UNIVERSITI PUTRA MALAYSIA

IMMUNISATION EFFECT IN HOST FOLLOWING ORAL EXPOSURE TO GRADED DOSES OF IMMUNOGENIC LIPOPOLYSACCHARIDE EXTRACTED FROM *PASTEURELLA MULTOCIDA* TYPE B:2

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A project paper submitted to the Faculty of Veterinary Medicine, Universiti Putra Malaysia

In partial fulfilment of the requirement for the

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It is hereby certified that we have read this project paper entitled "Immunisation effect in host following oral exposure to graded doses of immunogenic lipopolysaccharide extracted from *Pasteurella multocida* type B:2", by Sarah Helmy and in our opinion it is satisfactory in terms of scope, quality and presentation as partial fulfilment of the requirement for the course VPD 4999-Project.

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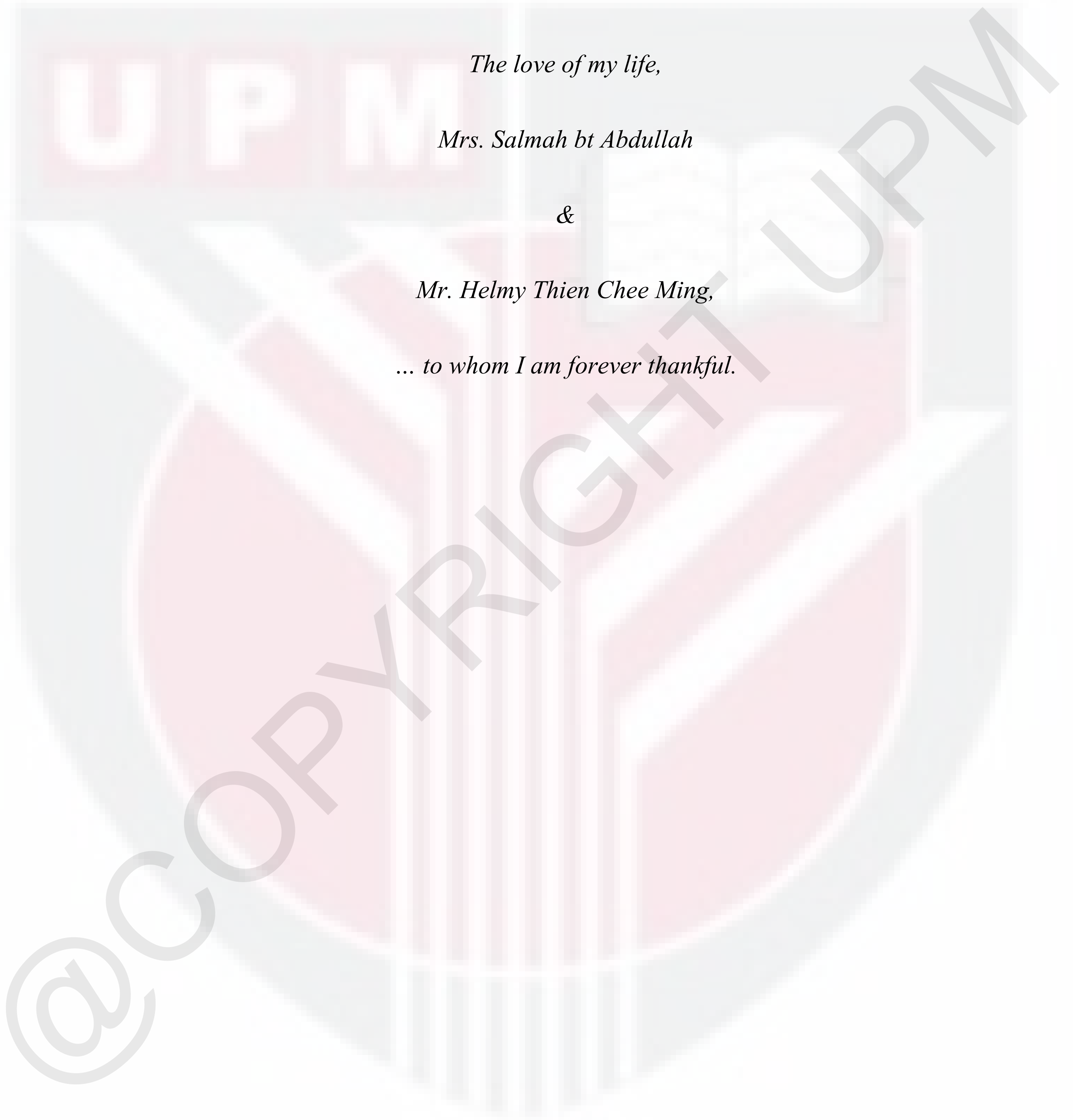
DEDICATION



The love of my life,

Mrs. Salmah bt Abdullah

&

Mr. Helmy Thien Chee Ming,

... to whom I am forever thankful.

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ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999 –Projek.

KESAN IMUNISASI TERHADAP HAIWAN BERIKUTAN INOKULASI MELALUI ORAL DENGAN LIPOPOLISAKARIDA DARIPADA PASTUERELLA MULTOCIDA JENIS B:2 YANG BERBEZA DOS

Oleh

Sarah Helmy

2016

Penyelia: Dr. Faez Firdaus Jesse Abdullah

Di Malaysia, penyakit hawar darah atau Haemorrhagic Septicaemia (HS), iaitu sejenis penyakit yang mempunyai kadar kematian yang tinggi di kalangan kerbau dan lembu, adalah disebabkan oleh bakteria *Pasteurella multocida* jenis B:2. Lipopolisakarida (LPS) merupakan salah satu faktor penting untuk *P. multocida* menghasilkan tindak balas imunisasi. Oleh itu, eskperimen ini dijalankan bagi mempelajari kesan imunisasi terhadap mencit yang diberi lipopolisakarida daripada *P. multocida* B:2 melalui mulut. Sebanyak 25 ekor mencit dibahagikan ke dalam lima kumpulan, dimana setiap kumpulan mempunyai lima ekor mencit. Kumpulan terkawal telah diberi 0.2 ml Phosphate Buffer Saline (PBS) pH 6.8 melalui oral, manakala Kumpulan 1, Kumpulan 2, Kumpulan 3 dan Kumpulan 4 masing-masing diberi 0.2ml lipopolisakarida daripada 10^3, 10^5, 10^7 dan 10^9 cfu *Pasteurella multocida* jenis B:2, melalui oral. Haiwan-haiwan tersebut diperhatikan setiap hari selama 14

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hari, untuk tanda-tanda penyakit yang penting. Selepas tujuh belas hari, haiwan-haiwan tersebut diberikan sebanyak 0.2 ml bakteria *Pasteurella multocida* yang mempunyai konsentrasi sebanyak 10^7 cfu. Sekali lagi, haiwan-haiwan tersebut diperhatikan untuk tanda-tanda penyakit penting. Selepas tujuh hari, mencit yang masih hidup, dimatikan dan organ di simpan untuk pemeriksaan histopathologi dan untuk mengasingkan dan mengesan kehadiran bakteria. 60% daripada haiwan daripada semua kumpulan mengalami cirit-birit sebelum mati, 38.5% daripadanya pula mempunyai luluhan okular dan 100% daripadanya mengalami kesukaran untuk bernafas. Terdapat sedikit sehingga sederhana lesi histopathologi di dalam kesemua organ yang dikaji yang merupakan jantung, paru-paru, hati, limpa, buah pinggang, usus besar, usus kecil dan perut di dalam kesemua kumpulan. Secara ketara ($P<0.05$), Kumpulan 1 mempunyai sedikit sel kerandangan di dalam limpa (1.4) dan usus kecil (0.7), manakala Kumpulan 4 mempunyai sedikit nekrosis di dalam buah pinggang (1.1) dan nekrosis sederhana di dalam perut (2.2). *P. multocida* telah dijumpai dari jantung, paru-paru, hati, limpa, buah pinggang, usus dan perut daripada kesemua kumpulan. Kadar kematian haiwan menujukkan tiada perbezaan ketara ($P>0.05$) diantara kumpulan terkawal (40% kematian), kumpulan konsentrasi rendah (Kumpulan 1 dan Kumpulan 2) (33.33% kematian) dan kumpulan konsentrasi tinggi (66.67%). Hal ini menunjukkan lipopolisakarida daripada *P. multocida* B:2, samada konsentrasi rendah atau konsentrasis tinggi gambar untuk memberi imunisasi untuk melindungi mencit daripada penyakit HS.

Kata kunci: Hawar darah, *Pasteurella multocida* jenis B, mencit, lipopolisakarida, lesi histopatologi

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ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfilment of the course VPD 4999 – Project.

IMMUNISATION EFFECT IN HOST FOLLOWING ORAL EXPOSURE OF GRADED DOSES OF IMMUNOGENIC LIPOPOLYSACCHARIDE EXTRACTED FROM PASTEURELLA MULTOCIDA TYPE B:2.

by

Sarah Helmy

2016

Supervisor: Dr. Faez Firdaus Jesse Abdullah

In Malaysia, *Pasteurella multocida* serotype B:2 is the causative agent of haemorrhagic septicaemia (HS), a highly fatal disease in buffalo and cattle. Lipopolysaccharide (LPS) is one of the major immunogen of *P. multocida.* This study aims to determine the protective effect due to oral inoculation of LPS extracted from *P. multocida* B:2 against HS disease. Twenty-five healthy mice were divided into five groups consisting of five animals each. The control group was inoculated orally with 0.2ml of Phosphate Buffer Saline (PBS) pH 6.8, whereas Group 1, Group 2, Group 3 and Group 4 were inoculated orally with 0.2ml lipopolysaccharides extracted from 10^3, 10^5, 10^7 and 10^9cfu of *Pasteurella multocida* serotypes B:2, respectively. The experimental animals were observed for clinical signs for seventeen days. All the groups were subjected for challenge with 0.2 mL of 10^7 wild type *Pasteurella multocida* B:2 after 17 days post LPS inoculation. Then, the groups were observed for

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clinical signs for seven days. After seven days, surviving mice were euthanised and the organs were collected for histopathological examination and bacterial isolation and identification. Before death, 60% of the animals from all group had diarrhoea, 38.5% had severe ocular discharge and 100% had laboured breathing. Mild to moderate histopathological lesions were observed in heart, lungs, liver, spleen, kidney, small intestine, large intestine, small intestine and stomach of all groups. Significantly ($P<0.05$), Group 1 had mild presence of inflammatory cells in spleen (1.4) and small intestine (0.7), whereas Group 4 had mild to moderate degeneration and necrosis of kidney (1.1) and stomach (2.2). *P. multocida* was isolated from heart, lung, liver, spleen, kidney and gastrointestinal tract of all groups. Rate of animal mortality showed no significant different ($P>0.05$) between control group (40% mortality), low concentration group (Group 1 and Group 2) (33.33% mortality) and high concentration group (Group 3 and Group 4) (66.67% mortality). This showed that oral inoculation of LPS extracted from *P. multocida*, both in low and high concentration, in mice, failed to give immunity to the host.

Keywords: Haemorrhagic septicaemia, *Pasteurella multocida* serotypes B:2, lipopolysaccharides, mice, histopathological lesions

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1.0 INTRODUCTION

World Organisation for Animal Health (OIE) defined Classical Haemorrhagic Septicaemia (HS) as a disease caused by *Pasteurella multocida* serotypes B:2 or E:2 (Mosier, 2014). HS is an acute, febrile and lethal disease, causing death in susceptible animals in less than 36 to 48 hours of exposure to the organism (Jamal, 2013). It is seen mainly in cattle and water buffalo with progressive clinical signs from dullness and fever to death within hours, and is transmitted via ingestion or inhalation of the organism (Spickler & Roth, 2009). HS causes high economic losses due to animal mortality.

Oil-Adjuvanted Vaccine (OAV), applied parenterally, is the main prophylactic agent used in Malaysia against HS infection (Chung *et al.,* 2015). However, only 17% of Malaysian buffaloes were vaccinated due to difficulty of vaccine administration (Zamri-saad, 2013). Animal needs to be individually restrained to apply parenteral vaccination, making vaccine application difficult. Studies by Abu Bakar *et al.* (2011) had shown that oral route inoculation of whole bacteria of *P. multocida* in buffaloes causes less severe clinical signs, compared to other routes, indicating that oral route might be a readily available route for effective vaccination administration (Chung, et al., 2015).

In addition, lipopolysaccharide of *Pasteurella multocida* type B was found to be protective against experimentally induced pasteurellosis in mice (Muniandy *et al.*, 1998). They are also good immunogens and can be used for subunit vaccine development (Sarangi, *et al.,* 2014). However, there is still a lack of understanding in the tissue changes of immune system organs with *P. multocida* infections and hence

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limited knowledge regarding the protective effect conferred by oral route inoculation of lipopolysaccharide of *Pasteurella multocida* type B:2 against HS disease.

Therefore, the aim of this study is to determine the protective effect on host upon oral inoculation with graded doses of immunogenic L extracted from *Pasteurella multocida* type B:2 against HS disease. The results and knowledge obtained from this study may serve as a future reference in improving the preventive measures and treatment of HS disease, and the development of oral subunit vaccine against HS applied in animal feed. Such application will be of more convenient to the farmers, and may greatly improve prophylactic measures and immunisation against HS in Malaysia.

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2.0 LITERATURE REVIEW

2.1 Haemorrhagic septicaemia (HS)

Haemorrhagic Septicaemia (HS) is a disease caused by *Pasteurella multocida* serotypes B:2 or E:2. Serotype B:2 have been identified in most endemic area, whereas E:2 is only found in Africa (Mosier, 2014). The most common hosts of HS are cattle and buffalo but some other animals such as goats, pigs, deer, sheep and camels are also susceptible to it (Ashraf *et al*., 2014). Farm with poor husbandry practices and practicing free-range system are more susceptible to HS (De Alwis, 1999). All age groups are affected by HS but in endemic areas, clinical cases tend to occur in young animals of 6 months to 2 years old (Spickler & Roth, 2009).

2.2 Importance of HS

The first case of HS was reported in Malaysia and dated as early as 1880s, and it remains endemic with HS until today. HS is also endemic in Bhutan, China, India, Indonesia, Mongolia, Myanmar, Phillipines, Sri Lanka and countries in Africa and America (Benkirane & De Alwis, 2002).

In South-East Asian region which include Malaysia, ranked HS as one of the economically important diseases of cattle and buffaloes (Benkirane & De Alwis, 2002). During 1980 to 1989, the losses due to HS in Malaysia were about 2.25 million ringgits (De Alwis, 1999). HS causes high economic losses to farmers via deterioration of productivity, impairment of reproductive potential of the animals, mortality and disease diagnosis (Benkirane & De Alwis, 2002). It is of a great economic importance for countries in Asia where the susceptible animals, such as cattle and buffaloes, are

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reared abundantly as source of food (meat and milk), income and draught power (De Alwis, 1999).

2.3 Aetiological agent of HS

Pasteurella multocida is a capsulated Gram-negative cocco-bacillus, and classified into 16 serovars based on lipopolysaccharide (LPS) antigens (Harper *et al.,* 2011). *P. multocida* is capable of causing different diseases in wide range of hosts, but only two serotypes, namely serotype B:2 and E:2 could cause classical HS (Spickler & Roth, 2009). It is also associated with atrophic rhinitis in Swine, fowl cholera in poultry (Ashraf *et al*., 2014), enzootic bronchopneumonia in cattle, sheep and goats and snuffles in rabbits (Sarangi, *et al.,* 2014).

2.4 Lipopolysaccharides

The outer membrane of gram-negative bacteria constitute mainly of lipopolysaccharides (LPS) which serves as a physical barrier to protect the bacteria against antibacterial agents (Rosenfeld & Shai, 2006). LPS generally functions in inducing a strong immune response in normal mammalian cells (Riesthel, 1994). In addition, LPS is an essential virulent factor and a major immunogen of *P. multocida* (Harper *et al.,* 2011).

2.5 Transmission route of HS

P. multocida is transmitted to susceptible animals via inhalation or ingestion, but survive poorly outside of the animal body (De Alwis, 1999). It can survives for hours and possibly days in damp soil or water, making these common transmission medium. Incubation period of serotypes that cause HS is usually three to five days, and it is believed to be shed into the oropharynx, and maintained in the lymphatic tissues associated with upper respiratory tract, which persist in crypt of tonsils in

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carrier animals (De Alwis, 1999), and shed periodically via nasal secretions (Spickler & Roth, 2009).

2.6 Diagnostic techniques of HS

The clinical, provisional diagnosis of HS is based on history (relevant epidemiological study of the farm or region), clinical signs and gross pathological lesions. A rapid diagnosis is important to control disease spread immediately (Benkirane & De Alwis, 2002). The differential diagnosis for HS includes lightning strikes, blackleg (*Clostridium cauveoi* infection), rinderpest, anthrax, acute salmonellosis and pneumonic pasteurellosis (Spickler & Roth, 2009).

2.6.1 Clinical signs of HS

In most cases, the disease state in cattle and water buffalos are acute or peracute. Buffaloes tend to have more severe clinical signs and shorter course of disease (Spickler & Roth, 2009). As the disease progress rapidly, clinical signs usually failed to be noticed, however it includes hyperthermia, inappetance, serous nasal discharges, hypersalivation, laboured breathing and swollen submandibular regions and death within hours (De Alwis, 1999).

2.6.2 Gross pathological changes of HS

Post mortem lesions include widespread haemorrhages as petechiae on many organs, widespread in thoracic cavity, and occasionally ecchymotic, especially on the heart, abomasal wall and mesentery. Moreover, oedema consisting of gelatinous mass with yellow or straw-coloured or bloodstained fluid at the subcutaneous of submandibular region and neck, musculature and organs such as lungs and pericardium) and hyperemia of the gastrointestinal tract may present (Spickler & Roth,

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2009). In addition, congested lungs with varying degrees of consolidation and marked thickening of interlobular septa and fibrinous pericarditis may present (De Alwis, 1999) with foam formation in the nasal cavity, trachea and bronchi (OIE, 2012).

2.6.3 Histopathological changes of HS

Histopathological changes of the lungs demonstrate thickening of pleura, interlobular septa and congested alveolar capillaries indicating generalised interstitial pneumonia. In addition, there are presence of hyperaemia, pulmonary oedema and focal infiltration of neutrophils and macrophages in many tissues which causes thickened alveolar septa and congested alveolar capillaries (OIE, 2012). The lymph nodes turn hyperaemic. The spleen and gastrointestinal tract have subserous haemorrhage and hyperaemia. In addition, the liver turns hyperaemic with cloudy swelling and fatty degeneration. The tubular epithelial cells of the kidneys demonstrate signs of swelling and pyknosis, whereas the heart indicates marked hyperaemia with subepicardial and subendocardial haemorrhages (De Alwis, 1999).

2.6.4 Identification and isolation of HS causative agent

Fresh blood samples from animal in terminal stage or swab collected from heart taken immediately after death or bone marrow from long bone (if animal died for a long time, where the bone's surface need to be prior freed of tissues and sterilised) can be used for bacterial culture and isolation. Nasal secretions however, are unreliable sample to demonstrate the presence of *P. multocida* B:2 or E:2 (OIE, 2012).

P. multocida forms smooth, non-haemolytic, greyish glistening translucent colonies of approximately 1 mm in diameter on blood agar after 24 hours incubation at 37°C. A microscopic examination of gram-stained blood smears will demonstrate

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Gram-negative, short or ovoid, bipolar-staining (more apparent with methylene blue or Leishman's stain) coccobacilli with a degree of pleomorphism (Spickler & Roth, 2009). Biological screening can be done by subcutaneous inoculation of mouse with about 0.1-0.2 ml of saline suspension of blood or bone marrow sample of suspected animal, and results in death within 24 hours with presence of HS (De Alwis, 1999).

Identification of the causative agent can be done via biochemical and serological tests (Spickler & Roth, 2009). Pasteurellae is oxidase and catalase positive, produce indole but not urease, utilise citrate, reduce nitrates, slow acid reaction with no gas in triple sugar iron (TSI) agar and able to grow on McConkey agar medium (De Alwis, 1999). HS causative agent, *P. multocida* serotypes B:2 and type E are hyaluronidase positive, compared to other *P. multocida* (OIE, 2012).

2.6.5 Serotyping of causative agent

There are two systems, namely 'capsular' typing and 'somatic' typing; where both systems are used in designation of serotypes of HS. Serological and molecular methods can be used for serotyping (OIE, 2012).

2.6.5.1 Serological tests

For serological tests, rapid slide agglutination for capsular typing (positive result will show floccular agglutination), indirect hemagglutination (IHA) test for capsular typing (positive result is shown by course agglutination of RBC), agglutination test for somatic typing (fine granular agglutination indicates a specific somatic agglutination), agar gel immunodifussion (AGID) for both capsular and somatic typing (all HS serotypes will react with type 2 antiserum) and counter-immunoelectrophoresis for rapid identification of the capsular types B and E (presence

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of distinct line between and antigen and antiserum wells considered as positive result) are used (Spickler & Roth, 2009; OIE, 2012).

In addition, IHA can be used for typing of unknown strains, and is efficient and reliable as quantitative test against HS strains. Other than that, IHA can used to detect antibodies against HS where high titers indicate recent exposure to HS (OIE, 2012).

2.6.5 Molecular methods

Polymerase chain reaction (PCR) can be used for rapid, sensitive and specific detection of *P. multocida* including strain differentiation within serotypes (De Alwis, 1999). Typing of some strains is done by *Pasteurella multocida* multiplex capsular PCR typing system, where conflicting results of some strains typing could be confirmed. Moreover, presumptive identification of HS strains can be done rapidly (within 3-4 hours) by PCR amplification (HS-causing type-B-specific PCR assay), where type B cultures with predominant somatic antigen of type 2 or 5 can be identified (OIE, 2012).

2.7 Treatment, control and prevention of HS

Only with prompt (early disease detection) and suitable treatment, that the animal may be fully recovered from clinical disease of HS (De Alwis, 1999). Effective treatment can be done by administration of antibiotics such as streptomycin or oxytetracycline via intramuscular route at a high dose in animals that has elevated rectal temperature (Benkirane & De Alwis, 2002). Infected animals may be treated with corticosteroid (such as dexamethasone), to avoid carrier animals from shedding (De Alwis, 1999).

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When there is an outbreak in vaccinated animals, the recommended protocol is to do whole herd vaccination by a dose of Alum-Precipitated Vaccine (APV) and followed by a dose of Oil-Adjuvanted Vaccine (OAV) (OIE, 2012). Other than that, rectal temperature of all animals in the herd are taken. Animals showing clinical signs are isolated and treated with parenteral broad-spectrum antibiotic. Moreover, animal movement in and out of the farm is restricted and diagnosis of disease is carried out rapidly when dead animal is discovered. Carcass are disposed in a proper manner (De Alwis, 1999).

In endemic regions, measures include routine vaccination (preferably two to three months prior to high-risk season, such a monsoon season), awareness of the disease among farmers, establishment of good reporting system and segregating animals to avoid contact of susceptible animals with carriers need to be carried out (Benkirane & De Alwis, 2002).

Prevention of HS can be done by strict exporting procedures such as ensuring animal originate from free-HS region, IHA testing of animals randomly prior to exportation, quarantine the animals before transportation and after arrival and vaccination of the animals (Benkirane & De Alwis, 2002).

2.7.1 Vaccination against HS

According to OIE (2012), effective vaccines against HS include formalin-killed bacterin or dense bacterins with adjuvants. Three common vaccine types used against HS include bacterins, APV and OAV, with OAV being the main prophylactic agent used in Malaysia (Chung *et al.,* 2015). A single dose of OAV to 4-6 months old calves will give immunity for 6-9 months (OIE, 2012).

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The vaccines available need to be applied via parenteral route, whereby animal need to be restrained individually, for vaccine application to be possible. This could account to occurrence of only 17% of buffaloes in Malaysia are vaccinated against HS (Zamri-saad, 2013). Other than that, although vaccination practice in endemic area has reduced disease incidence, the immunity duration is short and significant outbreaks still occur (Boyce & Adler, 2001).

2.8 Previous studies

2.8.1 Study in mice

Previous studies by Jesse et al., (2013) demonstrated that inoculation of *Pasteurella multocida* in mice via oral route causes less disease compared to intraperitoneal and intramuscular inoculation (Chung *et al.,* 2015). This show the possibility of developing vaccine that could be apply via oral route.

2.8.2 Study in other animals

A study in rabbit done by Ashraf *et al.,* (2014), demonstrated that an increased in dose of LPS antigen extracted from *Pasteurella multocida,* stimulate more antibody titre against HS. In another study carried out in calves by Jesse *et al.,* (2013), had revealed that calves inoculated with LPS of *P. multocida* B:2, showed similar clinical signs, post mortem lesions (moderate pulmonary oedema and severe haemorrhage in lung) and histopathological lesions (degeneration and necrosis of cells in the lung) but less severe (such as, no thrombus formation in lung) than those inoculated with whole bacteria of *P. multocida* B:2. Hence, these studies showed that there is possibility of developing vaccine using LPS extracted from *P. multocida* B:2 to provide protection against HS.

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3.0 MATERIALS AND METHOD

3.1 Mice

In the current study, 25 clinically healthy, non-pregnant ICR white mice, of three to four weeks old and mixed sex were used. Prior to experimentation, the mice were observed and acclimatised for seven days in the laboratory. The mice were placed into five plastic cages with each cage contained five mice. Wood shavings are placed as beddings. In addition, they were provided with water and pellets ad libitum.

3.2 Inoculums

Two types of inoculum were used which include wild type *Pasteurella multocida* type B:2 and its lipopolysaccharides. The *P. multocida* was obtained from stock culture from previous outbreak of HS in Kelantan, Malaysia; it was isolated and confirmed to be type B:2 by the Veterinary Research Institute (VRI) Ipoh, Perak. It was cultured in 5% horse blood agar and incubated at 37° C for 24 hours. Then, the bacteria was collected and diluted with distilled water and compared with McFarland Nephelometer Barium Sulphate standards (that were prepared a day prior) to produce 10^3, 10^5, 10^7 and 10^9 cfu of *P. multocida*.

Then, lipopolysaccharides were extracted from each bacterial concentration (10^3, 10^5, 10^7 and 10^9 cfu) using iNtRON Biotechnology, Inc. LPS (lipopolysaccharide) extraction kit. 5ml of bacteria was placed into each tube (20 tubes for each concentration) and centrifuge at 13,000rpm for 3-5 minutes at room temperature. After supernatant were removed, 1 ml of Lysis Buffer was added and vortex vigorously until all cell clump disappeared to lyse bacterial cell. Next, 200 µl of chloroform added to isolate RNA and genomic DNA or protein, and vortex

vigorously for 10-20 seconds, later incubated at room temperature for 5 minutes. After that, tubes are centrifuge at 13,000rpm for 10 minutes at 4ºC, and next 400 µl supernatants were transferred into a new 1.5 ml tube. Then, 800 µl of Purification Buffer were added, mixed well and incubated for 10 minutes at -20ºC, to purify LPS from other extract of cells. LPS pellet are then obtained by centrifugation at 13,000rpm for 15 minutes at 4ºC. 1 ml of 70% EtOH was added, centrifuged for 3 minutes at 13,000rpm at 4ºC. The upper layer were discarded and the remaining LPS pellet were dried. To remove impurities, 50 µl of PBS buffer was added, vortex and boiled for 2 minutes. Finally, LPS solution was obtained from 4 tubes (which each tube contained 50 µl) to obtain 0.2 ml LPS solution.

3.3 Study designs

The twenty-five mice were separated into five groups consisting of five mice each. The first group, which serves as the control group were inoculated orally with 0.2 ml of phosphate buffer solution (PBS) pH 6.8. Whereas, the other four groups which serve as treatment groups were inoculated orally with different concentration of lipopolysaccharides extracted from *Pasteurella multocida* type B:2. The second group (Group 1), was inoculated with 0.2 ml lipopolysaccharide with the concentration of 10^3 colony forming unit (cfu) of *Pasteurella multocida*. Whereas the third (Group 2), fourth (Group 3) and fifth (Group 4) group were inoculated with 0.2 ml lipopolysaccharides with the concentration of 10^5, 10^7 and 10^9 cfu of *Pasteurella multocida,* respectively. Group 1 and Group 2 are further classified as low concentration group, whereas Group 3 and Group 4 classified as high concentration group. The animals were observed for any significant changes in clinical signs, daily, for seventeen days.

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After seventeenth day post-infection, all the animals were inoculated orally with 0.2 mL of 10^7 wild type *Pasteurella multocida*. The animals were observed for any significant changes in clinical signs which include ruffled hair coat, alertness, ocular discharges, inappetance and mortality, for seven days. The observation was done hourly for the first 48 hours. After seven days, surviving mice were euthanised by cervical dislocation.

Post mortem was done on the animals immediately after death. Visceral organs were collected and processed for histopathological examination and bacterial isolation and identification. Histopathological scoring were done on the samples. Organ smears was used to isolate and identify bacteria using bacterial culture and microscopic examination. All the data were analysed with SPSS with data considered as significant at $P<0.05$.

3.4 Clinical signs scoring

There were six parameters accessed for clinical signs, which include food and water intake, ruffled hair coat, diarrhoea, responsiveness, ocular discharges and mortality. For food and water intake and responsiveness, animal is scored '0' for presence, and '1' for absence of each parameters. Whereas for diarrhoea and mortality, animal is scored '0 for absence, and '1' for presence of each parameters. For ocular discharges the animal is scored from 0-8 with '0' as absence of discharges, and one score for each region where the discharge is presence, which include either unilateral or bilateral, involvement of medial and lateral canthus, and involvement of upper and lower eyelids. Lastly, for ruffled hair coat, the animal was scored 0-6 with '0' as absence of ruffled hair coat. The animal body region was divided into six region: left

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and right, head, thorax and abdominal region, and ‘1’ score for each area having ruffled hair coat. Other than that, the rate of animal mortality was noted.

3.5 Histopathological lesions scoring

Immediately after the mice died, tissue samples from organs were collected and stored in 10% formalin solution. Tissues collected from organs, which include heart, lungs, liver, spleen, kidney, stomach, small intestine, and large intestines, were processed and examined under the microscope.

Three groups of lesion were examined, which include presence of inflammatory cells, degeneration and necrosis, and haemorrhage and congestion. For each tissue sample, six regions were examined for the stated lesion. Each region was scored 0-3, with ‘0’ as absence of lesion, ‘1’ for mild which less than 30% of the tissue region showing lesion, ‘2’ for moderate which 30-50% of the tissue region showing lesion, and ‘3’ is for severe which more than 50% is showing lesion.

The results obtained were analysed using IBM® SPSS® Statistics Version 20, whereby lesions of different organs were compared between experimental groups.

3.6 Bacterial isolation and identification

Organs which include heart, lungs, liver, spleen, kidney, small intestine, large intestine and stomach, were smeared properly unto blood agars media using the three-phase streaking pattern. The blood agars were incubated at 37°C for 24 hours. Then, the presence of bacterial growth on the media were observed. Isolation of smooth, non-haemolytic, greyish glistening translucent colonies of approximately 1 mm in diameter on the media was assumed as the causative agent. Next, bacterial colonies were stained with gram stain and Wright’s stain, to identify the organism.

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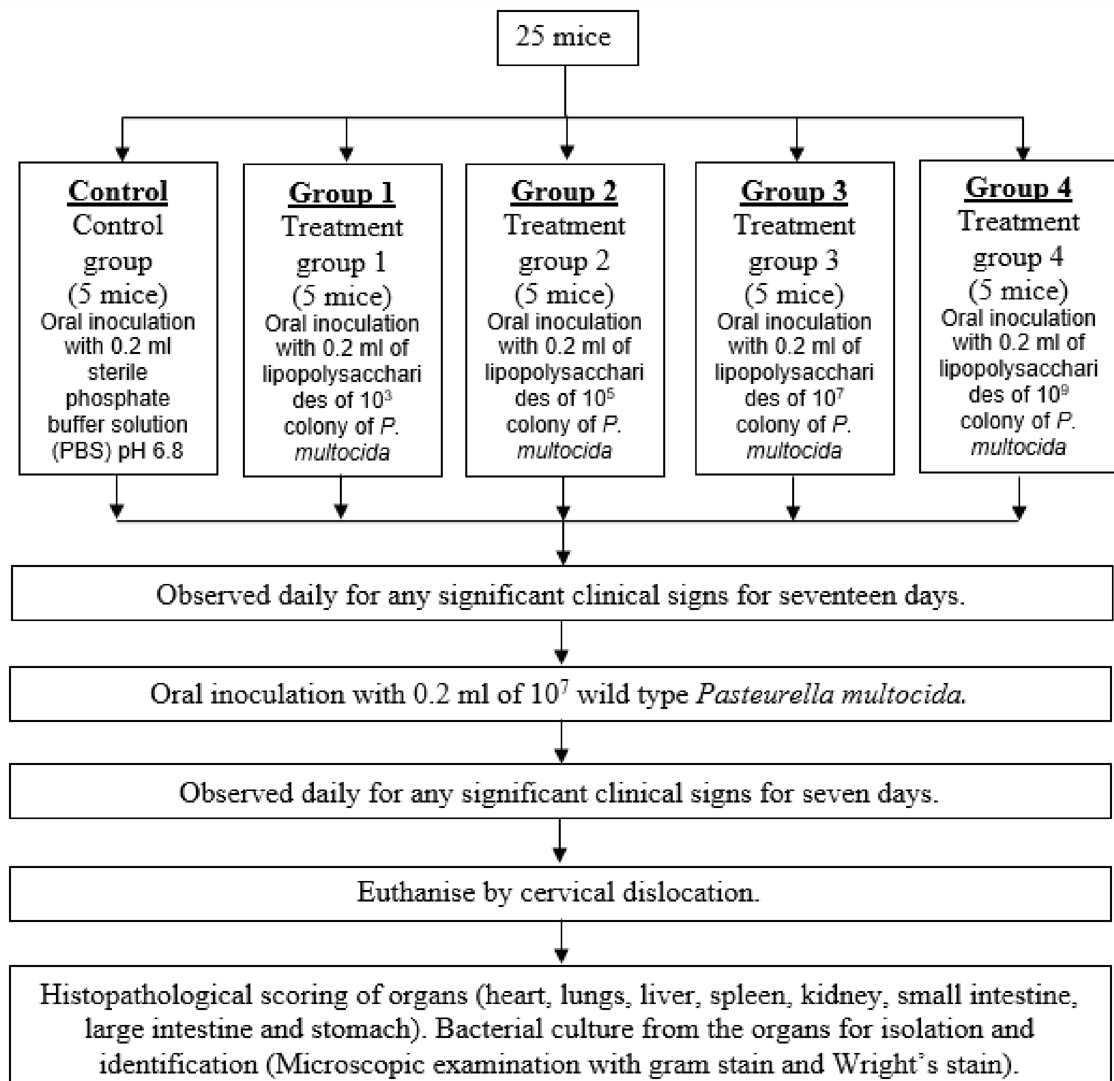
Figure 1: Flow chart of the study



4.0 RESULT AND ANALYSIS

4.1 Clinical signs

After inoculation with LPS, there was no significant findings (P>0.05) in clinical signs in between control group and treatment groups. Whereas one to two hours after challenged with whole bacteria, 60% of animals from all groups showed signs of diarrhoea. The clinical signs of animals that were euthanised for showing severe clinical signs, were analysed. Three to twenty hours before euthanised, 100% of the animals with severe clinical signs from all groups showed signs of laboured breathing and ruffled fur score of ‘6’. Moreover, 38.5% of the mice from all groups showed ocular discharges score of ‘8’ (the animals’ eyes were fully closed because of the discharges). Food and water intake and responsiveness were presence in animals from all groups.

The rate of mortality after the animals were inoculated with LPS extracted from *P. multocida* B:2 and whole bacteria of *P. multocida*, and the number of animals survived after seven days of bacterial inoculation are shown in Table 1. Mean of animal mortality (within the first 48 hours after inoculation with whole bacteria of *P. multocida*) were analysed by one-way ANOVA which showed no significant different (P>0.05) in between control (40%), low concentration (33.33%) and high concentration (66.67%) groups (Graph 1).

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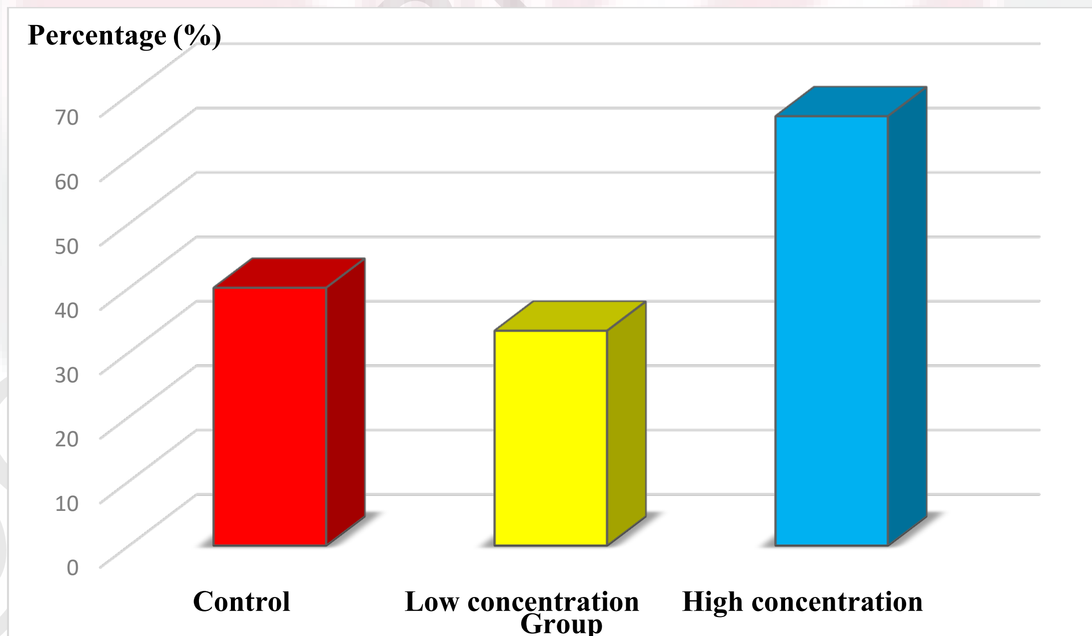
Table 1: Number of mortality of the animals

Group	Numbers of animal(s) died after LPS inoculation	Number of animal(s) died after *P. multocida* inoculation	Number of animal(s) survived
Control	-	2	3
Group 1	1	1	3
Group 2	0	4	1
Group 3	0	3	2
Group 4	1 (euthanised)	3	1

Graph 1: Percentage of animal mortality



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4.2 Histopathology

One-way ANOVA analysis was done to compare three groups of lesion (presence of inflammatory cells, degeneration and necrosis and haemorrhage and congestion) in each organ (heart, lung, liver, spleen, kidney, small intestine, large intestine and stomach) in each group.

Control Group had mild haemorrhagic and congestive lungs and kidney, mild to moderate degenerative, necrotic, haemorrhagic and congestive liver and spleen and mild degenerative and necrotic gastrointestinal tracts.

Group 1 had mild degenerative and necrotic heart and gastrointestinal tracts, mild degenerative, necrotic, haemorrhagic and congestive lungs, moderate degenerative and necrotic liver, and mild to moderate inflammatory cells, degenerative, necrotic, haemorrhagic and congestive spleen.

Group 2 had mild haemorrhagic and congestive heart and kidney, mild to moderate presence of inflammatory cells, degenerative, necrotic, haemorrhagic and congestive lungs and spleen, mild to moderate degenerative, necrotic, haemorrhagic and congestive liver, mild degenerative and necrotic large intestine and stomach.

Group 3 had mild haemorrhagic and congestive lungs and kidney, moderate degenerative and necrotic liver, mild to moderate degenerative, necrotic, haemorrhagic and congestive spleen, mild degenerative and necrotic gastrointestinal tract.

Group 4 had mild degenerative and necrotic heart and kidney, mild degenerative, necrotic, haemorrhagic and congestive lungs and liver, mild to moderate presence of inflammatory cells, degenerative, necrotic, haemorrhagic and congestive spleen, mild to moderate degenerative and necrotic gastrointestinal tracts.

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There is significant different (P<0.05) for the presence of inflammatory cells in spleen between Control Group (0.7) and Group 1 (1.4), where Group 1 has mild inflammatory cells in spleen. In addition, there is significant different (P<0.05) between degeneration and necrosis of the kidney between the groups. Group 4 (1.1) has mild degeneration and necrosis of kidney compared to Control Group (0.1), Group 1 (0.4) and Group 3 (0.6). Moreover, there is significant different (P<0.05) of inflammatory cells in the small intestine and large intestine between groups. Group 1 (0.7) has more inflammatory cells in the small intestine compared to Group 2 (0.03), Group 3 (0.02) and Group 4 (0.02). Whereas Control Group (0.4) has more inflammatory cells in the large intestine compared to Group 2 (0.1) and Group 4 (0.1). Lastly, there is also significant different (P<0.05) of degeneration and necrosis of stomach in between groups. Group 4 (2.2) has moderate degeneration and necrosis of stomach compared to the Control Group (1), Group 1 (1), and Group 2 (1).

Other than that, one-way ANOVA analysis was done to compare the histopathological lesions scoring in between animals that were euthanised within seven days post-challenged and animals that were euthanised after seven days post-challenged, as in graph 2. There were significant findings (P<0.05) of inflammatory cells in heart and lungs, of degeneration and necrosis in lungs and liver, and of haemorrhage and congestion in liver, kidney, small intestine and large intestine in between the two groups.

Animals that were euthanised within seven days post-inoculation had mild haemorrhagic and congestive heart, mild inflammatory cells, degenerative, necrotic, haemorrhagic and congestive lungs, mild to moderate degenerative, necrotic, haemorrhagic and congestive liver, mild to severe inflammatory cells, degenerative,

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necrotic, haemorrhagic and congestive spleen, mild haemorrhagic and congestive kidney, mild degenerative and congestive gastrointestinal tract.

Graph 2: Mean scores of histopathological lesions of animals euthanised within seven days post-challenged and after seven days post-challenged

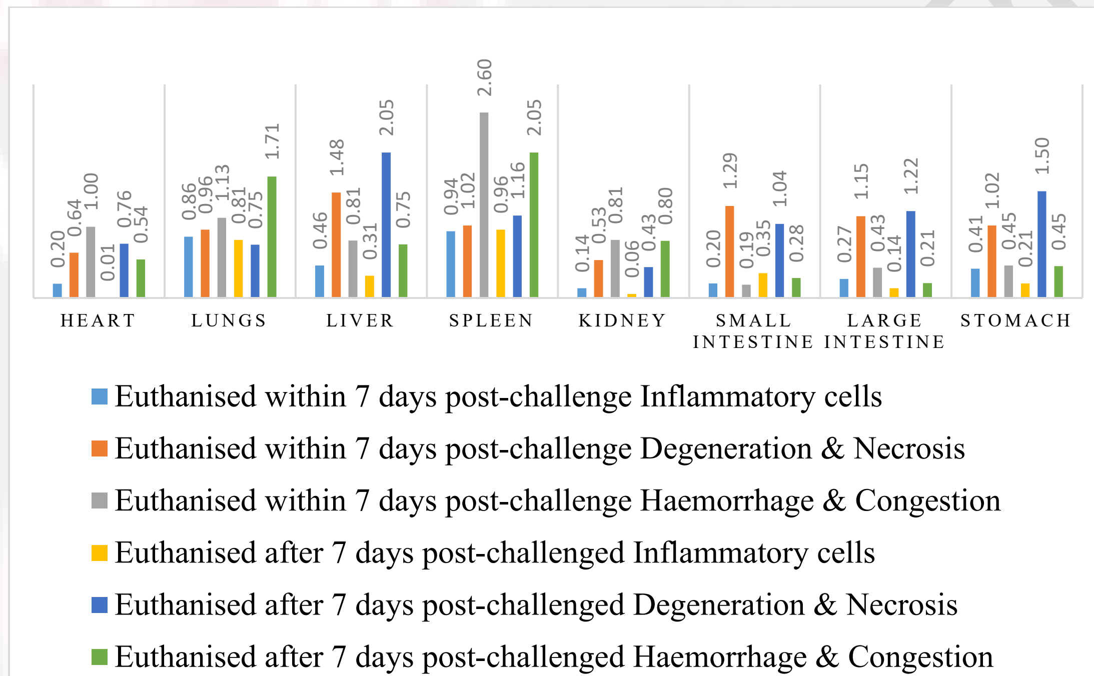

Table 2: Mean scores of inflammatory cells of animals euthanised within seven days post-challenged and after seven days post-challenged

Euthanised	Heart	Lungs	Liver	Spleen	Kidney	Small intestine	Large intestine	Stomach
Within 7 days	0.26±0.27[a]	0.99±0.5[b]	0.42±0.23	0.91±0.41	0.14±0.16	0.21±0.28	0.17±0.19	0.18±0.19
After 7 days	0.0.2±0.06[a]	0.27±0.25[b]	0.38±0.21	0.97±0.39	0.03±0.07	0.45±0.36	0.27±0.16	0.3±0.23

Values are expressed as mean ± SD, [a, b] Values within rows are significant at $P < 0.05$.

Table 3: Mean scores of degeneration and necrosis of animals euthanised within seven days post-challenged and after seven days post-challenged

Euthanised	Heart	Lungs	Liver	Spleen	Kidney	Small intestine	Large intestine	Stomach
Within 7 days	0.63±0.47	1.13±0.41[a]	1.29±0.84[b]	1.12±0.5	0.63±0.51	1.31±0.66	1.39±0.61	1.42±0.68
After 7 days	0.7±0.45	0.52±0.36[a]	2.68±0.37[b]	1.22±0.69	0.38±0.29	1.08±0.53	1.25±0.56	1.17±0.62

Values are expressed as mean ± SD, [a, b] Values within rows are significant at $P < 0.05$.

Table 4: Mean scores of haemorrhage and congestion of animals euthanised within seven days post-challenged and after seven days post-challenged

Euthanised	Heart	Lungs	Liver	Spleen	Kidney	Small intestine	Large intestine	Stomach
Within 7 days	0.91±0.51	1.58±0.73	1.01±0.3[a]	2.46±0.45	1.02±0.32[b]	0.31±0.23[c]	0.38±0.2[d]	0.49±0.27
After 7 days	0.53±0.39	1.00±0.6	0.43±0.42[a]	2.15±0.38	0.48±0.32[b]	0.13±0.13[c]	0.15±0.17[d]	0.3±0.27

Values are expressed as mean ± SD, [a, b,c,d] Values within rows are significant at $P < 0.05$.

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Graph 3: Mean score of histopathological lesions of heart

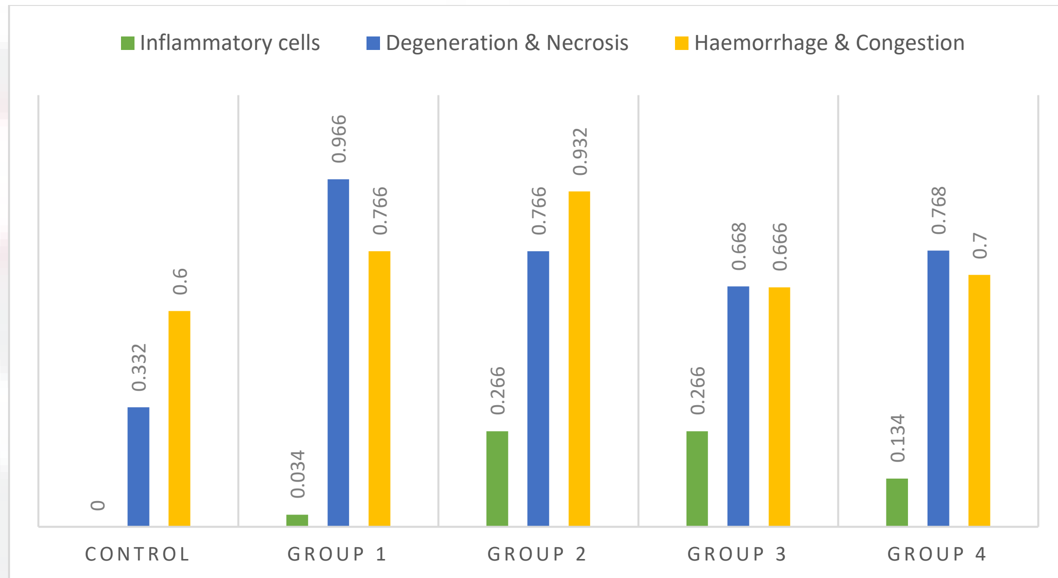

Table 5: Mean score of histopathological lesions of heart

Groups	**Control**	**Group 1**	**Group 2**	**Group 3**	**Group 4**
Inflammatory cells	0.00±0.00	0.03±0.08	0.27±0.24	0.27±0.38	0.13±0.14
Degeneration and necrosis	0.33±0.31	0.97±0.28	0.77±0.45	0.67±0.41	0.77±0.74
Haemorrhage and congestion	0.6±0.43	0.77±0.75	0.93±0.37	0.67±0.6	0.7±0.37

Values are expressed as mean ± SD, Values are significant at $P < 0.05$

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Graph 4: Mean score of histopathological lesion of lungs

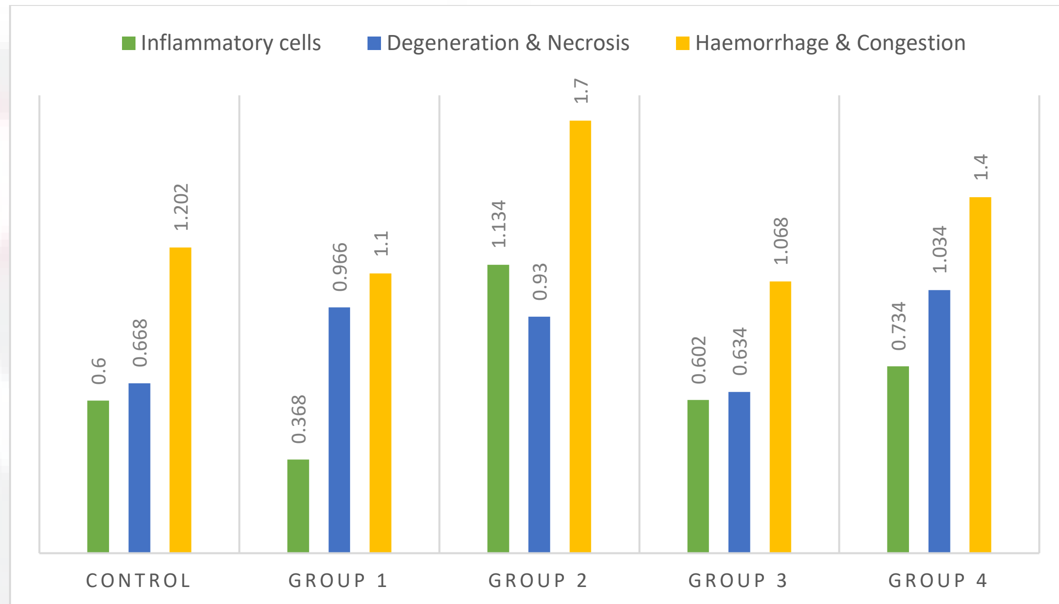

Table 6: Mean score of histopathological lesions of lungs

Groups	**Control**	**Group 1**	**Group 2**	**Group 3**	**Group 4**
Inflammatory cells	0.6±0.56	0.37±0.08	1.13±0.66	0.6±0.3	0.73±0.47
Degeneration and necrosis	0.67±0.61	0.97±0.35	0.93±0.42	0.63±0.42	1.03±0.57
Haemorrhage and congestion	1.2±0.58	1.1±0.44	1.7±0.74	1.07±0.74	1.4±1.11

Values are expressed as mean ± SD, Values are significant at $P < 0.05$

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Graph 5: Mean score of histopathological lesion of liver

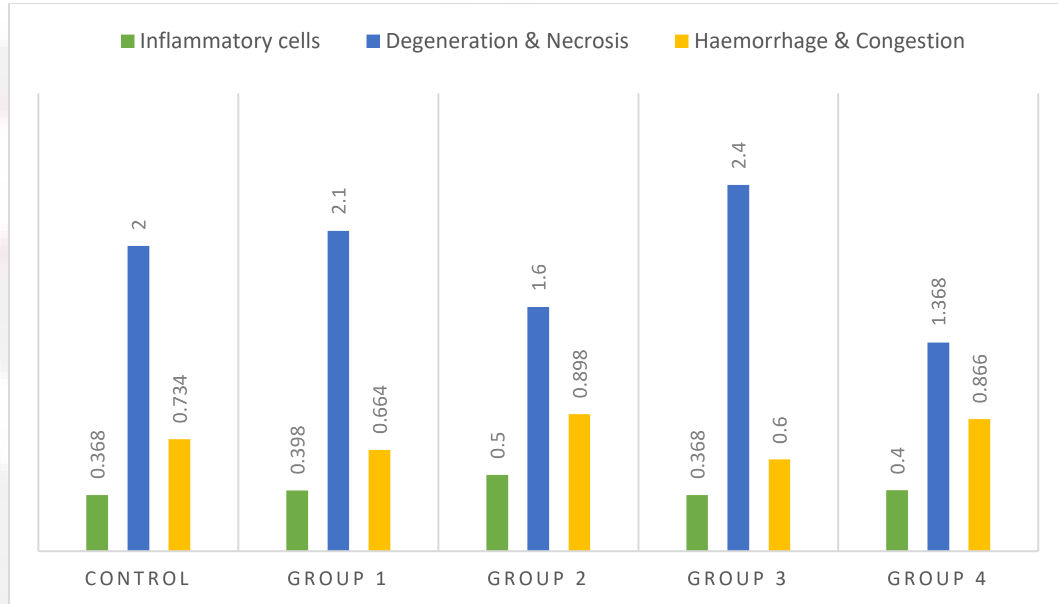

Table 7: Mean score of histopathological lesions of liver

Groups	Control	Group 1	Group 2	Group 3	Group 4
Inflammatory cells	0.37±0.22	0.42±0.10	0.50±0.26	0.37±0.30	0.38±0.21
Degeneration and necrosis	2.00±1.23	2.10±0.69	1.60±0.98	2.40±0.63	1.37±0.83
Haemorrhage and congestion	0.73±0.53	0.66±0.29	0.90±0.52	0.60±0.56	0.87±0.30

Values are expressed as mean ± SD, Values are significant at $P < 0.05$

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Graph 6: Mean score of histopathological lesion of spleen

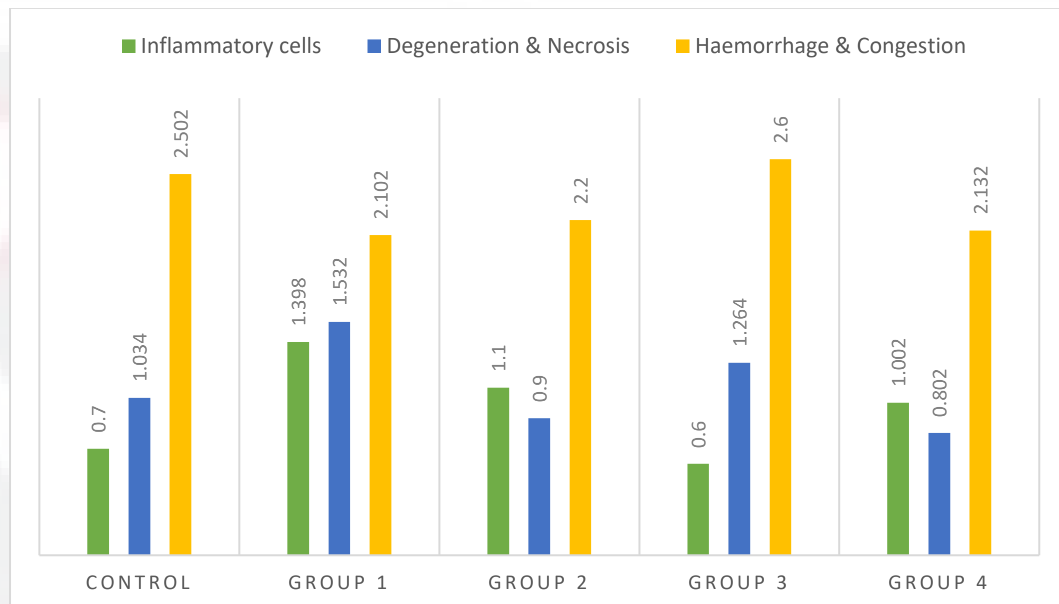

Table 8: Mean score of histopathological lesions of spleen

Groups	**Control**	**Group 1**	**Group 2**	**Group 3**	**Group 4**
Inflammatory cells	0.70±0.14[a]	1.40±2.89[a]	1.10±0.35	0.60±0.38	0.96±0.21
Degeneration and necrosis	1.03±0.43	1.53±0.16	0.90±0.38	1.26±0.69	0.80±0.96
Haemorrhage and congestion	2.50±0.35	2.10±0.34	2.20±0.36	2.60±0.48	2.13±0.42

Values are expressed as mean ± SD, [a]Values are significant at $P < 0.05$

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Graph 7: Mean score of histopathological lesion of kidney

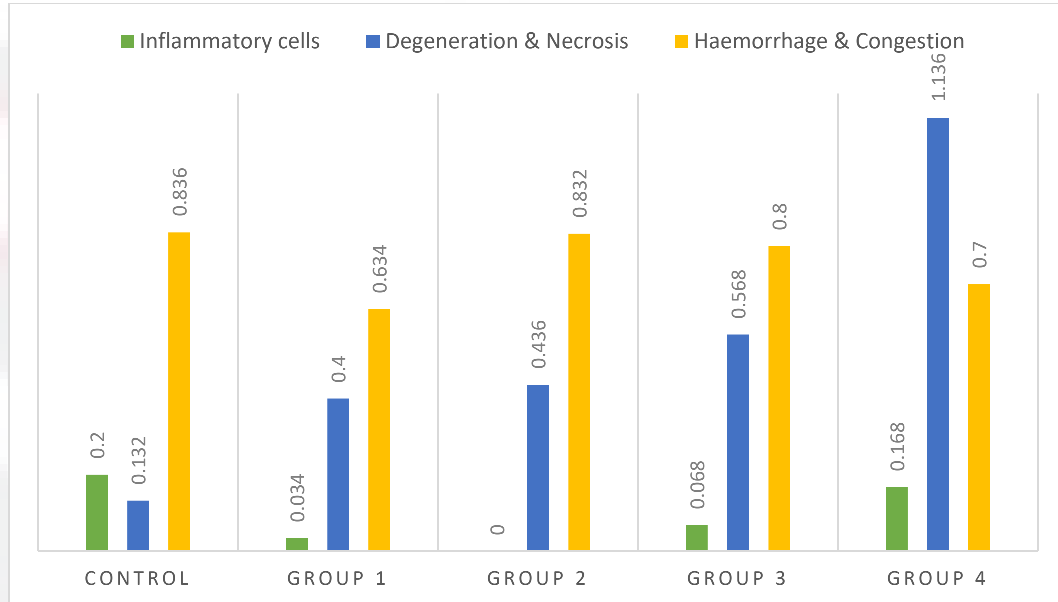

Table 9: Mean score of histopathological lesions of kidney

Groups	**Control**	**Group 1**	**Group 2**	**Group 3**	**Group 4**
Inflammatory cells	0.20±0.22	0.03±0.08	0.00±0.00	0.07±0.09	0.17±0.14
Degeneration and necrosis	0.13±0.18[a]	0.46±0.16[b]	0.44±0.25	0.57±0.52[c]	1.14±0.34[a,b,c]
Haemorrhage and congestion	0.84±0.66	0.63±0.16	0.83±0.35	0.80±0.55	0.70±0.24

Values are expressed as mean ± SD, [a,b,c]Values are significant at $P < 0.05$

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Graph 8: Mean score of histopathological lesion of small intestine

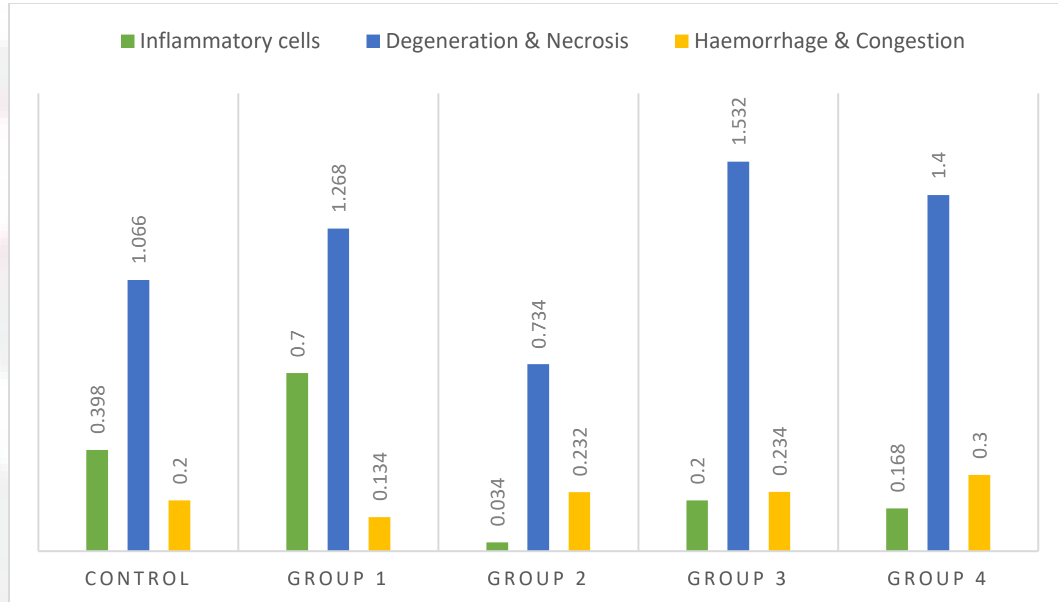

Table 10: Mean score of histopathological lesions of small intestine

Groups	**Control**	**Group 1**	**Group 2**	**Group 3**	**Group 4**
Inflammatory cells	0.40±0.30	0.79±0.37	0.03±0.07	0.20±0.21	0.17±0.08
Degeneration and necrosis	1.07±0.19	1.27±0.37[a]	0.73±0.61[b]	1.53±0.74[a,b,c]	1.40±0.52[c]
Haemorrhage and congestion	0.20±0.14	0.13±0.25	0.23±0.22	0.23±0.25	0.38±0.21

Values are expressed as mean ± SD, [a,b,c]Values are significant at $P < 0.05$

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Graph 9: Mean score of histopathological lesion of large intestine

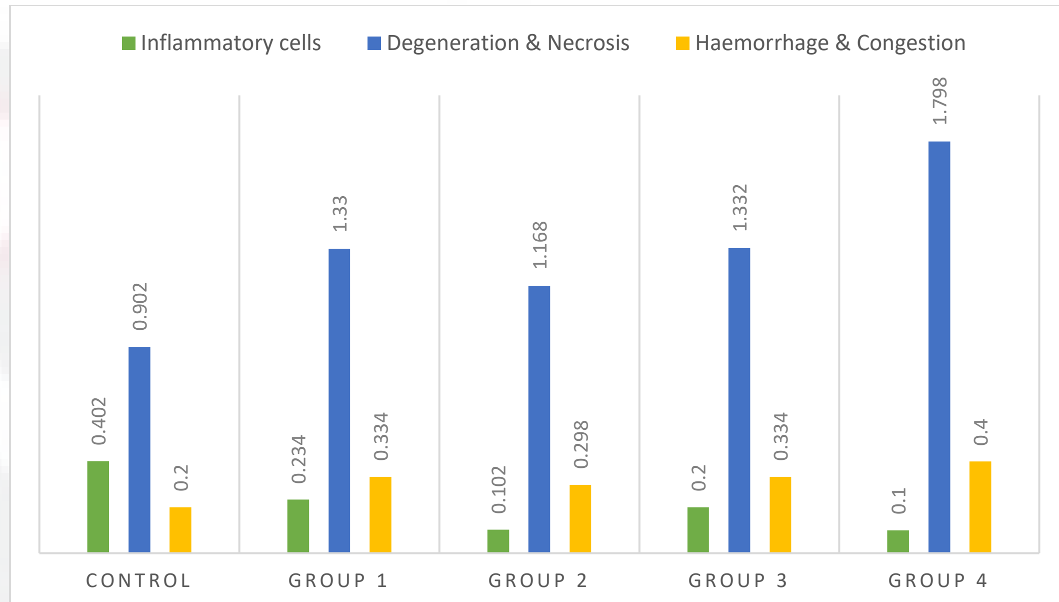

Table 11: Mean score of histopathological lesions of large intestine

Groups	**Control**	**Group 1**	**Group 2**	**Group 3**	**Group 4**
Inflammatory cells	0.40±0.25	0.23±0.08	0.10±0.09	0.20±0.14	0.13±0.16
Degeneration and necrosis	0.90±0.74	1.33±0.48	1.17±0.37	1.33±0.41	1.80±0.48
Haemorrhage and congestion	0.20±0.22	0.33±0.08	0.30±0.18	0.33±0.33	0.40±0.21

Values are expressed as mean ± SD, Values are significant at $P < 0.05$

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Graph 10: Mean score of histopathological lesion of stomach

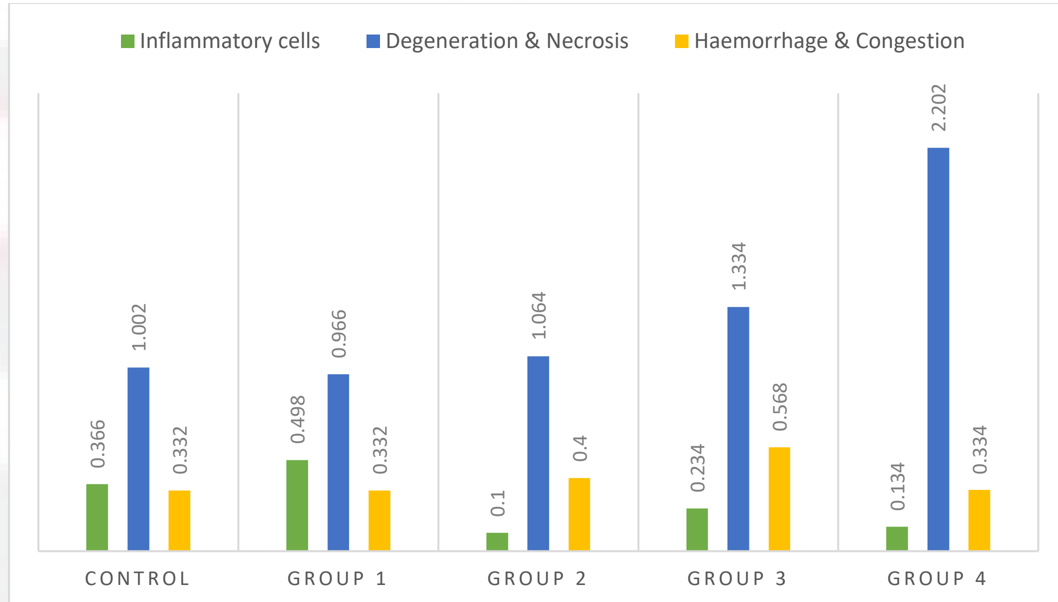

Table 12: Mean score of histopathological lesions of stomach

Groups	**Control**	**Group 1**	**Group 2**	**Group 3**	**Group 4**
Inflammatory cells	0.37±0.27	0.50±0.08	0.10±0.15	0.23±0.28	0.13±0.17
Degeneration and necrosis	1.00±0.72[a]	0.97±0.67[b]	1.06±0.48[c]	1.33±0.17	2.25±0.29[a,b,c]
Haemorrhage and congestion	0.33±0.35	0.33±0.24	0.40±0.25	0.57±0.30	0.33±0.28

Values are expressed as mean ± SD, [a,b,c]Values are significant at $P < 0.05$

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4.3 Bacterial isolation

Isolation and identification of *P. multocida* from organs (heart, lungs, spleen, liver, small intestine, large intestine, kidney and stomach) of each animal were analysed with one-way ANOVA. The analysis showed that there is no significant findings (P>0.05) in between groups. This show that bacteria was isolated from all stated organs in control and all treatment groups, showing that the animal mortality was due to *P. multocida*.

Other than that, one-way ANOVA analysis was done to compare isolation of *P. multocida* from animals that died naturally compared to animals that were euthanised. The analysis showed that there is no significant findings (P>0.05).

Table 13: Summary of isolation and identification of bacteria from organs

Time euthanised	Group	Heart	Lungs	Liver	Spleen	Kidney	Small intestine	Large intestine	Stomach
Within 7days	Control	+ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve
Within 7days	Control	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
After 7 days	Control	-ve	-ve	-ve	-ve	-ve	-ve	-ve	+ve
After 7 days	Control	-ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve
After 7 days	Control	+ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve
Within 7days	Group 1	+ve	+ve	+ve	-ve	-ve	+ve	+ve	+ve
After 7 days	Group 1	-ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve
After 7 days	Group 1	-ve	-ve	-ve	-ve	-ve	+ve	+ve	+ve
After 7 days	Group 1	+ve	+ve	-ve	-ve	+ve	+ve	-ve	+ve
Within 7days	Group 2	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve
Within 7days	Group 2	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve
Within 7days	Group 2	-ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve
Within 7days	Group 2	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve
After 7 days	Group 2	-ve	-ve	+ve	-ve	+ve	-ve	+ve	+ve
Within 7days	Group 3	-ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve
Within 7days	Group 3	-ve	+ve	-ve	+ve	-ve	+ve	-ve	-ve
Within 7days	Group 3	+ve	+ve	+ve	-ve	+ve	-ve	+ve	+ve
After 7 days	Group 3	-ve	-ve	-ve	-ve	-ve	+ve	+ve	-ve
After 7 days	Group 3	-ve	-ve	+ve	+ve	+ve	+ve	-ve	-ve
Within 7days	Group 4	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Within 7days	Group 4	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Within 7days	Group 4	+ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve
After 7 days	Group 4	-ve	-ve	-ve	-ve	-ve	-ve	-ve	+ve

+ve: Presence of *P. multocida* bacteria, -ve: Absence of *P. multocida* bacteria

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Table 14: Mean score of isolation and identification of bacteria in organs of animals

Group	Control	Group 1	Group 2	Group 3	Group 4
Heart	0.6±0.55	0.5±0.58	0.6±0.55	0.2±0.45	0.5±0.58
Lungs	0.6±0.55	0.75±0.5	0.6±0.55	0.6±0.55	0.5±0.58
Liver	0.6±0.55	0.5±0.58	0.8±0.45	0.6±0.55	0.5±0.58
Spleen	0.4±0.55	0.00±0.00	0.6±0.55	0.6±0.55	0.25±0.5
Kidney	0.6±0.55	0.25±0.5	1.00±0.00	0.6±0.55	0.5±0.58
Small intestine	0.4±0.55	0.75±0.5	0.4±0.55	0.5±0.45	0.5±0.58
Large intestine	0.6±0.55	0.5±0.58	0.4±0.55	0.6±0.55	0.5±0.58
Stomach	0.8±0.45	0.75±0.5	0.4±0.55	0.2±0.45	0.75±0.5

Values are expressed as mean ± SD, significant at $P < 0.05$

Table 15: Mean score of isolation and identification of bacteria in organs of animals euthanised within and after seven days post-challenged

Euthanised	Heart	Lungs	Liver	Spleen	Kidney	Small intestine	Large intestine	Stomach
Within 7 days	0.69±0.48[a]	0.85±0.38[b]	0.77±0.44	0.54±0.52	0.77±0.44	0.69±0.48	0.62±0.51	0.54±0.52
After 7 days	0.2±0.42[a]	0.3±0.48[b]	0.4±0.52	0.2±0.42	0.4±0.52	0.4±0.52	0.4±0.52	0.6±0.52

Values are expressed as mean ± SD, [a, b] Values within rows are significant at $P < 0.05$

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5.0 DISCUSSION

In this study, after inoculation with LPS, the animals from all treatment groups appeared to be normal compared to the control group (P>0.05). This showed that LPS extracted from *P. multocida* serotype B:2 inoculated orally into the animal, doesn't cause clinical disease. This is not consistent with a study done by Jesse *et al.,* (2013), that had demonstrated significant clinical signs of increased temperature, hypomotility and depression with intravascular inoculation of calves with LPS from *P. multocida.* This may be due to different route, animal host and method used, whereby in this experiment, the temperature of the mice were not monitored.

After oral inoculation with whole bacteria, animal from control and all treatment groups had exhibited clinical signs (P>0.05), histopathological lesions (P>0.05), and bacterial isolation and identification findings (P>0.05) that are consistent with the findings in animals with Haemorrhagic Septicaemia (HS). This showed that the animals from control and all treatment groups were successfully infected with HS and were showing clinical signs and lesions due to HS. This is consistent with another previous study done by Jesse *et al.,* (2013), whereby oral inoculation of *P. multocida* type B in mice exhibited significant clinical response (ruffled hair, laboured breathing and eye discharge) and cellular changes.

Other than that, isolation of *P. multocida* from animals that were euthanised within and after seven days post-infection with whole bacteria of *P. multocida* showed that there is no significant different (P>0.05). This showed that both animals that were euthanised within and after seven days post-infection with whole bacteria of *P. multocida.* However, there were significant different (P<0.05) in the histopathological

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findings in between animals of the two groups. The animals that were euthanised within seven days post-inoculation had mild degeneration, necrosis and inflammatory cells in lungs, mild to moderate degenerative, necrotic, haemorrhagic and congestive liver and mild haemorrhagic and congestive kidney, mild degenerative and congestive gastrointestinal tract, compared to euthanised animals. This shows possible presence of immunity, decreasing severity of disease, enabling animals to survive HS disease. This is consistent with previous study in rabbit done by Ashraf *et al.,* (2014) which demonstrated stimulation of antibody titre against HS with different dosage of LPS antigen extracted from *P. multocida.*

On the other hand, there was no significant different in rate of mortality of animal after being challenged with whole bacteria (P>0.05) in between control (40% mortality), low concentration (33.33% mortality) and high concentration (66.67% mortality) groups. This possibly show that there was no significant immunisation by oral inoculation of LPS, either of low concentration or high concentration, in the animal hosts. This is not consistent with the previously mentioned study done by Ashraf *et al.,* (2014), which may occur due to different inoculation route (subcutaneous in Ashraf's study), animal host and number of dosage given (in Ashraf's study, two dose of LPS were given with 15 days interval).

In addition, lack of significant different may also occur due to immunisation of the control group. This occur because of the poor experimental condition, whereby the control group were placed in close proximity with the treatment groups and groups from another experiment (which were inoculated whole bacteria *Pasteurella multocida* in animals within the seventeen days period of observation in this study),

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as there were limited space. As stated by De Alwis, (1999), *P. multocida* is naturally transmitted via inhalation or ingestion, hence, the control group in this study might had obtained immunisation via inhalation or fomites of causative organisms from those animals.

For future studies, it is recommended to add another control group, which will only be inoculated with PBS, without being challenged with *P. multocida*, unlike the presence positive control group used in this experiment. This control group could act as a negative control to ensure no exposure to causative agent via inhalation or contact in between different groups of animals. In addition, this group can be used as a guide in determining histopathological lesion scores of animals in treatment groups. In addition, repeated dosage of LPS, at least two to three times, with three to four days interval, should be given to the treatment groups, to ensure development of immunity, before being challenged with whole bacteria of *P. multocida* B:2. As this study had demonstrated that there is possible immune response against HS but fail to be significant. Hence, a larger sample size should be use to increase the estimation of mean, and possibly getting a significant result. Moreover, a similar study in mice should be carried out using outer membrane protein, instead of LPS, for possibility of producing better immune response in host against HS. Besides that, a better measures to control the experimental environment need to be practiced, to avoid the control groups from being exposed to causative agents, before being challenged.

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6.0 CONCLUSION

The animals in control group, low concentration groups (inoculated with LPS from 10^3 and 10^5 cfu of *Pasteurella multocida* serotype B:2) and high concentration groups (inoculated with LPS from 10^7 and 10^9cfu of *Pasteurella multocida* serotypes B:2) showed clinical signs (P>0.05), histopathological lesions (P>0.05) and bacterial isolation (P>0.05) consistent with haemorrhagic septicaemia disease. However, there was no significant different (P>0.05) in mortality percentage in between control, low concentration and high concentration groups. As a conclusion, there was no significant protective effect on host upon oral inoculation with low and high doses of lipopolysaccharide extracted from *Pasteurella multocida* type B:2 against haemorrhagic septicaemia disease

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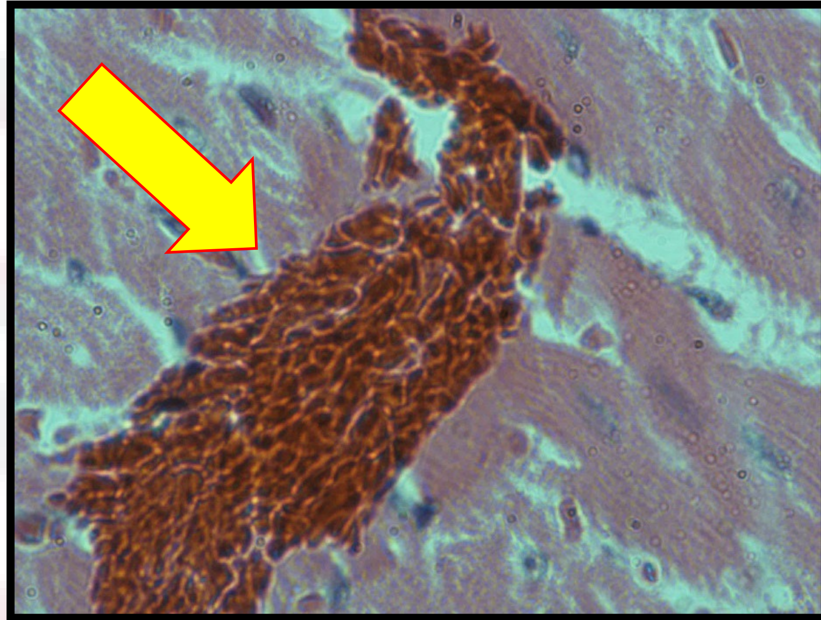
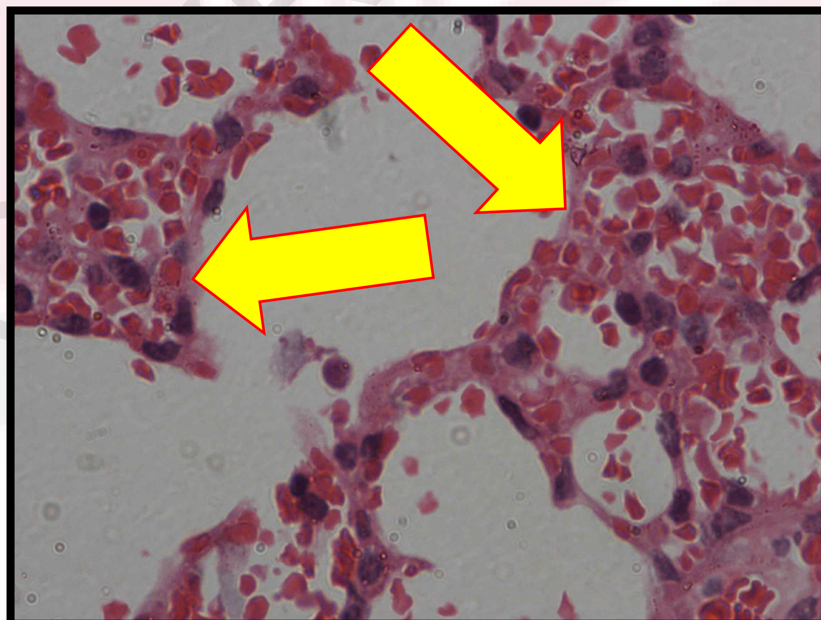
8.0 APPENDICES



Appendix 1: Micrograph of congestion and haemorrhage in the heart, H and E, x1000 (Group 2)



Appendix 2: Micrograph of congestion and haemorrhage in the lungs, H and E, x1000 (Group 2)

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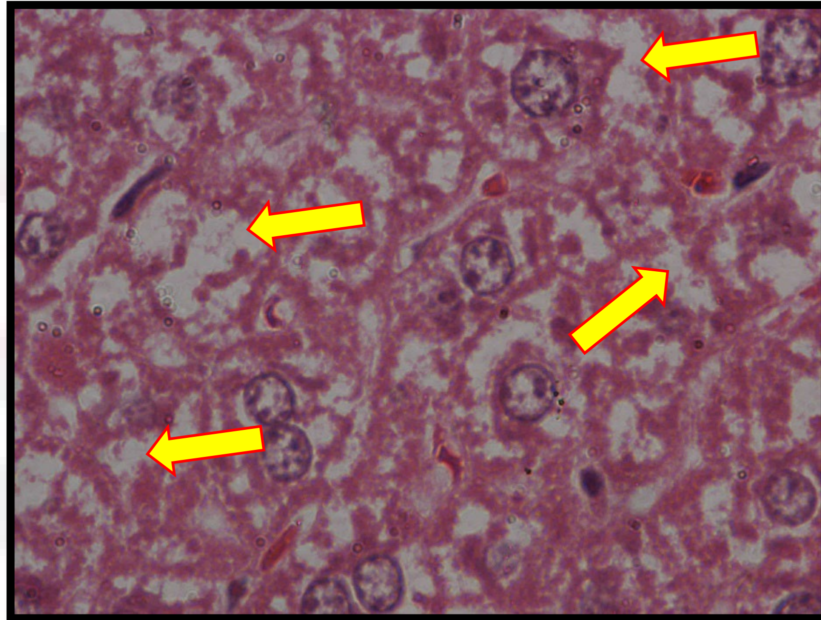
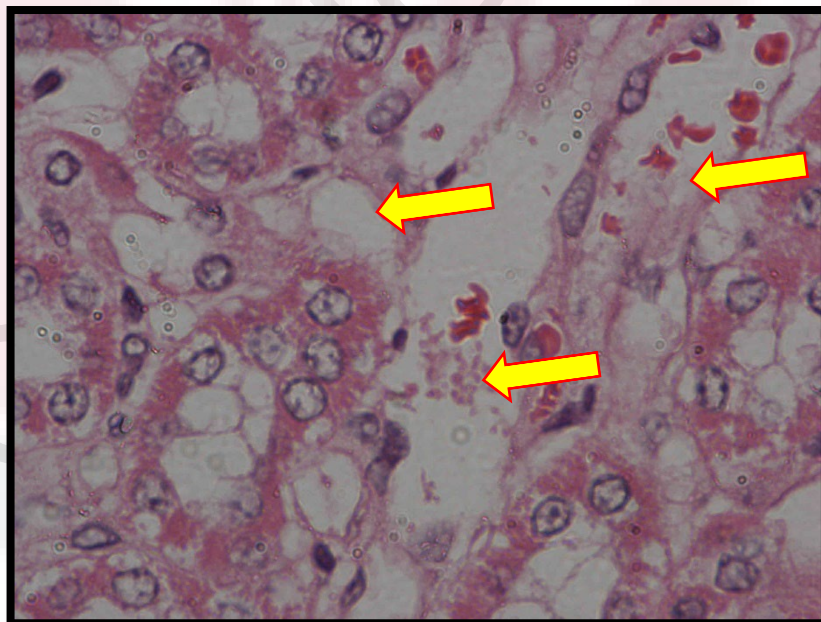


Appendix 3: Micrograph of degeneration and necrosis in liver, H and E, x1000 (Group 2)



Appendix 4: Micrograph of degeneration and necrosis in spleen, H and E, x1000 (Group 2)

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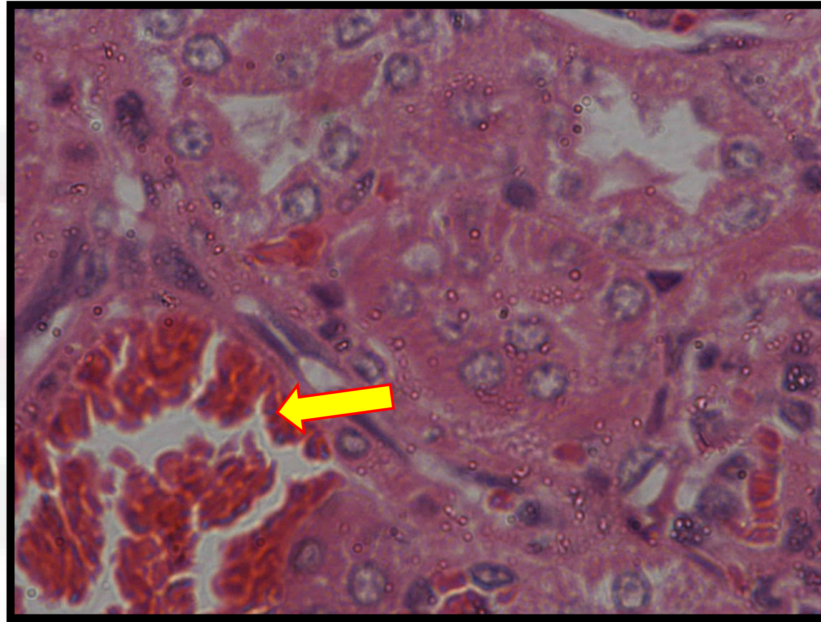
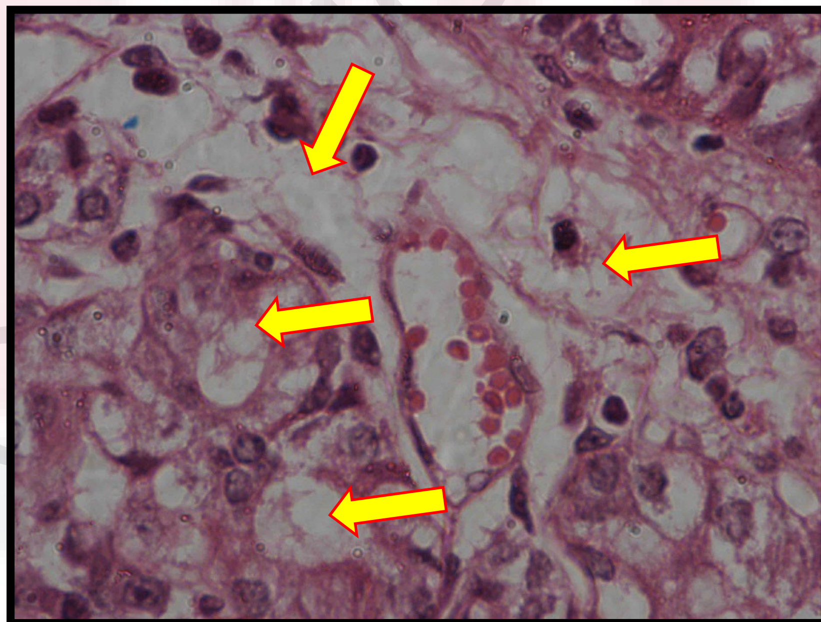


Appendix 5: Micrograph of haemorrhage and congestion in kidney, H and E, x1000 (Group 2)



Appendix 6: Micrograph of degeneration and necrosis in intestine. H and E, x1000 (Group 2)

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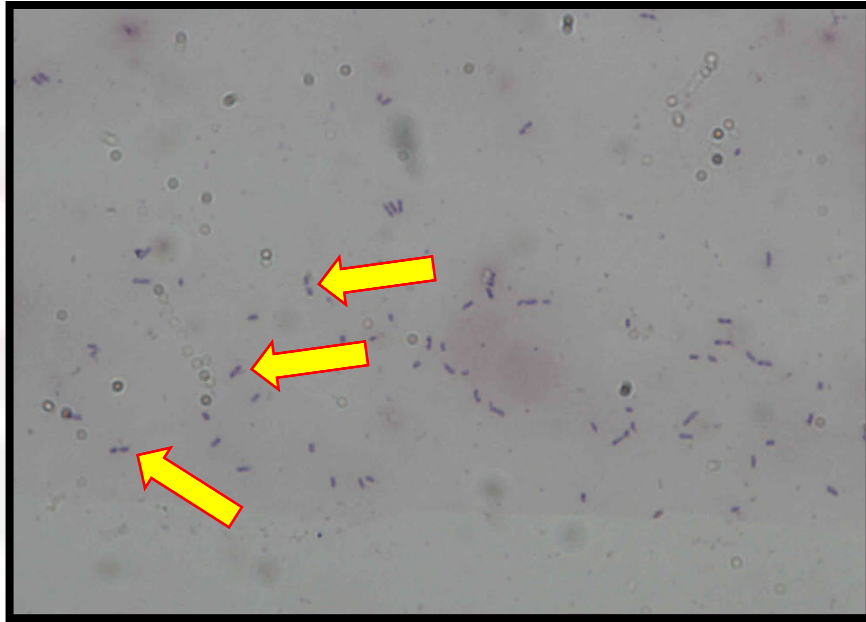
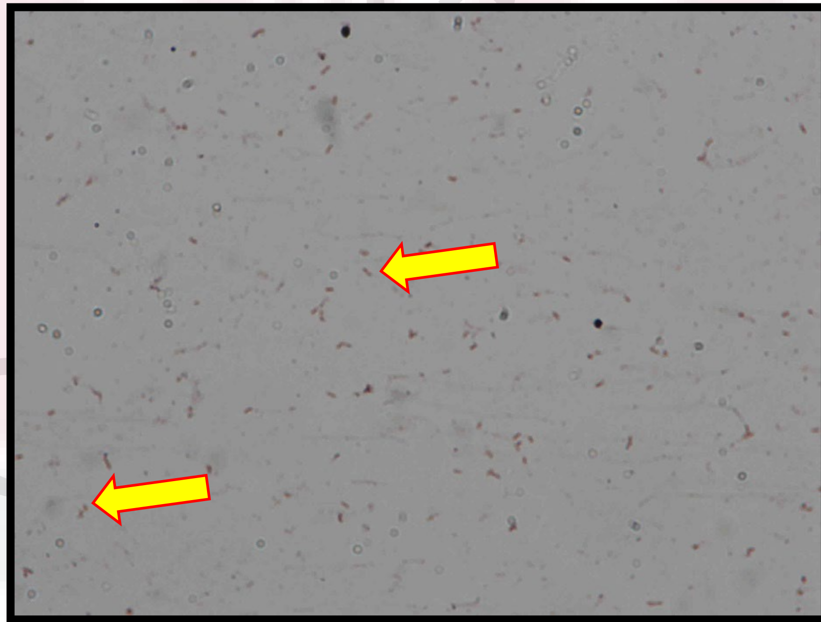


Appendix 7: Bipolar, cocco-bacillus organisms (*P. multocida)* with Wright's stain



Appendix 8: Gram-negative, rod organisms (*P. multocida)* with gram stain

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