UNIVERSITI PUTRA MALAYSIA

# IMMUNOLOGY OF THE OUTER MEMBRANE PROTEINS OF *PASTEURELLA HAEMOLYTICA* A2, A7 AND A9 IN SHEEP

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# IMMUNOLOGY OF THE OUTER MEMBRANE PROTEINS OF *PASTEURELLA HAEMOLYTICA* A2, A7 AND A9 IN SHEEP

By

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## TABLE OF CONTENTS


| | | |
|---|---|---|
| **ACKNOWLEDGEMENTS** | | ii |
| **LIST OF TABLES** | | vii |
| **LIST OF PLATES** | | viii |
| **LIST OF FIGURES** | | ix |
| **ABSTRACT** | | x |
| **ABSTRAK** | | xii |
| **CHAPTER** | | |
| **I** | **INTRODUCTION** | 1 |
| **II** | **LITERATURE REVIEW** | 4 |
| | Pneumonic pasteurellosis | 4 |
| | *Pasteurella haemolytica* | 7 |
| | The Potential Protective Antigens of *Pasteurella haemolytica* | 8 |
| | Outer Membrane Proteins | 8 |
| | Cytotoxin (Leukotoxin) | 12 |
| | Lipopolysaccharides (LPS) | 15 |
| | Iron-regulated Proteins | 17 |
| | Pasteurella Vaccine | 19 |
| **III** | **THE PROFILES OF OUTER MEMBRANE PROTEINS OF *PASTEURELLA HAEMOLYTICA* A2, A7 AND A9 USING SODIUM DODECYL SULFATE POLYACRYLAMIDE GEL ELECTROPHORESIS** | |
| | Introduction | 24 |
| | Materials and Methods | 27 |
| | Isolation and Purification of the Outer Membrane Proteins (OMP) | 27 |
| | SDS-PAGE | 28 |
| | Results | 29 |
| | Discussion | 31 |
| | Summary | 34 |

IV ANTIGENICITY AND CROSS-REACTION OF THE OUTER MEMBRANE PROTEINS OF *PASTEURELLA HAEMOLYTICA* A2, A7 AND A9 DETECTED BY IMMUNOBLOTTING
Introduction.......... 36
Material and Methods .......... 38
Preparation of Antisera Against *Pasteurella haemolytica* A2,A7 and A9 .......... 38
Immunoblotting of the OMPs .......... 40
Results .......... 41
Homologous Antigenicity .......... 41
Antigenic Cross-reaction .......... 42
Discussion .......... 46
Summary .......... 49

V EFFICACY OF THE OUTER MEMBRANE PROTEIN SUBUNIT VACCINE OF *PASTEURELLA HAEMOYTICA* A2, A7 AND A9 AGAINST INTRATRACHEAL CHALLENGE EXPOSURE IN SHEEP
Introduction .......... 50
Materials and Methods .......... 53
Animals .......... 53
Preparation of Outer Membrane Protein Vaccine .......... 53
Preparation of Bacteria Inocula for Challenge.......... 54
Vaccination, Bleeding and Challenge Procedure .......... 54
Serology .......... 57
Bacterial Isolations .......... 58
Statistical Analysis .......... 59
Results .......... 59
Clinical Observations.......... 59
Extent of Lung Lesions.......... 60
Serological Response.......... 62
Correlation between the Antibody Response and Extent of Lung Lesions.......... 66
Microbiological Isolations.......... 67
Discussion .......... 68
Summary .......... 71

**VI GENERAL DISCUSSION** ............................................ 73

**BIBLIOGRAPHY** ............................................................. 84

**APPENDIX** ...................................................................... 106
A ........................................................................... 106
B ........................................................................... 113
C ........................................................................... 116
D ........................................................................... 118

**VITA** ............................................................................. 119

## LIST OF TABLES


| Table | | Page |
|---|---|---|
| 5.1 | Experimental design | 56 |
| 5.2 | The extent of pneumonic lung lesions (average) following intratracheal challenge of vaccinated and unvaccinated animals with live Pasteurella haemolytica A2, A7 and A9 | 61 |

## LIST OF PLATES


| Plate | | Page |
|---|---|---|
| 3.1 | Coomassie Blue R-250 stained SDS-PAGE (12% slab gel) profile of outer membrane proteins from *Pasteurella haemolytica* A2, A7 and A9. Tracks contained 16 µg protein. Lane A – molecular weight standard in thousands. Lane B – OMPs of *P. haemolytica* A2. Lane C – OMPs of *P. haemolytica* A7. Lane D – OMPS of *P. haemolytica* A9 | 30 |
| 4.1 | Immunoblot banding profile of outer membrane proteins of *Pasteurella haemolytica* A2, A7 and A9 developed using hyperimmune serum to whole *P. haemolytica* A2. Lane A – molecular weight standard in thousands. Lane B – OMPs of *P. haemolytica* A2. Lane C – OMPs of *P. haemolytica* A7. Lane D – OMPs of *P. haemolytica* A9 | 43 |
| 4.2 | Immunoblot banding profile of outer membrane proteins of *Pasteurella haemolytica* A2, A7 and A9 developed using hyperimmune serum to whole *P. haemolytica* A7. Lane A – molecular weight standard in thousands. Lane B – OMPs of *P. haemolytica* A7. Lane C – OMPs of *P. haemolytica* A9. Lane D – OMPs of *P. haemolytica* A2 | 44 |
| 4.3 | Immunoblot banding profile of outer membrane proteins of *Pasteurella haemolytica* A2, A7 and A9 developed using hyperimmune serum to whole *P. haemolytica* A9. Lane A – molecular weight standard in thousands. Lane B – OMPs of *P. haemolytica* A9. Lane C – OMPs of *P. haemolytica* A2. Lane D – OMPs of *P. haemolytica* A7 | 45 |

## LIST OF FIGURES

| Figure | | Page |
|---|---|---|
| 5.1 | Antibody response in sheep towards the OMPs of *Pasteurella haemolytica* A2, A7 and A9 following vaccination with the OMPs of *Pasteurella haemolytica* A2 | 63 |
| 5.2 | Antibody response in sheep towards the OMPs of *Pasteurella haemolytica* A2, A7 and A9 following vaccination with the OMPs of *Pasteurella haemolytica* A7 | 64 |
| 5.3 | Antibody response in sheep towards the OMPs of *Pasteurella haemolytica* A2, A7 and A9 following vaccination with the OMPs of *Pasteurella haemolytica* A9 | 64 |

Abstract of thesis submitted to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the degree of Master of Science

**IMMUNOLOGY OF THE OUTER MEMBRANE PROTEINS OF *PASTEURELLA HAEMOLYTICA* A2, A7 AND A9 IN SHEEP**

By

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**JUNE 1999**

**Chairman: Associate Professor Dr. Mohd Zamri Saad, Ph.D**

**Faculty: Veterinary Medicine**

Pneumonic pasteurellosis is a common respiratory disease of goats and sheep throughout the world, including Malaysia. In Malaysia, *Pasteurella haemolytica* A2 is most commonly isolated from cases of pneumonic pasteurellosis in sheep and goats followed by *Pasteurella haemolytica* A7 and A9.

Vaccination has been used widely to control the disease with uncertain success rate. The reasons for vaccination failure in the field were due to incompatible strains, unsuitable antigen as vaccine component and improper vaccination programme. Therefore, the attentions have been focused on the concept of a novel vaccine, which includes subunit vaccine.

The outer membrane proteins (OMPs) of *Pasteurella haemolytica* A2, A7 and A9 have been extracted using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Each serotype gave two to three major polypeptide bands with some minor bands. Immunoblotting, carried out using homologous and heterologous antisera against the OMPs from all serotypes. The results showed that the 30 kDa band of *Pasteurella haemolytica* A7 could be recognised by all antisera, and was thus concluded as the major and common immunogen.

The *in vivo* tests using the OMPs of the three serotypes revealed that sheep injected with the 100 μg OMP followed by a booster dose on day 21 showed highest antibody level on day 28 post-injection. Animals vaccinated with the OMP of *Pasteurella haemolytica* A7 showed good immune response upon challenge with significantly ($p<0.05$) less severe lung lesions regardless whether challenged with *Pasteurella haemolytica* serotype A2, A7 or A9. Those animals vaccinated with the OMP of *Pasteurella haemolytica* A2 failed to protect against challenge with live *Pasteurella haemolytica* A9 while those vaccinated with the OMP of *Pasteurella haemolytica* A9 failed to protect against challenge with live *Pasteurella haemolytiva* A2 and A7. It is concluded that the OMP of *Pasteurella haemolytica* A7 provides cross-protection to challenges using live *Pasteurella haemolytica* A2, A7 and A9. Thus, the OMP of *Pasteurella haemolytica* A7, particularly the 30 kDa, could be the best candidate for a subunit vaccine against pneumonic pasteurellosis in sheep.

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**IMUNOLOGIKAL PROTEIN SELAPUT LUAR *PASTEURELLA HAEMOLYTICA* A2, A7 AND A9 KE ATAS BEBIRI**

Oleh

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**JUN 1999**

**Pengerusi:** **Professor Madya Dr. Mohd Zamri Saad, Ph.D**

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Pasteurelosis pneumonia merupakan penyakit pernafasan kambing dan bebiri yang lazim di serata dunia, termasuk Malaysia. Di Malaysia, *Pasteurella haemolytica* A2 adalah yang paling kerap diasingkan daripada kes pasteurelosis pneumonia kambing dan bebiri, diikuti oleh *Pasteurella haemolytica* A7 dan A9

Pemvaksinan diguna dengan meluas untuk mengawal penyakit ini tanpa kesan yang memuaskan. Sebab utama kegagalan pemvaksinan adalah kerana ketidak-keterampilan strain, ketidak-sesuaian antigen sebagai komponen vaksin dan program pemvaksinan yang kurang memuaskan. Maka, penumpuan telah dilakukan terhadap konsep vaksin novel, yang termasuk vaksin subunit

Protein selaput luar (OMP) *Pasteurella haemolytica* A2, A7 dan A9 telah diasing dengan menggunakan sodium dodecil sulfat-gel elektroforesis poliakrilamide (SDS-PAGE). Tiap-tiap serotip menghasilkan dua hingga tiga polipeptida major dengan beberapa gelung minor. Pemblotan imun yang dilakukan menggunakan antiserum homologus dan heterologus terhadap OMP kesemua serotip. Keputusan menunjukkan bahawa 30 kDa *Pasteurella haemolytica* A7 merupakan imunogen major dan lazim.

Ujian *in vivo* menggunakan OMP ketiga-tiga serotip menunjukkan yang bebiri yang disuntik dengan 100 µg OMP dan diransang lagi pada hari ke-21 mempunyai tahap antibodi yang paling tinggi pada hari ke 28 selepas suntikan. Haiwan yang disuntik dengan OMP *Pasteurella haemolytica* A7 menunjukkan gerakbalas imun yang baik dengan keterukan lesi peparu yang kurang ($p<0.05$) sama ada dicabar dengan *Pasteurella haemolytica* serotip A2, A7 atau A9. Haiwan-haiwan yang disuntik dengan OMP *Pasteurella haemolytica* A2 gagal melindung terhadap cabaran oleh *Pasteurella haemolytica* A9 sementara yang disuntik dengan OMP *Pasteurella haemolytica* A9 gagal melindung cabaran oleh *Pasteurella haemolytiva* A2 dan A7. Maka kesimpulannya menunjukkan bahawa OMP *Pasteurella haemolytica* A7 memberi perlindungan silang terhadap cabaran oleh *Pasteurella haemolytica* A2 dan A9. Maka, OMP *Pasteurella haemolytica* A7, terutama sekali 30 kDa, merupakan calon paling sesuai untuk vaksin subunit bagi menghalang pasteurellosis pneumonia pada bebiri.

# CHAPTER I

# INTRODUCTION

Pneumonic pasteurellosis is a common respiratory disease of goats and sheep (Gilmour, 1993). The disease was first reported early this century when enzootic pneumonia was reported in sheep in England and Wales (Montgomorie *et al*., 1938). *Pasteurella haemolytica* biotype A is the most common isolate from the pneumonic lungs of affected animals although *Pasteurella multocida* types A and D are occasionally isolated (Gilmour *et al*., 1991).

*Pasteurella haemolytica* has been recognised as a component of the normal flora of the nasopharynx and tonsils of apparently healthy animals of various species including sheep (Dungworth, 1985). Lambs are thought to acquire *Pasteurella haemolytica* in their nasal mucosa after birth, presumably by contact with the ewe (Shreeve and Thompson, 1970). Following stressful conditions, *Pasteurella haemolytica* proliferates in the upper respiratory tract and a great number of the organisms are inhaled into the lungs (Gonzalez and Maheswaran, 1991) leading to fibrinous pneumonia (Zamri-Saad, 1988; Jericho, 1989) and death (Zamri-Saad *et al*., 1983).

Vaccination is the most common method of controlling the disease worldwide (Mosier, 1993). Although the need for an efficacious *Pasteurella haemolytica* vaccine for goats and sheep is apparent, none of the commercially available pasteurella vaccines are able to provide good protection in the field (Wan Mohamed *et al*., 1988; Zamri-Saad *et al*., 1989a, b; Jamaludin, 1993). Recently, a new pasteurella spray vaccine was developed, which is easy to administer and provides good protection against experimental challenge (Effendy *et al*., 1998a, b). The vaccine, however, failed to provide cross-protection against *Pasteurella haemolytica* A7 and A9 (Zamirah, 1998).

Several antigens with high potential as components of a subunit vaccine have been identified in *Pasteurella haemolytica.* These include the iron-regulated proteins (IRPs) (Donachie and Gilmour, 1988; Ogunnariwo and Schryvers, 1990; Gilmour *et al*., 1991), the outer membrane proteins (OMPs) (Donachie *et al*., 1984), the lipopolysaccharide (LPS) (Fenwick, 1990), the capsular polysaccharide (CPS) (Czuprynski *et al*., 1991a) and the leukotoxin (LktA) (Lo *et al*., 1987). The antigenic similarity between the toxins produced by different serotypes of *Pasteurella haemolytica* has been shown to result in the production of cross-neutralizing antibodies (Shewen and Wilkie, 1988). This cross-neutralizing effect offers an antigenic advantage in the formulation of a subunit pasteurella vaccine (Fraser *et al*.,

1982). However, the toxins of *Pasteurella haemolytica* A2, which is the most commonly isolated serotype from pneumonic lungs of sheep and goats, do not posses cross-neutralization with other serotypes (Gilmour, 1993). This has lead to the formulation of many commercially available polyvalent pasteurella vaccines, which were aimed at providing a wide scope of protection (Gilmour *et al.*, 1979). The OMP's of *Pasteurella haemolytica* A2, A7 and A9 have not been analysed for their ability to evoke cross-neutralization and cross-protection. In consideration of the potential of OMP's as a vaccine candidate (Confer, 1993), the objectives of this research project were to:

1. determine the OMPs profiles of *Pasteurella haemolytica* A2, A7 and A9 isolates using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

2. determine the antigenicity of the OMPs of *Pasterurella haemolytica* A2, A7 and A9 isolates using Western blotting against homologous and heterologous antisera.

3. determine the *in vivo* immunogenicity of the OMPs of *Pasteurella haemolytica* A2, A7 and A9 and to assess their potential as a component of a subunit pasteurella vaccine in goats/sheep.

# CHAPTER II

# LITERATURE REVIEW

## Pneumonic pasteurellosis

Pneumonic pasteurellosis is an important respiratory disease of cattle, sheep and goats throughout the world. It is usually caused by *Pasteurella haemolytica* biotype A. *Pasteurella haemolytica* A1 is the most common causative agent in cattle while *Pasteurella haemolytica* A2 is the agent frequently isolated from sheep and goats (Gilmour, 1993). The disease occurs in sheep of all ages. In young animals of less than 3 weeks old, the disease is hyperacute with generalised infection, while animals between 3 to 12 weeks of age suffer from acute infection characterised by pleurisy and pericarditis for 2-3 days (Gilmour, 1993).

*Pasteurella haemolytica* is part of the nasal bacterial flora of sheep and goats. The prevalence and numbers of *Pasteurella haemolytica* in the nasopharynx increase during stressful conditions and predispose the host to pneumonic pasteurellosis (Gilmour, 1993). There are several predisposing

factors that lead to the development of this disease. The predisposing factors fall into two main categories. The first category is the management and environment factor, in which proof is circumstantial (Jasni *et al*., 1990; Zamri-Saad *et al*., 1991; Gilmour, 1993). The second category is the infectious agents such as the parainfluenza virus type 3 (Gilmour *et al*., 1991), herpesvirus (Buddle *et al*., 1990) and *Haemonchus contortus* (Zamri-Saad *et al*., 1994).

Upon entrance of a large number of *Pasteurella haemolytica* into the lungs, they produce leukotoxin, which is either toxic to the alveolar macrophage or has the ability to reduce the efficiency of phagocytosis by neutrophilic leucocytes and alveolar macrophages. The adverse effects of leukotoxin on inflammatory cells of the lungs enhance colonisation of *Pasteurella haemolytica* onto the alveolar epithelium (Effendy *et al*., 1998a) and minimise phagocytosis of bacterial cells by the alveolar macrophage (Zamri-Saad *et al*., 1996; Maswati, 1998). Following successful colonisation onto the lung surface, the bacteria enter the pneumocytes causing the formation of cytoplasmic vacuoles and necrosis of the affected pneumocytes (Maswati, 1998). At the same time, endotoxin (lipopolysaccharide) of *Pasteurella haemolytica* causes severe lesions, particularly on the pulmonary blood vessels (Heng *et al*., 1996). Typical fibrinous bronchopneumonia comprised of fibrin and the presence of numerous macrophages and oat cells in the alveolar space has been reported in animals infected with both

*Pasteurella haemolytica* and *Pasteurella multocida* (Zamri-Saad, 1987; Loganathan and Chandrasekaran, 1992).

There are no clear clinical signs for pneumonic pasteurellosis. Studies revealed that signs of respiratory tract infection, such as coughing, nasal discharge and dyspnoea are poorly correlated with the severity of lung lesions following infection by *Pasteurella haemolytica* compared to *Pasteurella multocida* (Zamri-Saad *et al*., 1996; Effendy *et al*., 1998b). The mortality rate, however, ranges from 10 to 30% (Jasni *et al*., 1990; Fatimah *et al*., 1992; Gilmour, 1993).

In Malaysia, since *Pasteurella haemolytica* was confirmed as the most common cause of pneumonia in sheep and goats with a 40 to 43% isolation rate followed by *Pasteurella multocida* with 24 to 48% isolation rate (Sheikh-Omar *et al*., 1989, 1993), the disease was considered endemic (Saharee and Fatimah, 1993). The disease has become increasingly important (Jamaludin, 1993) following attempts to increase the sheep population in Malaysia in 1987 (Hadi, 1988) and is recognised as one of the major causes of death of sheep and goats in this country (Zamri-Saad *et al*., 1987; Jasni *et al*., 1990; Fatimah *et al*., 1992).

### *Pasteurella haemolytica*

*Pasteurella haemolytica* is a Gram-negative bacterium, which has been identified as the aetiological agent of bovine pneumonic pasteurellosis or shipping fever and ovine pneumonic pasteurellosis and septicaemic pasteurellosis (DeAlwis, 1993). These diseases have been known to produce significant economic losses (Gilmour, 1993). *Pasteurella haemolytica* comprises two biotypes; A and T, based on their fermentation of arabinose and trehalose. Of the two biotypes of *Pasteurella haemolytica*, 16 serotypes were identified, based on their soluble capsular antigen (Biberstein, 1978; Fodor *et al*., 1988). Since *Pasteurella haemolytica* biotype T ferments trehalose, it has been proposed that the biotype be re-named *Pasteurella trehalosi* (Sneath and Stevens, 1990).

*Pasteurella haemolytica* biotype A comprises 13 serotypes while *Pasteurella haemolytica* biotype T comprises 3 serotypes (Adlam, 1989; Younan and Fodor, 1995). The serotypes of *Pasteurella haemolytica* biotype A include A1, A2, A5, A6, A7, A8, A9, A11, A12, A13, A14, A16, and A17. Frederiksen (1973) discovered that *Pasteurella haemolytica* serotype A11 isolates did not fit easily into biotype A and proposed a third biotype to accommodate them. In addition to recognised serotypes, approximately 10% of the isolates of *Pasteurella haemolytica* obtained from cattle and sheep are untypable (Fraser *et al*., 1982; Quirie *et al*., 1986). Although some untypable

isolates are found to be closely related to those of biotype A, others represent different species (Mutters *et al*., 1986; Davies *et al*., 1996).

*Pasteurella haemolytica* A1 is the predominant serotype recovered from cattle with pneumonic pasteurellosis. Serotype A2 is less frequently isolated from pneumonic lungs of cattle even though it is often isolated from the naso-pharynx of healthy animals (Frank, 1989). *Pasteurella haemolytica* serotype A2, however, is the predominant serotype recovered from cases of pneumonic pasteurellosis in sheep while serotype A1 is less frequently isolated from this animal species (Gilmour and Gilmour, 1989; Bahaman *et al*., 1991; Mohamad *et al*., 1993).

## The Potential Protective Antigens of *Pasteurella haemolytica*

### Outer Membrane Proteins (OMPs)

Separation of the inner and outer membranes of Gram-negative bacteria has been carried out successfully using several methods (Hancock, 1991). Both inner and outer membrane structures are relatively rich in proteins. The outer membrane is composed of a small number of major proteins; four to five prominent proteins in some bacterial species (Hancock, 1991).

Studies aimed at determining immunogenic components of *Pasteurella haemolytica* and *Pasteurella multocida* have focused mainly on the outer membrane proteins. This is because the outer membrane of Gram-negative bacteria is the cellular component, which is in direct contact with the host (Squire *et al*., 1984; Owen, 1991). It has been demonstrated to be involved in the protection against the bacteria itself (Squire *et al*., 1984; Owen, 1991). The size of the outer membrane is approximately 10 nm in diameter and its structure, composition and functions are different from the cells' cytoplasm or the inner membrane. The membrane is a fully asymmetrical bilayer, composed of the outer membrane proteins (approximately 44%), phospholipids (approximately 13%) and carbohydrate polymers (approximately 43%) (Squire *et al*., 1984; Adlam, 1989; Owen, 1991). The outer layer of the OMP contains lipopolysaccharide (LPS) whereas the inner layer is composed largely of phospholipid and the acyl chains of lipoproteins (Squire *et al*., 1984; Owen, 1991). The major OMPs are limited in number and range between three to eight protein bands, present in high copy numbers between 50,000 to 750,000 copies/cell (Donachie *et al*., 1984; Squire *et al*., 1984).

Research on the outer membrane has greatly contributed to an understanding of the pathogenesis of many Gram-negative bacterial infections (Beachey, 1981; Squire *et al*., 1984; Lu *et al*., 1988; Botcher *et al*., 1991; Sherman *et al*., 1991; Weiser and Gotschlich, 1991). Many of these

studies have demonstrated that the major OMPs (MOMP), which represent the most abundant OMP in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), are protective and showed some degree of antigenic heterogeneity among different strains (Munson *et al*., 1983; Virji *et al*., 1986). One of the major outer membrane proteins (MOMPs) is called porin, which is identifiable when solubilized in sample buffer at 37°C and appears at relatively low electrophoretic mobility (Lugtenberg and Van Alphen, 1983). Another MOMP is known as the heat-modifiable protein (OmpA), and is classically recognised by a current charge in its mobility on SDS-PAGE when solubilized in sample buffer at a different temperature. When heat-modifiable OMP is solubilized at 88°C or less, it showed a relatively low molecular mass, whereas the apparent molecular mass increased when it was solubilized at 100°C (Hancock and Carey, 1979; Tagawa *et al*., 1993a). The heat-modifiable OMP is necessary for the maintenance of the structural integrity of the cell envelope (Sonntag *et al*., 1978), bacterial conjugation (Schweizer and Herning, 1977), bacteriophage attachment (Datta *et al*., 1977) and porin activity (Sugawara and Nikaido, 1992). The antigenicity of the heat-modifiable OMP and its composition are conserved during the evolution among the Gram-negative bacteria (Beher *et al*., 1980). The heat-modifiable OMP also determines the resistance of the organism to complement–mediated serum killing (Weiser and Gotschlich, 1991). Bacterial OMPs are important for attachment to the host cells and for the transport of materials through the membrane. On the other hand,

antibodies against the OMPs of Gram-negative bacteria are expected to provide protection against infection by these Gram-negative microorganisms (Kuusi *et al*., 1979; Zollinger *et al*., 1979; Winter *et al*., 1983; Hedstrom *et al*., 1984). In fact, the importance of using OMPs to stimulate immunity has been shown for many Gram-negative bacteria (Dubray and Bezard, 1990; Adamus *et al*., 1980).

Ali (1992) and McCluskey (1994) described the variations in LPS and OMP profiles of a small number of isolates of *Pasteurella haemolytica* serotypes A1 and A2, which were associated with pneumonic pasteurellosis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting techniques have been widely used to analyse the OMPs and LPS profiles to determine strain variations, epidemiology and virulence of bacterial pathogens. The OMP patterns in SDS-polyacrylamide gels have been used to differentiate both human (Loeb and Smith, 1980; Mocca and Frasch, 1982; Achtman *et al*., 1983; Blaser *et al*., 1983; Odumeru *et al*., 1983) and veterinary (Lugtenberg *et al*., 1984; Rapp *et al*., 1986; Davies, 1991) pathogens. Analyses of both OMP and LPS profiles by SDS-PAGE, however, are less frequently carried out even though such study is performed routinely on *Escherichia coli* (Achtman *et al*., 1986) and *Pasteurella multocida* (Lugtenberg *et al*., 1984). The Western blotting has been used successfully on both Gram-negative (Bolstad *et al*., 1990; Hasman and