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IN VITRO AND *IN VIVO* ESTABLISHMENT OF *PASTEURELLA HAEMOLYTICA* A2 IN THE LUNGS OF GOATS

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By

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Thesis Submitted in Fulfilment of the Requirements for the Degree of Master of Science in the Faculty of Veterinary Medicine and Animal Science Universiti Putra Malaysia

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UPM

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By

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JULY 1998

Chairman: Assoc. Prof. Dr. Mohd Zamri Saad

Faculty: Veterinary Medicine and Animal Science

Pneumonic pasteurellosis is an important respiratory disease of sheep and goats throughout the world. It is mainly caused by *Pasteurella haemolytica* even though *Pasteurella multocida* has occasionally been associated with the same disease. *In vitro* challenge of lung tissues with *Pasteurella haemolytica* A2 revealed an early colonization of the bacteria onto the lung tissue as early as 1 hour post-challenge. The severity of colonization increased with time of challenge and reached a maximum rate at 6 hours post-challenge. Similar *in vitro* challenge on the lung tissues derived from goats that were exposed earlier to intranasal sprays of formalin-killed *Pasteurella haemolytica* A2, however, revealed a less severe colonization by 6 hours post-challenge.

Following intratracheal challenge of goats with 10^8 /ml colony forming units of *Pasteurella haemolytica* A2, 20% of the goats succumbed to peracute infection in which they died within 12 hours post-challenge. Examinations of the lungs revealed classical toxaemic lesions consisted of severe pulmonary oedema, pulmonary congestion and haemorrhage, and thrombosis with a few neutrophils in the alveoli. The lesions were remarkably similar to those peracute infections caused by *Pasteurella multocida* types A and D. The only difference was the absence of *Pasteurella haemolytica* A2 organisms in the heart blood samples compared to the infections by *Pasteurella multocida* types A and D.

Goats that survived the peracute episode developed pneumonic lesion. Phagocytic activity by the bronchoalveolar macrophages was obvious by 4 days post-challenge and by day 7 post-challenge, the goats which were unable to phagocytose most of the bacterial cells succumbed to severe pneumonia in which the bacteria proliferated and overloaded the lungs leading to the invasion of the bacteria into the pneumocytes and spreading the infection further. Goats with efficient phagocytosis were able to reduce the number of bacterial cells in the lungs leading to failure of bacterial establishment in the lungs.

Infections by *Pasteurella haemolytica* A2 isolated from nasal mucosa produced an insignificantly ($p>0.05$) less extensive lung lesions compared to infections by *Pasteurella haemolytica* A2 isolated from pneumonic lungs. The pulmonary responses and pattern of lesion development, however, remained similar for both isolates.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia bagi memenuhi syarat-syarat ijazah Master Sains

PENGHASILAN JANGKITAN *IN VITRO* DAN *IN VIVO* OLEH *PASTEURELLA HAEMOLYTICA* A2 KE ATAS PEPARU KAMBING

Oleh

MASWATI MAT AMIN

JULAI 1998

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Pasteurella pneumonia merupakan satu penyakit pernafasan penting pada kambing dan bebiri di seluruh dunia. Ia lazimnya disebabkan oleh *Pasteurella haemolytica* walaupun kadang-kadang *Pasteurella multocida* juga dikaitkan dengan penyakit ini. Cabaran *in vitro* ke atas tisu peparu menggunakan *Pasteurella haemolytica* A2 menunjukkan penjajahan bakteria ke atas tisu peparu berlaku seawal 1 jam selepas cabaran. Keterukan penjajahan bertambah dengan bertambahnya masa dan mencapai kadar maksima selepas 6 jam. Cabaran *in vitro* yang sama ke atas tisu peparu yang diambil daripada bebiri yang telah didedahkan kepada semburan intranasum *Pasteurella haemolytica* A2 pula menunjukkan kadar penjajahan yang kurang teruk selepas 6 jam.

Selepas kambing dicabar dengan 10^8/ml unit pembentuk koloni *Pasteurella haemolytica* A2, kira-kira 20% daripada kambing-kambing tersebut terkena jangkitan perakut di mana kambing mati dalam masa 12 jam. Pemeriksaan ke atas peparu

menunjukkan lesi toksemia klasik yang terdiri daripada edema pulmonari yang teruk, pendarahan dan kongesi pulmonari, trombosis dan terdapat sedikit sel neutrofil dalam alveolus. Lesi tersebut adalah sama dengan lesi jangkitan perakut oleh *Pasteurella multocida* jenis A dan D.

Kambing yang terselamat daripada jangkitan perakut menunjukkan lesi pneumonia. Aktiviti fagositosis oleh sel makrofaj bronkoalveolus jelas kelihatan pada hari ke 4 pasca cabaran dan pada hari ke 7, kambing yang gagal untuk membunuh kebanyakan bakteria mengalami lesi pneumonia yang lebih teruk di mana bakteria membiak dan membanjiri peparu sehingga memasuki sel pneumosit di samping tersebar dengan lebih meluas. Kambing yang menunjukkan aktiviti fagositosis yang cekap dapat mengawal bilangan bakteria dalam peparu menyebabkan kegagalan bakteria untuk menjangkiti peparu.

Jangkitan oleh *Pasteurella haemolytica* A2 yang diasingkan daripada mukosa hidung menghasilkan lesi peparu yang kurang meluas tetapi tidak bermakna ($p>0.05$) berbanding jangkitan oleh *Pasteurella haemolytica* A2 yang diasingkan daripada peparu. Walau bagaimanapun, gerakbalas pulmonari dan corak perkembangan lesi yang dihasilkan oleh kedua-dua isolat tersebut adalah sama.

CHAPTER 1

INTRODUCTION

Pasteurellosis is one of the most common bacterial diseases affecting most animal species including production animals in Malaysia as well as world wide. It is caused by a microorganism belonging to the genus *Pasteurella*, named after Louis Pasteur, who in 1880 was the first person to show that the organism caused a disease which was later to be known as fowl cholera (Trevisan, 1887). Two major species of *Pasteurella* are *Pasteurella multocida* and *Pasteurella haemolytica* which are able to produce either the septicaemic or the pneumonic infection in various animal species (Gilmour, 1993).

Apart from fowl cholera which is a septicaemic *Pasteurella multocida* infection of poultry leading to high and sudden mortality, *Pasteurella multocida* and *Pasteurella haemolytica* have also been reported to infect other animal species such as cats, dogs, pigs, horses, camels, minks and monkeys (Carter, 1959). Recent reports of pasteurella infections include donkeys, horses (Parvi and Apte, 1967) and deer (Jones and Hussaini, 1982; Carringan *et al*., 1991). The infection has also been recorded in wildlife such as elephants, bison and snow leopard (Carter, 1957; Carter, 1959; De Alwis and

Thambithurai, 1965; Bain *et al.,* 1982; De Alwis, 1982; Wicknemasuriya and Kendanagama, 1982; Chaudhuri *et al.,* 1992).

Both *Pasteurella multocida* and *Pasteurella haemolytica* have been associated with diseases of ruminants. In cattle and buffalo, *Pasteurella multocida* type B has been associated with an important septicaemic disease known as haemorrhagic septicaemia while both *Pasteurella multocida*, particularly types A and D and *Pasteurella haemolytica* have been associated with a pneumonic disease known as pneumonic pasteurellosis or `shipping fever'.

Similarly in sheep and goats both the septicaemic and the pneumonic infections by either *Pasteurella haemolytica* or *Pasteurella multocida* have been reported throughout the world (Gilmour, 1993; Zamri-Saad *et al.*, 1996). However, pneumonic pasteurellosis has been recognised as a more important and commonly observed pasteurella infection in sheep (Gilmour *et al.,* 1991). In their study, up to 30% of the herd have been reported to either been infected or died of the disease, leading to great economic loss through loss of production, death and costs of treatment (Gilmour *et al.*, 1991).

Pasteurella haemolytica especially of serotypes A2, A7 and A9 are most frequently isolated from cases of pneumonic pasteurellosis not only in Malaysia, but throughout the

world (Davies *et al.*, 1982; Bahaman *et al.*, 1991). The organism has also been recognised as one of the commensals of the nasopharynx of many animals (Dungworth, 1985). It is able to multiply in the nasopharynx (Jasni *et al.*, 1991), transformed to become the invasive and pathogenic strain (Gonzalez and Maheswaran, 1993) and invades the lungs to produce the disease when the host is subjected to stressful conditions such as transportation, overcrowding, malnutrition, weaning as well as following concurrent viral infection and other diseases (Davies *et al.*, 1982; Buddle *et al.*, 1990; Gilmour *et al.*, 1991; Zamri-Saad *et al.*, 1994a, 1994b).

Although the disease is commonly encountered throughout the world, little has been known about the pathogenesis of the disease. Apart from the role played by stressful conditions (Zamri-Saad *et al.*, 1991) and concurrent diseases in the development of the disease (Buddle *et al.*, 1986; Zamri-Saad *et al.*, 1994a, 1994b), other information about the development of the disease in sheep and goats such as the duration required for the organism to establish itself in the lungs and the lung reactions to the infection are still not well understood. Similarlr, the pathogenicity of *Pasteurella haemolytica* A2 isolated from the nasal mucosa and those isolated from the pneumonic lungs were not thoroughly examined.

The objectives of this study are:

i. to study the *in vitro* establishment of *Pasteurella haemolytica* A2 infection in the lung tissue of goats.

ii. to study the clinical and pathological changes in the respiratory tract of goats following peracute infection by *Pasteurella haemolytica* A2.

iii. to study the *in vivo* establishment of *Pasteurella haemolytica* A2 and the lungs reactions following intratracheal infection by *Pasteurella haemolytica* A2.

CHAPTER 2

LITERATURE REVIEW

Pneumonic pasteurellosis is a respiratory disease of sheep and goats that has been reported throughout the world. It affects the lungs of animals under stressful conditions or following concurrent diseases such as respiratory viral infection or haemonchosis (Davies *et al.*, 1986; Zamri-Saad *et al.*, 1994b). Both *Pasteurella multocida* type A and D, and *Pasteurella haemolytica* type A have been associated with the disease in sheep and goats, but *Pasteurella haemolytica,* particularly *P. haemolytica* A2, A7 and A9 are more frequently isolated (Davies et al., 1986, Bahaman *et al.*, 1991).

The Aetiological Agent

In the early years, species names of Pasteurellae were given according to the host animal that they infected. After several name changes, Rosenbusch and Merchant (1939) proposed a species name of *Pasteurella multocida* for the organism under the genus *Pasteurella*. In the subsequent years, new species of pasteurella were added to the list. *Pasteurella haemolytica* was first identified and recognised in 1932 (Newson and Cross, 1932), *Pasteurella pneumotropica* in 1950 (Jawetz, 1950), *Pasteurella gallinarum* in 1955

(Hall *et al.*, 1955), *Pasteurella urae* in 1962 (Jones, 1962) and recently the gas-producing *Pasteurella aerogenes* in 1974 (McAllister and Carter, 1974).

Pasteurella haemolytica is a small encapsulated Gram negative cocco-bacillus bacteria. It is non motile, exhibiting slight pleomorphism with occasional bipolar staining (Adlam, 1989). It forms a narrow zone of haemolysis on 7% ovine or bovine blood agar which is used to distinguish *P. haemolytica* from other pasteurella species (Adlam, 1989). Sometimes observation of the haemolytic zone can only be seen when the colonies were scraped off the surface of the plate (Biberstein *et al.*, 1960). The ability to grow on MacConkey agar, the lack of urease and the inability to produce indole are additional tests to distinguish *P. haemolytica* from other pasteurella species (Adlam, 1989).

P. haemolytica has the serotype specific capsular polysaccharides, lipopolysaccharides, outer and inner membrane proteins and peptidoglycan. The organism also produces enzymes such as neuraminidase which is an extracellular enzyme and proteins such as cytotoxin which play a role in its virulence.

Species of Pasteurella

The disease pneumonic pasteurellosis, observed in small ruminants in the temperate climate is commonly caused by *P. haemolytica* and rarely caused by *P. multocida*

(Gilmour and Gilmour, 1989). There are generally two biotypes of *P.haemolytica* that causes the disease, the biotype A and biotype T identified based on the carbohydrate fermentation reactions against arabinose and trehalose (Smith, 1961). After 24 hours of incubation, the colonies of biotype A strain appeared evenly coloured grey while the biotype T strain appeared larger with large brownish colonial centre (Adlam, 1989). Each biotype has been associated with a distinct clinical syndrome shown by the affected animals. Biotype A strains of *P. haemolytica* usually cause pneumonia in all ages and occasionally cause septicaemia in young lambs. In contrast, biotype T strains of *P. haemolytica* cause a well-defined acute systemic disease in young adult sheep (Gilmour and Gilmour, 1989).

Among the biotype A strains of *P. haemolytica*, serotype A2 is the most commonly isolated strain from pneumonic lungs of small ruminants in Malaysia (Bahaman *et al.*, 1991), Europe (Gilmour *et al.*, 1991), New Zealand (Prince *et al.*, 1985) as well as from healthy sheep flocks in the United States of America (Frank and Smith,1983).

Pasteurellosis in Sheep and Goats

There are two forms of pasteurellosis in small ruminants; the septicaemic form caused by *P. haemolytica* biotype T and the pneumonic form caused by *P. haemolytica* biotype A. Septicaemic pasteurellosis affects mainly young lambs in which the affected

animals showed an outbreak of sudden onset of fever followed by sudden deaths. Post mortem examinations revealed severe and generalised congestion of organs with pin point necrosis which appeared as white spots in the liver (Gilmour and Gilmour, 1989). The causative organism can be isolated from various organs particularly the heart blood, lungs and liver.

The most common form of pasteurellosis in small ruminants is the pneumonic pasteurellosis. One of the earliest reports on pneumonia in sheep came from Iceland where Dungal (1931) described an outbreak of pneumonia in housed sheep. Although his description of the causal organism was slightly different from the present description of *P. haemolytica*, there was little doubt that the lesions described was of pneumonic pasteurellosis. Later, Montgomerie *et al.* (1938) tried to correlate the stressful conditions with pneumonia in sheep while describing outbreaks of enzootic pneumonia in North Wales and East Anglia when they noted that the disease appeared to be precipitated by sudden environmental changes.

In sheep and goat throughout the world, pneumonic pasteurellosis is caused by *P.haemolytica* A2 which is present in the upper respiratory tract of sheep of all ages. In lambs less than 3 weeks old the infection is hyperacute with generalised infection; the infection in lambs between 3 to 12 weeks old is usually acute which lasted between 2 to 3 days and characterised by pleurisy and pericarditis. In older sheep, the subacute to

chronic fibrinous pneumonia usually predominates (Jericho, 1989; Gilmour *et al.*, 1991; Gilmour, 1993).

Epidemiology of Pneumonic Pasteurellosis in Sheep and Goats

Pasteurella haemolytica has been known to be present in the nasopharynx and tonsils of apparently healthy animals (Dungworth, 1985; Gonzalez and Maheswaran, 1993). Lambs acquire the infection soon after birth, probably transmitted by close contact with their dams (Shreeve and Thompson, 1970). However, normal healthy flocks have a lower nasal carrier rate than those flocks undergoing outbreaks of pasteurellosis (Biberstein and Thompson, 1966). This was further confirmed in a survey of nasal carriers that revealed that the number of carriers peaked coinciding with the increased incidence of the disease (Biberstein *et al.*, 1970).

A study carried out by British Veterinary Investigation Centre on *P. haemolytica* from cases of ovine pasteurellosis revealed different serotypes isolated from these cases. Biotype A comprised 65%, biotype T comprised 28% while 7% were untypeable. Several serotypes have been reported under the biotypes A and T based on the indirect hemagglutanation test (Fraser *et al.*, 1983). Among the reported serotypes for biotype A that affected sheep and goats were serotypes 1, 2, 5, 6, 7, 8, 9, 11 and 12 while the serotypes for biotype T were serotypes 3, 4 and 10 (Fraser *et al.*, 1982).

The prevalence of the untypeable strains remained low throughout the year (Gilmour and Gilmour, 1989) while serotype A2 was the most common serotype isolated in sheep and goats with pneumonic pasteurellosis (Gilmour *et al.*, 1991). Other than serotype A2, Serotypes A7, A9 and A1 were also isolated from pneumonic lung but none of serotype T predominated in pneumonic pasteurellosis (Gilmour and Gilmour, 1989).

Pneumonic pasteurellosis of sheep and goats caused by *P. haemolytica* usually is endemic with occasional sporadic outbreaks, involving animals of all ages (Gilmour, 1993). Affected herds usually showed sudden death particularly in young lambs and kids. The dead lambs or kids usually showed evidence of acute pneumonia involving the antero-ventral portion of the lungs. Older animals that usually survived the acute phase showed signs related to pneumonia such as coughing, respiratory difficulties and nasal discharge. Mortality varied from 5 to 30% while the mobidity from 10 to 60% (Gilmour, 1993).

Pathogenesis of Pneumonic Pasteurellosis

It is believed that *P. haemolytica* which is a part of the normal flora of nasopharynx of animals (Dungworth, 1985) proliferates in the nasal cavity following stressful conditions. Isolation of *P. haemolytica* from the nasal cavity showed seasonal

pattern with highest rate of isolation in rainy season and lowest isolation in dry season (Jasni *et al.*, 1990). Stressful conditions caused by transportation and administration of dexamethasone also lead to increased isolation rates of *P. haemolytica* (Jasni *et al.*, 1991; Zamri-Saad *et al.,* 1991).

In healthy animals, the mucociliary ladder, the cellular and humoral defense mechanisms of the respiratory tract serve in clearing the pasteurella (Gilmour, 1993). Stress factors cause immunosuppression (Chiang *et al.*, 1990) through the release of steroids from the adrenal cortex inhibiting the leucocyte production that lead to marked increased in circulating leucocytes and significant reduction of the leucocyte numbers in tissues (Schalm *et al.,* 1975).

The suppression of respiratory defense mechanism leads to multiplication of resident *P. haemolytica* in the upper respiratory tract (Baskerville, 1981), transformation of the organism to become the pathogenic strain (Gonzalez and Maheswaran, 1993), invade the lung tissue (Shewen, 1994) and initiate severe fibrinous pneumonic lesions (Jericho, 1989).

Once the lungs are invaded, there will be adhesion of *P. haemolytica* onto the respiratory alveolar surface by its large fimbrae which is rigid and small fimbrae which is flexible (Morck *et al.,* 1987). Other than the fimbrae, there is a capsular material

known as glycocalyx, which facilitates the adhesion of *P. haemolytica* onto the pulmonary epithelial surface. The glycocalyx is a polysaccharide, which is produced during the logarithmic phase of growth (Morck *et al.*, 1987) and will form a complex with pulmonary surfactant to facilitate local adherence of the organism (Brogden *et al.*, 1989).

The nature of pneumonia that developed following *P. haemolytica* infection depends on the rate and extent of bacterial proliferation and the virulence of the organism. The virulent determinances of *P. haemolytica* exert their influence not only to produce lesions which include alveolar oedema, exudative inflammatory reactions and inter-alveolar haemorrhages (Jericho, 1989) but also to maintain the presence of the organism in the respiratory tract by preventing phagocytosis and increasing resistance to complement and bacteriocidal effects of the host defense mechanism (Gilmour, 1993).

Among the virulence determinances recognised in *P. haemolytica* include lipopolysaccharides which induce pulmonary reactions in the form of neutrophil and alveolar macrophage reaction, changes on the blood capillaries which lead to thrombosis and pulmonary oedema as well as the damages on the pulmonary epithelium (Breider *et al.*, 1990; Heng *et al.*, 1996).

Apart from the lipopolysaccharide, a protein toxin known as cytotoxin, which is produced during the logarithmic phase of the growth, acts as a pore-forming cytolysin on