enhance resistance against challenges with *M. haemolytica*. Batra *et al*. (2016) constructed chimeric protein comprising of the leukotoxin-neutralizing epitopes and immuno-dominant epitopes of outer membrane protein PlpE. Immunogenicity of the chimeric protein was evaluated in mice. Yektaseresht *et al*. (2019) produced rPlpE and assessed immunogenicity of the recombinant protein in goats. In this study, we observed that rPlpE of ovine *M. haemolytica* isolate provided 90% protection to the vaccinated mice against lethal challenge with virulent *M. haemolyticai* culture. Anti-rPlpE Polysera showed enhanced viability against challenge with *M. haemolytica*. Western blot analysis using anti-rPlpE Polysera showed specific reactivity with PlpE from whole *M. haemolytica*, suggesting possible maintenance of the native epitope structure in the recombinant protein (rPlpE). Our results indicated that protection conferred by rPlpE against lethal challenge with virulent *M. haemolytica* was induced by the antibody response.

Anti-rPlpE antibodies had higher protective index against *M. haemolytica* infection. This suggests that the protective activity of anti-rPlpE antibodies may be due to the direct blocking of the function of PlpE homologous proteins. The present study showed the potential of recombinant PlpE of the local ovine *M. haemolytica* isolate as a vaccine candidate to protect sheep against *M. haemolytica* infection.

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## Conflict of interest

The authors do not have any conflicts of interest to declare.

## References

**Aubry, P; Warnick, LD; Guard, CL; Hill, BW and Witt, MF** (2001). Health and performance of young dairy calves vaccinated with a modified live *Mannheimia haemolytica* and *Pasteurella multocida* vaccine. J. Am. Vet. Med. Assoc., 219: 1739-1742.

**Ayalew, S; Confer, AW; Payton, ME; Garrels, KD; Shrestha, B; Ingram, KR; Montelongo, MA and Taylor, JD** (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: 4955-4961.

**Batra, SA; Shanthalingam, S; Donofrio, G and Srikumaran, S** (2016). A chimeric protein comprising the immunogenic domains of *Mannheimia haemolytica* leukotoxin and outer membrane protein PlpE induces antibodies against leukotoxin and PlpE. Vet. Immunol. Immunopathol., 175: 36-41.

**Carpenter, T; Khalid, S and Sansom, MS** (2007). A multi domain outer membrane protein from *Pasteurella multocida*: modelling and simulation studies of PmOmpA. Biochim. Biophys. Acta. 1768: 2831-2840.

**Clinkenbeard, KD; Mosier, DA and Confer, AW** (1989). Transmembrane pore size and role of cell swelling in cytotoxicity caused by *Pasteurella haemolytica* leukotoxin. Infect. Immun., 57: 420-425.

**Confer, AW and Ayalew, S** (2018). *Mannheimia haemolytica* in bovine respiratory disease: immunogens, potential immunogens, and vaccines. Anim. Health Res. Rev., 19: 79-99.

**Confer, AW; Ayalew, S; Montelongo, M; Step, DL; Wray, JH; Hansen, RD and Panciera, RJ** (2009). Immunity of cattle following vaccination with a *Mannheimia haemolytica* chimeric PlpE-LKT (SAC89) protein. Vaccine. 27: 1771-1776.

**Confer, AW; Ayalew, S; Panciera, RJ; Montelongo, M; Whitworth, LC and Hammer, JD** (2003). Immunogenicity of recombinant *Mannheimia haemolytica* serotype 1 outer membrane protein PlpE and augmentation of a commercial vaccine. Vaccine. 21: 2821-2829.

**Confer, AW; Ayalew, S; Panciera, RJ; Montelongo, M and Wray, JH** (2006). Recombinant *Mannheimia haemolytica* serotype 1 outer membrane protein PlpE enhances commercial *M. haemolytica* vaccine-induced resistance against serotype 6 challenge. Vaccine. 24: 2248-2255.

**Confer, AW; McCraw, RD; Durham, JA; Morton, RJ and Panciera, RJ** (1995). Serum antibody responses of cattle to iron-regulated outer membrane proteins of *Pasteurella haemolytica* A1. Vet. Immunol. Immunopathol., 47: 101-110.

**Conlon, JA**; **Shewen, PE and Lo, RY** (1991). Efficacy of recombinant leukotoxin in protection against pneumonic challenge with live *Pasteurella haemolytica* A1. Infect. Immun., 59: 587-591.

**Gatto, NT; Dabo, SM; Hancock, RE and Confer, AW** (2002). Characterization of, and immune responses of mice to, the purified OmpA-equivalent outer membrane protein

of *Pasteurella multocida* serotype A:3 (Omp28). Vet. Microbiol., 87: 221-235.

**Guzman-Brambila, C; Quintero-Fabian, S; Gonzalez-Castillo, C; de Obeso-Fernandez del Valle, A; Flores-Samaniego, B; de la Mora, G; Rojas-Mayorquin, AE and Ortuno-Sahagun, D** (2012). LKTA and PlpE small fragments fusion protein protect against *Mannheimia haemolytica* challenge. Res. Vet. Sci., 93: 1293-1300.

**Hsuan, SL; Kannan, MS; Jeyaseelan, S; Prakash, YS; Malazdrewich, C; Abrahamsen, MS; Sieck, GC and Maheswaran, SK** (1999). *Pasteurella haemolytica* leukotoxin and endotoxin induced cytokine gene expression in bovine alveolar macrophages requires NF-kappaB activation and calcium elevation. Microb. Pathogenesis. 26: 263-273.

**Lee, HY; Kehrli Jr, ME; Brogden, KA; Gallup, JM and Ackermann, MR** (2000). Influence of beta(2)-integrin adhesion molecule expression and pulmonary infection with *Pasteurella haemolytica* on cytokine gene expression in cattle. Infect. Immun., 68: 4274-4281.

**Maheswaran, SK; Weiss, DJ; Kannan, MS; Townsend, EL; Reddy, K; Rwhiteley, LO and Srikumaran, S** (1992). Effects *of Pasteurella haemolytica* A1 leukotoxin on bovine neutrophils: degranulation and generation of oxygen-derived free radicals. Vet. Immunol. Immunop., 33: 51-68.

**Morton, RJ; Panciera, RJ; Fulton, RW; Frank, GH; Ewing, SA; Homer, JT and Confer, AW** (1995). Vaccination of cattle with outer membrane protein-enriched fractions of *Pasteurella haemolytica* and resistance against experimental challenge exposure. Am. J. Vet. Res., 56: 875-879.

**Ozbey, G and Muz, A** (2006). Isolation of aerobic bacteria from the lungs of chickens showing respiratory disorders and confirmation of *Pasteurella multocida* by polymerase chain reaction (PCR). Vet. Arh., 76: 217-225.

**Pandher, K; Confer, AW and Murphy, GL** (1998). Genetic and immunologic analyses of PlpE, a lipoprotein important in complement-mediated killing of *Pasteurella haemolytica* serotype 1. Infect. Immun., 66: 5613-5619.

**Pandher, K; Murphy, GL and Confer, AW** (1999). Identification of immunogenic, surface-exposed outer membrane proteins of *Pasteurella haemolytica* serotype 1. Vet. Microbiol., 65: 215-226.

**Purdy, CW; Straus, DC; Sutherland, RJ and Ayres, JR** (1996). Efficacy of a subcutaneously administered, ultraviolet light-killed *Pasteurella haemolytica* A1-containing vaccine against transthoracic challenge exposure in goats. Am. J. Vet. Res., 57: 1168-1174.

**Quinn, PJ; Markey, BK; Leonard, FC; Hartigan, P; Fanning, S and Patrick, ESF** (2011). *Veterinary microbiology and microbial disease*. UK, John Willy & Son Ltd.

**Rajeev, S; Kania, SA; Nair, RV; McPherson, JT; Moore, RN and Bemis, DA** (2001). *Bordetella bronchiseptica* fimbrial protein-enhanced immunogenicity of a *Mannheimia haemolytica* leukotoxin fragment. Vaccine. 19: 4842-4850.

**Shewen, PE; Lee, CW; Perets, A; Hodgins, DC; Baldwin, K and Lo, RY** (2003). Efficacy of recombinant sialoglycoprotease in protection of cattle against pneumonic challenge with *Mannheimia (Pasteurella) haemolytica* A1. Vaccine. 21: 1901-1906.

**Shewen, PE and Wilkie, BN** (1985). Evidence for the *Pasteurella haemolytica* cytotoxin as a product of actively growing bacteria. Am. J. Vet. Res., 46: 1212-1214.

**Yektaseresht, A; Sabet Sarvestani, F; Dordani, M and Hosseini, A** (2019). Cloning and expression of *Mannheimia haemolytica* PlpE gene in *Escherichia coli* and its immunogenicity assessment. Arch. Razi Inst., 74: 111-118.