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ESTABLISHMENT OF *PASTEURELLA HAEMOLYTICA* A2 IN THE LUNGS OF GOATS FOLLOWING INTRATRACHEAL EXPOSURE

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SUMMARY

Twenty healthy goats were divided into three groups of eight goats in each of the first two groups and four goats in group 3. All goats were transport stressed by road before goats in group 1 were challenged intratracheally with *Pasteurella haemolytica* A2 isolated earlier from the nasal mucosa of a healthy goat and group 2 with *P. haemolytica* A2 isolated from pneumonic lungs of a goat. Group 3 was the uninfected control. The goats were serially slaughtered at 1, 4, 7 and 11 days post challenge to study the bacterial establishment and to compare the extent of lung lesions produced by the two isolates. The gross lung lesions showed significant ($P<0.05$) and progressive development from an average of 6.3 ± 2.1 per cent at 24 h post challenge to 26.8 ± 13.1 per cent after 11 days of challenge. *P. haemolytica* A2 isolated from lungs consistently produced insignificantly ($P>0.05$) more extensive lesions compared to *P. haemolytica* A2 isolated from the nasal mucosa. There were small numbers of neutrophils present in the lung washing fluid at 24 h post challenge before a mixture of neutrophils and macrophages with phagocytic activity was observed at day 4 post challenge. At day 7 post challenge, goats that showed more severe lung lesions had numerous bacteria cells in the lung either floating freely in the alveolar spaces or attached to the pneumocytes. At day 11 post challenge, the bacteria had penetrated the cell wall and were found in the vacuoles of pneumocytes.

Keywords: *Pasteurella hemolytica* A2, establishment, lungs, goats.

INTRODUCTION

Pneumonic pasteurellosis is a common disease of sheep and goats throughout the world. It has frequently been associated with *P. haemolytica* biotype A, and *P. haemolytica* serotype A2 is the most frequently isolated organism from pneumonic lungs of small ruminants (Gilmour *et al.*, 1991). *P. haemolytica* has been considered as a normal flora in the upper respiratory tract of animals, and isolations have been made from various sites particularly from the nasopharynges, tonsils and lungs of sheep and goats (Dungworth, 1985). Isolates from the upper respiratory tract are thought to multiply under stressful conditions before becoming pathogenic and invade the lungs to produce the disease (Gilmour *et al.*, 1991). Isolates from the nasal cavity of healthy animals are suspected to be of less virulent and must convert to a more selective, explosive growth and upper respiratory tract colonisation strain before lung lesions can be produced (Gonzalez and Maheswaran, 1993).

Apart from a study using goats as the animal model (Debey *et al.*, 1992), most studies on pneumonic pasteurellosis particularly the establishment and pathological studies were carried out in cattle and sheep (Rushton *et al.*, 1979; Jericho, 1989). This report describes the pulmonary establishment of *Pasteurella haemolytica* A2 and compares the lung lesions in goats following intratracheal inoculation of *P. haemolytica A2* isolated earlier from the nasal cavity of a healthy goat and the pneumonic lungs of a goat.

MATERIALS AND METHODS

Animals

Twenty clinically healthy goats of about 7 months old were selected. They were divided into three groups of eight goats in each of the groups 1 and 2 and four goats in group 3, and kept in separate rooms. Cut grass and supplemented feed were provided daily while drinking water was available *ad libitum*. Upon arrival, nasal swabs were collected for bacteriological cultures to ensure that the goats were free of *P. haemolytica* for at least two weeks prior to the start of the experiment.

Experimental procedures

At the start of the experiment, all goats were transport stressed by road for a distance of 400 km to predispose animals to infection (Zamri-Saad *et al.*, 1991) and immediately the goats in group 1 were challenged intratracheally with 4 mL inoculum containing 1.5×10^8 colony forming unit (cfu) of *P. haemolytica* A2 isolated earlier from the nasal cavity of a healthy goat. Goats in group 2 were similarly

isolated from pneumonic lungs of a goat whereas goats in group 3 were the uninfected control.

The animals were observed daily for signs of pneumonic pasteurellosis such as nasal discharge, pyrexia (rectal temperature exceeding 40°C), dullness or lethargy, abnormal respiration or death. Surviving goats were slaughtered, two goats each from groups 1 and 2, and one goat from group 3 on days 1, 4, 7 and 11 post challenge. Fifty mL of normal saline was introduced into every lung before the fluid was re-collected for cytological examinations.

Detailed post mortem examinations on the respiratory tract were carried out. Lung lesions were measured and the extent of lung lesions produced by the two *P. haemolytica* A2 isolates were compared (Gilmour *et al.*, 1983). During the post mortem examination, the lung samples were collected for bacteriology, histopathology and electron microscopy examinations.

Sample processing

The lung washing fluid was centrifuged at 200 x g for 15 minutes before smears were prepared from the sediment, stained with Giemsa and examined for cytological study.

The lung samples for histopathological examinations were embedded in Historesin (Cambridge Instruments, UK), sectioned at 2 mm and stained with Giemsa to observe the bacterial establishment, and with hematoxylin and eosin to observe the inflammatory cell response (Newman and Hobot, 1993). For the ultrastructural study, the samples were fixed in 4 per cent glutaraldehyde for 4 h, post fixed in 1 per cent osmium tetroxide, dehydrated, infiltrated and sectioned at 30 nm thick.

Samples for bacteriological isolations were cultured on blood agar and incubated at 37°C for 24 h before colonies suspected of *P. haemolytica* were re-cultured in MacConkey agar and identified using the triple sugar iron, oxidase, urease and motility tests (Zamri-Saad *et al.*, 1991).

Statistical analysis

The differences in the extent of pneumonic lesions between the groups were statistically analysed by the ANOVA repeated measure test.

RESULTS

Clinical findings

One goat from group 2, challenged earlier with *P. haemolytica* A2 isolated from the caprine pneumonic lung was found dead 8 h post challenge. Four other goats from the same group showed slight to moderate nasal discharge between 4 to 10 days post challenge, but remained bright and alert with normal body temperatures. None of the goats in groups 1 and 3 showed clinical signs of pneumonic pasteurellosis.

Disease establishment

Five (62 per cent) goats from each of the groups 1 and 2 had pneumonic lung lesions typical of pneumonic pasteurellosis. *P. haemolytica* A2 isolated from the lungs produced insignificantly ($P>0.05$) more extensive lesions than those isolated from the nasal mucosa (Table 1). The average lung area affected was 8.0 ± 6.9 per cent for group 1 and 19.1 ± 14.0 per cent for group 2. Most lesions appeared as patches of dark red and firm lesions on the antero-ventral portion of the lungs. None of the goats in group 3 showed lung lesions.

Table 1. Percentage of lung area of each goat showing pneumonic lesions following infection with *P. haemolytica* A2 isolated from nasal mucosa and lungs

Days post infection‡	Percentage of lung lesions*	
	P. haemolytica nasal	*P. haemolytica* lungs
1	5	5
	5	10
4	7	5
	5	40
7	10	0
	0	18
11	7	25
	25	50

‡ The differences in lung lesions against time post infection are significant ($P<0.05$)

* The differences in lung lesions between the two isolates are insignificant ($P>0.05$)

At 24 h, the goats had an average of 6.3 ± 2.1 per cent lung showing lesions of acute pneumonia mostly on the right anterior lobes. Lung washing fluid had numerous neutrophils with occasional macrophages. Most cells appeared normal. Histological lesions showed evidence of acute inflammatory reactions. The interalveolar septa were thickened with congestion of the capillaries. Small numbers of neutrophils were observed in the interalveolar septa, particularly near the capillaries and in the alveoli close to the alveolar wall. Many bacterial cells were observed in the alveoli among the neutrophils without evidence of phagocytosis. Electron microscopic examinations revealed congested capillaries with the presence of neutrophils and bacteria cells found floating freely in the lumen of the alveoli. The thin film of surfactant which covered the alveolar wall, particularly near the bacteria cells was found disrupted.

After 4 days, the extent of gross lung lesions generally increased to an average of 14.3 ± 14.8 per cent, affecting mostly the antero-ventral portion of the

lungs. The lung washing fluid consisted of a mixture of neutrophils and macrophages; many of these cells had pycnotic nuclei. Histological lesions showed evidence of subacute reactions. There were several congested capillaries with a mixture of neutrophils and macrophages filled the alveoli and bronchioles. The bacteria were again observed in the alveolar spaces, some had been phagocytised by the macrophages. Ultrastructurally, numerous macrophages were observed in the alveoli. Some bacteria were found either attached to the wall of alveoli or being phagocytised by macrophages (Figure 1). The macrophages that involved in phagocytic activity contained many large vacuoles while the cell wall at the point of bacterial entry was disrupted and degenerated.

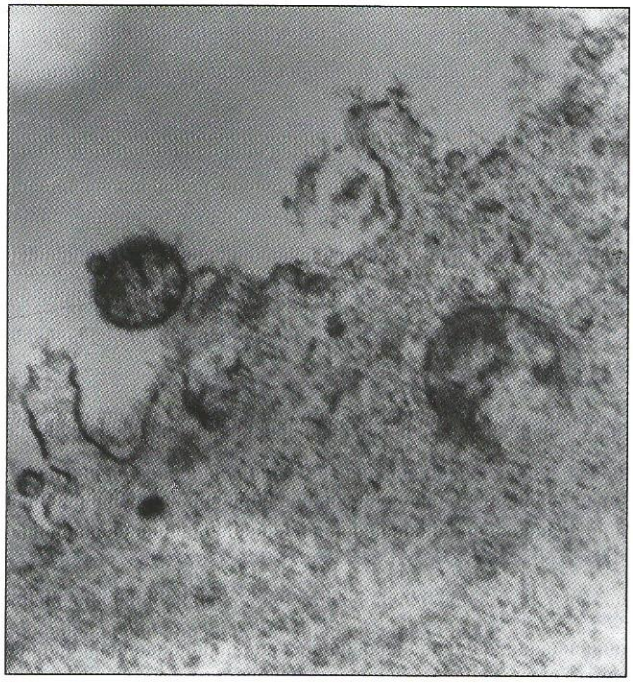

Figure 1. Electron micrograph of lung at 4 days post challenge with *Pasteurella haemolytica* A2 showing a macrophage in the process of phagocytosis

Only two (50 per cent) of the goats (one from each group) showed gross lung lesions at 7 days post infection, affecting an average of 7.0 per cent lung area (Table 1). The content of lung washing fluid of goats showing gross lung lesions was mainly macrophages with few neutrophils. Almost 50 per cent of these neutrophils and few macrophages showed eosinophilic cytoplasm and pycnotic nuclei. Similar exudate consisted of mostly macrophages was observed in the alveoli and bronchioles. Histologically many bacteria cells were observed in the cytoplasmic vacuoles of the swollen, degenerated macrophages but many more were found floating freely in the alveolar spaces. The presence of numerous freely floating bacteria cells were confirmed ultrastructurally. Most bacteria cells were observed to be closely associated to the pneumocytes (Figure 2). Although the other two goats had no gross lung lesions at 7 days post infection, cytological and histological examinations revealed the presence of many macrophages and bacteria. Most bacteria cells were being successfully phagocytised by these macrophages with little numbers of the non-phagocytised bacteria found freely in the alveoli.

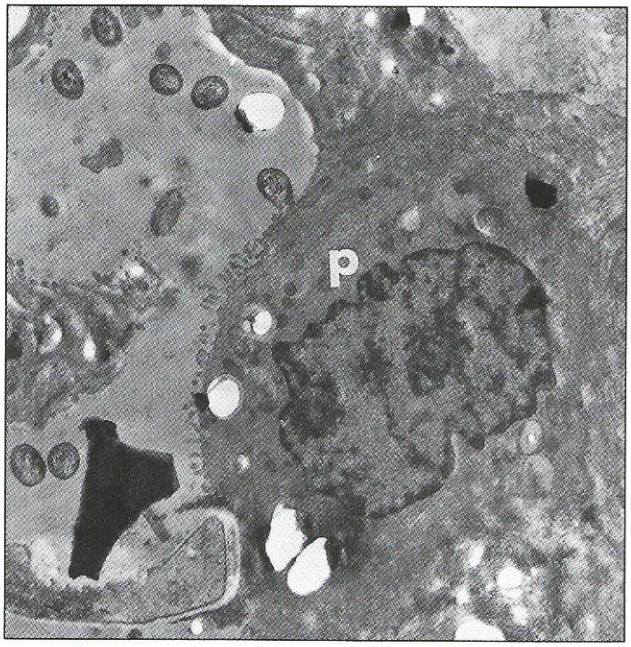

Figure 2. Electron micrograph of lung 7 days post challenge with *Pasteurella haemolytica* A2 showing many bacteria cells in alveolar spaces with a bacteria cell in close association to the wall of pneumocyte (P).

The increasing pattern of lung area showing lesions was observed again at day 11 post challenge. An average of 26.8 ± 12.3 per cent of lung area, particularly the antero-ventral and anterior diaphragmatic lobes were affected. The lung washing fluid contained mostly macrophages; some of which were found phagocytising bacteria cells. Between 40 to 50 per cent of the macrophages were degenerated, swollen with cytoplasmic vacuoles and pycnotic nuclei. Ultrastructurally, many pneumocytes showed degenerative changes with large cytoplasmic vacuoles containing many bacteria cells. The large vacuole seemed to push the degenerating nucleus to the periphery of the affected pneumocytes.

Lung washing fluid from uninfected control goats in group 3 revealed the presence of several tall columnar cells of bronchi without the presence of any bacteria or inflammatory cells.

Bacteriology

P. haemolytica A2 was successfully re-isolated from goats in groups 1 and 2 that showed pneumonic lung lesions. None of the uninfected control goats had *P. haemolytica*.

DISCUSSION

Pathological responses observed at certain time post exposure to *P. haemolytica* had been described in lungs of calves, lambs and mice (Jericho, 1989; Gilmour *et al.*, 1991; Pace *et al.*, 1994) but serial pulmonary pathological responses had not been described. In this study, the presence of bacterial cells in the alveoli of goats at 24 h post challenge stimulated an acute inflammatory reaction similar to those described in mice (Pace *et al.*, 1994). At this stage, almost all bacteria cells were observed freely in the alveolar spaces without being phagocytised. It had been reported that in the first 24 h post infection, both neutrophils and monocytes exit the circulatory system and migrate by chemotaxis toward the inflammatory site (Corey and Rosoff, 1989). The results suggested that the period between 4 to 7 days post challenge was critical for the establishment of *P. haemolytica* infection in goats since those goats that had a successful phagocytosis by the macrophages during the 4 to 7 day period post challenge showed no gross lung lesions. This is in agreement with an earlier suggestion that clearance of particles deposited in alveoli takes from several days to months depending on their physical nature and irritant capability (Dungworth, 1985).

Those goats in which their pulmonary macrophages were unsuccessful in phagocytosis during this period either due to the ability of the bacteria to survive intracellular antimicrobial function, or being insensitive to the macrophage antimicrobial mechanisms, or able to escape from phagocytome developed further lesions (Ho, 1989). The bacteria started to adhere to the pneumocytes by day 7 resulted in degenerated pneumocytes and macrophages similar to those described in lambs and calves (Jericho, 1989; Gilmour *et al.*, 1991). At 11 days post challenge, the bacterial infection had been established in the lungs and the phagocytic activities were further intensified. At this stage, the bacteria were observed to penetrate the lung parenchyma through their presence in cytoplasmic vacuoles of pneumocytes. Endocytosis across the alveolar type I epithelial cells is believed to be the method of bacterial penetration into the lung parenchyma (Dungworth, 1985). This penetration indicated the increasing bacterial load and firm establishment of *P. haemolytica* infection in the lungs of goats by 11 days post challenge. Parenchyma or interstitial penetration becomes increasingly important as the bacterial load increases (Dungworth, 1985).

Donachie *et al.* (1983) had reported the significance of *P. haemolytica* strain resulting in different extent of pneumonic lung lesions in sheep. This was later found otherwise (Gilmour *et al.*, 1986). As suggested by Gonzalez and Maheswaran (1993) the *P. haemolytica* A2 isolated from nasal mucosa was less virulent and produced a marked but insignificantly less extent of gross lung lesions, but the pulmonary responses and the lesion development patterns remained remarkably similar.

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RINGKASAN

PENUBUHAN PASTEURELLA HEAMOLYTICA A2 *DALAM PEPARU KAMBING BERIKUTAN PENDEDAHAN INTRATRAKEA*

Dua puluh ekor kambing sihat telah dibahagikan kepada tiga kumpulan terdiri daripada lapan ekor kambing dalam setiap satu daripada dua kumpulan pertama dan empat dalam kumpulan 3. Kesemua kambing telah ditekan-angkut secara perjalanan sebelum kambing dalam kumpulan 1 dicabar secara intratrakea dengan Pasteurella haemolytica *A2 yang telah dipencil daripada mukosa nasum kambing sihat dan kumpulan 2 dicabar dengan* P. haemolytica *A2 yang dipencil daripada peparu pneumonia kambing. Kumpulan 3 merupakan kawalan bukan terjanghkit. Kambing-kambing ini disembelih secara bersiri pada hari 1, 4, 7 dan 11 pascacabaran untuk mengkaji penubuhan bakteria dan untuk membanding kadar lesi peparu yang dihasilkan oleh kedua-dua pencilan tersebut. Lesi peparu kasar menunjukkan perkembangan progresif dan tererti* ($P<0.05$) *daripada purata* 6.3 ± 2.1 *peratus pada 24 jam pascacabaran kepada* 26.8 ± 13.1 *peratus selepas 11 hari dicabar.* P. haemolytica *A2 yang dipencil daripada peparu sentiasa menghasilkan lesi lebih teruk bukan tererti* ($P>0.05$) *berbanding* P. haemolytica *A2 yang dipencil daripada mukosa nasum. Sebilangan kecil neutrofil wujud dalam bendalir basuhan peparu pada 24 jam pascacabaran sebelum campuran neutrofil dan makrofaj berkegiatan fagositosis dapat dicerapkan pada hari 4 pascacabaran. Pada hari 7 pascacabaran, kambing yang menunjukkan lesi peparu lebih teruk mempunyai banyak sel bakteria dalam peparu sama ada terapung bebas dalam ruang alveolus atau terlekat pada pneumosit. Pada hari 11 pascacabaran, bakteria telah mula menusuk masuk dinding sel dan terdapat dalam vakuol sel pneumosit.*