UNIVERSITI PUTRA MALAYSIA

COMPARISON OF PULMONARY MACROPHAGE RESPONSE BETWEEN EXPERIMENTAL BUFFALO WITH ACUTE HAEMORRHAGIC SEPTICAEMIA AND CARRIER BUFFALO

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FACULTY OF VETERINARY MEDICINE
UNIVERSITI PUTRA MALAYSIA
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THANUSHA RAJU

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CERTIFICATION

It is hereby certified that we have read this project paper entitled "Comparison of Pulmonary Macrophage Response between Experimental Buffalo with Acute Haemorrhagic Septicaemia and Carrier Buffalo" by Thanusha Raju and in our opinion, it is satisfactory in terms of scope, quality and presentation as partial fulfillment of the requirement for the course VPD 4999 – Final Year Project.

DR. ANNAS SALLEH

DVM (UPM), PhD (UPM),

Senior Lecturer,
Faculty of Veterinary Medicine,
Universiti Putra Malaysia.
(Supervisor)

PROF DR. MOHD ZAMRI SAAD

DVM (UPM), PhD (University of Liverpool,UK),

Senior Lecturer,
Faculty of Veterinary Medicine,
Universiti Putra Malaysia.
(Co-Supervisor)

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PERBANDINGAN RESPONS MAKROFAG ANTARA KERBAU EKSPERIMEN HAWAR BERDARAH AKUT DAN PEMBAWA

Oleh

Thanusha a/p Raju

2020

Penyelia: Dr Annas Salleh

Penyelia Bersama: Prof Dr. Zamri Saad

Pasteurella multocida merupakan flora biasa di saluran pernafasan atas dalam banyak haiwan termasuk bovid domestic dan liar. Seperti yang diketahui, *P.multocida* serotaip spesifik B:2 atau E:2 adalah punca kepada penyakit hawar berdarah atau *haemorrhagic septicaemia (HS)*. Penyakit ini bersifat pasteurellosis yang akut dan memudaratkan dimana ia melibatkan kerbau, lembu dan bison. Objektif pertama kajian ini adalah untuk membandingkan populasi makrofag antara kerbau HS akut dan pembawa. Objektif kedua pula adalah untuk membandingkan kadar fagositosis makrofag paru-paru antara kerbau HS akut dan

pembawa. HS ialah penyakit bakteria kerbau air dan lembu yang penting dari segi ekonomi terutamanya di Asia Tenggara di mana populasi kerbau air adalah tinggi. Kajian ini menggunakan sampel slaid histologi dari kajian yang pernah dilakukan di mana kajian ini melibatkan enam kerbau yang dibahagi kepada dua kumpulan. Kumpulan 1 telah diinokulatkan dengan 1 ml inokulum yang mengandungi 10^5 cfu/ml *P. multocida* B:2 secara 'intranasal' (kumpulan akut). Kerbau kumpulan 2 pula tidak diinokulat tetapi dibenarkan untuk berinteraksi dengan kerbau dari kumpulan 1 untuk mewujudkan kumpulan haiwan pembawa. Kerbau dari kumpulan 1 telah mati dalam masa 68 hingga 72 jam selepas inokulasi dilakukan dan haiwan pembawa dari kumpulan 2 telah disembelih. Sampel paru-paru telah difiksasi, diproses dan pengwarnaan dengan H&E, dan immunoperoxidase (IP) terhadap sel B:2 *P. multocida* telah dilakukan. Slaid-slaid ini diguna untuk membezakan tindak balas makrofag paru-paru antara kerbau akut dan pembawa. Slaid yang telah distain dengan H&E digunakan untuk mendiskripsi lesi dan skor diberi terhadap populasi makrofag paru-paru di 10 hpf. Slaid yang telah distain dengan IP pula digunakan untuk memberi skor terhadap makrofag yang mempunyai stain IP yang positif dan tidak mempunyai stain IP yang positif bagi menentukan kadar fagositosis makrofag. Keputusan kajian ini menunjukkan kerbau akut mempunyai populasi dan kadar fagositosis makrofag yang lebih tinggi berbanding kerbau pembawa walaupun signifikan data tidak dapat dibuktikan. Populasi dan kadar fagositosis makrofag boleh menjadi faktor penentu infeksi *P. multocida* B:2 dalam kerbau.

Kata Kunci: *Haemorrhagic septicaemia, P.multocida*, Kerbau, Makrofag Paru-Paru

ABSTRACT

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

COMPARISON OF PULMONARY MACROPHAGE RESPONSE BETWEEN EXPERIMENTAL BUFFALO WITH ACUTE HAEMORRHAGIC SEPTICAEMIA AND CARRIER BUFFALOES

by

Thanusha Raju

2020

Supervisor: Dr Annas Salleh

Co-supervisor: Prof Dr. Zamri Saad

Pasteurella multocida is a normal flora of the upper respiratory tract of many animals including the domestic and wild bovids. *P.multocida* of specific serotype B:2 or E:2 are known to cause hemorrhagic septicaemia (HS). This disease is an acute and deadly form of pasteurellosis that affects mainly water buffalo, cattle, and bison. The first objective of this study was to compare the population of pulmonary macrophage between buffalo with acute and carrier HS. The second objective was to compare the phagocytosis rate of pulmonary macrophage between buffalo with acute and carrier HS. This study was done using histology slide samples from previous study where 6 buffaloes were used which

were divided into 2 groups. Group 1 was infected with 1 ml of inoculum containing 10^5 cfu/ml of *P. multocida* B:2, intranasally (acute group). Group 2 were buffaloes which were not inoculated, but allowed to co-mingle with Group 1 buffaloes in order to create carrier animals. Buffaloes from Group 1 died within 68 to 72 hours post-inoculation and carrier animals were slaughtered. Samples of the lungs were previously fixed, processed, and stained with hematoxylin and eosin (H&E) and immunoperoxidase (IP) against whole cell *P. multocida* B:2. These slides were used in this study where the pulmonary macrophage response were compared with acute and carrier buffaloes. The slides stained with H&E were used to describe the lesions, and to score the population of pulmonary macrophages at 10 random high power fields (hpf). On the other hand, the slides stained with immunoperoxidase (IP) were used to score the population of macrophages with and without the positive immunostaining against *P. multocida* B:2 to determine the phagocytosis rate. Results showed that the acutely infected buffaloes had a higher macrophage population and phagocytosis rate compared to carrier buffaloes eventhough the significance of the data was not proven due to lack of data. The macrophage population and phagocytosis rate may play an important role in deciding the course of *P.multocida* B:2 infection in buffaloes.

Keywords: *Haemorrhagic septicaemia, P.multocida,* Buffaloes , Pulmonary Macrophage

1.0 INTRODUCTION

Pasteurella multocida is a normal flora of the upper respiratory tract of many animals including the domestic and wild bovids. *P.multocida* of specific serotype B: 2 or E: 2 are known to cause hemorrhagic septicaemia (HS). This disease is an acute and deadly form of pasteurellosis that affects mainly water buffalo, cattle, and bison (MSD Veterinary Manual, 2014). Buffaloes are more prone to HS compared to cattle. HS is uncommon in deer, camels, elephants, horses, donkeys and yaks (Oie.int, 2020), while there was no report of natural infection in sheep, goats, and swine.

HS is an economically important bacterial disease of water buffalo and cattle especially in Southeast Asia, where water buffalo are highly populated (MSD Veterinary Manual, 2014). The disease is endemic in India and Southeast Asia with high mortality rate (Khin et *al*., 2010). Buffalo and cattle that survive the acute infection usually will become carriers of the disease. They usually harbor the pathogen in the tonsils. Up to 5% of healthy buffalo are colonized by small numbers of *P. multocida* serotype B:2 or E:2 (MSD Veterinary Manual, 2014), which can be shed with the presence of stressors such as excessive temperature and humidity, coexistent diseases, nutrient deficiency or work stress, mostly during the rainy season (MSD Veterinary Manual, 2014). The infection occurs either through direct contact with the oral or nasal droplets or by ingestion of contaminated feed or water (MSD Veterinary Manual, 2014). When *P. multocida* B:2 enters through respiratory tract, there is mild lesions in the lungs prior entering

the blood circulation through the pulmonary capillaries which will eventually cause severe septicaemia (Khin et *al.*, 2010).

The clinical signs of HS are death within 8-24 hours, ptyalism, nasal discharge, and respiratory difficulties (MSD Veterinary Manual, 2014). In infected animal, there are four clinical syndromes. Firstly, the animal will become hyperthermic with body temperature above 40°C, then develops submandibular edema and later respiratory difficulties with copious nasal discharge, and finally recumbency and death. The histopathological lesions such as haemorrhage, hyperemia, edema and white blood cells infiltration were observed in the lungs, lymph nodes, spleen, gastrointestinal tract, liver, kidney and the heart (Chung *et a*l., 2015).

It was previously proven that infection could occur via the respiratory and gastrointestinal tract, with the former being the most important organ in the pathogenesis of HS. The lungs, where *P. multocida* B:2 could be isolated in abundance in cases of HS, possess residence macrophage that are vital for the pulmonary immunity. The response of pulmonary macrophage is known to determine the outcome of a disease, including septicaemic diseases. However, the response of pulmonary macrophage in septicaemic and carrier buffaloes of HS is currently unknown.

Thus, the objectives of this study are:

1. To compare the population of pulmonary macrophage between buffalo with acute HS and HS-carrier buffalo.

2. To compare the phagocytosis rate of pulmonary macrophage between a buffalo with acute and carrier HS buffalo.

While the hypothesis for this study are:

H_O1: Pulmonary macrophage population is comparable between the acutely infected buffalo and the carrier buffalo.

H_A1: Pulmonary macrophage population is higher in the acutely infected buffalo compared to the carrier buffalo.

H_O2: The macrophage phagocytosis rate is similar in the acutely infected buffalo compared to the carrier buffalo.

H_A2: The macrophage phagocytosis rate is higher in the acutely infected buffalo compared to the carrier buffalo.

2.0 LITERATURE REVIEW

2.1 Water Buffaloes

Water buffalo (*Bubalus bubalis*) is the scientific name for water buffaloes. The suspected indigenous domain of *B.bubalis* was from Central India to southern Nepal in the west to Vietnam and East Malaysia. They are herbivorous mammals that mostly consume grass but also eats many other vegetation that grows in or along rivers and streams. Bulls have horns that are huge, curved backwards and crescent-shaped and cows have smaller horns compared to the bulls. The feral bull may have a body weight to almost 1,200 kg, and feral cows reaching 800 kg

whereas tamed water buffalo range from 250 to 550 kg. Their hooves are gaped widely to help them from sinking into the mud and eases their mobilization through wetlands and swamps. The water buffaloes are known to be land animals however it spends most of its time in rivers or mud holes. Since water buffaloes have lesser sweat glands than other bovids, they are delicate to heat which may cause heat stress. Thus, they cool themselves by wallowing in the mud or finds a shelter. Cows have a gestation period 9 to 11 months where it calves down every alternate year (Water Buffalo | National Geographic, 2020).

5,000 years ago, water buffalo was domesticated making them economically important. Not only that, 5% of the global milk supply are from water buffaloes. The milk compositions are more in fat, lactose, protein and lesser water compared to cow's milk. The examples of byproducts are butter,oil butter oil (ghee), high grade cheeses, and many more. Also, it has soft and tasty meat which is impossible to compare with beef, leather can be produced from the skin, feces used as fertilizer and is used as a draught animal (Roth, J. 2004).

2.2 *Pasteurella multocida*

Pasteurella multocida, a Gram-negative, non-motile coccobacillus, causes fowl cholera in poultry, hemorrhagic septicemia in cattle, atrophic rhinitis in swine and snuffles in rabbits. There are five serogroups (A, B, D, E, and F) and 16 somatic serotypes (1–16) . The virulence factors of *P.multocida* strains that have been found before are hemagglutinins, fimbriae, lipopolysaccharides (LPS), hyaluronidase, iron regulated and iron acquisition proteins, the capsule, and a dermonecrotic toxin found in type D strains (Guo et al., 2012).

Buffaloes in stressful environment may cause the agent to proliferate which leads to the deadly pasteurellosis. Nearly 100% death rate may occur once clinical manifestation is shown as buffaloes are the native host for *P.multocida* (Rajagopal et *al*., 2010).

Lungs, nasopharynx and tonsils are infected with *P.multocida* prior to spreading to the other organs. Animals undergoing stress from transportation, high density population, poor nutrition and ventilation succumbs the disease easily as *P.multocida* is a commensal of the buccal-pharyngeal region where the agent multiplies and spreads to the lower respiratory tract leading to HS or pneumonic pasteurellosis. An important feature in all forms of pasteurellosis is septicemia. The incubation period lasts from 3 to 5 days. Sudden death with absence of clinical manifestations happens in peracute cases (Mohammed et *al.,* 2018).

2.3 Haemorrhagic septicaemia

P.multocida serotype B: 2 and E: 2 results in HS which is an acute, fatal and septicaemic pasteurellosis (Ara, MS et *al.*, 2016 & Annas et *al.,* 2015) predominantly in water buffalo, cattle, and bison. (MSD Veterinary Manual, 2014). Buffaloes are more prone to get infected with HS but less likely in sheep, goats and swine whereas rare in deer, camels, elephants, horses, donkeys and yaks (Oie.int, 2020).

HS often occurs in poor farm managed environment and where HS monitoring is not well evolved. Besides that, fatigue, chilling, malnourishment, changes of feed, lack of water, captivated, weakened state and overpopulated environment are the other stressors contributing to HS (Chung *et al.,* 2015).

Merging clinical manifestation, gross pathology lesions, herd background, morbidity and mortality pattern, species predisposition ,age factor and laboratory diagnosis aids in HS diagnosis. Isolation and identification of *P.multocida* can be carried out for morphological, cultural and biochemistry characteristic. Serological, biochemistry and molecular tests such as Polymerase Chain Reaction (PCR) was used in a mice to identify *P.multocida* B:2 which has been infected through contaminated river stream (Chung *et al*., 2015 & Oie, 2009). *P.multocida* can be confirmed after fresh blood smear is stained with Gram's, Leishman's or Methylene Blue *demonstrates* a Gram negative, bipolar, pleomorphic bacterial cells (Shivachandra, Viswas and Kumar., 2020).

HS has three forms which are acute, sub-acute and carrier forms. Acute HS demonstrates immediate onset and death within 1 day (Shivachandra, Viswas and Kumar., 2020). Affected animals exhibit hyperthermia about 41–42 ℃, faint pulse with fast, shallow breathing and cyanotic mucous membrane. Besides that, other signs that can be seen are anxious, mild colic pain, muscle tremors, tearing, nasal discharge and diarrhea. Next, animals in the sub-acute form shows throat and brisket edema swelling and bronchopneumonia where the animal lives for 2–3 days. Then, animals in the chronic form which has the prolonged infection demonstrates short, fast hurting respiration together with mucopurulent or blood-stained nasal discharge (Shivachandra, Viswas and Kumar., 2020 & OIE, 2009). When necropsy is performed, subcutaneous edema, especially in the mandibular and brisket regions is apparent. Petechial-to-echymotic hemorrhages, congestion and/or lungs consolidation, fibrinous pneumonia, pleurisy and pericarditis are other lesions seen (Shivachandra, Viswas and Kumar., 2020). The lungs, lymph nodes, spleen,

gastrointestinal tract, liver, kidney and heart showed reddening, oedema and infiltration of leukocytes which was observed in a bison calf induced with HS B:2 experimentally. Few experiments were carried out where different inoculation routes were used and it showed that exposure of agent via oral route had milder clinical manifestation compared to intratracheal or respiratory routes (Chung *et al*., 2015).

Treatment of HS is of no value due to the acute nature of the disease leads to inability for early detection of the disease. However, sulfonomides, streptomycin and oxytetracycline are examples of antimicrobial *P.multocida* is sensitive to. Fusing penicillin-streptomycin or one dose of long acting oxytetracycline was shown previously to be successful in managing the clinical manifestation and death rate (Chung et *al.,* 2015).

Vaccination is an important measure in order to prevent and control HS. Bacterins, Alum-Precipitated Vaccine (APV) and Oil-Adjuvanted Vaccine (OAV) are routinely used against HS (Chung *et a*l., 2015 & OIE, 2008). Since 1966, OAV was used in Malaysia to act as an preventive method whereas when outbreak occurs, broth bacterin is administered for immediate protection to vulnerable. HS is impossible to be eradicated because of the presence of latent carriers (Chung *et a*l., 2015). Although these vaccines are known to provide high antibody titre, the biggest challenge is to have wide vaccination coverage. This is highly related to the laborious intramuscular administration of vaccine, and the extensive rearing system of buffalo in South and South East Asian countries.

2.4 Economic Importance

HS emerges after the successful eradication of rinderpest in almost all Asian countries where it is rated as an important fatal disease (DE ALWIS, 1984). The economic importance is higher in Asia compared to Africa (Benkirane & MCL De., 2012). HS has a significant economic importance in countries such as Malaysia and the whole Southeast Asia where cattle and buffaloes produce plenty beef and milk. The rice production can also be affected because the HS occurrence among working animals are high (Chung *et al*., 2015).

In 1900, the first HS epidemic was reported in Malaysia where the meat and dairy industry was badly affected. Approximately RM2.4 million loss happens yearly as an impact of HS to the cattle and buffalo related industry . insufficient vaccination and poor veterinary assistance in Peninsular Malaysia lead to high death rate (D.V, R., 2018).

A total of 432 million cattle and 146 million buffaloes are predisposed to HS in Asia which is 30% and 95% of the world's cattle and buffalo population, respectively. The highest milk production in Asia is India from which about 50% of the milk is produced by the more susceptible buffaloes. Milk production by buffaloes throughout Asia is 37%. HS causes economic losses because there is high population of buffalo in Asia, thus higher predisposition to HS leading to elevated fatality case. HS is categorized as economically important disease of cattle and buffaloes in Southeast Asia countries such as Indonesia, Malaysia, Thailand, Myanmar, Laos, Cambodia and the Philippines. In Malaysia there are 735 000 cattle

and 186 000 buffaloes with a loss of RM2.25 million (Benkirane & MCL De., 2012).

2.5 Immunity

The immune system is comprised of the innate and humoral immunity where it defends the body against foreign organism or antigens. Innate immunity acts as a first line defence that blocks pathogen entry whereas the humoral immunity produces specific antibodies against pathogens. The ratio of immune to non immune animals will directly affect the morbidity and mortality of HS. So, naturally gained immunity is significant in determining the different patterns of morbidity and mortality although there is also relation between naturally acquired immunity and carrier animals. Persistent carriers are animals that have high antibody titre post infection where their survival is responsible outbreaks to occur from time to time (Chung *et a*l., 2015).

2.6 Bovine Respiratory Tract

The respiratory system is divided into the upper part where it is comprised of nose, nasal cavity, pharnyx and larynx and the lower part where it comprised of trachea and the lungs (bronchi, bronchiole and alveoli). There are 8 lung lobes in bovids and they are right cranial cranial, right cranial caudal, right middle, right caudal, left cranial, left middle, left caudal and accessory lobe. The respiratory tract is an essential path for infection in animals and humans. For instance, HS in buffaloes and cattle. Thus, the respiratory system has its own defence mechanism

such as mucous production, presence of cilia at the epithelium and nasal associated lymphoid tissue (NALT) to protect against any infection.

2.7 Alveolar Macrophage

Alveolar macrophage resides in the lungs and it has contrast functions and immunological characteristics which makes it different from the rest of its kind. The alveolus has an aerobic surrounding and the pulmonary macrophage has suited itself accordingly. In addition, alveolar macrophage carries out phagocytosis, produces lysozyme and interferon, and cytotoxicity for cells infected with virus which helps in clearance of the lungs and host resistance against bacterial and viral invasion. It engages in the immune reaction as well (N.J. Khadom et al., 1985).

The innate immunity consists of macrophages and neutrophils which eliminate and destroys foreign invaders during an infection as it is the first line of defense. As for now, adhesion and phagocytosis followed by bactericidal activity of *P. multocida* by macrophage has been experimented in turkeys, chicken, mouse and bovine. It has been reported before in a study the reaction of leukotoxin from *M. haemolytica* and lipopolyscharide (LPS) endotoxin from *P. multocida* with bovine white blood cells developed in high number of cell death including apoptosis and necrosis. Data from in-vivo experiment proposes that during pasteurellosis, endotoxin from *P. multocida* may lead to drastic inflammatory changes and tissue pathology. Despite this, the studies on alveolar macrophage on *P. multocida* is very little where the ability of this cells are not known clearly (Abubakar and Zamri-Saad., 2011). Taking into account the gap of knowledge in this matter, this study

was conducted to compare the phagocytosis rate and the population of pulmonary macrophage in acute and carrier buffaloes infected with *P. multocida* serotype B:2 and the factors that determine the outcome (acute mortality or development of carrier) of *P. multocida* B:2 infection is unknown.

3.0 MATERIALS AND METHODS

3.1 EXPERIMENTAL DESIGN

This study was conducted using histology slide samples obtained from a previous study (Annas. S., 2015). Briefly, six healthy buffaloes were divided into two groups. Group 1 was infected with 1 ml of inoculum containing 10^5 cfu/ml of *P. multocida* B:2, intranasally. Group 2 buffaloes were not inoculated, but allowed to co-mingle with Group 1 buffaloes in order to create carrier animals. Buffaloes of Group 1 succumbed to acute HS. On the other hand, all buffaloes of Group 2 survived and were confirmed as carrier buffaloes based on the clinical examination, positive isolation of *P. multocida* B:2 from deep nasal swab samples, and from detection of the pathogen in the tonsil, lungs, and gastrointestinal tract using PCR and immunohistochemistry. Samples of the lungs were previously fixed, processed, and stained with H&E, and immunoperoxidase (IP) against whole cell *P. multocida* B:2. These slides were used in this study.

3.2 EXPERIMENTAL PROCEDURE:

There were three sets of slides for the acute group and two for the carrier group where all the eight lung lobes had to be viewed for both H&E and IP stained slides. The eight lung lobes are right cranial cranial, right cranial caudal, right

middle, right caudal, left cranial, left middle, left caudal and accessory lobe. For the H&E stain, the lesions were described and scored based on Abu Bakar et *al.*, 2011. Also, the macrophage population were calculated by counting macrophage based on ten random high power field (hpf) under 100x magnification.

Then, for the slides stained with IP, the evaluation was done based on presence and intensity of the immunoreactivity and its distribution where the intensity for IP was described as no detectable reaction, light brown, brown and golden brown with the guidance of Abu Bakar et *al.*, 2011.

Also, the macrophage with and without the positive immunostaining were counted based on ten random hpf under 100x magnification to determine the phagocytosis rate. Calculation was based on the following formula:

Phagocytosis rate = (number of macrophage with positive immunostaining) / (total number of macrophage) x 100%.

3.3 DATA ANALYSIS

All the data were recorded and tabulated to make a descriptive analysis based on the comparison of pulmonary macrophage response between acutely infected buffaloes and carrier buffaloes.

4.0 RESULTS

4.1 Lesion Description

Histopathological evaluation of the sections of the eight lung lobes in the acutely infected buffaloes showed severe thickening of the interalveolar septa and

interlobular septa. Thickening of the interlobular septa was largely contributed by interlobular oedema. On the other hand, thickening of the interalveolar septa occurred due to severe congestion of the interalveolar capillaries. Oedema fluid was observed in most alveolar spaces. The capillaries adjacent to the bronchus was severely congested whereas mildly congested in capillaries adjacent to the bronchiole. There was presence of moderate fibrin in the lumen of bronchus and bronchioles. Alveolar macrophage was present in all lung lobes where severe distribution was seen in right cranial cranial , right cranial caudal and left caudal lung lobe. The rest of the lung lobes had moderately distributed macrophages.

The carrier buffaloes on the other hand showed severe interlobular and interalveolar septa thickness. There were certain places where the interalveolar septa was not thickened. The alveolar interstitium was mildly congested. Mild oedema was seen in the alveolar spaces. The capillaries adjacent to the bronchus was mildly congested but not detectable in bronchiole. The bronchus and bronchiole had mild presence of fibrin. Alveolar macrophage was moderately present in all lung lobes but severely present in right cranial cranial , right cranial caudal and right caudal lung lobe.

Next, the immunoreactivity in the carrier buffaloes showed diffuse light brown intensity at the interalveolar septa and space. Similarly, was observed at the interlobular septa. The lumen and connective tissues surrounding the bronchus and bronchiole showed diffuse light brown intensity. The capillaries adjacent to the bronchus and bronchiole had a multifocal light brown distribution at its lumen and connective tissue. However, there was an exception for animal 3 where the left

middle lung lobe had diffuse dark brown intensity throughout the slide. The alveolar macrophage had diffuse light brown to brown intensity which was seen at the cytoplasm of the alveolar macrophage.

The acutely infected buffaloes showed a variation of diffuse light brown, brown and golden brown intensity at the interalveolar and interlobular septa. The alveolar space had multifocal brown and light brown intensity. There was a mixture of multifocal and diffuse brown in the blood vessel lumen. Also, the tunica intima of the blood vessel had a diffuse dark brown intensity, tunica media with diffuse brown intensity and diffuse light brown to brown intensity at the tunica adventitia. The lumen of the bronchus had a diffuse light brown intensity. Likewise, diffuse dark brown intensity was observed at the ciliated pseudostratified epithelium of the bronchus. The lamina propria had multifocal brown intensity whereas the submucosa and the loose connective tissue surrounding the bronchus had diffuse brown intensity. The muscularis layer had multifocal light brown intensity. The chondrocytes also were seen to have multifocal brown to golden brown intensity. The bronchiole had diffuse brown to golden brown intensity at the simple cuboidal epithelium. The lumen of the bronchiole had multifocal light brown intensity. Diffuse brown intensity was seen at the lamina propria whereas the submucosa had multifocal brown intensity. The muscularis layer had focal light brown intensity. The loose connective tissue surrounding the bronchiole had diffuse brown intensity. The alveolar macrophage had diffuse brown to golden brown intensity at the cytoplasm.

4.2 Macrophage Population

In general, all lung lobes of the acutely infected buffalo showed higher number of alveolar macrophage compared to the carrier buffaloes.

In the acute group, animal 2 had the highest macrophage population in the right cranial cranial and left caudal lung lobe whereas least macrophage population in the right caudal lung lobe. Animal 3 had the highest macrophage population in the right cranial caudal lung lobe and least macrophage population in the accessory lung lobe. Animal 4 had the highest macrophage population in the accessory lung lobe and least macrophage population in the left middle lung lobe.

In the carrier group, animal 3 had the highest macrophage population in the right caudal lung lobe and least macrophage population in the left middle lung lobe. Animal 6 had the highest macrophage population in the right cranial cranial and cranial caudal lung lobe and the least macrophage population in the right middle lung lobe.

Although statistical analysis was not done to proof the statistical significance, highest alveolar macrophage population between the acute and carrier animals was observed in the right cranial cranial and right cranial caudal lung lobe whereas lowest in the left middle lung lobes.

Table 1 summarizes the alveolar macrophage population between the acutely infected buffalo and the carrier buffalo.

Table 1: Comparison of macrophage population in different lungs lobes between acutely infected buffalo with *P. multocida* B:2 and the carrier buffaloes.

Lung lobe	Acute (2)	Acute (3)	Acute (4)	Carrier (3)	Carrier (6)
R CRCR	39	32	39	19	21
R CRCAU	30	35	42	10	21
R MID	23	28	21	16	9
RCAU	20	26	15	23	10
L CR	32	19	40	13	12
L MID	35	33	14	6	10
L CAU	39	17	17	20	10
ACC	27	11	49	10	11

Red = highest in macrophage population
Green = lowest in macrophage population

4.3 Macrophage phagocytosis rate

In general, acutely infected buffalo showed higher macrophage phagocytosis rate compared to the carrier buffaloes.

In the acute group, animal 2 had the highest macrophage phagocytosis rate in the right cranial cranial and the least macrophage phagocytosis rate in the accessory lung lobe. Animal 3 had the highest macrophage phagocytosis rate in the left caudal lung lobe and the least macrophage phagocytosis rate in the left middle lung lobe. Animal 4 had the highest macrophage phagocytosis rate in the left middle lung lobe and the least macrophage phagocytosis rate in the right caudal lung lobe.

In the carrier group, animal 3 had the highest macrophage phagocytosis rate in the right caudal lung lobe and least macrophage phagocytosis rate in the left cranial

lung lobe. Animal 6 had the highest macrophage phagocytosis rate in the left middle and the least macrophage phagocytosis rate in the accessory lung lobe.

Although statistical analysis was not done to indicate the statistical significance, highest alveolar macrophage phagocytosis rate between the acute and carrier animals was observed highest in the right cranial caudal lung lobe whereas lowest in the accessory lung lobe.

Table 2 summarizes the phagocytosis rate between the acutely infected buffalo and the carrier buffalo.

Table 2: Comparison of phagocytosis rate in the different lungs lobes between acutely infected buffalo with *P. multocida* B:2 and the carrier buffaloes.

Lung lobe	Acute			Lung lobe	Carrier		
	Positive stain	Total	Phagocytosis rate		Positive stain	Total	Phagocytosis rate
2 R CRCR	17	21	81	3 R CRCR	66	79	84
R CRCAU	15	17	88	R CRCAU	52	77	68
R MID	11	16	69	R MID	15	18	83
R CAU	34	45	76	R CAU	50	50	100
L CR	34	57	60	L CR	38	68	56
L MID	26	38	69	L MID	26	30	87
L CAU	25	42	60	L CAU	29	37	78
ACC	10	17	59	ACC	22	22	95
3 R CRCR	82	98	84	6 R CRCR	67	77	87
R CRCAU	57	80	71	R CRCAU	20	27	74
R MID	4	14	29	R MID	24	37	65
R CAU	7	14	50	R CAU	40	41	98
L CR	6	11	55	L CR	25	42	60
L MID	0	9	0	L MID	9	9	100
L CAU	52	52	100	L CAU	35	45	78
ACC	60	93	65	ACC	29	57	51
4 R CRCR	2	7	29				
R CRCAU	7	26	27				
R MID	22	36	61				
R CAU	9	41	22				
L CR	12	23	52				
L MID	23	29	79				
L CAU	8	19	42				
ACC	5	19	26				

Red = highest phagocytosis rate in acute buffaloes
Green = highest phagocytosis rate in carrier buffaloes

4.4 Descriptive statistics

Table 3 summarizes the mean and standard deviation of the macrophage population in the acutely infected buffalo and the carrier buffalo. From the table below, it can be concluded that both acute and carrier buffaloes had the highest mean and standard deviation at the right cranial cranial lobe whereas lowest in the right caudal lung lobe for the acute buffaloes and left middle lung lobe for the carrier buffaloes.

Table 3: Mean ± SD of number of alveolar macrophage in different lung lobes of buffaloes with acute HS and carrier buffaloes H&E

Lung lobe	Acute	Carrier
R CRCR	36.67 ± 4.04	20.00 ± 1.41
R CRCAU	35.67 ± 6.03	15.50 ± 7.78
R MID	24.00 ± 3.61	12.50 ± 4.95
R CAU	20.33 ± 5.51	16.50 ± 9.19
L CR	30.33 ± 10.60	12.50 ± 0.71
L MID	27.33 ± 11.60	8.00 ± 2.83
L CAU	24.33 ± 12.70	15.00 ± 7.07
ACC	29.00 ± 19.08	10.50 ± 0.71
TOTAL	**28.46 ± 5.70**	**13.81 ± 3.74**

Red = Highest Mean ± Standard Deviation of macrophage population
Green = Lowest Mean ± Standard Deviation of macrophage population

Table 4 summarizes the mean and standard deviation of the phagocytosis rate in the acutely infected buffalo and the carrier buffalo. From the table below, the acute buffaloes showed highest mean and standard deviation at the left caudal lung lobe where as the carrier buffaloes showed highest at the right caudal lung lobe. The lowest mean and standard deviation can be seen at the right caudal lung lobe in the acute buffaloes where as lowest at left cranial lung lobe in carrier buffaloes.

Table 4: Mean ± SD of percentage of phagocytosis rate of alveolar macrophages in different lung lobes of buffaloes with acute HS and carrier buffaloes

Lung lobe	**Acute**	**Carrier**
R CRCR	64.67 ± 30.93	85.50 ± 2.12
R CRCAU	62.00 ± 31.48	71.00 ± 4.24
R MID	53.00 ± 21.17	74.00 ± 12.73
R CAU	49.33 ± 27.01	99.00 ± 1.41
L CR	55.67 ± 4.04	58.00 ± 2.83
L MID	49.33 ± 43.02	93.50 ± 9.19
L CAU	67.33 ± 29.69	78.00 ± 0.00
ACC	50.00 ± 21.00	73.00 ± 31.11
TOTAL	**56.42 ± 7.29**	**79.00 ± 13.20**

Red = Highest Mean ± Standard Deviation of macrophage phagocytosis rate
Green = Lowest Mean ± Standard Deviation of macrophage phagocytosis rate

gure 1: Arrowhead shows macrophage that has positive immunostain from the IP stained slide.

Figure 1: Arrowhead shows macrophage that has positive immunostain from the IP stained slide.

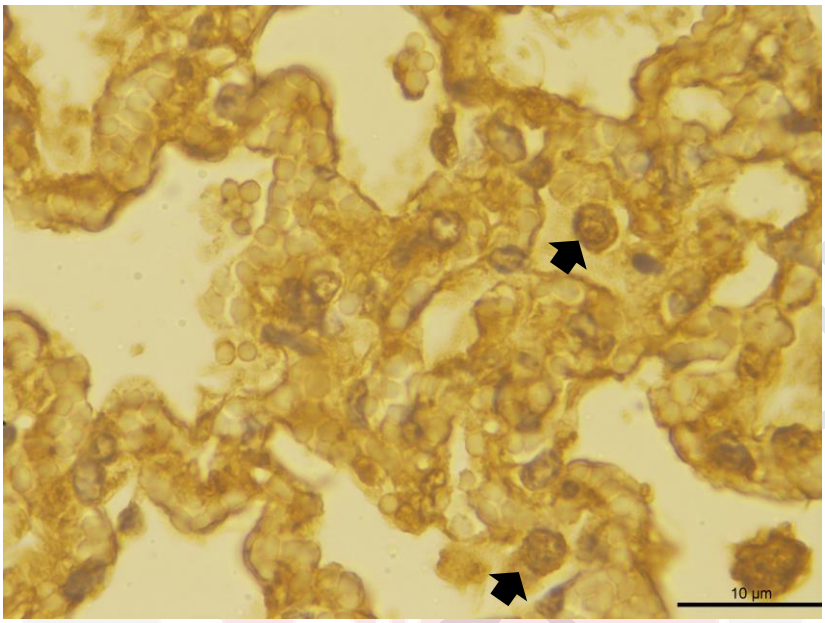
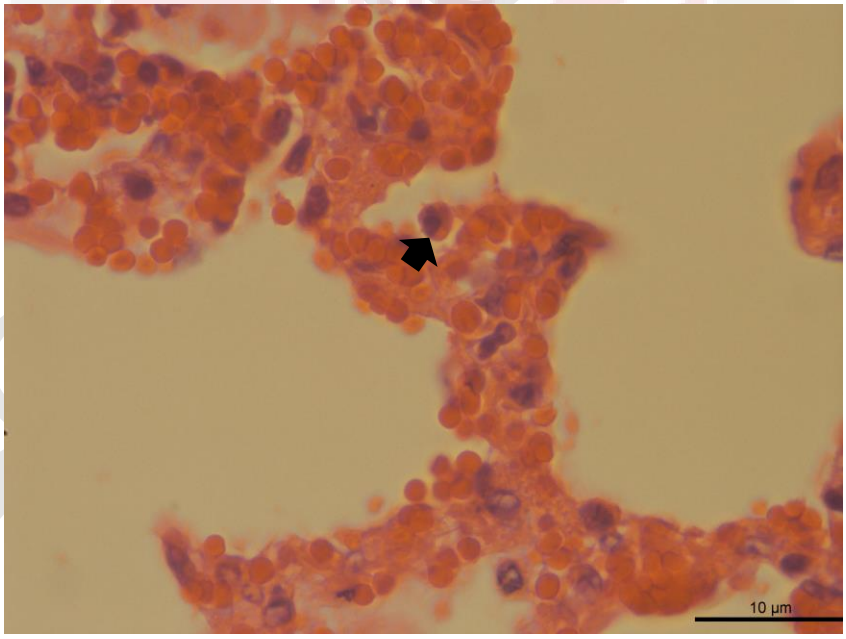


Figure 2: Arrowhead shows macrophage from H&E stained slide



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5.0 DISCUSSION

The findings from both H&E and IP stained slides were in agreement with a study done by Annas, 2015. The histopathological lesions and immunoreactivity were observed to be more severe in acute buffaloes compared to the carrier buffaloes as seen similarly in the previous study done by Annas, 2015.

From the results, the acute HS buffaloes showed a high macrophage population but a low phagocytosis rate. This is merely because in an early stage infection, there is high multiplication of agent and release of toxins which causes the body to respond by directing the macrophage to the particular site (Annas, 2015). Furthermore, the phagocytosis rate is low due to vacuolation and lysis of the macrophage which was caused by the HS serotype B:2 as mentioned by Alwis, M. C.,1999. Also, Alwis, M. C., 1999 and Shivachandra et *al.*, 2020 have stated that capsule, polysaccharide and lipopolysaccharide are the virulence factors that inhibits phagocytosis. Hence, reducing the phagocytosis rate which was observed similarly in this study.

Conversely, the carrier HS buffaloes showed a low macrophage population but a high phagocytosis rate. When an infection occurs, the defense mechanism dominates the agent which results in an arrested infection. Now, the buffaloes have become a carrier animal and there is absence of ongoing infection. Therefore, the macrophage population is low as observed in this study (Alwis, M. C., 1999).

Moving on, both acute and carrier buffaloes have shown highest pulmonary macrophage population similarly at the right cranial cranial and right cranial caudal lung lobe and highest phagocytosis rate similarly at the right cranial cranial lung

lobe. The macrophage population was seen lowest similarly at the left middle lung lobe whereas similarly lowest phagocytosis rate at the accessory lung lobe.

A previous study done by Annas, 2015, concluded that the right middle lung lobe have the highest *P.multocida* concentration because the right middle lung lobe is located closely to the bifurcation of the trachea and the hilum of the pulmonary vessels. However, the right middle lung lobe was not observed to have the highest pulmonary macrophage population or phagocytosis rate. This is because the recruitment of the macrophage does not depend solely on the site where there is presence of highest concentration of the agent. The cytokines and toxin release plays an important role in recruiting the macrophage as well.

Not only that, animal 3 from the carrier group have highest macrophage population and highest phagocytosis rate on the same lung lobe which is the right caudal lung lobe. Similarly, was seen in animal 4 from the acute group, where the lowest macrophage population and phagocytosis rate was on the same lung lobe which is the L middle lung lobe. The rest of the animals did not exhibit results as mentioned above.

6.0 CONCLUSIONS

In a conclusion, it can be said that acute and carrier buffaloes have different macrophage population and phagocytosis rate. The macrophage plays an important role in the outcome of the *P.multocida* B:2 infection in buffaloes.

7.0 RECOMMENDATIONS

For future studies, it is recommended that more replicates of each group (acute and carrier) should be included in order to carry out statistical analysis on the results. Power analysis was used by Charan and Kantharia (2013) to calculate sample size using power analysis where the appropriate sample size for a study can be obtained.

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