UNIVERSITI PUTRA MALAYSIA

ULTRASTRUCTURE EVALUATION OF *PASTEURELLA MULTOCIDA* B:2 IN RESPIRATORY ORGANS OF AEROSOLLY-INFECTED MICE

RAZILA RAAZALI

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SERDANG SELANGOR

ULTRASTRUCTURE EVALUATION OF *PASTEURELLA MULTOCIDA* B:2 IN RESPIRATORY ORGANS OF AEROSOLLY-INFECTED MICE

RAZILA RAAZALI

A project paper submitted to the
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In partial fulfilment of the requirement for the
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CERTIFICATION

It is hereby certified that we have read this project paper entitled "Ultrastructure Evaluation of *Pasteurella Multocida* B:2 in Respiratory Organs of Aerosolly-Infected Mice", by Razila Binti Raazali and in our opinion it is satisfactory in terms of scope, quality, and presentation as partial fulfilment of the requirement of the course VPD 4901 – Project.

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DEDICATION

Alhamdullilah to Allah SWT.

I dedicate this thesis to my beloved parents and family,

Lecturers,

Loved ones,

Friends,

Thank you for your endless love and support

and everyone who reads and benefits from this thesis.

ACKNOWLEDGEMENTS

Alhamdulillah, greatest thanks to Allah, with all His blessing, I able to complete my final year project successfully. Special thanks to my dedicated supervisor Dr. Annas Salleh for his unwavering guidance, patience and knowledge he poured to me throughout this project. For my co- supervisor Prof. Dato' Dr. Tengku Azmi and Associate Prof. Dr. Firdaus Jesse, million thank you for all your guidance, times and support to complete this project.

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My deepest gratitude goes to my beloved family for the utmost moral, emotional and physical support that bring me here today. Only Allah can repay all your love and warmth for me. To my dearest Syafiq Shahudin, thank you for being one of the important person in my thick and thin journey. Words not enough to thank your unconditional love and support.

I also would like to place my sincere gratitude to one and all who, directly and indirectly have lent their helping hand in completion of this project. Thank you.

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ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine, UPM in partial requirement to fulfil the course of VPD 4999- Final Year Project.

ULTRASTRUCTURE EVALUATION OF *PASTEURELLA MULTOCIDA* B:2 IN RESPIRATORY ORGANS OF AEROSOLLY-INFECTED MICE

By

Razila Raazali

2017

Supervisor: Dr Annas Salleh

Haemorrhagic septicaemia (HS) is an acute, fatal, septicaemic disease of buffaloes and cattle caused by *Pasteurella multocida* B:2. Natural cases of HS occur primarily via the aerogenous route of susceptible animals. This study was conducted to determine and evaluate ultrastructure of respiratory organ with respect to aerosol inoculation of *P. multocida* in mice which mimics natural HS transmission. A group of 30 healthy female mice were divided into two groups, control (n=5) and treatment. The treatment groups were further divided equally into 5 groups (n=5). All treatment group were inoculated aerosolly with 10 ml of 1 X 10^9 cfu/mL of *P. multocida* for 20 minutes. The control group were euthanised at 6 h prior to inoculation, while the treatment groups were euthanised by cervical dislocation at 0, 6, 12, 18, 24 h pi. Clinical signs were observed for 24 h pi. Lungs tissue were collected for ultrastructural examination. Clinical signs of reduced activity were observed starting from 12 to 24 h pi. The ultrastructure evaluation for the treatment groups showed mild to severe degree of pathological changes in the pneumocyte and endothelial cell. Overall, inoculation of

P. multocida Type B:2 via aerogenous route revealed significant ($p<0.05$) cellular changes on the lungs.

Keywords: Haemorrhagic septicaemia, *Pasteurella multocida* serotypes B:2, electron microscope examination, aerosol, mice

ABSTRAK

Abstrak daripada kertas project yang dikemukakan kepada Fakulti Perubatan Veterinar UPM untuk memenuhi sebahagian daripada keperluan kursus VPD 4999-Projek Tahun Akhir.

PENILAIAN ULTRASTUKTUR PASTUERELLA MULTOCIDA JENIS B:2 DALAM ORGAN RESPIRASI BERIKUTAN JANGKITAN MELALUI AEROSOL MENCIT

Oleh

Razila Raazali

2017

Penyelia: Dr Annas Salleh

Penyakit hawar berdarah disebabkan oleh *Pasteurella multocida* merupakan penyakit septisemia akut dalam kerbau dan lembu yang berlaku sepanjang tahun di Malaysia. Kajian ini dilakukan untuk menentukan dan menilai ultrastuktur organ respirasi menggunakan jangkitan melalui aerosol dalam mencit dimana menyerupai natural transmisi penyakit hawar berdarah. Sekumpulan 30 mencit betina yang sihat dibahagikan kepada dua kumpulan, kawalan (n=5) dan rawatan (Kumpulan 1, 2, 3, 4, 5). Kesemua mencit kumpulan rawatan diinokulasi melalui aerosol dengan 10 ml *P. multocida* B:2 dengan konsentrasi 3.7 X 10^9 jajahan membentuk unit (CFU)/mL selama 20 minit. Kemudian, mencit tersebut dibahagikan kepada lima kumpulan. Mencit dari kumpulan kawalan dan rawatan dieuthanisia pada -6, 0, 6, 12, 18, 24 jam. Tanda-tanda klinikal diperhatikan sepanjang 24 jam selepas jangkitan. Paru-paru dikumpulkan untuk pengasingan-pengesahan bacteria dan pemeriksaan elekton mikroskop. Mencit dari Kumpulan 1 dan 2 tidak menunjukkan sebarang perubahan dalam tingkah laku. Walau bagaimanapun, mencit dalam Kumpulan 3, 4 dan 5 mendedahkan pengurangan dalam tingkah laku. Evaluasi ultrastruktur untuk

kumpulan rawatan menunjukkan sedikit kepada teruk darjah lesi di dalam pneumosit dan sel endothelial. Keseluruhanya, inokulasi *P. multocida* jenis B:2 melalui laluan aerogenus mendedahkan perrubahan ketara sel paru paru.

Katakunci: hawar berdarah, *Pasteurella multocida* jenis B:2, mencit, electron mikroskop

INTRODUCTION

Pasteurella is a genus from Pasteurellaceae family. It is a Gram-negative, small, pleomorphic, non-flagellated coccobacillus. *P. multocida* can be divided into serogroups and serotypes. The serogroups (A, B, D, E and F) are divided based on their antigenicity of the capsule. Meanwhile, the serotypes (1-16) were divided based on the lipopolysaccharide (LPS) antigens which also known to release endotoxin (De Alwis, 1992; Wilkie *et al.,* 2012). *P. multocida* is a causative agent that associated with wide range of veterinary diseases in domestic animals, feral animals and also agricultural species (De Alwis, 1999). This organism can become primary pathogen or can also play a role as secondary pathogen of a disease.

HS is an acute, highly fatal disease that can impose a huge economic loss in livestock industry that is caused by *P. multocida* Type B:2 (Dziva *et al.,* 2008). This organism can be transmitted via inhalation and ingestion of contaminated water or feed with *P. multocida*. In most cases, rapid spread of HS is due to close-contact of infected animals with affected animals within or between the herds and also inhalation of causative agent which originate in nasopharynx of carrier animal (OIE, 2013). Thus, pathological signs mainly in respiratory tract are observed in this disease. According to the Tabatabaei *et al.* (2007), infected animals may show short clinical signs such as depression, high temperature, dyspnoea and mainly respiratory signs and followed by recumbent then lead to death.

In an acute disease, early disease detection is crucial for the treatment to be effective and antibacterial therapy is usually effective at this stage (Benkirine & De Alwis, 2002). However, the most important measures in order to protect animals

against HS is by practicing routine vaccination besides increase hygiene and reduced epizootical factors (Shah *et al.*, 1997). According to the study done by Zamri *et al.* (2007), the animals were succumbed to this organism and produced peracute and acute infection with respect to inoculation of *P. multocida* via subcutaneous (S.Q) and intratracheal (I.T) route. Nevertheless, the study had fail to prove that intranasal (I.N) inoculation can recreate the clinical response of HS in the experimental goat. Therefore, this study was undertaken to evaluate the effect in the ultrastructure of mice lungs exposed with *P. multocida* B:2 via aerogenous route.

2.0 LITERATURE REVIEW

2.1 *Pasteurella multocida*

Pasteurella genus is in Pasteurellaceae family is a Gram negative, and non-endospore forming bacteria with small round, yellowish or greyish, glistening, translucent, non- haemolytic with diameter of 1-2 mm on blood agar. *P. multocida* is a non-motile, small and pleomorphic cell which can occurs singly, in pairs or short chains (Shivachandra *et al.*, 2011) According to Namioka-Carter system, this septicaemic disease is caused by specific serotypes 6: B or 6: E which happen in buffaloes and cattle as Asian and African serotypes respectively. Meanwhile, as stated in Carter-Heddleston system, *P. multocida* serotype B: 2 in Asian and E: 2 as African serotype (De Alwis, 1984). This organism is easily transmitted via inhalation or ingestion (Iowa, 2009). *P. multocida* is the causative agent that responsible for several veterinary diseases. HS in ungulates, atrophic rhinitis in pigs, snuffles in rabbit and fowl cholera in poultry are examples of primary diseases caused by this organism. Meanwhile, other problem for instance lower respiratory tract infection in ungulates and also sporadic septicaemias possibly happen due to underlying infection that caused weakening of the immune system.

2.2 Pathogenicity of *P. multocida* B:2

Post-infected animals with low number of virulent *P. multocida* will show a rapid onset of the clinical signs due to its acute disease in nature. Most studies related to this disease used mice as a host animal as *P. multocida* are highly virulent to the laboratory animals such as mice and rabbit through inoculation from several routes (Shivachandra *et al.,* 2011). Different methods of inoculation including intraperitoneal (I.P), S.C, and intramuscular (I.M) routes were effectively causing different pathological changes in the mice that resembles same pathological effect as in cattle and buffalo. The suitability of detection of HS using mice inoculation was evaluated via many criteria such as critical pathogenic dose, kinetics of infection, pathology of the disease and also resistance of the animals to reinfection (Ramdani *et al.,* 1990; Wilkie *et al., 2012*).

2.3 Haemorrhagic Septicaemia

Haemorrhagic septicaemia (HS) is caused by *P. multocida* Type B:2, a Gram-negative coccobacillus bacterium. This fatal disease can occur in ungulates at which buffalo (*Bubalus spp.)* appears to be more susceptible species compared to other domesticated animals (Wilkie *et al*., 2012). Studies revealed that, this disease associated with humid and wet weather where during this time, *P. multocida* will have longer survival time causing more outbreak incidence of this organism. Aside from seasonal incidence and stress factor, this disease also happens in situation whereby

there is poor husbandry practice, and not well-developed disease surveillance system in that area (De Alwis, 1992; Chaturvedi *et al.,* 2002).

2.4 Diagnosis

HS is an acute nature disease thus, provisional diagnosis is crucial because, immediate disease confirmation is important to control the disease spread (Benkirane & De Alwis, 2002). In clinical diagnosis of the disease, it is usually based on the history of earlier outbreaks, failure to vaccinate animals recently, species affected, age of the animal, season of disease occurred which is usually during wet season, rapid course of disease, high herd morbidity and also high mortality. Besides that, early disease determination is important to rule out other possible disease that exhibit same clinical signs such as Shipping Fever in cattle, Acute Salmonellosis, Mycoplasmosis, and Pneumonic Pasturellosis (OIE,2013). HS can be confirmed through clinical findings of the animals, pathological lesions during post mortem and also laboratory diagnosis of the samples.

2.4.1 Clinical signs of HS

Buffalo and cattle that are infected with HS will show rapid signs that usually last 1-3 days before death. Visible clinical signs initially increased in temperature, submandibular oedema that may spread to the brisket region and into respiratory phase in which the animal shows respiratory signs such as difficulty in breathing. Then,

termination phase where animals show recumbent and dies. As buffalo is more susceptible that cattle, thus buffaloes die quicker (De Alwis, 1999).

2.4.2 Pathological changes in HS

According to Annas *et. al* (2014), the pathological lesions observed on the respiratory tract were more severe compared to gastrointestinal tract and kidney. Animals that died within 24-36 hours of HS infection had petechial haemorrhages lesion on organs. Gross pathological lesion on the animals that lasted for 48 hours had more severe ecchymotic haemorrhage with appearance of fibrinous pericarditis. When the infection extended to 72 hours, the animals showed extensive lungs consolidation with thickening of interlobular septa. At this stage also, there was pleurisy and serofibrinous pericarditis with adhesions between pleural layer and pericardium. (De Alwis, 1984).

2.4.3 Identification and Isolation of *P. multocida*

Species identification is done using cultural and biochemical tests (De Alwis, 1992). This organism can grow mostly on most usual laboratory media for example nutrient agar, blood agar and casein-sucrose-yeast (CSY) agar together with 5% blood (bovine, sheep). Optimal temperature for the growth of *P. multocida* is between 35-37°C for 16-48 hours (ACIAR, 2017). The colonies appeared as grey, viscous but rough and irregular on blood agar. *P. multocida* do not growth on MacConkey agar but poorly grow on some cysteine lactose electrolytedeficient (CLED) agars.

Microscopic appearance of *P. multocida* using Wright's stain are spherical, ovoid, rod or coccobacilli-shaped Gram negative which may occur singly, pairs or short chains (SMI, 2015).

In biochemical tests, Pasteurella species give positive oxidase (except *P. bettyae)*, catalase positive (except *P. bettyae, P. caballi* and few strains of *A. hominis* which are catalase negative (SMI, 2015).

2.4.4 Molecular Identification and Differentiation of *P. multocida*

Detection and identification of organism using specific Polymerase Chain Reaction (PCR) assays in order to detect infectious agent in this case is *P. multocida* gives advantages includes less time-consuming, more sensitivity, specificity and also simplicity. In P. multocida-specific identification from mixed cultures or clinical samples, species-specific PCR assay was used (Hunt, 2000). According to Dviza *et al.* (2007), there are primers based on a clone of KMT1 which indicating specificity for type B only. Detection for type B:2 strains of *P. multocida* later was designed using 16S-23S rDNA universal primers thus become a diagnostic marker for HS detection in Asia.

2.5 Treatment, Prevention, and Control

In order to preserve the animal's health, it is suggestive to practice metaphylaxis treatment which is administration of antimicrobial to all animals that shows suggestive clinical signs of the disease during the early phase of physical examination (Ferran, 2011). However, treatment approach is usually considered as of little value as HS is a peracute to acute nature disease. Therefore, it is crucial for early detection in order for the treatment to be effective. One of the practical method in early detection is to check for increase in rectal temperature of all animals that starts to show clinical signs and also all in-contact animals. According to Jesse *et al.* (2013), acute phase protein (APPs) which are group of serum protein; Haptoglobin (Hp) and Serum Amyloid A (SAA) are sensitive biomarkers in detection of HS. Different in concentration of APPs during infection of disease can be related to the time course of the inflammatory response and tissue damage. SAA and Hp which known to be major APPs will increase to 1000 fold from its normal plasma concentration when there is infection (Eckersall *et al*., 2007).

In early detection animals, antibacterial therapy is the most effective. Choice of antibacterial, Sulphadimidine 33.3%, administered Intravenously (I.V). But, this route of administration is not practical in a large herd. Therefore, other choices of broad antibacterial that are more convenient in which can be administered I.M are also effective (De Alwis, 1992). Reducing stress and also increase sanitation during wet season can greatly reduce HS incidence as this disease is considered as stress-induced disease that can be due to the poor feed quality, climate change, transportation stress or closed-herd system (OIE, 2013). Other than that, vaccination is also crucial. There

are two types of common vaccines that usually be used; inactivated and live attenuated vaccine. In endemic area, inactivated vaccine is used as prophylactic to provide protection up to one year. In order to control the disease over sixth month old cattle and buffalo, live attenuated vaccine can be administered by intranasal aerosol (Benkirane, 2002). According to Derek (2016), a modified-live vaccine seems effective and recommended by Food and Agricultural Organization of the United Nations (FAO) in southeast Asia. However, modified-live vaccine derived from a virulent *P. multocida* B:2 isolate by deletion of *aroA gene* is effective when administered via intramuscular route (Zamri and Annas, 2016).

2.6 Species susceptibility towards *P. multocida*

2.6.1 Cattle and Buffalo

Both cattle and buffalo will show typical clinical lesion related to respiratory tract as well as septicaemia infection once infected with *P. multocida* through IV, IM, SC, and IP (De Alwis, 1984). Annas *et al.* (2014) described that, inoculation of this organism via SC in buffalo calves suggested involvement of respiratory, gastrointestinal tract and urinary tract towards the infection as the animals exhibited classical clinical signs of HS infection at which the respiratory tract showed to be most affected.

2.6.2 Goat and Sheep

Studies regarding pathological changes in the respiratory tract of goats that were experimentally infected by *P. multocida* through few different routes including intratracheal, subcutaneous or intranasal routes showed that, intratracheal and subcutaneous route of inoculation tend to result in clinico-pathological lesion similar to natural infection. However, intranasal route failed to show any respiratory tract infection or septicaemia (Zamri and Shafarin, 2007). Clinico-pathological lesions on lambs inoculated with *P. multocida* via intratracheal route revealed, there were respiratory tract lesions p.i which similar with HS signs observed in cattle and buffalo (Odugbo *et al.*, 2005).

2.6.3 Mice

Previous study related to the HS inoculation route in mice is usually through intraperitoneal and subcutaneously (Ramdani *et al.*, 1990; Al-Maaly *et al.*, 2014). According to this study, various pathological lesion especially respiratory tract infection showed in organs of infected mice that inoculated with 10^2, 10^4 CFU and septicaemia in 20^1 cfu. Oral inoculation of HS in mice was done and revealed similar signs as in intraperitoneal inoculation (Jesse *et al.*, 2013).

3.0 MATERIALS AND METHOD

3.1 Mice

A total of 30 clinically healthy mice were selected. All of the selected mice were four to five weeks old. These mice were obtained from Animal Resources Unit, Faculty of Veterinary Medicine, University Putra Malaysia (UPM) and were kept at the Animal Research Facility. Upon the arrival at the research facility, all the mice were acclimatised for five days. The mice were randomly and equally divided into three different plastic containers with 10 mice per container. All of the containers were bedded with wood shavings that was changed every two days. Throughout the acclimatisation and the experiment period, commercial pellet and clean water were provided *ad libitum*.

3.2 Preparation of *Pasteurella multocida* B:2 Inoculum

Wild-type *P. multocida* serotype B:2 which was obtained from stock culture which was isolated from previous outbreak of HS. 1 ml of *P. multocida* B:2 was added with 500 ml of brain-heart infusion(BHI) broth then shook at 150 rpm for 18 hours and incubated at 37°C for 24 hours. 1 ml of the seeded BHI than mixed with 9 ml of PBS. 1µl from previous solution was cultured onto blood agar and incubated at 37°C for 24 hours. After that, serial dilution of 1 ml of seeded BHI together with 2.5 ml of PBS was done to produce desired bacterial concentration with estimated infection dose of 1.0 X 10^9 colony forming unit (cfu) of *P. multocida* B:2/ml. The organism from

diluted solution in tube number sixth until tenth were cultured on blood agar, and prepared in duplicate then, incubated for 24 hours at 37°C.

3.3 Study Design

The mice were divided into two groups which were control (n=5) and treatment (n=25). Under treatment groups, the mice were further sub-divided into five groups with five mice each. All the mice in the treatment group were grouped together in one large plastic container. Five mice were randomly chosen for temperature checking before the inoculation. Then, the treatment groups were inoculated with 10 ml of *P. multocida* B:2 through aerosol, with concentration of 1 X 10^{9} cfu for 20 minutes in the nebulizer chamber. The chamber was covered with cloth to prevent the air escaped out and the aerosolised procedure was done in the safety-fumed cabinet level 2 with present of bunsen burner to maintain the septic area. After 20 minutes, all mice were divided into five treatment groups (Group 1, 2, 3, 4, 5).

Subsequently, the mice were observed for clinical signs and recorded throughout 24 hours. Clinical signs include level of alertness, ruffled hair, mice huddle together, respiratory signs such as ocular discharge and/or laboured breathing (Jesse *et al.*, 2013). The mice were euthanised by cervical dislocation at predetermined interval which were at -6 (control), 0 (Group 1), 6 (Group 2), 12 (Group 3), 18 (Group 4), 24 (Group 5) hours post-infection.

3.4 Sampling

Clinical signs such as level of alertness, ruffled hair, mice huddle together, respiratory signs such as ocular discharge and/or laboured breathing were observed (Jesse *et al.,* 2013). Respective group were euthanised by cervical dislocation at predetermined period then, necropsy was performed and lungs samples were aseptically collected and preserved in the 4 % Glutaraldehyde for 16-24 hours at 4°C for electron microscope examination (Periasamy *et al.*, 2010).

3.5 Ultrastructural Examination and Evaluation

The lungs samples were used for electron microscopic examination. Electron microscope sample processing were done according to the established routine protocol (Lovy and Wadowska, 2014). The lung samples were trimmed into 1 mm^3 then fixed in 4% glutaraldehyde followed by 0.1 M Sodium Cacodylate Buffer for 18-24 hours at 4°C. Routine process of the samples for TEM were done as described in Figure 1. Samples were then trimmed and undergo ultrathin sectioning using ultra-microtome at 1µm thick sections. Then, the samples were placed onto copper grids and stained with Uranyl acetate for 15 minutes and lead citrate. After that, all samples were examined and photographed under transmission electron microscope (Leo 912 AB EFTEM, Omega Filtering system, Germany). The ultrastructural changes following the cellular response were described and scored based on the ultrastructural scoring table in Table 1 (Annas *et al.*, 2014).

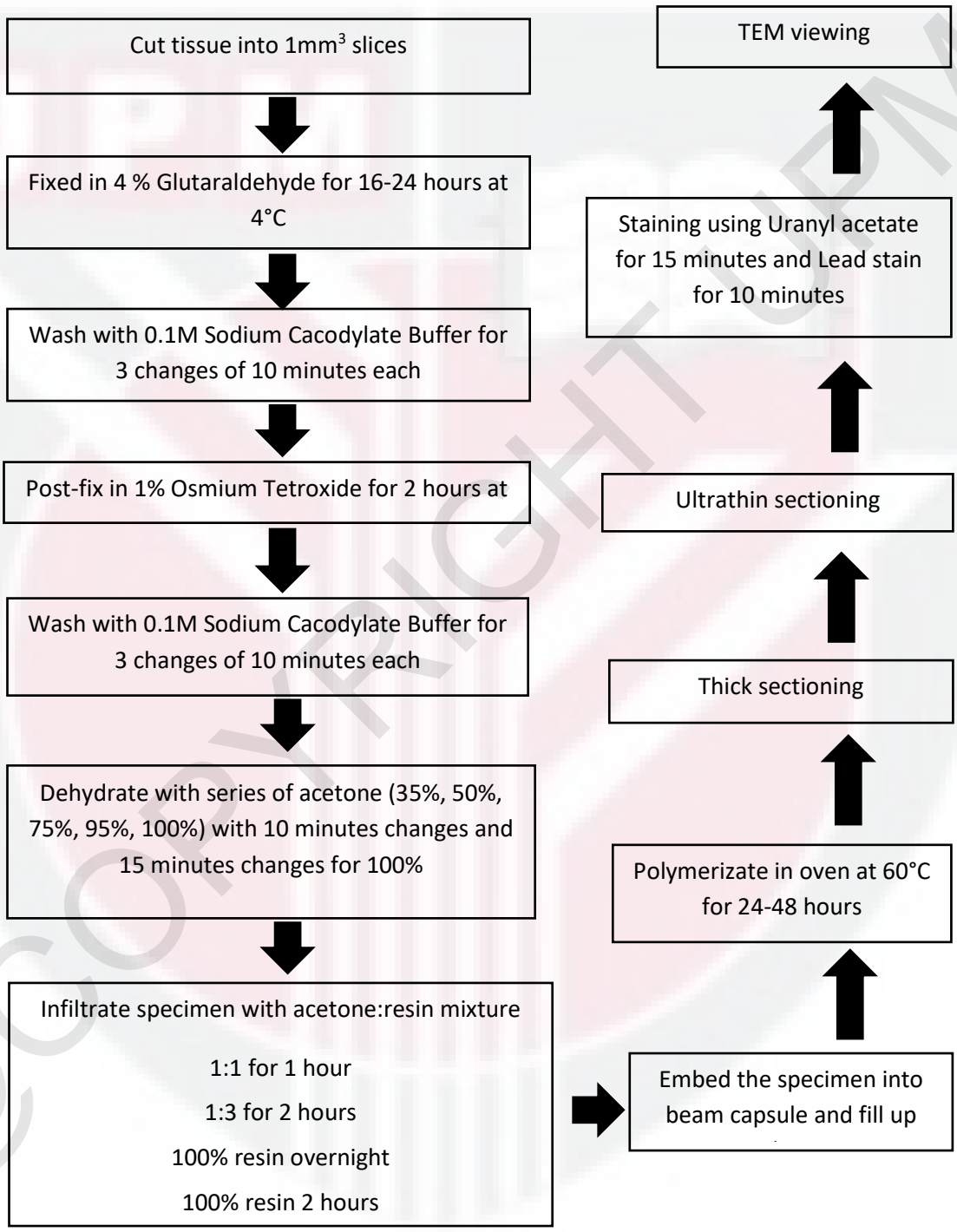
Table 1: Ultrastructural Scoring

Normal **Score 0**	**Mild** **Score 1**	**Moderate** **Score 3**	**Severe** **Score 4**
Normal organelles	Mitochondria swelling	Mitochondrial cristolysis	Mitochondrial vacuoles
	Cytocavitary network dilation	Cytocavitary network membrane fragmentation	Cytoplasmic vesiculation
	Nuclear envelope dilation	Nuclear envelope collapse & invagination	Nuclear envelope disintegration
	Eccentric nuclei	Nucleoplasm rarefaction	Nuclear chromatin peripheralisation

3.6 Statistical Analysis

All data obtained regarding severity of ultrastructural changes in samples were analysed using one-way ANOVA. The statistical analysis was analysed by IBM ® SPSS® Statistic Version 22.

Figure 1: Routine process of samples for TEM



4.0 RESULT AND ANALYSIS

4.1 Clinical Response

Clinical response towards *P. multocida* B:2 were observed in both control and treatment group. Following aerosol inoculation of *P. multocida* at 0 hour, all mice in treatment group 1 were euthanized. All mice in treatment group 1 and 2 showed no changes in behaviour. All appeared no depression, actively running and no huddling was observed. Upon euthanisation at 6 hours p.i, mice in treatment group 2 showed no changes in behaviour. At 12 hours p.i, all treatment group 3 mice appeared less active. However, no huddling, no ruffled hair coat, or no respiratory distress were observed. At 18 hours p.i, treatment group 4 mice become less active with no other abnormalities observed. All mice in treatment group 5 appeared less active and no other abnormalities were observed. Control group mice showed no clinical signs, and were euthanized on the last day.

4.2 Ultrastructural Lesions

There are many ultrastructural changes seen in endothelium and alveolar epithelium of lungs in treatment group mice. All these changes showed mild to severe changes which indicates acute cellular injury. Different group revealed different ultrastructural severity of the lungs tissue.

In this study, mice were inoculated with *P. multocida* B:2 resulted in pathological changes in ultrastructure of the lungs tissue. In the control group mice, normal ultrastructure of pneumocyte and endothelial cell were observed with intact

nuclear membrane as well as other organelles. Ultrastructure lesions of endothelial cell as well as pneumocyte observed indicating acute cell injury with appearance of mild to severe cytoplasmic vesiculation (Figure 2) and also nuclear disintegration. In early inoculation which at 0 and 6-hours group, mild lesions occurred mostly involving mitochondria. Nonetheless, starting from 12-hours group, the ultrastructural scores ranging from moderate to severe lesions which involved mitochondria, cytocavitary network, nuclear membrane and nuclei. Type I pneumocyte showed nuclear invagination, extensive nuclear disintegration with some of the nuclear content leaked out (Figure 3) and swollen of mitochondria at 24-hour group. There were also mild cytoplasmic vesiculation, and cytocavitary network membrane fragmentation observed in this group mice. Type II pneumocyte showed various stages of lesion ranging from mild to severe. At 12-hour group mice, there were peripheral nuclear condensation, swollen and some cristolysis of mitochondria (Figure 5) with intact cytonetwork observed. Other than that, for group 3 at 12 hours, the endothelial cell showed irregular nuclear envelope, nuclear invagination and some has peripheral nucleus condensation. Nonetheless, mild cytoplasmic vesiculation can also be observed at 18-hour and 6-hour group mice. The blood capillaries were congested with red blood cell and some has blood platelet aggregation inside the capillaries of 18-hour group mice (Figure 4). The mitochondria were swollen, with some had mitochondrial vacuoles (Figure 6) and cristolysis of cristae (Figure 5).

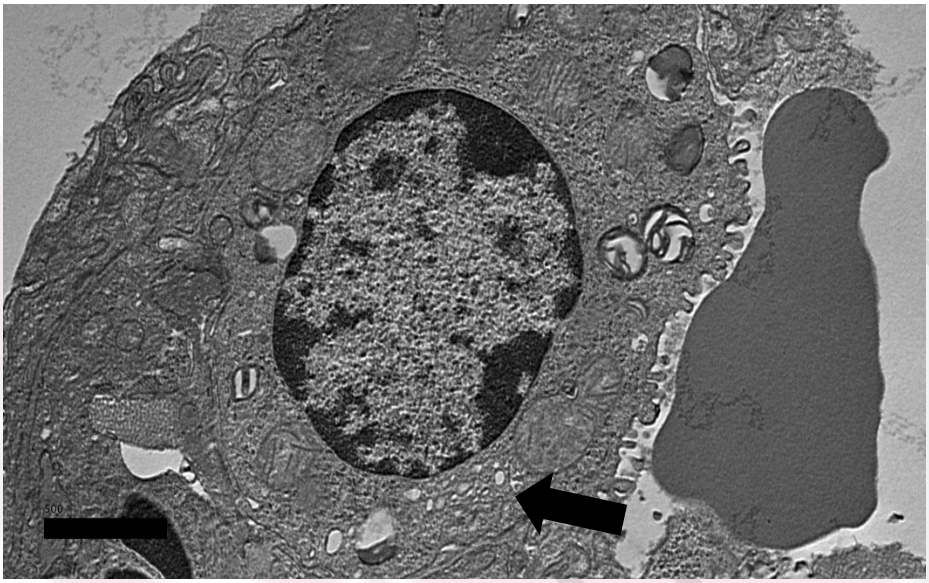


Figure 2: Cytoplasmic vesiculation of pneumocyte (long arrow) (bar=1µm, TEM).

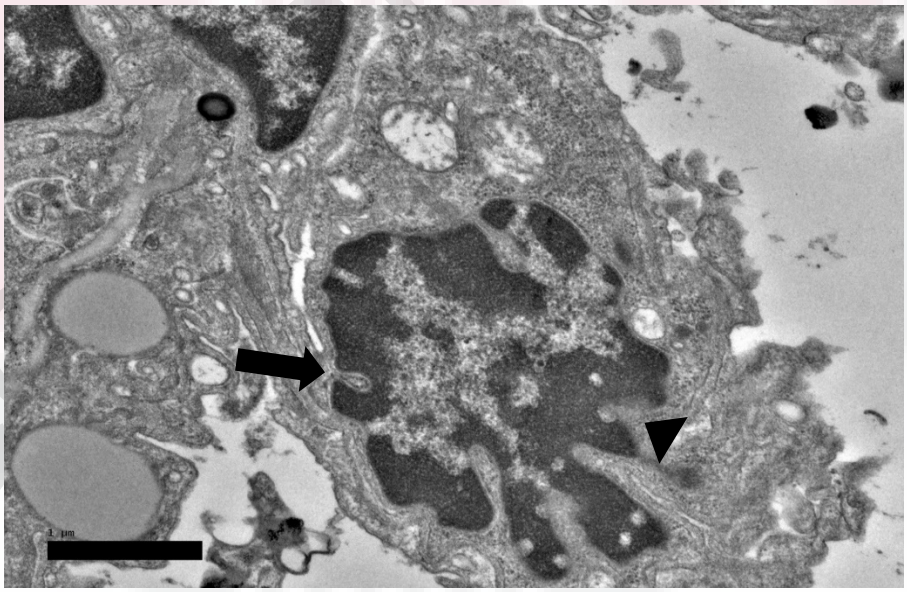


Figure 3: Nuclear disintegration (arrow head) with slight leakage of nuclear content and nuclear invagination of pneumocyte (long arrow) (bar=1µm, TEM).

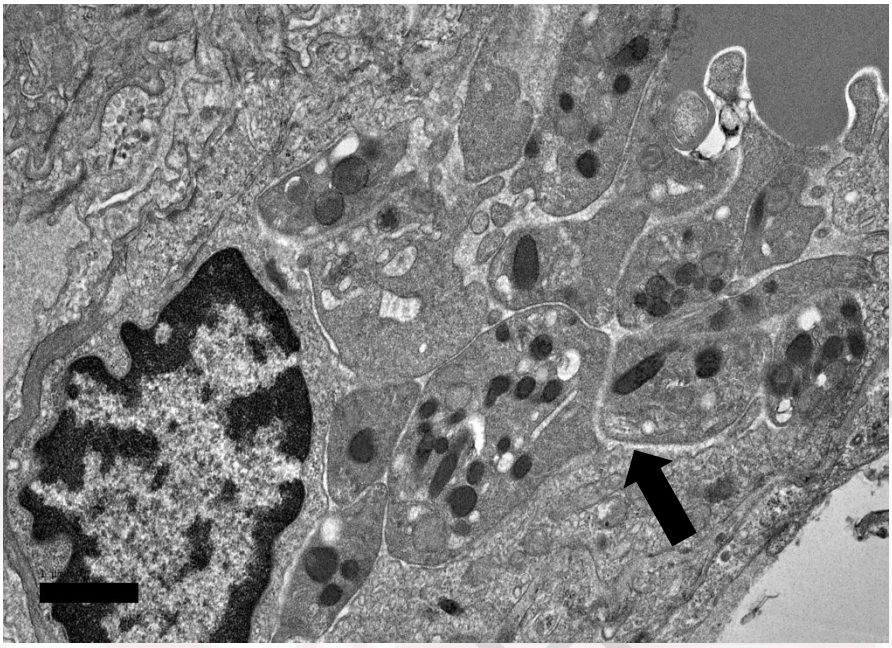


Figure 4: Aggregation of platelets in blood capillary (long arrow) (bar=1µm, TEM).

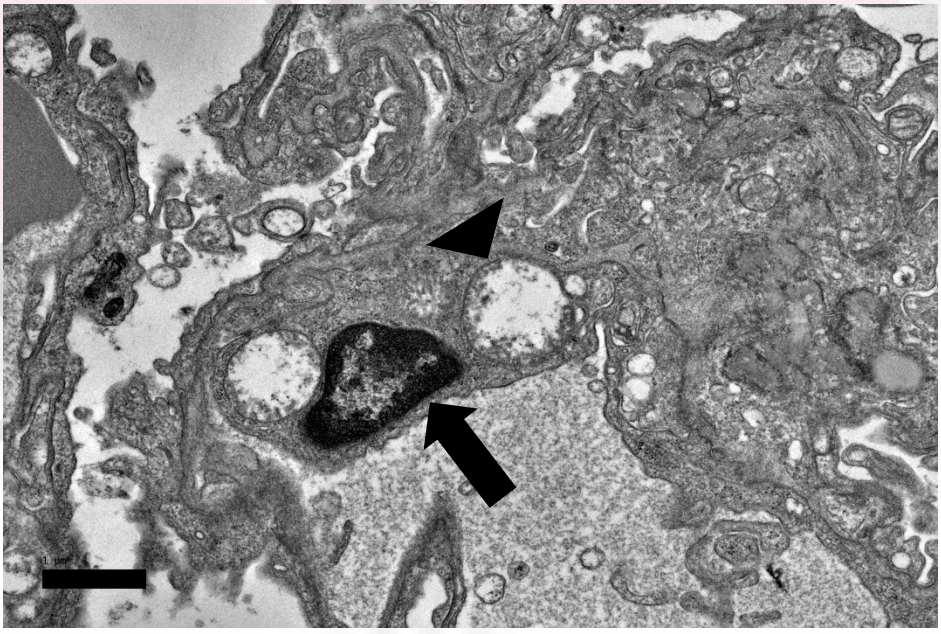


Figure 5: Nuclear of endothelial cell undergone nuclear condensation (long arrow). Swollen of mitochondria and cristolysis of cristae (arrow head) (bar=1µm, TEM).

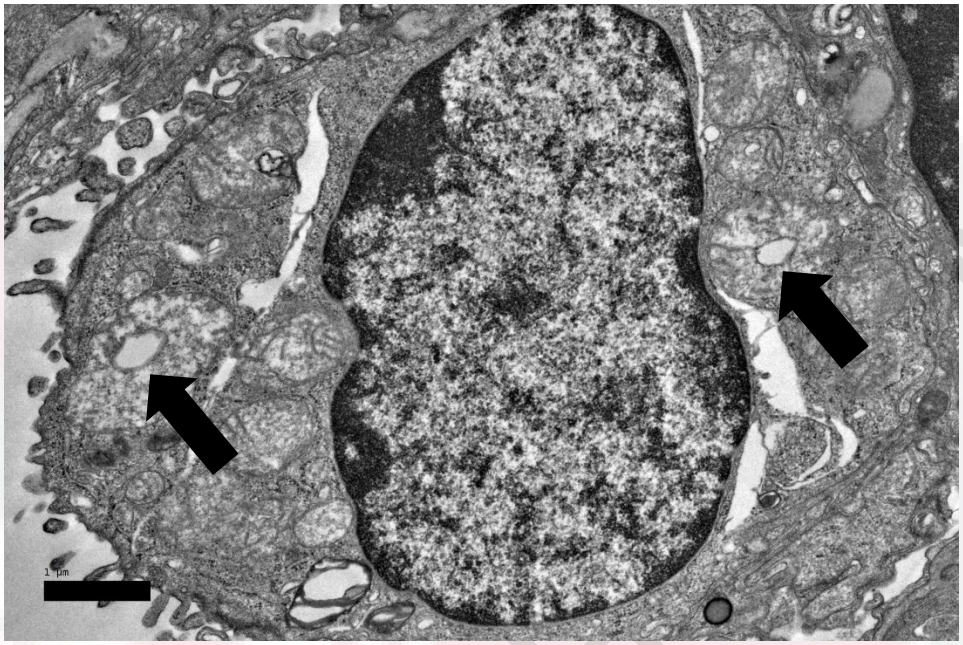
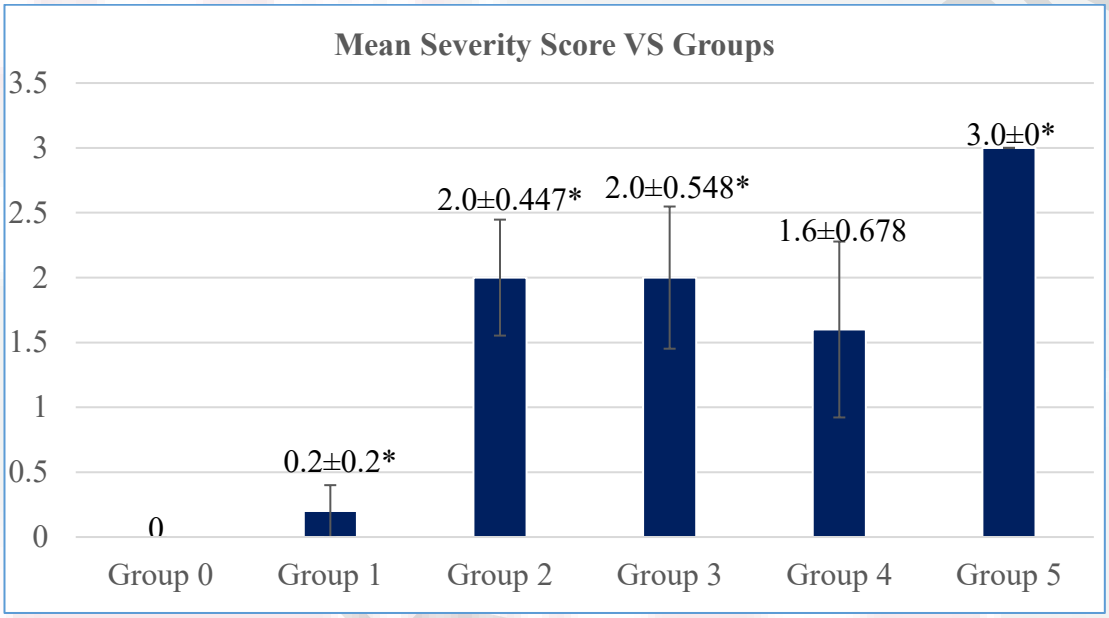


Figure 6: Mitochondrial vacuoles in the pneumocyte (long arrows) (bar=1µm, TEM).

4.3 Severity of Ultrastructural Lesions

Figure 7: Mean scoring graph of ultrastructural changes of lungs tissue sample of aerosolly-infected mice with *Pasteurella multocida* B:2.



***Values are significant at P< 0.05 between respective groups against control group**

Table 2: Mean scoring table of ultrastructural changes of lungs tissue sample of aerosolly-infected mice with *Pasteurella multocida* B:2.

Groups	Ultrastructural Severity Score (Mean±SD)
Control	0.00±0.00*
Group 1	0.20±0.447*
Group 2	2.00±1.00*
Group 3	2.00±1.225*
Group 4	1.60±1.517
Group 5	3.00±0.00*

Values are expressed as mean ± SD, *Values are significant at P< 0.05 between respective groups against control group

5.0 DISCUSSION

Nature of immune response toward *P. multocida* which caused fatal acute disease especially in buffalo and cattle is still poorly understood. The natural transmission route of *P. multocida* is via inhalation and ingestion (De Alwis, 1999). This study revealed that, inoculation of *P. multocida* B:2 via intranasal which mimics the natural transmission route of infection with concentration of 1 X 10^{9} cfu for 20 minutes did not showed any classical clinical signs. As stated in previous study, the classical clinical signs for HS including ruffled hair, recumbent, and respiratory distress can be observed in mice inoculated via other routes such as S.C, I.P, and oral as early as 12 hours p.i and mortality can be observed in 24 hours p.i (Ramdani *et al.,* 1990; Al-Maaly *et al.*, 2014; Jesse *et al*., 2013).

Despite absence of respiratory signs, the treatment group mice did show reduced in activity. Although, it is not possible to claim that the animals did not develop the disease later. Al-Maaly *et al.* (2014) stated that, the clinical signs usually occur starting from 6 hours p.i and result in mortality within 24-72-hour infection. This due to the fact that, mice are experimentally susceptible to this organism (Barthold *et al.*, 2016). Other than that, the infection also very rapid in vivo multiplication even with small amount of inoculum concentration (Al-Maaly *et al.,* 2014). However, as stated by Gardenhire *et al.* (2013), aerosol-device such as nebulizer needs 12 times more drugs or inhalant to be well deposited in the lungs. This is because, the dose can be loss in the device, exhaled breath, and via oropharynx. Therefore, from this study, the concentration of the inoculum may not be enough to develop classical clinical signs of HS in the mice within 24 hours. Nonetheless,

absence of classical clinical signs and death in this study could also be related to the protracted time frame.

These are important preliminary findings as it revealed that inoculation of *P. multocida* B:2 via aerogenous route produced the same pathological changes in the lungs which confirmed the development of the disease. As expected, the exposure towards this organism via aerogenous route of inoculation was able to produce the development of the disease in the respiratory organ and provide wide range of lungs lesions ranging from mild to severe ultrastructural lesions. The ultrastructural lesions seen from this study were similar as previous study done in rabbit which inoculated via intranasal (Al-Haddawi *et al.*, 2000). Not only that, the same lesions were also appreciated from the study done in calves through S.C route of inoculation which revealed acute cellular injury causing damages to the pneumocyte and endothelial cell resulted from the infection (Annas *et al.*, 2014). In this study, there were present of aggregation of blood platelet in the blood capillary. As stated by Löffler *et al.* (2017) and Garroud *et al.* (2015), evidence of aggregated platelet indicates inflammatory response triggered by an infectious organism in the blood capillaries at which in this study might suggestive due to *P. multocida* B:2.

6.0 CONCLUSION

This study proved that *Pasteurella multocida* Type B:2 has the ability to produce classical pathological changes in ultrastructure of lungs tissue of mice as observed in cattle and buffalo that infected with HS with respect to intranasal route of inoculation. However, no classical clinical signs can be observed within 24 hours p.i that might be due to not enough inoculum concentration used in aerosolised infection. Thus, it is suggestive that aerogenous inoculation of this organism can positively establish the HS infection in mice model.

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