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Ultrastructural pathology of nasal and tracheal mucosa of rabbits experimentally infected with *Pasteurella multocida* serotype D:1

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SUMMARY

Sixteen 8- to 9-week-old *Pasteurella multocida*-free New Zealand White rabbits were divided into two equal groups. The first group was inoculated intranasally with *P multocida* serotype D:1 strain and the second group that was inoculated with phosphate-buffered saline (PBS) only was used as a control group. *Pasteurella multocida* was isolated from the nasal cavity of all infected rabbits in group 1 and from tracheal swabs of seven rabbits in this group. Four rabbits in group 1 died with clinical signs of septicaemia, two rabbits had mucopurulent nasal discharge and pneumonic lesions and the other two did not show any clinical signs or gross lesions. The ultrastructural changes detected were deciliation or clumping of cilia of ciliated epithelium, cellular swelling, vacuolation and sloughing. The subepithelial capillaries showed congestion, intravascular fibrin deposition, platelets aggregation and endothelial injury. *Pasteurella multocida* was observed attached to the injured endothelial cells. Heterophils, mast cells, vacuolated monocytes and macrophages infiltrated the lamina propria and between the degenerated epithelial cells. ©2001 Harcourt Publishers Ltd

PASTERURELLOSIS is considered to be one of the most important diseases of rabbits with different clinical forms (Flatt 1974). The causative agent, *Pasteurella multocida*, can cause high morbidity and mortality in laboratory and commercial rabbit colonies and results in unsuitability of the affected rabbits for research use.

Type D strain is frequently isolated from septicaemic and pneumonic cases (Flatt and Dungworth 1971a; Okerman et al 1979). A number of investigators have reported purification of a heat labile-toxin from the virulent type-D strain of *P multocida* in rabbits and pigs (Nakai et al 1984, Chrisp and Foged 1991). The lower and the upper respiratory tract lesions, particularly the ultrastructural changes, caused by *P multocida* type D isolates from septicaemic or pneumonic cases have not been widely studied although a few experimental studies have been conducted on the pathogenicity of capsular type D of unknown somatic types in rabbits (Flatt and Dungworth 1971b, Percy et al 1986) and its toxin in pigs and rabbits (Rutter and Mackenzie 1984, Chrisp and Foged 1991).

The purpose of this study was to observe the ultrastructural changes in the upper respiratory tract of rabbits infected intranasally with toxigenic *P multocida* type D:1 strain.

MATERIAL AND METHODS

Animals

Sixteen 8- to 9-week-old New Zealand White rabbits were obtained from the Animal Resource Centre, Universiti Putra Malaysia. The rabbits were negative for *P multocida* following three attempts at isolation from the nasal cavity and detection of antibodies against *P multocida* in their sera using enzyme-linked immnosorbent assay (ELISA). They were housed individually in stainless steel cages and provided with water and rabbit pellets ad libitum.

ELISA

The test was conducted to assay immunoglobulin (IgG) to *P multocida* in the sera of the experimental animals prior to inoculation. *Pasteurella multocida*-boiled cell extract antigen was prepared as described by Kawamoto et al (1994). The ELISA procedure was conducted using the method of Klaassan et al (1985).

Pasteurella multocida *inoculum*

The *P multocida* serotype D:1 used in this experiment was isolated from an Angora rabbit that had died of septicaemia. The isolate was maintained on nutrient agar slant (Oxoid) at 25˚C and was subcultured on 5 per cent horse-blood agar at 37˚C overnight before one colony was selected and grown in brain–heart infusion (BHI) broth (Oxoid) at 37˚C for 18 hours. To recover the pathogenicity of the isolate, a mouse was inoculated intraperitoneally with 0·1 ml of the cultured broth before the micro-organism was re-isolated from the heart blood of the dead mouse after 24 hours. The isolate was identified as type D by the acriflavine flocculation test (Carter and Subronto 1973). Somatic serotyping was conducted by Dr Thula Wijewardena at the Veterinary Research Institute, Sri Lanka. The bacteria were quantitated by diluting a 1·0 ml sample of the broth (BHI

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growth), 10-fold in sterile phosphate-buffered saline (PBS), pH 7·4. Each dilution was streaked onto 5 per cent horse-blood agar and counted after incubation for 24 hours at 37˚C. Samples for inoculation were diluted in PBS to 2×10^8 colony forming units per ml (cfu ml^{-1}).

Detection of dermonecrotic toxin (DNT)

The procedure for demonstrating toxin production was according to the method described by Carter (1990). *Pasteurella multocida* isolates were inoculated into 5 ml BHI broth containing 0·3 per cent yeast extract. The inoculated broth was incubated at 37˚C for 6 hours. The culture was then sonicated in an ice bath for 5 minutes at an intensity of 55 (Vibra Cell, Sonic & Material Inco, USA). Following sonication, the broth culture was centrifuged at 2000 *g* for 20 minutes at 4˚C. The supernatant was filtered through a 0·22 µm membrane and the filtrate was used immediately. The skin of Guinea pigs was clipped and shaved before injection. A Guinea pig was injected intradermally with 100 µl of the filtrate of each strain. Filtrate of a strain of *P multocida* known to be negative for the toxin was used as a control. The necrotic zone that developed was 5 cm in diameter after 48 hours.

Experimental design

The 16 *P multocida*-free rabbits were divided into two equal groups. Rabbits in group 1 were inoculated intranasally with *P multocida* at a dose rate of 2×10^8 cells in 1 ml of PBS (Al-Haddawi et al 1999). Inoculation was conducted by inserting the end of 1 ml syringe without needle into the nares of rabbits and instilling the inocula gently. Rabbits in group 2 were similarly inoculated intranasally with only PBS, pH 7·4 as control. Two rabbits were killed on days 14 and 21 post-inoculation each from group 1 while four rabbits from group 2 were killed on days 14 and 21 post-inoculation each, by severing the jugular vein following anaesthesia with 0·5 ml Ketavet (Ketamin hydrochloride 100 mg ml^{-1}; Delta Vet, Laboratories Pty. Ltd., Australia) and 0·2 ml Romazine (Xylazine 20 mg ml^{-1}; Jurox Pty. Ltd., Australia) i.m. Other rabbits from group 1 died on days 6, 7, 12 and 19 post-inoculation.

Bacteriology

Nasal swabs were collected at 3-day intervals for bacterial isolation during the time of the experiment. Swabs from nasal and tracheal mucosa and samples from lung, liver, kidney, heart and brain were collected also for bacterial isolation at postmortem. Bacterial isolation was conducted aseptically in the laboratory using sterile instruments for each sample.

Pathology

Samples from three portions (cranial, middle and caudal) of nasal mucosa (both sides) and middle part of trachea that yielded heavy to pure growth of *P multocida* were collected for transmission electron microscopy (TEM). The samples were collected from six rabbits from both infected groups. The samples of nasal mucosa were fixed for 8 hours with 2·5 per cent glutaraldehyde and that of trachea were fixed with 4 per cent glutaraldehyde, using 0·1 M sodium cacodylate buffer. Samples were washed three times in 0·1 M cacodylate buffer for 10 minutes each, post-fixed for 2 hours with 1 per cent osmium tetroxide. Fixed samples were rinsed, dehydrated in a graded acetone series and embedded in resin. Ultrathin sections were cut on Ultracut E (Reichert-Jung, Austria) microtome and mounted on 200-mesh-copper grids. The sections were stained with uranyl acetate and lead citrate and examined using Hitachi transmission electron microscope H 7100.

RESULTS

Bacterial isolation

Pasteurella multocida was isolated from the nasal cavity of all sacrificed and dead rabbits from group 1 starting from day 2 post-inoculation, and from the trachea of seven rabbits in group 1. Heavy to pure growth of *P multocida* was obtained from the nasal swabs of six infected rabbits in group 1. In addition, tracheal swabs cultures of seven rabbits in group 1 yielded heavy to pure growth of *P multocida*. *Pasteurella multocida* was not isolated from rabbits in group 2. Other bacteria were also isolated from the nasal cavity prior and post-inoculation from rabbits of groups 1 and 2. These bacteria were *Staphylococcus aureus*, *Escherichia coli*, *Citrobacter* spp, *Bacillus subtilus*, *Aeromonas* spp and *Pseudomonas aeruginosa*. *Pasteurella multocida* was isolated from all the organs sampled from dead rabbits.

Clinical signs

Four rabbits from group 1 died on days 6, 7, 12 and 19 post-inoculation. These animals were depressed, anorectic and dyspnoeic before death. Serous nasal exudate was evident in these rabbits four days post-inoculation which became mucopurulent five to eight days post-inoculation until the rabbits died. Two surviving rabbits in this group that were killed at 14 and 21 days post-inoculation, showed increased rectal temperature and mucopurulent nasal discharge. The two remaining rabbits in group 1 and all the rabbits in group 2 killed at 14 and 21 day post-inoculation, did not show any clinical signs.

Gross findings

Rabbits that died in group 1 showed signs of septicaemia and pneumonia. There were congestion and haemorrhages of nasal and tracheal mucosa, lungs, heart, liver, kidneys and meninges. The gross pathological findings in dead rabbits from group 1 were characteristic of septicaemia. In group 1, one rabbit that died at six days post-inoculation showed cyanosis of the mucosal membranes and congestion of the nasal and tracheal mucosa and the meninges. The lungs were congested and oedematous. Mucopurulent exudate was found in the nasal cavity of rabbits that died seven days post-inoculation. Two rabbits died seven and 12 days post-inoculation, respectively, showed congested tracheal mucosa, liver, meninges and lungs. Subcapsular haemorrhages were observed on the surface of the kidneys. Two rabbits in group 1 that were killed 14 and 21 and one rabbit that died 19 days post-inoculation had pneumonic lungs with presence of fibrin deposition on the pleura.

Transmission electron microscopy

Nasal mucosa. The ultrastructural changes in the nasal mucosa of infected rabbits in group 1 that died 6–12 days post-inoculation were degeneration of the surface epithelium with inflammatory response. The ciliated cells showed either loss of cilia or clumping of their cilia (Fig 1). Vacuolation of the cytoplasm and dilatation of the organelles of degenerated cells were also observed. The mitochondria showed dilatation and loss of their cristae, and the rough endoplasmic reticulum (rER) were swollen. Gaps had formed between the degenerated basal cell, which had numerous intracytoplasmic vacuoles and sloughing of many degenerated cells into the nasal cavity were evident. Heterophils infiltrated in between the degenerated cells and in subepithelial area near the basement membrane (Fig 2).

The blood vessels in the lamina propria were congested and there was intravascular fibrin deposition. Vacuolated monocytes were also seen attached to the wall of blood capillaries. Many granulated mast cells and monocytes had infiltrated the lamina propria and near blood capillaries in rabbit that died six days post-inoculation. The mast cells were laden with large granules (Fig 3).

Degenerative changes were also seen in the endothelial cells of blood capillaries. These cells displayed an irregular luminal surface. Some cells formed a thin layer, while others were swollen and contained vacuoles with large osmiophilic materials in their cytoplasm. Some swollen endothelial cells projected into the blood vessels (Fig 4). Intravascular polymorphonuclear neutrophils were prominent and some were attached to the degenerated endothelial cells. These polymorphonuclear neutrophils had cytoplasmic projections toward the injured endothelium and large dark vacuoles near the cell membrane. The pericytes adjacent to those

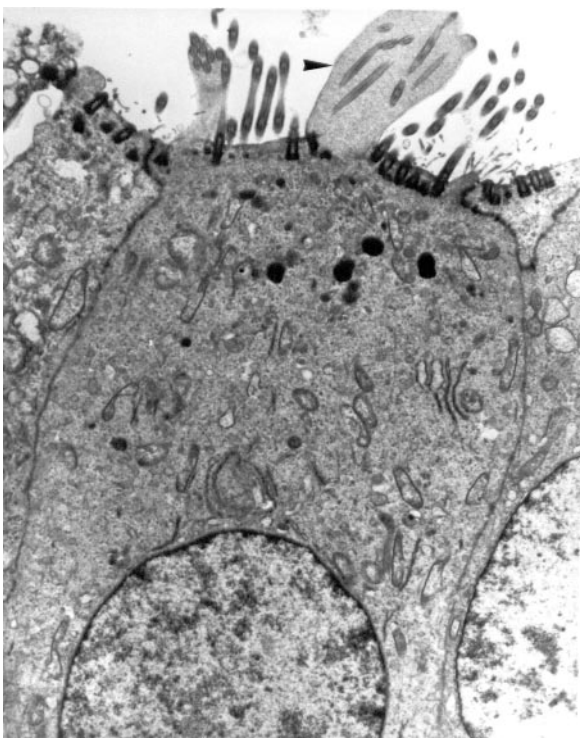

FIG 1: Electron micrograph. Nasal mucosa of a rabbit from group 1 infected with *Pasteurella multocida* type D:1 that died seven days post-inoculation. Clumping of cilia (arrow head) of the ciliated nasal epithelium. TEM × 7 500

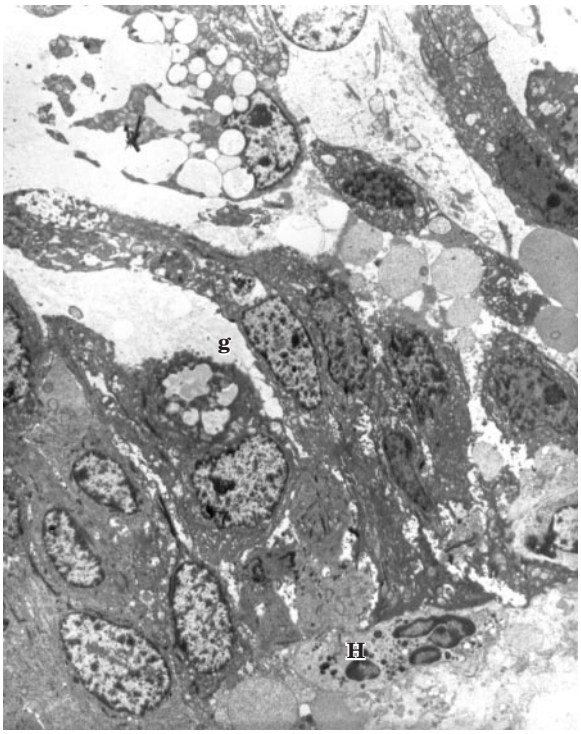

FIG 2: Electron micrograph. Nasal mucosa of a rabbit from group 1 infected with *Pasteurella multocida* type D:1 that died 12 days post-inoculation. Note gaps (g) between degenerated epithelial cells, which have intracytoplasmic vacuoles, sloughing of degenerated epithelial cells and infiltration of heterophils (H) near basement membrane TEM × 2 200

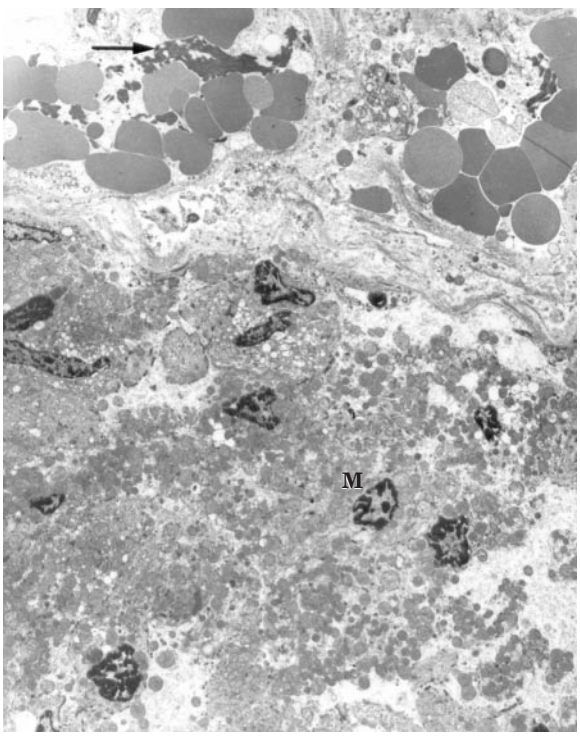

FIG 3: Electron micrograph. Nasal mucosa of a rabbit from group 1 infected with *Pasteurella multocida* type D:1 that died six days post-inoculation. Note the congestion of subepithelial capillaries with intravascular fibrin (arrow) deposition. Many granulated mast (M) cells have infiltrated adjacent the capillaries. TEM × 2 000

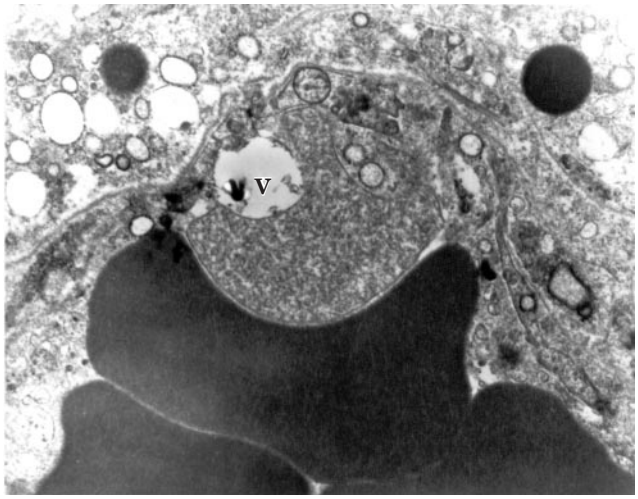

FIG 4: Electron micrograph. Nasal mucosa of a rabbit from group 1 infected with *Pasteurella multocida* type D:1 that died seven days post-inoculation. Note swelling of the endothelial cell with vacuolation (V) of the cytoplasm. TEM × 13 500

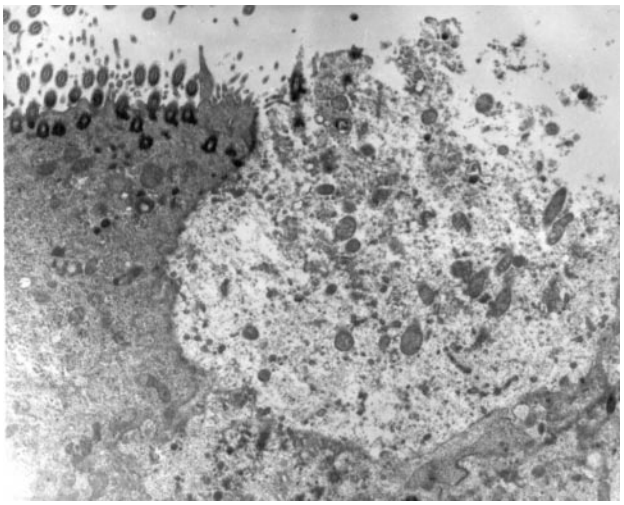

FIG 5: Electron micrograph. Trachea of a rabbit from group 1 infected with *Pasteurella multocida* type D:1 that died six days post-inoculation. Note the degenerated epithelial cell with ruptured cell membrane and the organelles. TEM × 7 440

capillaries had dilated mitochondria and pyknotic nuclei, which indicated degeneration of these cells.

Mucosal glands showed vacuolation of the cytoplasm, karyolysis of nuclei and atrophy of the epithelial lining.

Rabbits that were killed 21 days post-inoculation had sloughing of the degenerated epithelial cells into the nasal cavity leaving a layer of the basal cells. Disintegration of the intracellular structures was seen in the sloughed and degenerated basal cells. Many macrophages and plasma cells and a few heterophils had infiltrated the subepithelial area. Fibroblast proliferation and collagen fibre formation were also evident in this area.

Trachea. The ultrastructural changes in the epithelial cells of the trachea of infected rabbits that died 6–12 days post-inoculation were degeneration and sloughing of the surface epithelium. Some degenerate cells had ruptured cell walls (Fig 5). The sloughing of the surface epithelium resulted in only one layer of basal cells remaining. The basal cells showed disintegration of intracellular structures and some of these cells had pyknotic nuclei and damaged cell walls

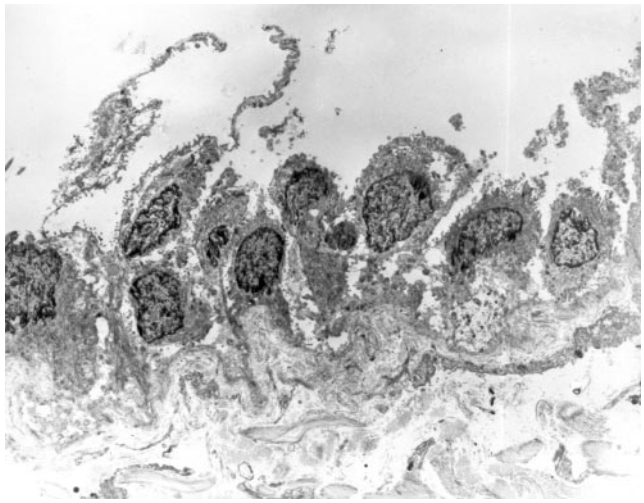

FIG 6: Electron micrograph. Trachea of a rabbit from group 1 infected with *Pasteurella multocida* type D:1 that died seven days post-inoculation. Note that the surface epithelium has sloughed and only one layer of basal cells with disintegrated intracellular structures has remained. TEM × 2 100

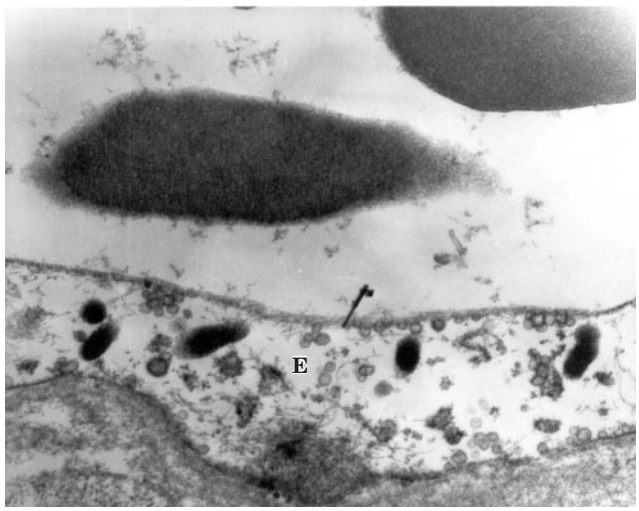

FIG 7: Electron micrograph. Trachea of a rabbit from group 1 infected with *Pasteurella multocida* type D:1 that died 12 days post-inoculation. Note vacuolation of cytoplasm of an endothelial (E) cell and the organelles scattered within the cytoplasm. TEM × 27 000

(Fig 6). Gaps between the basal cells were evident, which led to detachment of the degenerate cells.

The endothelial cells were swollen and had large nuclei and vacuoles. Some degenerate cells had scattered organelles within their cytoplasm (Fig 7). Other endothelial cells showed ruptured cell walls and nuclear membranes. Aggregation of intravascular platelets that attached to the damaged endothelial cell together with fibrin deposition and heterophils (Fig 8) were evident. Intravascular aggregates of bacterial cells were seen adhered to the degenerate endothelial cells and bacteria under division were also seen (Fig 9). Vacuolated mononuclear cells were seen around the capillaries. In rabbits which died or were killed on days 19 and 21 post-inoculation, sloughing of the epithelial cells was noted and infiltration of macrophages, plasma cells and heterophils into the subepithelial area. Degenerative changes of the endothelial cells were also observed.

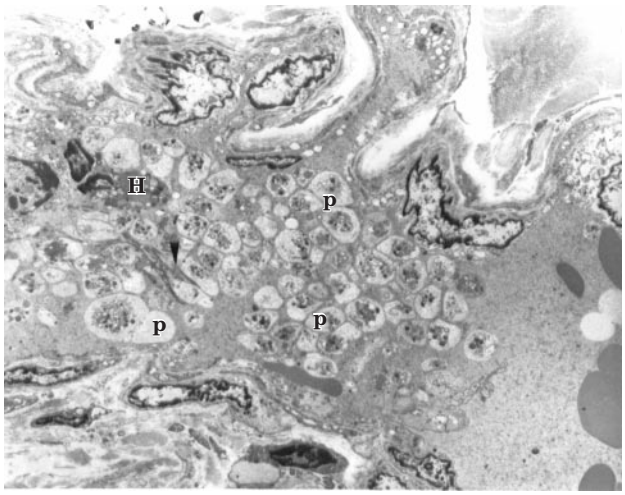

FIG 8: Electron micrograph. Trachea of a rabbit from group 1 infected with *Pasteurella multocida* type D:1 that died six days post-inoculation. Note platelet aggregation (P) in a capillary together with fibrin (arrow head) deposition and heterophils H). TEM × 2 100

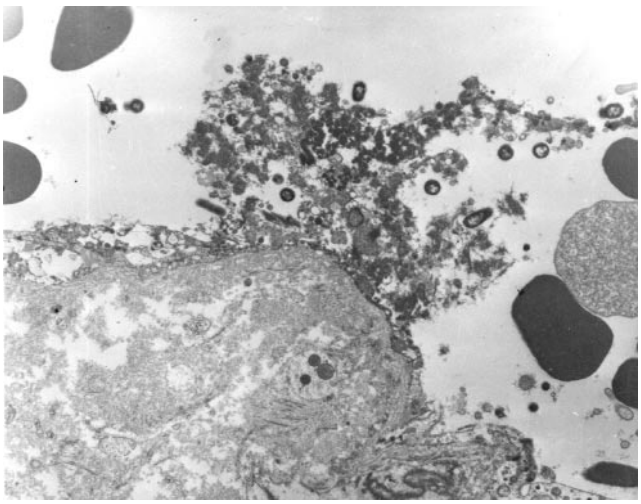

FIG 9: Electron micrograph. Trachea of a rabbit from group 1 infected with *Pasteurella multocida* type D:1 that died six days post-inoculation. Note the bacterial colonization on the damaged endothelial cell. TEM × 5 300

DISCUSSION

The clinical signs and pathological observations in this study were consistent with septicaemia, which caused death starting from day 6 post-inoculation, while the surviving rabbits showed acute, subacute and chronic infections. Studies on the pathogenicity of *P multocida* type D:1 strain in rabbits have not been reported before. However, similar results were observed in other studies conducted in rabbits using *P multocida* type D isolates of unknown serotype (Flatt and Dungworth 1971b). On the other hand, *P multocida* type D:3 was reported earlier as not highly virulent in rabbits, where rabbits infected with a higher dose of inocula than the present study, developed only pneumonic lesion (Percy et al 1986). This study demonstrated that the *P multocida* type D:1 strain isolated from a rabbit was pathogenic and highly virulent in experimentally infected rabbits when compared to type A:3 strain (Al-Haddawi et al 1999). Although a similar dose (10^8 cfu ml^{-1}) and age of rabbits (8–9 weeks old) were used in both studies no deaths were reported in rabbits infected with the type A:3 strain.

The bacterial isolation results showed that *P multocida* type D:1 can simultaneously colonize the internal organs as well as the nasal cavity, trachea and lungs resulting in peracute infection and death. Variations in the virulence of *Pasteurella* isolates in animals depends on many bacterial products including endotoxin, nuraminidase, adhesin and antiphagocytic capsule (Glorioso et al 1982, Anderson et al 1984). The clinical signs observed in the present study were depression, anorexia and dyspnoea before death. These clinical signs might be attributed to the effects of endotoxin. It has been reported that endotoxin in rabbits is associated with fever, central nervous system depression, disseminated intravascular coagulopathy, leukopenia, thrombocytopenia and death (Heng et al 1996). The *P multocida* endotoxin has also been detected free in the plasma of septicaemic rabbits (Rebers et al 1967; Corrigan and Kiernat 1979).

The results of the present study showed that the filtrate of *P multocida* serotypes D:1 strain caused skin lesions at the site of injection in the guinea pigs. These results revealed that this isolate produced a dermonecrotic toxin and thus is toxigenic. A heat labile toxin produced by virulent strains of *P multocida* type D has been identified. A number of its biological activities, including dermonecrosis in Guinea pigs skin and lethality for mice after intraperitoneal inoculation have been determined (Nakai et al 1984, Kamp et al 1987). This toxin is also suggested to be responsible for pulmonary lesions and it is likely that the pulmonary lesions are induced primarily by circulating toxin followed by bacterial infection (Ringler et al 1985). These findings are supported by other investigators who reported that inoculation of *P multocida* toxin intranasally in rabbits could cause pneumonia with pleuritis (Chrisp and Foged 1991). These researchers suggested that pneumonia and pleuritis might be the result of a systemic rather than inhaled toxin.

The ultrastructural changes in the nasal and tracheal epithelial cells, cilia, microvilli and mitochondria of affected epithelia indicate impaired synthesis in these cells. This impairment is due apparently to the bacteria or their products like exotoxin and endotoxin (Salyers and Whitt 1994). *Pasteurella multocida* or its toxin can induce rhinitis and pneumonia in rabbits (Ringler et al 1985, Chrisp and Foged 1991) and it is cytotoxic (Suckow et al 1991). Toxigenic *P multocida* strains can naturally and experimentally cause atrophic rhinitis in pigs and rabbits (Rimler and Brogden 1986, Sukano et al 1992). The lesions due to *P multocida* type D strains were more severe in pigs pre-treated or infected either with acetic acid or toxigenic *Bordetella bronchiseptica* (Chanter and Rutter 1989, Hamilton et al 1998). The damage caused by acetic acid or *B bronchiseptica* toxin may facilitate the colonization of *P multocida*. Acetic acid alone can cause epithelial cell degeneration, cilia loss, goblet cell hyperplasia and inflammatory reaction in the nasal mucosa of pigs (Gagne and Martineau-Doize 1993).

In the present studies, although the rabbits were not pretreated with intranasal inoculation of acetic acid or *B bronchiseptica*, similar changes were found in the nasal and tracheal mucosa. These findings indicate that toxigenic *P multocida* type D strain alone is capable of inducing

severe lesions in the nasal and tracheal mucosa of rabbits. It seems possible that the dermonecrotic toxin produced by toxigenic *P multocida* can induce lesions in the nasal mucosa similar to those produced by acetic acid or *B bronchiseptica* toxin, which subsequently facilitates colonization of the nasal mucosa by the bacteria. Although the infected (group 1) and control (group 2) rabbits were housed in the same environment, the ultrastructural changes were observed only in the nasal mucosa of infected rabbits but not of control rabbits. Additionally, other bacterial species were isolated from the nasal cavity of control rabbits. These results indicate that neither the environment nor the presence of other bacteria contributed to the development of lesions in the nasal mucosa caused by this toxigenic *P multocida* type D strain.

It was remarkable that by day 21 post-inoculation, *P multocida* serotype D:1 could be detected by conventional bacteriological procedures from the nasal mucosa but not from the tracheal mucosa of all rabbits. The finding that *P multocida* was not recovered from tracheal lesion of two rabbits infected with type D:1 suggested that *P multocida* existed in the nasal mucosa and produced its toxins which led to lesions in other organs (Kamp and Kimman 1988).

Aggregation of swollen reactive macrophages with intracytoplasmic vacuoles and dense cytoplasmic particles, which was attributed to toxic effect of *P multocida* isolated from buffalo and cattle in vitro in cultured mouse macrophages (Shah et al 1996) was also observed in rabbits killed or died 14, 19, 21 days post-inoculation in this study.

Intravascular aggregation of platelets observed in this study may have been related to increased level of platelet activating factor and thromboxane due to release of endotoxin (Koltai et al 1993). Oedematous separation of basal cells and condensed nuclei of pericytes adjacent the injured capillaries indicated a direct effect of lipopolysaccharides (LPS) on the integrity of endothelium, causing increased microvascular permeability (Meyrick et al 1986). Additionally, intravascular macrophages or monocytes activated by endotoxin can elaborate toxic oxygenated metabolites, which directly injured the local endothelium (Warner et al 1988).

Bacterial cells were observed attached to the wall of capillaries of septicaemic rabbits. Presence of bacteria in the blood vessels and its ability to produce toxins confirmed the signs of septicaemia on the infected rabbits. Endothelial cell injury observed in these studies was believed the result of direct toxic effect on the endothelium (Meyrick et al 1986).

In conclusion, *P multocida* type D:1 was pathogenic to the infected rabbits and highly virulent. Dermonecrotic toxin may play a role in colonisation of *P multocida* type D strain by causing damage to the ciliary system of the nasal mucosa. Colonised *P multocida* type D:1 strain caused septicaemia in infected rabbits with high mortality rate. The most severe consequence of septicaemia was the development of septic shock, which was associated with coagulopathy, vasomotor collapse, organ failure, and death.

ACKNOWLEDGEMENT

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