UNIVERSITI PUTRA MALAYSIA

STUDY OF POSSIBLE IMMUNE PROTECTION WITH GRADED DOSES OF *PASTEURELLA MULTOCIDA* TYPE B:2 INOCULATED ORALLY IN MICE .

TAI SHEN RONG

STUDY OF POSSIBLE IMMUNE PROTECTION WITH GRADED DOSES OF *PASTEURELLA MULTOCIDA* TYPE B:2 INOCULATED ORALLY IN MICE .

TAI SHEN RONG

A project paper submitted to the

Faculty of Veterinary Medicine, University Putra Malaysia

In partial fulfillment of the requirement for the

DEGREE OF DOCTOR OF VETERINATY MEDICINE

University Putra Malaysia

Serdang, Selangor Darul Ehsan.

March 2016

It is hereby certified that we have read this project paper entitled "Study of Possible Immune Protection with Graded Dose of *Pasteurella multocida* Type B:2 Inoculated Orally in Mice", by Tai Shen Rong and in our opinion it is satisfactory in term of scope, quality, and presentation as partial fulfillment of the requirement for the course VPD 4999-Project.

DR. FAEZ FIRDAUS JESSE ABDULLAH

D.V.M (UPM), Ph.D. (UPM),

Lecturer,

Faculty of Veterinary Medicine

University Putra Malaysia

(Supervisor)

PROF. DR. MOHD ZAMRI SAAD

D.V.M (UPM), Ph.D. (UK),

Lecturer,

Faculty of Veterinary Medicine

University Putra Malaysia

(Co- Supervisor)

DR. ANNAS SALLEH

D.V.M (UPM), Ph.D. (UPM),

Lecturer,

Faculty of Veterinary Medicine

University Putra Malaysia

(Co- Supervisor)

.

ACKNOWLEDGEMENT

I would like to extend my gratitude to Dr. Faez Firdaus Jesse Abdullah, as my supervisor, for his invaluable time, guidance, knowledge, and advice for completing this project.

Acknowledgement also goes to Dr. Eric Lim and Dr. Nagachandra who helped and guided me on my clinical works during my project time.

A word of thanks is also conveyed to Mr. Jefri from Department of Veterinary Clinical Studies and Madam Latifah from Histopathology Laboratory, Universiti Putra Malaysia for their technical assistance and support. This paper would not have completed without the help of them.

I would also like to use this opportunity to thanks my family and friend, who gave me metal support to finish my project in time.

Lastly, acknowledgment also goes to those involve directly and indirectly in the completion of this study.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Haemorrhagic septicaemia (HS) is an acute fatal septicaemic disease in cattle and buffaloes caused by *Pasteurella multocida* Type B:2 in Malaysia. There is need to develop a vaccine easily administrated compared to those currently used. This study describes the possibility of using graded doses of oral bacterium to induce immunity. A total of 26 mice were divided into treatment groups (Group 1, 2, 3, and 4) each with 5 mice and control (n=6) groups. Treatment groups were inoculated orally with 0.2ml of 10^3, 10^5, 10^7 and 10^9 colonies forming units (CFU) respectively, while control received 0.2ml of phosphate buffered saline (PBS). Surviving mice were re-challenged with 0.2 ml of 10^7 CFU orally and observed for another 7 days. Clinical signs, mortality rate and histopathological lesions were examined. All clinical signs were observed to be not significantly ($P > 0.05$) observed in Group 1 and 2 except (level of alertness and ocular discharges) in Group 3 and 4. Presence of inflammatory cells, haemorrhages and congestions were mild to moderately observed in all treatment groups. However, degeneration and necrosis were observed to be moderate to severe in Group 4. All mice in Group 3 and 4 were euthanized at early stage after second challenged, except Group 1 and 2 with 20% of survival. Bacterial culture from survived mice was significantly lower ($P < 0.05$) in heart, lung, liver, spleen and kidney. *Pasteurella multocida* was confirmed by Gram and Wright's stains from all positive organ cultures. In conclusion, better survivability was observed in oral bacterium of 10^3 and 10^5 CFU with milder clinical signs and histological lesions, while 10^7 and 10^9 CFU resulted in detrimental effects on mice. Thus, low but not high dose of oral inoculum was believed to induce immunity and possible to use as oral vaccine.

Keywords: Pasteurella multocida Type B:2, mice, oral inoculation, clinical signs, histological signs, oral vaccine.

ABSTRAK

Penyakit hawar berdarah adalah penyakit septicaemic maut akut pada lembu dan kerbau yang disebabkan oleh jangkitan *Pasteurella multocida* Jenis B: 2 di Malaysia. Terdapat keperluan untuk membangunkan satu jenis vaksin mudah diaplikasikan berbanding dengan mereka yang kini digunakan. Kajian ini menerangkan kemungkinan menggunakan dos bergred oral bakteria untuk mendorong imuniti. Seramai 26 mencit dibahagikan kepada kumpulan rawatan (Kumpulan 1, 2, 3, dan 4) masing-masing dengan 5 mencit dan kumpulan kawalan (n = 6). Kumpulan rawatan disuntik secara oral dengan 0.2ml 10^3, 10^5, 10^7 dan 10^9 jajahan membentuk unit (CFU) masing-masing, manakala kawalan menerima 0.2ml fosfat buffered masin (PBS). Mencit yang hidup pada cabaran pertama telah dicabar semula dengan 0.2 ml 10^7 CFU secara oral dan diperhatikan untuk 7 hari lagi. Tanda-tanda klinikal, kadar kematian dan lesi histopatologi telah diperiksa. Semua tanda-tanda klinikal yang dapat diperhatikan sebagai tidak bererti (P> 0.05) bagi Kumpulan 1 dan 2 tetapi tidak (tahap kewaspadaan dan cecair okular) dalam Kumpulan 3 dan 4. Kehadiran sel-sel inflamasi, hemoraj dan kongesi yang ringan ke sederhana diperhatikan dalam semua kumpulan rawatan. Walau bagaimanapun, degenerasi dan nekrosis adalah didapati sederhana kepada teruk dalam Kumpulan 4. Semua mencit dalam Kumpulan 3 dan 4 telah eutanasia pada peringkat awal selepas cabaran kedua, kecuali Kumpulan 1 dan 2 yang didapati 20% hidup. Bakteria kaltur dari tikus terselamat adalah jauh lebih rendah (P <0.05) di dalam hati, paru-paru, hati, limpa dan buah pinggang. *Pasteurella multocida* telah disahkan oleh kesan Gram dan Wright dari semua organ positif. Kesimpulannya, kemandirian lebih baik diperhatikan dalam bakteria oral 10^3 dan 10^5 CFU dengan tanda-tanda

klinikal yang lebih ringan dan lesi histologi, manakala 10^7 dan 10^9 CFU mengakibatkan kesan memudaratkan mencit. Oleh itu, dos rendah tetapi tidak dos tinggi inokulum oral dipercayai mendorong imuniti dan mungkin untuk digunakan sebagai vaksin oral.

Katakunci: *Pasteurella multocida* jenis B:2, mencit, oral inokulum, tanda-tanda klinikal, tanda histological, vaksin oral.

1.0 INTRODUCTION

Pasteurella multocida is a non-motile, gram-negative, coccobacillus that is found in the nasopharynx and gastrointestinal tract of many wild and domesticated animals (Sugan *et al*., 2013). It is the aetiological agent for an acute, fatal septicaemic disease known as haemorrhagic septicaemia (HS). The disease mainly in found South and Southeast Asia, Africa and India (Hussaini & Jumahat, 2014), which include Malaysia. The death usually occurs quickly and mortality rate without prompt antibiotic treatment in a naïve population is close to 100% (Aktories *et al*., 2012; Jumahat *et al.*, 2015). However, treatments of infected animals with *P. multocida* are complex and unsuccessful due to increasing antibiotic resistance strains. Vaccination is the principle method of controlling the disease (Zamri *et al*., 2006) but difficulties in vaccine administration lead to low vaccination coverage and disease outbreaks (Saharee *et al*., 1993). Moreover, the efficacy and safety of available vaccines are limited (Hussaini *et al.,* 2012). Large-scale vaccination of cattle against HS is not practiced in many countries of Africa (FAO, 2005), which is the same scenario in Malaysia. This could be due to the laborious process such as herding and restraining, which involved when vaccinating the cattle by injection. In 2013, Zamri had reported the vaccine coverage of HS for buffaloes in Malaysia is just 17% and it was most probably due to difficulty in vaccine administration. Besides, vaccination via injection might result in adverse reaction such as lumps and abscess at injection sites (Verma and Jaiswal, 1998). Although intranasal HS vaccine has been developed in Myanmar, however, it is still laborious to perform in large scale. Safe and effective vaccines against pasteurellosis are still lacking (Hunt *et al.,* 2000). Therefore there is a need to improve the vaccination approaches. There have been previous studies done by (Jesse *et al.,* 2013) on oral inoculation of *P. multocida* using mice model and shows that it can produce similar clinical signs and pathological lesion in the real host. However, there is still lack of research in immunization against pasteurellosis via oral exposure to the animals. Most of the study is either via subcutaneous route or intraparitoneal route.

Due to the huge economic loses, many research have been carried out to determine which is the protective antigens found in the bacteria. For now, the identified protective antigens are the outer membrane protein-H (OmpH) (Luo *et al.,* 1999), lipoprotein B (Tabatabai and Zehr, 2004), lipopolysaccharide, and one or more iron-responsive OMPs (Ruffolo *et al.,* 1998). However, Boyce and Adler (2006) state that there are "host-respond-proteins" that expressed only during *in vivo* situation and these proteins might be the antigens that able to stimulate full protective immunity against both homogenous and heterogenous infections.

Studying the possible oral immunization of live bacteria in animals can give valuable information regarding the minimal dosage that will protect the host from getting the disease. Besides, this study might result in a new vaccination route where HS vaccine can be given in feed and water. Therefore, this study was conducted to examine the possibility of using oral live bacterium of *P. multocida* to induce immune-protection in mice and the minimal dosage that will protect the host from HS disease.

2.0 LITERATURE REVIEW

2.1 *Pasteurella multocida*

Pasteurella multocida is a gram negative bacterial from the family *Pasteurellaceae*. The bacteria can be found in the nasopharynx, respiratory tract and the gastrointestinal tract of animals and causes a disease generally term as pasteurellosis (Ahmad *et al.*, 2014). Pathogenicity of the bacteria depends on the host involved and the bacteria serotype. Carter's and Heddleston (capsular-somatic combination) typing systems have been generally adopted for serotyping of the bacteria (OIE, 2008). In the system, bacteria is divided into 5 serogroups (Group A, B, D, E and F) and 16 serotypes based on their capsule and lipopolysaccharide respectively. *Pasteurella multocida* has a broad host range, as a primary pathogen it is responsible for HS in cattle and bufalloes, fowl cholera in poultry, and snuffles in rabbits. As secondary or opportunist pathogens it causes atrophic rhinitis in swine, pneumonia in cattle, sheep, goats and small laboratory rodents. Each disease is a result of different serotype and is most commonly associated with diseases of economic important. Serotyping of *P. multocida* strains is essential to establish correlation between organism and the disease (Kalorey *et al.,* 2008). It is considered to be an important pathogenic bacterium of domestic animals worldwide and impose a huge economic losses in livestock industries (Dziva *et al,*. 2008).

2.2 Haemorrhagic septicaemia

Capsular serotypes B and E are associated with haemorrhagic septicaemia in Asian and African countries respectively (Jumahat *et al*., 2015). It is an acute, highly fatal septicaemic disease of cattle and buffaloes, characterized by high morbidity and mortality. The disease affecting predominantly cattle but buffaloes are more susceptible. The major pathogenic components identified were bacterial capsules, outer membrane protein (OMP), lipopolysaccharides (LPS), surface adhesions and iron acquisition proteins (Kharb and Charan, 2013; Seleim, 2003; Chung *et al.,* 2016). Among which LPS is the major virulence factor and played a significant role in causing HS diseases (Seleim, 2003). The clinical signs seen are often include pyrexia, respiratory distress with nasal discharge, froth from the mouth, recumbent and eventually death (OIE, 2008; Ali *et al,*. 2015). Healthy carriers or latent infection are common and play a significant role in epidemiology of HS disease (Dziva *et al,* 2008). It was believed that the transmission of the disease can occur via inhalation or ingestion of contaminated feed and water (Annas *et al.,* 2014; Benkirane and De Alwis, 2002). Most Asian countries consider HS as the most important bacterial contagious disease of large ruminants (Benkirane and De Alwis, 2002). In Malaysia, most outbreaks occur during the raining season as it induces stress to the animals (Ali *et al.,* 2015). It was supported by study done by Bisht *et al* (2006) where he found that high seasonal outbreak might be due to climatic changes, vaccination activities and other factors.

2.3 Diagnostic technique

Clinic-pathological findings coupled with laboratory analysis of samples are needed to confirm the disease. For laboratory analysis, polymerase chain reaction (PCR) assays is a useful diagnostic tools to detect and characterization of the bacteria (Kalorey *et al.,* 2008). *Pasteurella multocida* specific PCR (PM-PCR) by Townsend *et al.* (1998), allowed detection of the bacteria in mixed cultures or clinical samples. With slight modification Lee *et al.* (2000) able to detect *P. multocida* in intestinal contents of orally infected chickens. However, a presumptive diagnosis can be made based on the clinical signs seen and minimal laboratory findings which include: growth characteristic, colony morphology, odour, bipolar staining, positive catalase, and oxidase reaction and failure to grow in MacConkey agar (Dziva *et al.,* 2008).

2.4 Treatment, Control and Prevention

Since it is a bacterial infection, antibiotic treatment should be able to control the infection. However, the treatment is generally expensive, lengthy and ineffective for pasteurellosis (Coudert *et al.*, 2006; Ahmad *et al.*, 2014). For HS infection, it usually is peracute in nature and treatment is often of limited value (De Alwis, 1992) as the animal might in its terminal stage by the time the disease is diagnosed. Benkirane and De Alwis (2002) also stated that animals can only be cured by antibiotics if in the very early stages of disease. Besides, antimicrobial resistant strains toward commonly used tetracyclines have been reported (Kehrenberg *et al,* 2001). The key factors for prevention and control would be timely reporting, rapid diagnosis and strategic use of vaccine (Benkirane and De Alwis, 2002). Thus, vaccination by meant is the most economic and effective method to control and protect the animals from the disease (OIE, 2012). Currently the commonly used vaccines are bacterins, alum-precipitated vaccine and oil adjuvant vaccine (OAV). Of these, OAV is preferred over other vaccines (Pati *et al.*, 1996); however, due to its preparation that was viscous and required deep intramuscular injection (De Alwis, 1984), it is not user friendly during the field.

2.5 Previous Studies

2.5.1 Cattle

Chung *et al* (2016) in his experiment proof that LPS extract of *P. multocida* Type B:2 can stimulate host response and shows clinico-pathological response that resemble the natural infection. Besides, Abubakar and Zamri (2011) in their experimental oral exposure of *P.*

multocida B:2 in buffalo calves resulted in development of syndromes and lesions typical of HS disease. They were also able to re-isolate the causative bacteria from the gastro-intestinal tract of infected calves. Experimental studies on oral inoculation of *P. multocida* Type B:2 and its LPS have proven that it could be one of the natural route of infection.

2.5.2 Chicken

A well-developed oral vaccine that used in drinking water has been used for control of fowl cholera disease, a septicaemic pasteurellosis in avian species. The used of avirulent Clemson University strain of *P. multocida* as oral vaccine able to stimulate high protective immunity without adverse effects in chicken and turkeys (Dua and Maheswaran, 1978; Ahmad *et al.,* 2014). Although it was caused by a different serotype of pasteurella bacteria, there might be a possibility that HS vaccine can followed the same approach.

2.5.3 Mice

The study of pathogenesis and immunity against *P. multocida* can often be conducted in white mice as they are low priced but give high sensitivity to infection of the bacteria (Youssef, 2011; Kharb and Charan, 2013). Abdullah *et* al (2013) have proved that oral route inoculation in mice able to infect the animal and able to result in clinico-pathological lesion similar to natural infection (Jesse *et al.,* 2013; Abdullah *et* al., 2014). In Abdullah study in 2013, only 25% of mice inoculated with 10^9 CFU survived for 120 hours, while 12.5% mice died per-acutely within 24 hours and 62.5% died within 48 hours. By using contaminated river water with pasteurella bacteria, Khaleel *et al.* (2014) shows that mice also able to develop similar histopathological changes as in natural HS infection.

3.0 MATERIALS AND METHODS

3.1 Mice

A total of 26 clinically healthy ICR mice with mixed gender, at ages around 4 to 5 weeks old were used in this study. These mice were obtained from Institute of Cancer Research (ICR) and were kept at the Animal Research Centre, University Putra Malaysia (UPM). The mice were allowed to acclimatize in the new environment for one week before experiment begun. They were kept in a plastic container with wood shavings bedding that was changed twice a week. During the whole period of experiment, commercial pellet and clean water were provided *ad libitum.*

3.2 Inoculums

The inoculum used in this study was a wild-type *P. multocida type* B:2 which obtained from stock culture. It was previously identified as *P. multocida* bacteria by using gram-staining method and biochemical characterization of oxidase, urea broth, suplhur indole motility (SIM), triple sugar iron (TSI) and citrate tests. Bacterial typing was done in Veterinary Research Institute (VRI) Ipoh, Perak and confirmed as *P. multocida* type B:2. From the stock culture, the bacterium was sub-cultured into 5 % horse blood agar for 24 hours at 37 ^{0}C. Colonies from the blood agar plate were then mixed with sterile distilled water, after which the concentration was determined by comparing the turbidity using McFarland Nephelometer Barium Sulfate Standards.

3.3 Study Designs

The mice were divided into control (n=6) and treatment group (n=20). For the treatment group, the mice were sub-divided into 4 groups with 5 mice each. Mice in the treatment group (Group 1, 2, 3 and 4) were inoculated with 0.2 ml of *P. multocida* type B bacterium orally, with concentrations ranging from 10^3, 10^5, 10^7 and 10^9 colony forming unit (CFU) respectively. Mice in control group were then inoculated with 0.2ml of sterile phosphate buffered saline (PBS) orally as placebo.

Clinical signs such as alertness, ruffled fur, ocular discharges and laboured breathing observed in both the treatment and control groups were observed and recorded for a period of 16 days. Scoring value was given for each of the assessing parameter and the mean scores (in percentage) were used to judge the severity of clinical signs. Mean scores less than 30 % indicate mild abnormalities, 30 – 60% indicate moderate abnormalities, while values above 60% meant severe abnormalities.

All survived mice in treatment groups and 4 control group mice were then challenged second time with 0.2ml of 10^7 CFU of bacterium orally and observed for another 7 days. Mice that survived after 7days post-challenge were deemed immune. The clinical signs and mortality were observed and recorded as in first challenge.

3.4 Sampling and Culture

Mice that survived after the 7 days post-challenge were euthanized via cervical dislocation. Together with the mice that were euthanized due to severe clinical signs during the experiment, post-mortem examinations were done. Affected organs such as lungs, heart, liver, spleen, kidney and the gastrointestinal tracts were aseptically sampled for bacterial cultures on blood agar at 37 ^{0}C for 24hours. From the culture, colony that resembles and suggestive that one of *P. multocida* (small rounded, greyish translucent colony) was selected and sub-cultured into blood agar to obtain pure colonies. The colonies were then stained with Gram and Wright's stain to detect the present of gram-negative coccobacillus and bipolar stained bacteria respectively.

3.5 Histopathology Lesion Scoring

The same organs taken for bacteria culture were also sampled for histological slides preparation. Organ samples were collected and fixed in 10% buffered formalin. The samples were then processed and stained with Haematoxylin and eosin (H & E) before examined under light microscope at 400X magnification. The histopathology lesion was examined for the presence of inflammatory cells; degeneration and necrosis; haemorrhages and congestions. Six different views of the slides were examined and scoring was given to each view as below:

Table 1: Scoring system for histopathological lesions.

Score	Indications
0	Normal; no lesion visible on field
1	Mild; less than 30% of field affected
2	Moderate; between 30 - 60 % field affected
3	Severe; more than 60% of the field affected

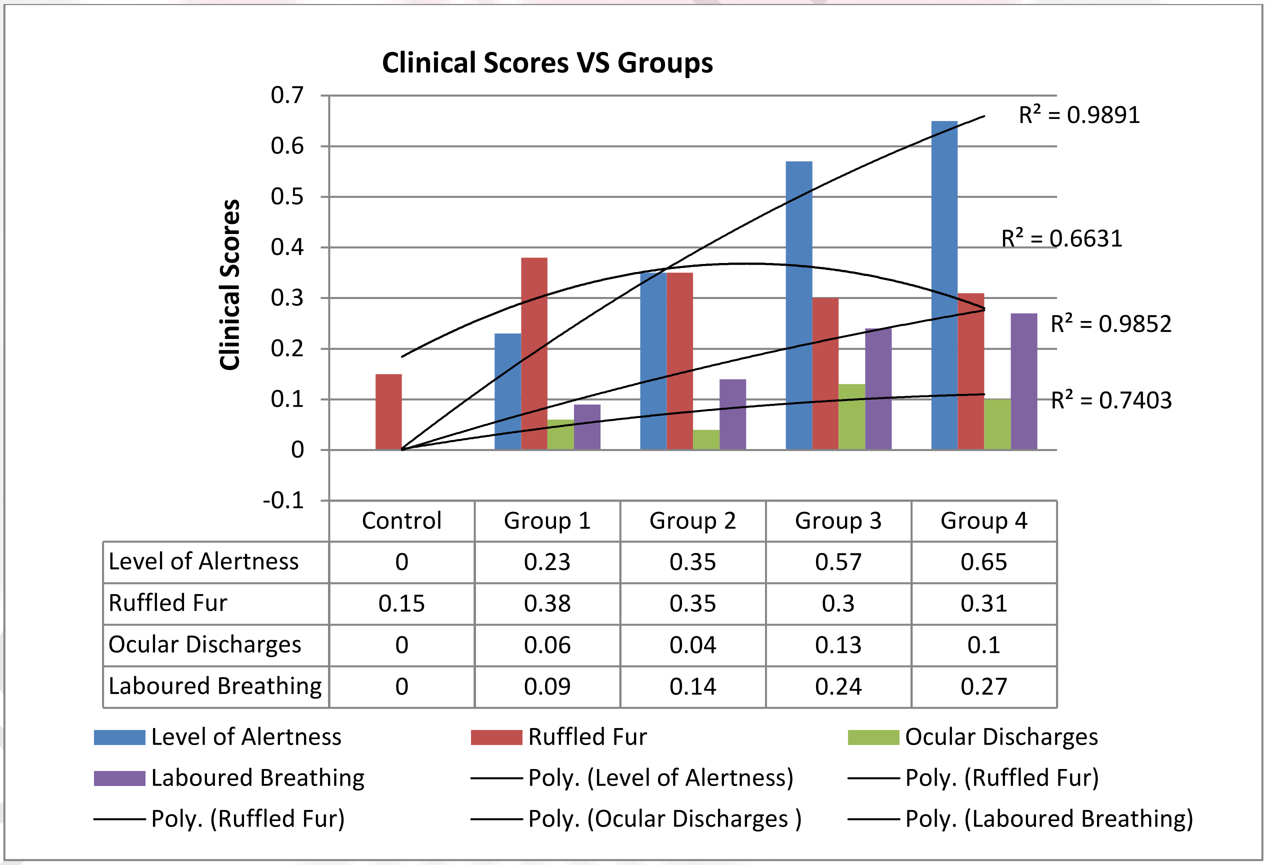
3.6 Statistical analysis

All the findings in the study were analyzed using IBM SPSS Version 20. One-way ANOVA test was used to analyzed difference of means between groups for their clinical signs, general histological lesions of groups and bacteria culture between survived and died mice at the end of challenges at level of significance $P < 0.05$. As for survivability, Mann-Whitney U-Test was used to test the significant different between groups at $P < 0.05$ level.

4.0 RESULTS

4.1 Clinical Observation

All clinical signs observed were showed to be mild to moderate in all treatment groups except Group 3 and 4 showed significant (P <0.05) moderate to severe reduction in level of alertness. As for ocular discharges, it was found to be mild in all treatment groups, but were significantly higher (P < 0.05) in Group 3 and 4. Although ruffled fur and laboured breathing were not significant (P > 0.05) in between groups, but the mean scores were observed to gradually increase across the treatment groups with Group 1 showed about 2x lower than Group 4. Generally all clinical signs observed were gradually increased in severity from mild to moderate to severe across treatment groups.



	Control	Group 1	Group 2	Group 3	Group 4
Level of Alertness	0	0.23	0.35	0.57	0.65
Ruffled Fur	0.15	0.38	0.35	0.3	0.31
Ocular Discharges	0	0.06	0.04	0.13	0.1
Laboured Breathing	0	0.09	0.14	0.24	0.27

Figure 1: Comparison between mean clinical scores of different groups.

4.2 Lethality and survivability rate between groups

Generally the condition of the mice stable after 48 hours in both first and second challenged group. Mortality in first challenged experiment Groups 1, 2, 3 and 4 were 20%, 40%, 100% and 80% respectively. While in second challenge, all mice died except 20% of mice founded to be survive from Group 1 and 2. Statistical analysis for mortality between first challenge and second challenged was not done due to insufficient data. However, overall mean time of death between groups were tested to be no significant different ($P > 0.05$), which ranged from 14 - 46 hours. For the single mean value analyze, mice from Group 1 after second challenge took longer time (15.7 hours) to show severe clinical signs prior euthanized when compared to first challenge (9 hours). The survival time for Group 3 and 4 were shorter from Group 1, 2 and Control. However, it was only significantly ($P < 0.05$) shorter than Control.

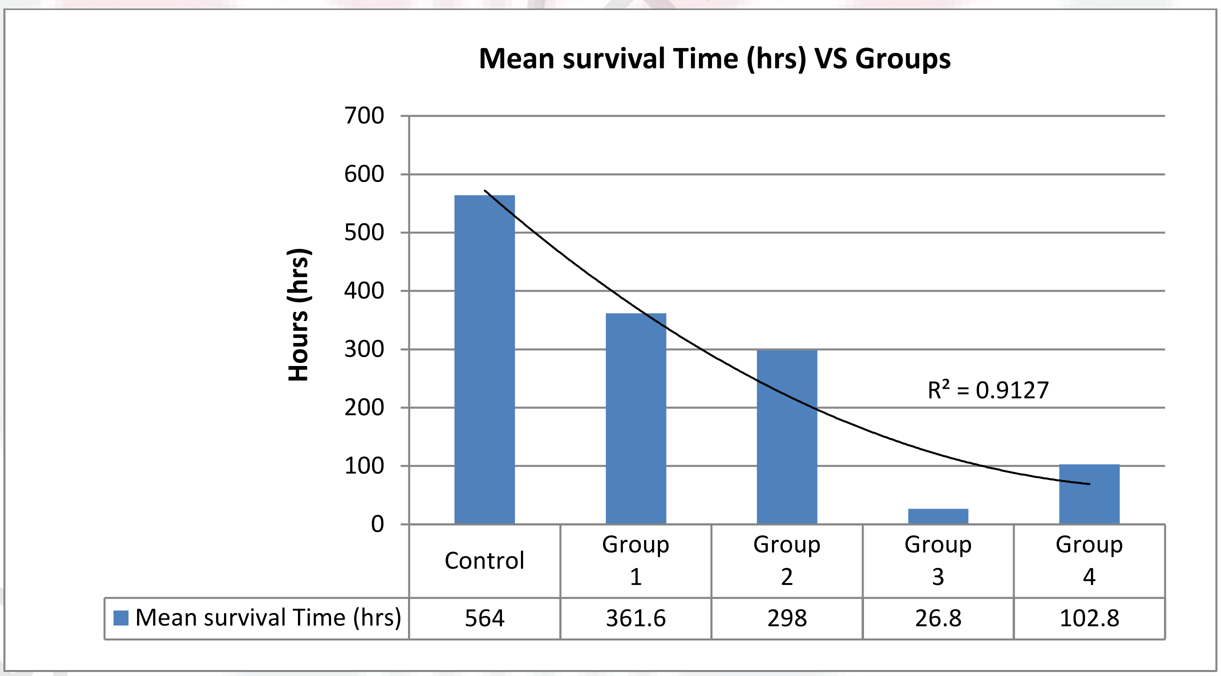


Figure 2: Mean survival times in different groups.

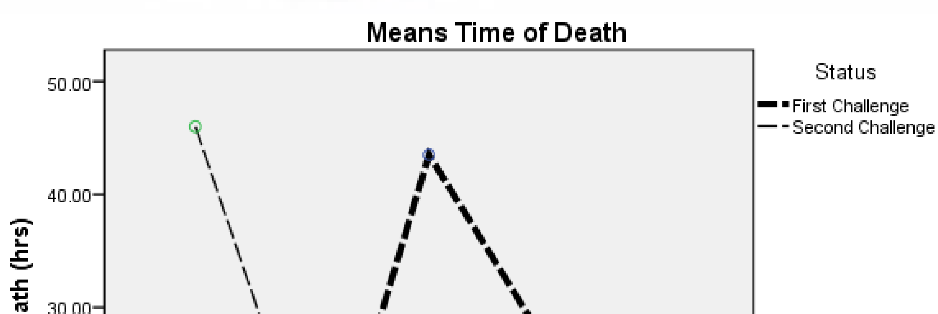


Figure 3: Mean time of death between groups for first and second challenge.

4.3 Detection of organism from cultures and microscope

Colony and bacterial morphology from the sub-cultured samples were examined. Most of the colonies were round greyish mucoid colony with smooth edges which size was ranged from 1 to 2 mm in diameter. All the plates observed have colonies which were non-haemolytic properties on blood agar and gave typical smell that resembles *P. multocida*. Only those colonies that fit the above characteristic were selected and staining was performed. Gram and Wright's staining reviewed present of gram negative coccobacillus bacteria with characteristic of bipolar staining in all the suspected colonies. Bacterial cultures from organs of survived mice from second challenge was significantly lower ($P < 0.05$) in heart, lung, liver, and spleen.

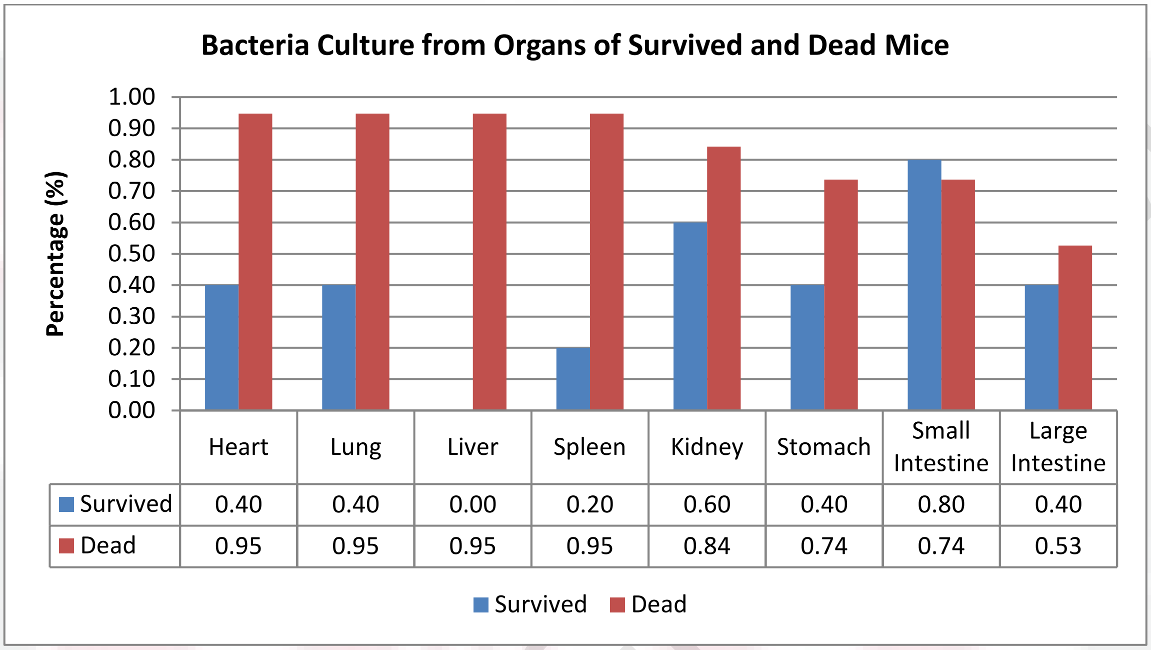


Figure 4: Bacteria Culture from Organs of Survived and Dead Mice.

4.4 Post Mortem and Histopathological Findings

Upon post mortem examination, all of the treatment mice have congested superficial blood vessels, heart, lung, liver spleen and kidney. Splenomegaly and nephromegaly were also commonly seen in all the death mice. Pin-point also haemorrhages were observed on lungs, liver, and kidneys in all the mice. Grossly, the serosa surfaces of the entire length of gastro-intestinal tract of all treatment groups look normal.

Inflammatory cells

In general, there were no significant different ($P > 0.05$) between inflammatory cells infiltration in heart, lung, kidney, stomach, small intestine and large intestine of treatment groups from control, all showing mild to moderate inflammatory response. However, mean score values were generally higher in most organs (heart, liver, kidney, and stomach) of

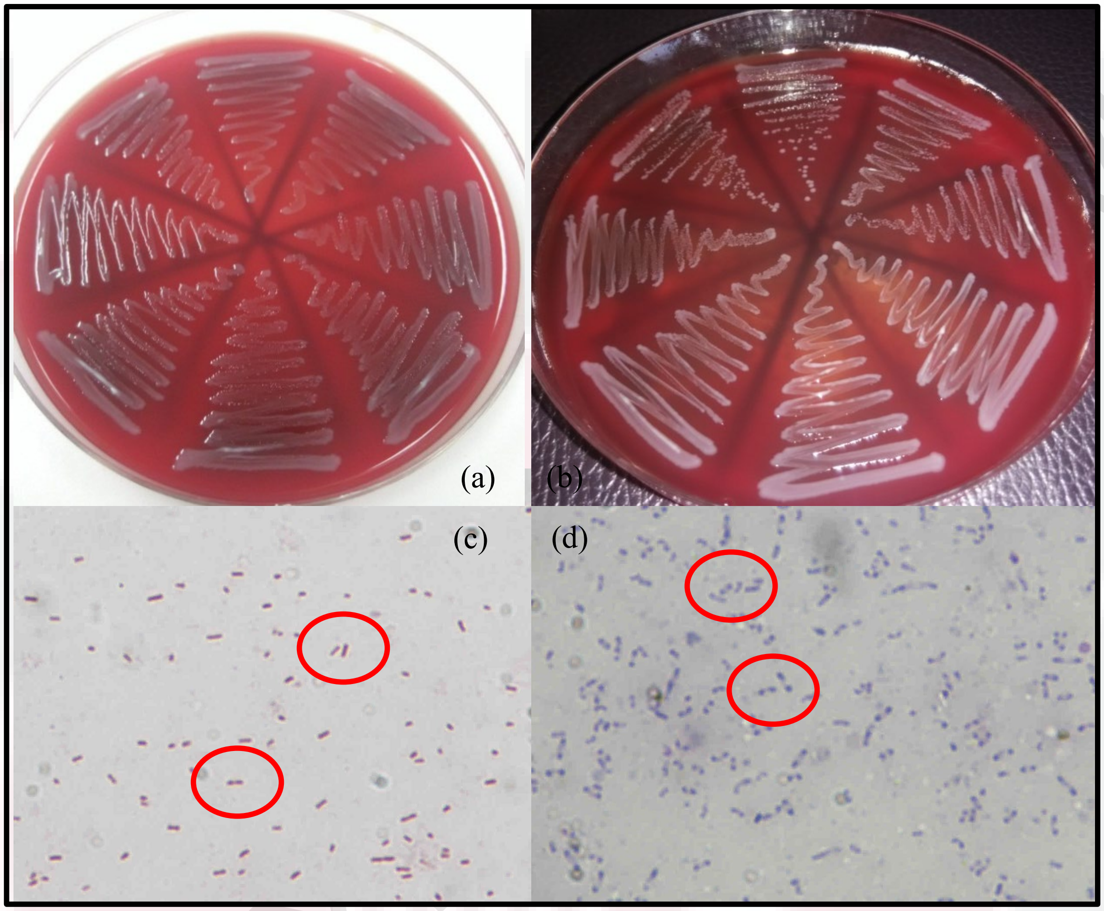
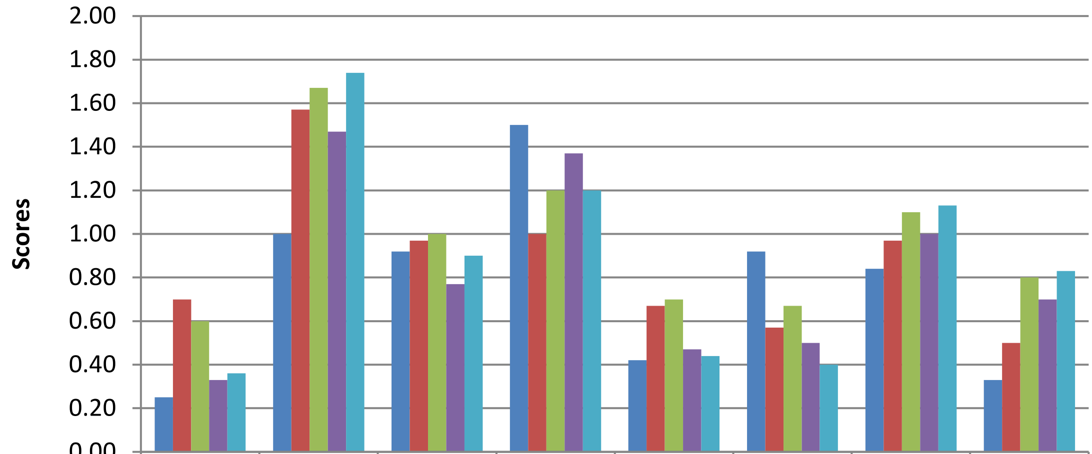


Figure 5: (a) blood culture showing greyish mucoid colonies; (b) blood culture showing rounded greyish colonies; (c) Gram negative rod bacteria; (d) Wright's stain showing bipolar rod bacteria.

Group 1 and 2 when compared to Group 3 and 4. Inflammatory cells found in liver of Group 2 were significantly higher ($P < 0.05$) than those in Group 3, while in spleen; it was significantly lower in Group 1 when compared to Group 2 and 4.



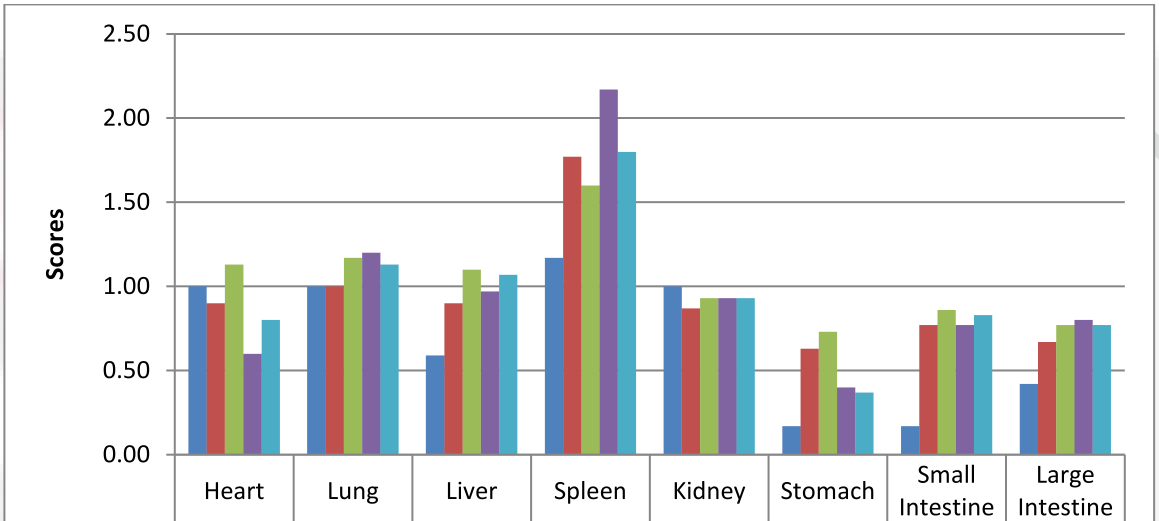
	Heart	Lung	Liver	Spleen	Kidney	Stomach	Small Intestine	Large Intestine
Control	0.25	1.00	0.92	1.50	0.42	0.92	0.84	0.33
Group 1	0.70	1.57	0.97	1.00	0.67	0.57	0.97	0.50
Group 2	0.60	1.67	1.00	1.20	0.70	0.67	1.10	0.80
Group 3	0.33	1.47	0.77	1.37	0.47	0.50	1.00	0.70
Group 4	0.36	1.74	0.90	1.20	0.44	0.40	1.13	0.83

Organs

Figure 6: Mean lesion scores of inflammatory cells in different organs of different groups

Haemorrhages and Congestions

Haemorrhage and congestion in all treatment groups showed to be mild to moderate. Although no statistical significant ($P > 0.05$) was proven, haemorrhage and congestion observed in all organs of Group 1 were generally mildest when compared to those in Group 2, 3 and 4, except heart and stomach. Heart of Group 2 was significantly more haemorrhagic and congested than Group 3. Mean lesion scores in liver and small intestine of Group 2 were significantly ($P <$ 0.05) congested when compared to control.

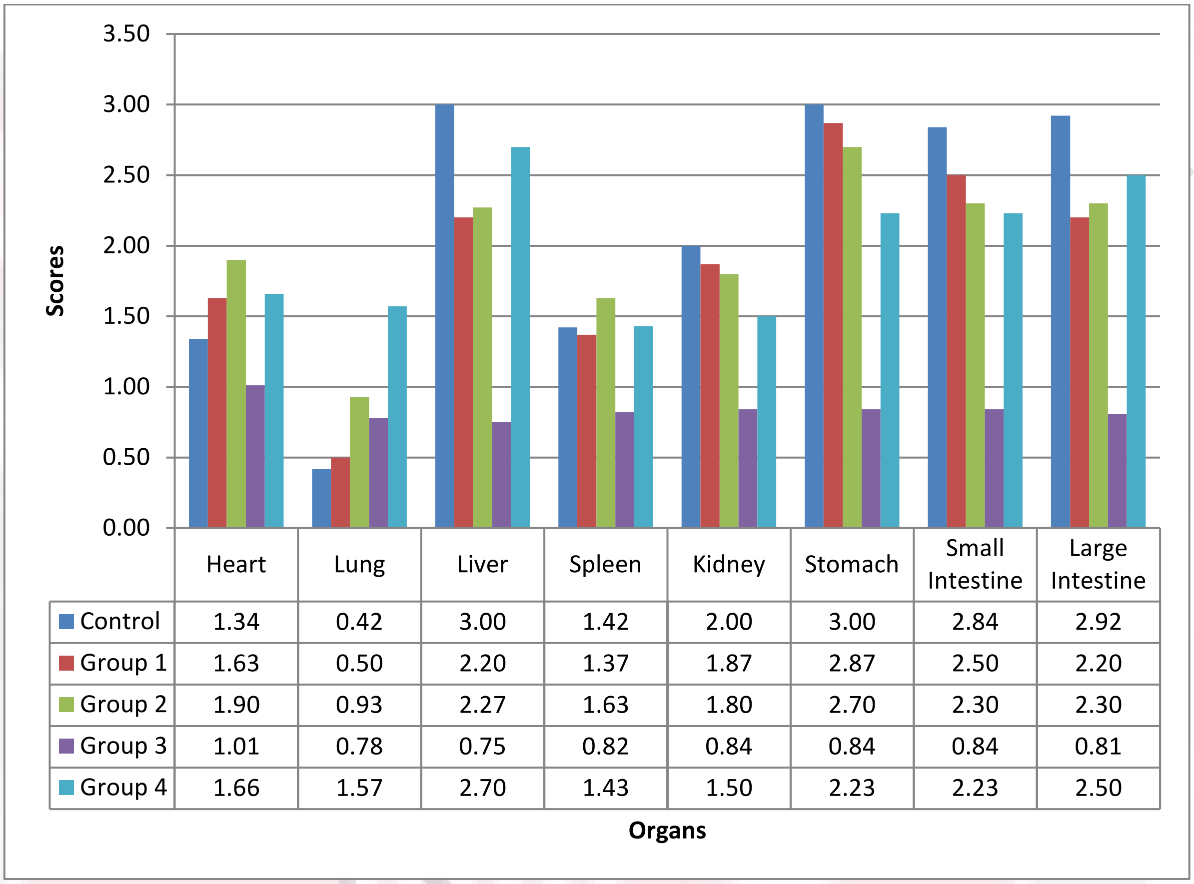


	Heart	Lung	Liver	Spleen	Kidney	Stomach	Small Intestine	Large Intestine
Control	1.00	1.00	0.59	1.17	1.00	0.17	0.17	0.42
Group 1	0.90	1.00	0.90	1.77	0.87	0.63	0.77	0.67
Group 2	1.13	1.17	1.10	1.60	0.93	0.73	0.86	0.77
Group 3	0.60	1.20	0.97	2.17	0.93	0.40	0.77	0.80
Group 4	0.80	1.13	1.07	1.80	0.93	0.37	0.83	0.77

Figure 7: Mean lesion scores of haemorrhage and congestions in different organs of different groups

Degeneration and necrosis

In general, only organs from Group 3 were showing mild degeneration and necrosis, all organs from other groups (except lung) showed moderate to severe degeneration and necrosis. The only organs which showed to be similar ($P > 0.05$) between all groups were the heart. Lungs of Group 4 were significantly ($P < 0.05$) more severe than Control, Group 1 and 3. Degeneration and necrosis from liver, spleen, kidney, stomach, small intestine and large intestine of Group 3 were all mildest among the treatment groups, however only liver, stomach, small intestine and large intestine were significantly milder ($P < 0.05$) when compared to control and other treatment groups.



	Heart	Lung	Liver	Spleen	Kidney	Stomach	Small Intestine	Large Intestine
Control	1.34	0.42	3.00	1.42	2.00	3.00	2.84	2.92
Group 1	1.63	0.50	2.20	1.37	1.87	2.87	2.50	2.20
Group 2	1.90	0.93	2.27	1.63	1.80	2.70	2.30	2.30
Group 3	1.01	0.78	0.75	0.82	0.84	0.84	0.84	0.81
Group 4	1.66	1.57	2.70	1.43	1.50	2.23	2.23	2.50

Figure 8: Mean lesion scores of degenerations and necrosis in different organs of different groups.

Oedema in Lungs

All treatment groups have mild oedema in their lungs. The oedema observed was not significantly ($P > 0.05$) different from the control, but the mean values were observed to be gradually decreased over groups, with Group 1 showed to be higher than Group 4.

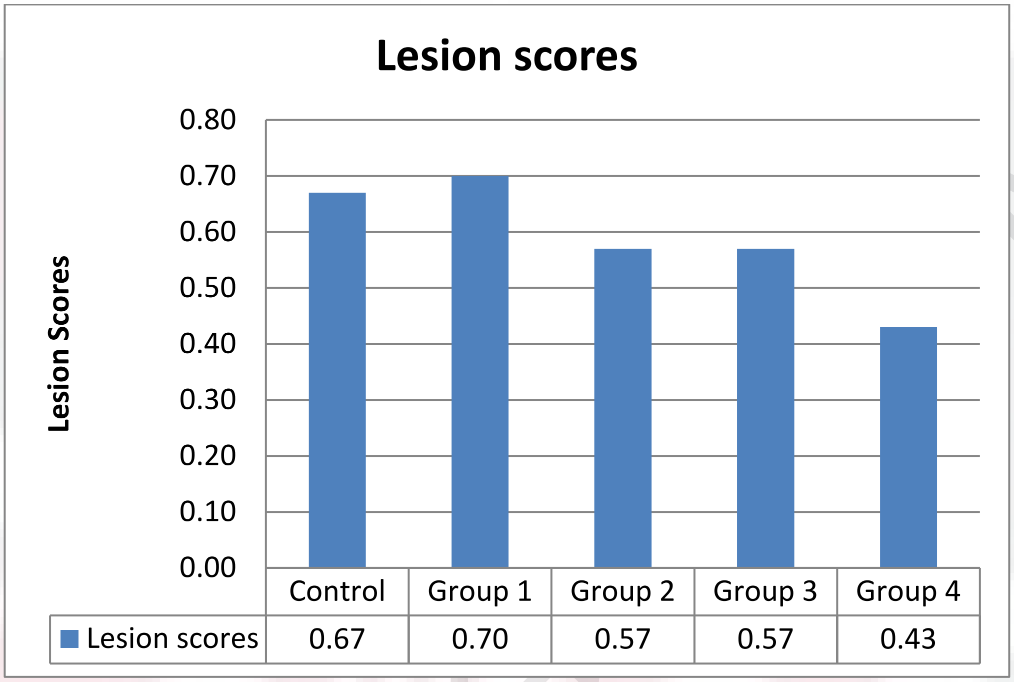


Figure 9: Mean lesion scores for oedema in different organs of different groups.

5.0 DISCUSSION

The pathogenesis of *P. multocida* is complex and varies depend on host and bacteria virulence factors (Boyce and Adler, 2006; Hunt *et al.,* 2000). Host factors can be include the species, age and immune status. Although the virulence factors of *P. multocida* has been identified as capsules and endotoxin, the mechanism by which these bacteria develop disease are poorly understood (Marza *et al.,* 2015). Due to the dynamic interaction between strains and host immune status, different severity of disease may result. In present studies, higher dose of oral bacterium (10^7 and 10^9 CFU) result in reduced in alertness and developed mild ocular discharges, but not in low dose of bacterium (10^3 and 10^5 CFU). The present of variation in severity of clinical signs observed indicate that it was dose dependent. These findings were different than those reported by Abdullah *et al.* (2014), where only ocular discharges was significantly higher in mice inoculated orally with 0.4ml of 10^7 CFU. The differences can be explained as most of the mice in our experiment were around 4 weeks old but those used by Addullah *et al.* (2014) was 5 weeks old.

In this study, mortality rate was observed to be in range between 20 to 100% in first challenged groups, while the rate increased to 66.6 to 100% when the mice were re-challenged with bacteria. Our finding was contradicting to those reported by Kharb and Chara (2013), they stated that regardless of route of inoculation, mortality in mice was 80% in all groups studied. The different could be due to the routes of inoculation in their study where the oral route was not included. Besides that, increased in mortality rate was also observed to be higher in higher dose of oral challenges. However, in Kharb and Charan (2013) experiment with 10^1, 10^3 and 10^5 CFU doses *P. multocida* B:2 inoculated subcutaneously and intra-nasally in mice model, found that the mice mortality were independent of the dose of inoculums. Similar finding was also reported by Annas *et al.* (2014) in calves experimentally infected subcutaneously with 10^5 and 10^9 CFU of *P. multocida* B:2. Our finding was totally contradicting to those reported by both of them, where mice mortality was dependent of the dose of oral inoculums. The increased in mortality could be due to the accumulation effect from the virulence factor from previous challenged which make the mice more immunosuppressed.

The most common histopathological lesions observed in mice after oral inoculation were inflammatory cells infiltrations; haemorrhages and congestions; degeneration and necrosis and oedema. The findings were similar to those reported in Khaleel *et al.* (2014) and Ali *et al.* (2015). All the histopathological lesions were generally found in all visceral organs (heart, lung, liver, spleen, kidney, stomach, small intestine and large intestine). Jesse *et* al (2013) found histopathological lesion in small intestine following oral inoculation of *P. multocida* B:2 in mice and speculated that the bacteria have certain affinity towards the enterocytes. Similar findings reported by Annas *et* al (2014) in buffaloes following subcutaneous inoculation.

From the result we found that heart, liver, spleen, kidney and stomach of Group 1 and 2 generally have higher number of inflammatory cells infiltration when compared to those found in Group 3 and 4. It was significantly higher in liver of Group 1 and spleen of Group 2 when compared to Group 3 and 4 respectively. The significantly higher in number of inflammatory cells response indicated that some immune responses have been developed and the memory cells was activated to release more inflammatory cells during the second challenge. As for haemorrhages and congestions, there were no significant different in all treatment groups.

For degenerations and necrosis, only Group 3 showed to develop mild lesions while others showed mild to moderate lesions. The degeneration and necrosis in liver, stomach, small and large intestines of Group 3 were significantly milder compared to other groups. When the survival time was analyzed, Group 3 showed to have lowest mean survival time among all groups. Due acute death of mice from endotoxin shock, the lesion might not have developed yet. Although pathogenesis of HS was not clear but it was believed that septicaemia developed leading to acute death due to endotoxaemia (Horadagoda et al. 2001). Generally all organs in Group 4 (mean survival time = 102 hrs), showed moderate to severe degeneration and necrosis when compared to other groups, but only lungs were showed to be statistically significant higher when compared to control. This shows that the extent of lesion manifestation depends on duration of the disease and the dose of bacterial inoculums.

Pasteurellosis can be easily treated by preparing autogenous vaccines from frequently isolated strains with most immunogenic characteristic (Youssef, 2011). Thus, it is crucial to use the strains that isolated in Kelantan during the HS outbreak and develop as vaccine. Ahmad *et al* (2014) stated that to achieve desired protection against *P. multocida*, vaccine program should be a combination of initial well adjuvanted killed vaccine and followed by lifetime oral doses of attenuated vaccine. There is tendency of farmers to only have their animals vaccinated during HS outbreak but not the years after the outbreak resulted in poor herd immunity (Bisht *et al.,* 2006). Thus, if oral vaccine can be developed and incorporated into drinking water or feed, the above situation can be avoided.

6.0 CONCLUSION

High dose of oral *P. multocida* Type B:2 will result in high mortality in mice, while mortality observed in lower dose is lesser. The present of survival mice in second challenged group indicated that they are indeed possible immunized to the bacteria. With the current study, we suggest that there is possibility of immune protection present in mice challenged with low dose of bacterium oral. However, further study on the serological differences induced by different graded dose of oral bacterium is needed to confirm it to make the dream of developing oral vaccine to become reality. In conclusion, better survivability was observed in oral bacterium of 10^3 and 10^5 CFU with milder clinical signs and histological lesions, while 10^7 and 10^9 CFU resulted in detrimental effects on mice. Thus, low but not high dose of oral inoculum was believed to induce immunity and possible to use as oral vaccine.

7.0 REFERENCES

Abdullah, J. F. F., Osman, A. Y., Adamu, L., Yusof, M., Syamil, M., Omar, A. R., & Saad, M. Z. (2013). Polymerase chain reaction detection of Pasteurella multocida type B: 2 in mice following oral inoculation. *Asian Journal of Animal and Veterinary Advances*, *8*(3), 493-501.

Abdullah, J., Firdaus, F., Adamu, L., Abba, Y., Tijjani, A., Mohammed, K., & Saharee, A. A. (2014). Effect of dose dependent oral inoculation of Pasteurella multocida type B: 2 in mice: Molecular detection and histopathological evaluation. *Research Opinions in Animal & Veterinary Sciences*, *4*(10).

Abubakar, M. S., & Zamri-Saad, M. (2011). Clinico-pathological changes in buffalo calves following oral exposure to Pasteurella multocida B: 2. *Basic and Applied Pathology*, *4*(4), 130-135.

Ahmad, T. A., Rammah, S. S., Sheweita, S. A., Haroun, M., & El-Sayed, L. H. (2014). Development of immunization trials against Pasteurella multocida.*Vaccine*, *32*(8), 909-917.

Aktories, Klaus, Joachim H. C Orth, and Ben Adler. 'Pasteurella Multocida'. Google Books. N.p., 2012.

Ali, O. S., Adamu, L., Abdullah, F. F. J., Abba, Y., Hamzah, H. B., Mohd-Azmi, M. L., ... & Zamri-Saad, M. (2015). Haematological and Histopathological Vicissitudes Following Oral Inoculation of Graded Doses of Pasteurella multocida Type B: 2 and its Lipopolysaccharide in Mice.*Veterinary Science & Technology*, *6*(2), 1.

Annas S, Zamri-Saad M, Abubakar MS, Jesse FFA, Zunita Z (2014) Distribution of *Pasteurella multocida* B:2 in the Respiratory, Gastrointestinal and Urinary Tracts of Buffaloes Following Experimental Subcutaneous Inoculation. J Veterinar Sci Technol 5: 177.

Benkirane A, De Alwis MCL (2002) Haemorrhagic septicaemia, its significance, prevention and control in Asia. Vet Med – Czech 47: 234-240

Bisht, K. S., Salim, N., Hassan, L., Zunita, Z., Kamarudin, M. I., & Saharee, A. A. (2006). Temporal Patterns of Haemorrhagic Septicaemia Mortalities Ion Cattle and Buffaloes in Peninsular Malaysia, 1993-2003. *Journal of Animal and Veterinary Advances*.

Boyce, J. D., & Adler, B. (2006). How does *Pasteurella multocida* respond to the host environment?. *Current opinion in microbiology*, *9*(1), 117-122.

Chung, E. L. T., Abdullah, F. F. J., Ibrahim, H. H., Marza, A. D., Zamri-Saad, M., Haron, A.

W., ... & Norsidin, M. J. (2016). Clinico-pathology, hematology and biochemistry responses in buffaloes towards Pasteurella multocida type B: 2 immunogen lypopolysaccharide via oral and intravenous routes of infection. *Microbial pathogenesis*, *91*, 141-154.

De Alwis, M . C . (1984) . Haemorrhagic septicaemia in cattle and buffaloes . Rev. Sci. Tech . Off. Int. Epiz . 3, 707-30.

De Alwis, M. C. (1982). Immune status of buffalo calves exposed to natural infection with haemorrhagic septicaemia. *Tropical animal health and production*, *14*(1), 29-30.

De Alwis, M. C. L. (1992, August). Pasteurellosis in production animals: a review. In *ACIAR proceedings* (Vol. 43, pp. 11-22).

Dua, S. K., & Maheswaran, S. K. (1978). Studies on Pasteurella multocida. VI. Nature of systemic immunity and analysis of the correlation between levels of immunity induced by various fowl cholera vaccines and protection against challenge. *Avian diseases*, 748-764.

Dziva, F., Muhairwa, A. P., Bisgaard, M., & Christensen, H. (2008). Diagnostic and typing options for investigating diseases associated with Pasteurella multocida. *Veterinary Microbiology*, *128*(1), 1-22.

Fao.org,. 'EMPRES Transboundary Animal Diseases Bulletin No. 27-2005'. N.p., 2005.

Homchampa, P., Strugnell, R. A., & Adler, B. (1997). Cross protective immunity conferred by a marker-free aroA mutant of Pasteurella multocida. *Vaccine*, *15*(2), 203-208.

Horadagoda, N. U., Hodgson, J. C., Moon, G. M., Wijewardana, T. G., & Eckersall, P. D. (2001). Role of endotoxin in the pathogenesis of haemorrhagic septicaemia in the buffalo. Microbial pathogenesis, 30(3), 171-178.

Hunt, M. L., Adler, B., & Townsend, K. M. (2000). The molecular biology of Pasteurella multocida. *Veterinary microbiology*, *72*(1), 3-25.

Hussaini, J., Abdullah, M. A., & Ismail, S. (2012). Expression and immunogenicity determination of recombinant clone of Pasteurella multocida serotype B against hemorrhagic septicaemia towards a vaccine development. Journal of Animal and Veterinary Advances, 11(3), 315–356.

Hussaini, Jamal, and Noor Masyitah Jumahat. 'Characterization Of Recombinant Protein Of Pasteurella Multocida Serotype B'. N.p., 2014

Jesse, F. F., Affandi, S. A., Osman, A. Y., Adamu, L., Saad, M. Z., Haron, A. W., ... & Saharee, A. A. (2013). Clinico-pathological features in mice following oral exposure to Pasteurella multocida B: 2. *IOSR J. Agric. Vet. Sci*, *3*(4), 35-39.

Jumahat, Noor, Zaini Zain, and Jamal Hussaini. 'Identification Of Immunogenic Soluble Protein Of Pasteurella Multocida'. Journal of Advanced Biomedical & Pathobiology Research 5.1 (2015): n. pag.

Kalorey, D. R., Yuvaraj, S., Vanjari, S. S., Gunjal, P. S., Dhanawade, N. B., Barbuddhe, S. B., & Bhandarkar, A. G. (2008). PCR analysis of Pasteurella multocida isolates from an outbreak of pasteurellosis in Indian pigs.*Comparative immunology, microbiology and infectious diseases*, *31*(6), 459-465.

Kehrenberg, C., Salmon, S. A., Watts, J. L., & Schwarz, S. (2001). Tetracycline resistance genes in isolates of Pasteurella multocida, Mannheimia haemolytica, Mannheimia glucosida and Mannheimia varigena from bovine and swine respiratory disease: intergeneric spread of the tet (H) plasmid pMHT1. *Journal of Antimicrobial Chemotherapy*, *48*(5), 631-640.

Khaleel, M. M., Abdullah, F. F. J., Adamu, L., Abba, Y., Haron, A. W., Saad, M. Z., & Omar, A.

R. (2014). Histopathological changes in mice infected with river water contaminated by Pasteurella multocida type B: 2. *American Journal of Animal and Veterinary Sciences*, *9*(2), 71.

Kharb, S., & Charan, S. (2013). Mouse model of haemorrhagic septicaemia: dissemination and multiplication of Pasteurella multocida B: 2 in vital organs after intranasal and subcutaneous challenge in mice. *Veterinary research communications*, *37*(1), 59-63.

Luo, Y., Zeng, Q., Glisson, J. R., Jackwood, M. W., Cheng, I. H. N., & Wang, C. (1999). Sequence analysis of Pasteurella multocida major outer membrane protein (OmpH) and application of synthetic peptides in vaccination of chickens against homologous strain challenge. *Vaccine*,*17*(7), 821-831.

Marza, A. D., Abdullah, F. F. J., Ahmed, I. M., Chung, E. L. T., Ibrahim, H. H., Zamri-Saad, M., ... & Lila, M. A. M. (2015). Involvement of nervous system in cattle and buffaloes due to *Pasteurella multocida* B: 2 infection: A review of clinicopathological and pathophysiological changes. *Journal of Advanced Veterinary and Animal Research*, *2*(3), 252-262.

OIE, 2008. Chapter 2.4.12- Haemorrhagic septicaemia. Terrestrial Manual

OIE, 2012. Haemorrhagic septicemia. Terrestrial Manual.

Pati, U. S., Srivastava, S. K., Roy, S. C., & More, T. (1996). Immunogenicity of outer membrane protein of *Pasteurella multocida* in buffalo calves. *Veterinary Microbiology*, *52*(3), 301-311.

Ruffolo, C. G., Jost, B. H., & Adler, B. (1998). Iron-regulated outer membrane proteins of Pasteurella multocida and their role in immunity.*Veterinary microbiology*, *59*(2), 123-137.

Saharee, A.A., N.B. Salim, A. Rasedee and M.R. Jainudeen,1993. Haemorrhagic septicaemia carriers among cattle and buffaloes in Malaysia. In: Patten, B.E., T.L. Spencer, R.B. Johnson, D. Hoffmann and L. Lehane (Eds.), Pasteurellosis in production animals. Proceeding of An International Workshop. Bali, Indonesia, August 10-13, 1992. ACIAR Proceedings No. 43: 89-91.

Seleim, R. S. (2003). Review: Major Pathogenic Components Of Pasteurella multocida And Mannheimia (Pasteurella) Haemolytica Isolated From Animal Origin.

Shivachandra, S. B., Viswas, K. N., & Kumar, A. A. (2011). A review of hemorrhagic septicemia in cattle and buffalo. *Animal Health Research Reviews*, *12*(01), 67-82.

Sugun, Manasa et al. 'Isolation And In Vitro Antibiotic Susceptibility Of Pasteurella Multocida From Cattle Origin. International Research Journal Of Microbiology'. ResearchGate. N.p., 2013.

Tabatabai, L. B., & Zehr, E. S. (2004). Identification of five outer membrane-associated proteins among cross-protective factor proteins of Pasteurella multocida. *Infection and immunity*, *72*(2), 1195-1198.

Verma, R., & Jaiswal, T. N. (1998). Haemorrhagic septicaemia vaccines.*Vaccine*, *16*(11), 1184-1192.

Youssef, E. A. (2011). Study of pathogenicity and immunogenicity of Pasteurella multocida recently isolated from infected rabbits. *Egyptian Journal of Agricultural Research*.

Zamri-Saad, Haemorrhagic Septicaemia of Cattle & Buffaloes in Asia, Universiti Putra Malaysia Press, Selangor, 2013.

8.0 APPENDICES

Table 2: Mean scores of clinical signs observed in different groups

	Control	Group 1	Group 2	Group 3	Group 4
Level of Alertness	0.00±0.00[a]	0.23±0.37	0.35±0.37	0.57±0.18[a]	0.65±0.38[a]
Ruffled Fur	0.15±0.18	0.38±0.26	0.35±0.22	0.30±0.20	0.31±0.30
Ocular Discharges	0.00±0.00[b]	0.06±0.03	0.04±0.02	0.13±0.08[b]	0.10±0.06[b]
Laboured Breathing	0.00±0.00	0.09±0.12	0.14±0.15	0.24±0.20	0.27±0.31

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

Table 3: Survival times in between different groups

Groups	**Survived Time (hrs)**						**Mean survived time (hrs)**
	Mice 1	**Mice 2**	**Mice 3**	**Mice 4**	**Mice 5**	**Mice 6**	
Control	564	564	35*	57*	564*	564*	564.00±0.00[a]
Group 1	9	405*	413*	417*	564*	-	361.60±207.90
Group 2	12	75	412*	427*	564*	-	298.00±240.79
Group 3	4	19	24	41	46	-	26.80±17.02[a]
Group 4	10	19	37	44	404*	-	102.80±168.93[a]

[a] The mean difference is significant at 0.05 level.
Value with * indicate survival time from second challenge.

Table 4: Time of Death between Groups

Groups	**Mice 1**	**Mice 2**	**Mice 3**	**Mice 4**	**Mice 5**	**Mice 6**	**Mean Time of Death (hrs)**
	First Challenge						
Control	na	na	na	na	na	na	na
1	9	na	na	na	na	-	9
2	12	75	na	na	na	-	43.5
3	4	19	24	41	46	-	26.8
4	10	19	37	44	na	-	27.5
	Second Challenge						**Mean Time of Death (hrs)**
Control	35	57	na	na	na	na	46
1		9	17	21	na	-	15.67
2			16	31	na	-	23.50
3						-	-
4					8	-	8.00

na; not available as mice survived

Table 5: Distribution of inflammatory cell response in different organs of different groups.

Organs	Control	Group 1 (10^3 CFU)	Group 2 (10^5 CFU)	Group 3 (10^7 CFU)	Group 4 (10^9 CFU)
Heart	0.25±0.11	0.70±0.34	0.60±0.15	0.33±0.31	0.36±0.76
Lung	1.00±0.24	1.57±0.37	1.67±0.52	1.47±0.53	1.74±0.25
Liver	0.92±0.12	0.97±0.08	1.00±0.00^{a}	0.77±0.19^{a}	0.90±0.09
Spleen	1.50±0.00	1.90±0.42^{a,b}	1.20±0.28^{a}	1.37±0.48	1.20±0.22^{b}
Kidney	0.42±0.12	0.67±0.20	0.70±0.22	0.47±0.32	0.44±0.25
Stomach	0.92±0.12	0.57±0.19	0.67±0.26	0.50±0.26	0.40±0.38
Small Intestine	0.84±0.23	0.97±0.27	1.10±0.25	1.00±0.43	1.13±0.32
Large Intestine	0.33±0.00	0.50±0.26	0.80±0.22	0.70±0.18	0.83±0.31

Values are expressed as mean ± SD, ^{a,b} Values within rows are significant at $P < 0.05$.

Table 6: Distribution of haemorrhages and congestion in different organs of different groups.

Organs	Control	Group 1 (10^3 CFU)	Group 2 (10^5 CFU)	Group 3 (10^7 CFU)	Group 4 (10^9 CFU)
Heart	1.00±0.00	0.90±0.15	1.13±0.30^{a}	0.60±0.34^{a}	0.80±0.18
Lung	1.00±0.00	1.00±0.00	1.17±0.24	1.20±0.14	1.13±0.14
Liver	0.59±0.12^{a}	0.93±0.25	1.10±0.22^{a}	0.97±0.14	1.07±0.09^{a}
Spleen	1.17±0.23	1.77±0.53	1.60±0.35	2.17±0.39	1.80±0.49
Kidney	1.00±0.00	0.87±0.22	0.93±0.09	0.93±0.15	0.93±0.15
Stomach	0.17±0.00	0.63±0.27	0.73±0.15	0.40±0.42	0.37±0.14
Small Intestine	0.17±0.00^{a}	0.77±0.28	0.86±0.08^{a}	0.77±0.15	0.83±0.17
Large Intestine	0.42±0.12	0.67±0.20	0.77±0.25	0.80±0.14	0.77±0.33

Values are expressed as mean ± SD, ^{a}Values within rows are significant at $P < 0.05$.

Table 7: Distribution of degenerations and necrosis in different organs of different groups.

Organs	Control	Group 1 (10^3 CFU)	Group 2 (10^5 CFU)	Group 3 (10^7 CFU)	Group 4 (10^9 CFU)
Heart	1.34±0.94	1.63±0.50	1.90±0.68	1.01±0.13	1.66±0.42
Lung	0.42±0.12^{a}	0.50±0.37^{b}	0.93±0.48^{c}	0.78±0.16^{d}	1.57±0.23^{a,b,c,d}
Liver	3.00±0.00^{a}	2.20±0.69^{b}	2.27±0.63^{c}	0.75±0.17^{a,b,c,d}	2.70±0.25^{d}
Spleen	1.42±0.35	1.37±0.27^{a}	1.63±0.32^{b}	0.82±0.12^{a,b,c}	1.43±0.30^{c}
Kidney	2.00±0.24^{a}	1.87±0.30^{b}	1.80±0.30^{c}	0.84±0.12^{a,b,c,d}	1.50±0.26^{d}
Stomach	3.00±0.00^{a}	2.87±0.22^{b}	2.70±0.22^{c}	0.84±0.14^{a,b,c,d}	2.23±0.58^{d}
Small Intestine	2.84±0.23^{a}	2.50±0.33^{b}	2.30±0.22^{c}	0.84±0.14^{a,b,c,d}	2.23±0.70^{d}
Large Intestine	2.92±0.12^{a}	2.20±0.49^{b}	2.20±0.46^{c}	0.81±0.14^{a,b,c,d}	2.50±0.33^{d}

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

Table 8: Distribution of oedema in lung of different groups.

Groups	Control	Group 1 (10^3 CFU)	Group 2 (10^5 CFU)	Group 3 (10^7 CFU)	Group 4 (10^9 CFU)
Lesion scores	0.67±0.24	0.70±0.25	0.57±0.19	0.57±0.52	0.43±0.22

Values are expressed as mean ± SD. No significant different between groups.

	Survived			Die		
Organs	**Postive**	**Negative**	**Total**	**Postive**	**Negative**	**Total**
Heart	2	3	5	18	1	19
Lung	2	3	5	18	1	19
Liver	0	1	1	18	1	19
Spleen	1	4	5	18	1	19
Kidney	3	2	5	16	3	19
Stomach	2	3	5	14	5	19
Small Intestine	4	1	5	14	5	19
Large Intestine	2	3	5	10	9	19

Table 9: Total of positive and negative bacterial culture from organs of survived and dead mice.

\

Figure 10: Showed one mouse reduced alertness and isolate from others.

Figure 11: Mice showed ruffled fur and huddle together.

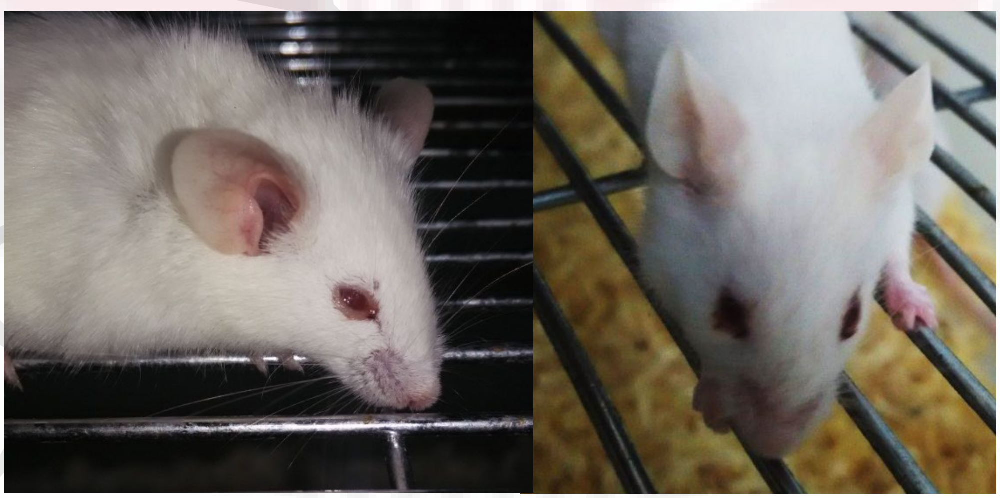


Figure 12: Mice showed severe ocular discharges.

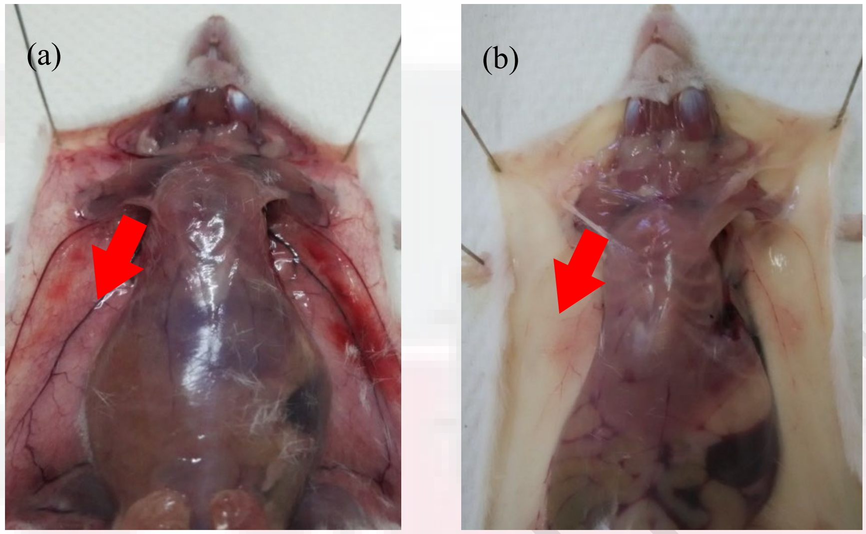


Figure 13:(a) Congestion of superficial blood vessels;(b) normal superficial blood vessels

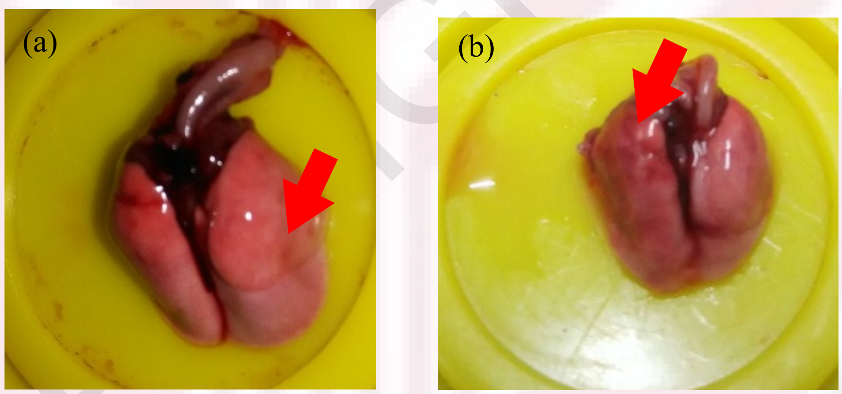


Figure 14a,b: Haemorrhage and congested lungs.

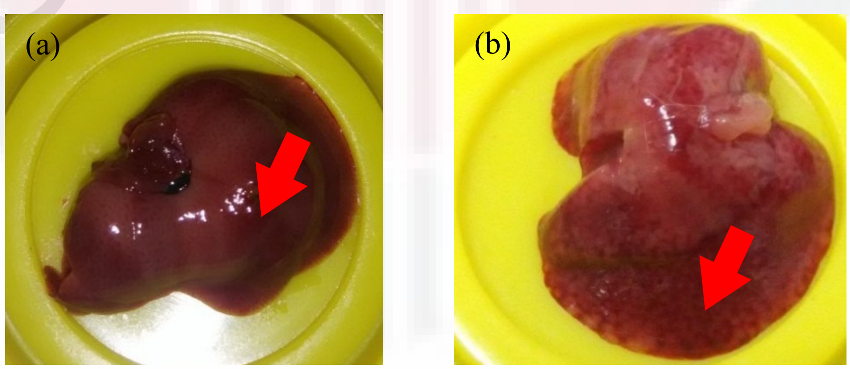


Figure 15a,b: Haemorrhage and congested liver.

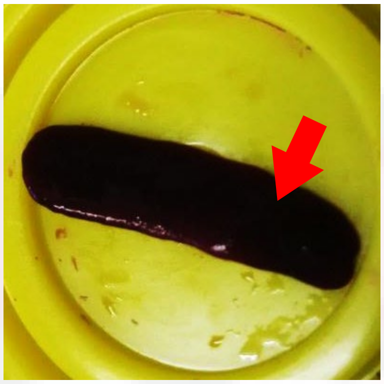


Figure 16: Congested and enlarged spleen.

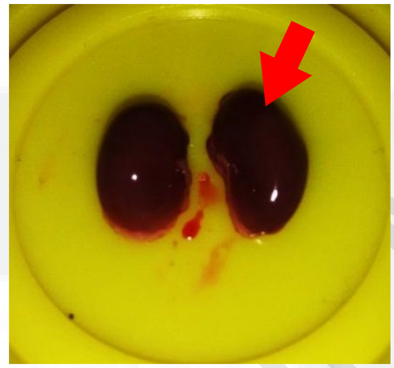


Figure 17: Congested and enlarged kidneys.

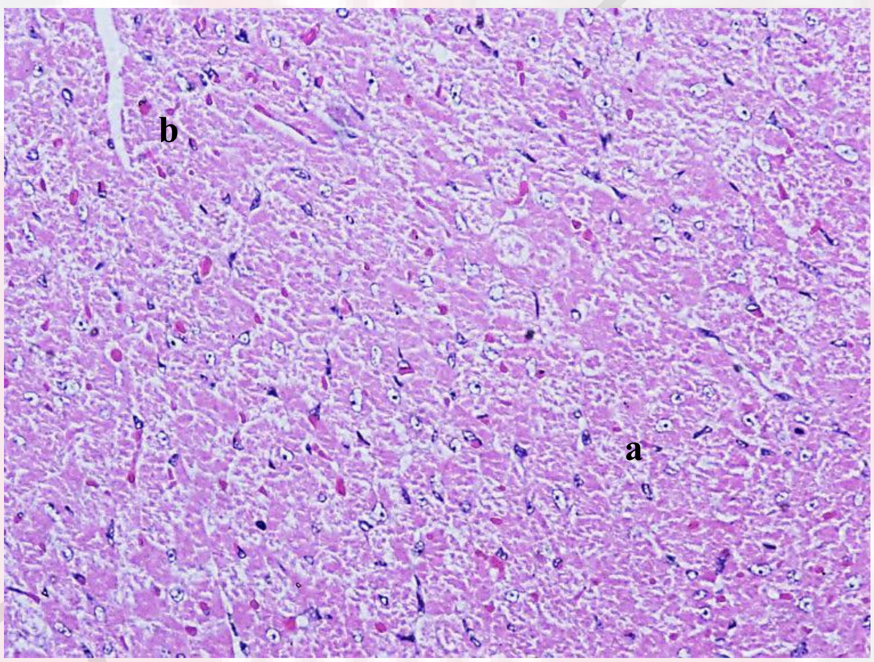


Figure 18: Photomicrograph of the heart, H&E, 400X. a) Degeneration and necrosis; b) inflammatory cells infiltration

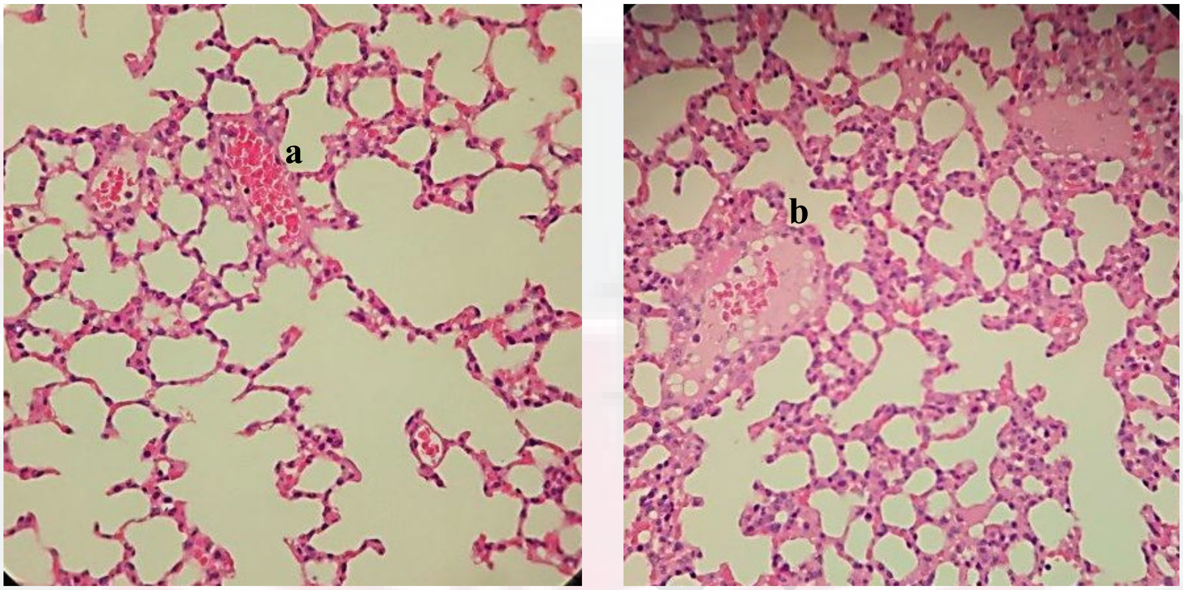


Figure 19: Photomicrograph of the lung, H&E, 400X. a) Mild inflammatory cells infiltration and mild congestion; b) Moderate inflammatory cells infiltration with mild congestion and oedema

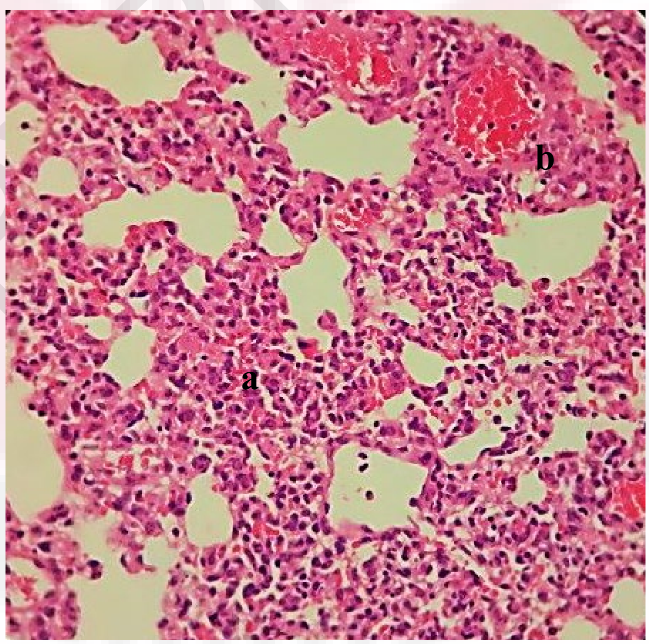


Figure 20: Photomicrograph of the lung, H&E, 400X. a) Severe inflammatory cells infiltration; b) Mild haemorrhage and congestion

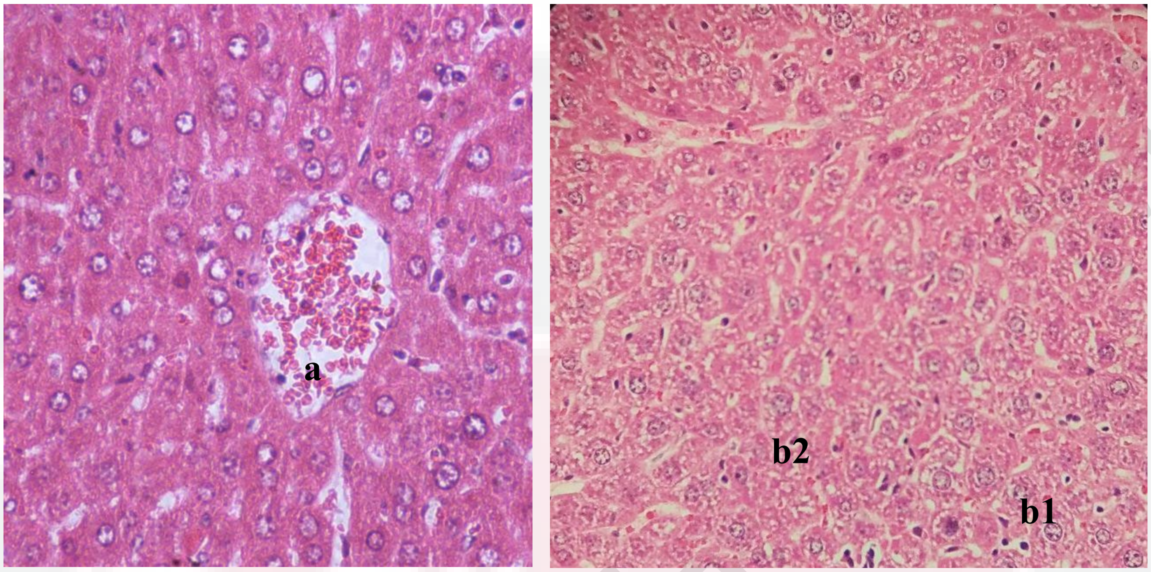


Figure 21: Photomicrograph of the liver, H&E, 400X. a) Congested central vein; b1) inflammatory cells infiltration; b2) degeneration and necrosis

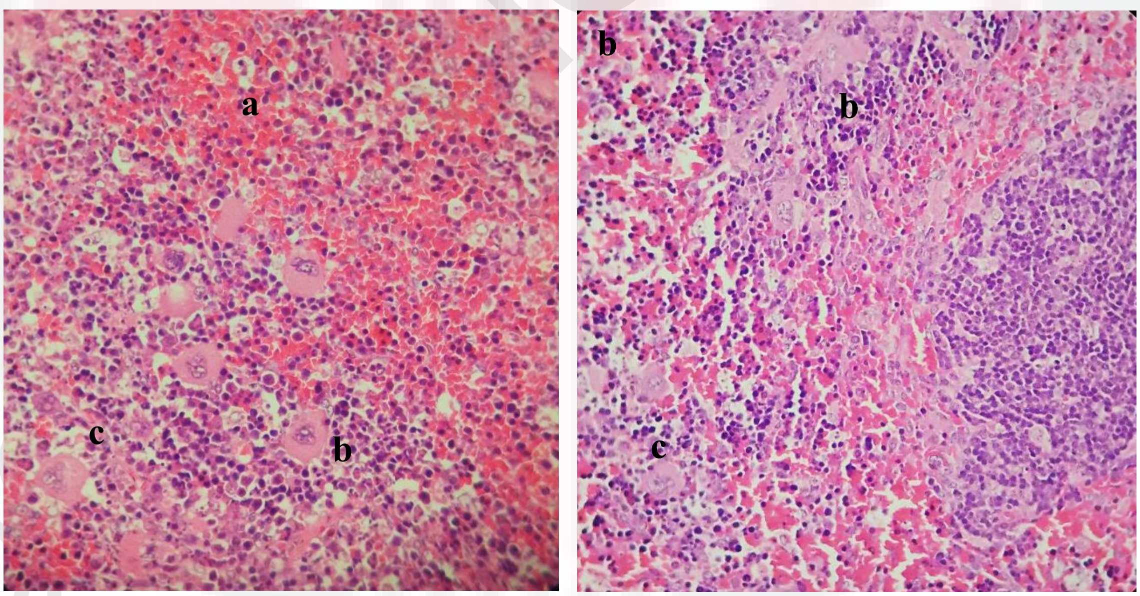


Figure 22: Photomicrograph of the spleen, H&E, 400X. a) Haemorrhage and congestion; b) inflammatory cells infiltration; c) degeneration and necrosis

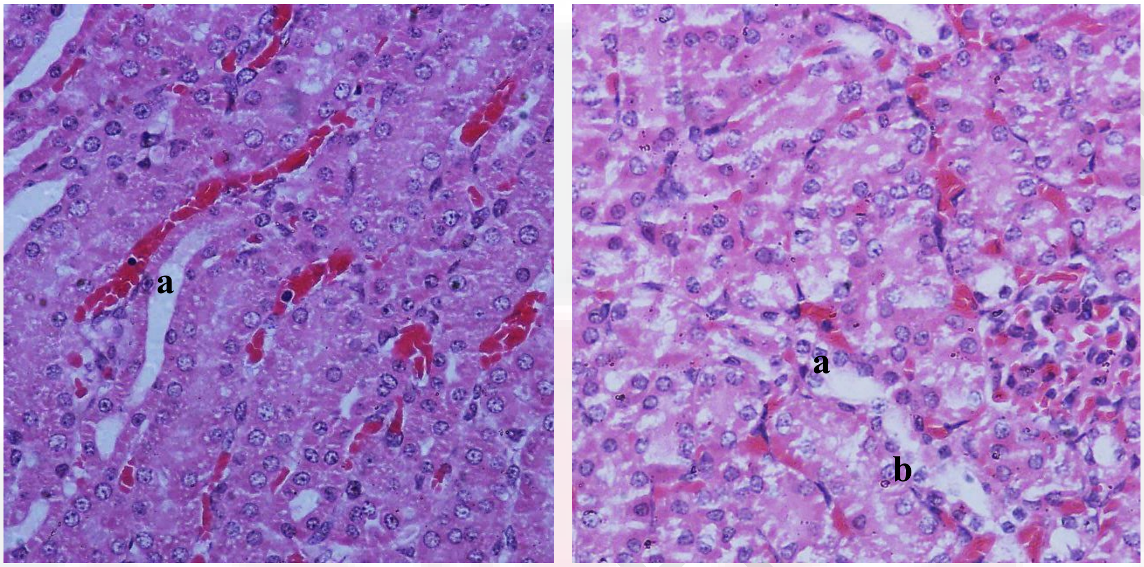


Figure 23: Photomicrograph of the kidney, H&E, 400X. a) Mild haemorrhage and congestion; b) Mild degeneration and necrosis

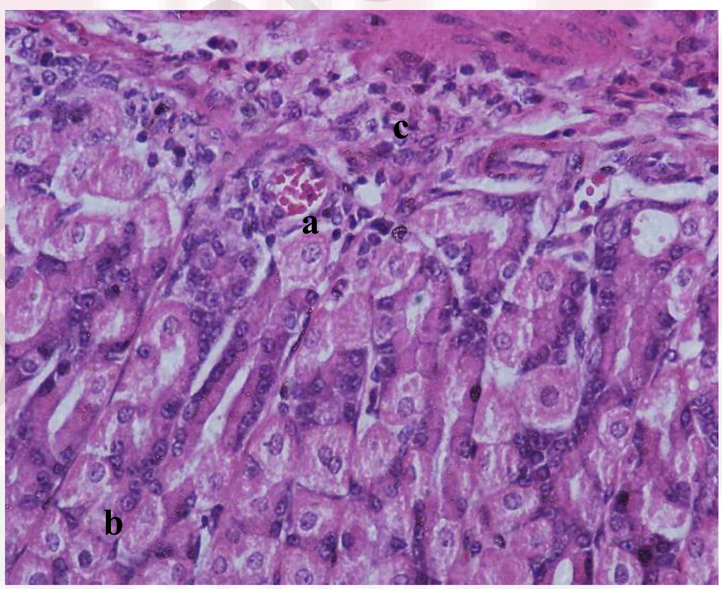


Figure 24: Photomicrograph of the stomach, H&E, 400X. a) Mild haemorrhage and congestion; b) Mild degeneration and necrosis; c) mild inflammatory cell infiltration

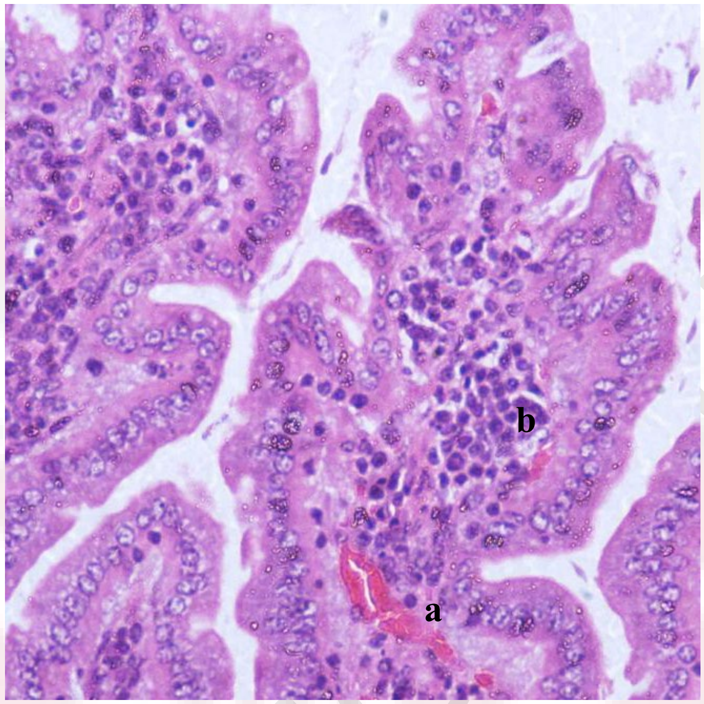


Figure 25: Photomicrograph of the small intestine, H&E, 400X. a) Mild haemorrhage and congestion; b) Mild inflammatory cell infiltration

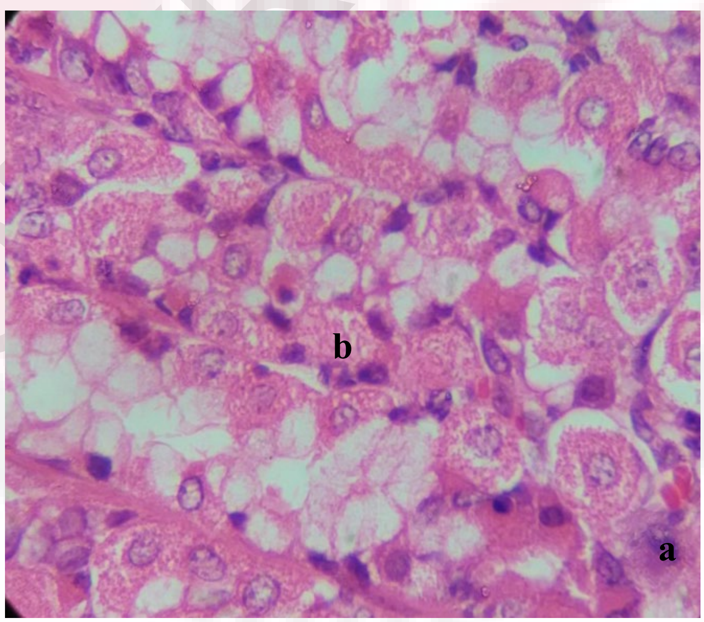


Figure 26: Photomicrograph of the large intestine, H&E, 400X. a) Mild haemorrhage and congestion; b) Mild degeneration and necrosis