COMPARATIVE In-Vitro PATHOGENESIS OF Pasteurella multocida B:2 INFECTION IN BUFFALO

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By

YULIANNA PUSPITASARI

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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March 2020

Chairman : Professor Mohd Zamri bin Saad, DVM, PhD
Faculty : Veterinary Medicine

Haemorrhagic septicaemia (HS) is a septicaemic disease caused by *Pasteurella multocida* serotype B:2. It leads to outbreaks with high mortality among cattle and buffalo. The organism enters the host via inhalation or oral routes since *P. multocida* B:2 has been isolated in both the nasopharynx and intestine of dead cattle and buffaloes. Furthermore, urinary tract was also revealed to play significant role in the development and transmission of HS. Thus, understanding the interactions of *P. multocida* B:2 with the host cells at the point of entry and excretion, and at the endothelial cells that leads to severe damage and destruction are important. Subsequently, following entrance into blood circulation, survival of *P. multocida* B:2 against phagocytic cells to overwhelm the host is critical. There are few studies involving *in-vitro* pathogenesis but none was done using buffalo cells, organs or tissues. Since buffalo is more susceptible to HS, this study uses buffalo for evaluating the attachment and distribution of *P. multocida* B:2 onto cells of respiratory and urinary tracts, the survival of *P. multocida* B:2 in phagocytic cells and the severity of endothelial cell damage by *P. multocida* B:2. To achieve these objectives, explants from the repiratory and urinary tracts were prepared and challenged with *P. multocida* B:2, the neutrophil and macrophage were harvested and culture before challenge while the endothelial monolayers were prepared and similarly challenged. The attachment and distribution, survival within phagocytic cells, and endothelial destruction were determined using a scoring system.

Three healthy buffaloes with no history of vaccination against haemorrhagic septicaemia were killed before the lung and bladder explants were prepared. The explants were then challenged with 10^9 cfu/ml of live *P. multocida* B:2. At the same time, a known septicaemic organism, *Escherichia coli* and a known respiratory organism, *Mannhaemia haemolytica* A:2 were used as comparison, since HS is a septicaemic disease and the respiratory tract is the preferred sites

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for *P. multocida* B:2. The explants were harvested at 2-h intervals until 12 hours before the rate of attachment was scored using scanning electron microscopy while the distribution was determined using immunoperoxidase staining. All bacterial strains showed similar attachment and distribution patterns. *Pasteurella multocida* B:2 and *M. haemolytica* A:2 showed significantly ($p<0.05$) increasing attachment and distribution with time to reach peak at 8-10 h and 12 h post-inoculation, respectively. On the other hand, *E. coli* showed significantly ($p<0.05$) increasing attachment and distribution with time to reach peak at 12 h post-inoculation. There were significant ($p<0.05$) correlations between the rate of attachment and distribution of all bacteria. In general, the attachment and distribution of *P. multocida* B:2 in the lungs and urinary bladder of buffalo were much better than the known respiratory bacterium, the *M. haemolytica* A2, and as good as the known septicaemic bacterium, the *E. coli.* Therefore, *P. multocida* B:2 is, in fact, a septicaemic bacterium with high potential of causing septicaemic disease.

Neutrophils and monocytes were harvested from healthy buffaloes to evaluate the *in-vitro* efficacy of phagocytosis and bacterial killing. The neutrophils were prepared as cell culture while the monocytes were allowed to mature into macrophages before being prepared as cell culture. These cells were divided into 3 groups. Group 1 was inoculated with *P. multocida* B:2, Group 2 with *E. coli* while Group 3 with *M. haemolytica* A:2 at 10^7 cfu/ml of the respective bacterium. The inoculated cell cultures were harvested at 0, 30, 60 and 120 min post-exposure and the rates of phagocytic, killing and phagocytic cell death were determined. Both phagocytosis and killing rates of all bacteria increased over incubation time. Phagocytosis involved between 71% and 73% of the neutrophils and between 60% and 64% of the macrophages at 120 min. Successful bacterial killings were shown by between 76% and 79% of the neutrophils and between 70% and 74% of the macrophages at 120 min. Death rate of the neutrophils ranged between 67% in Group 3, and 88% in Group 1 at 120 min, significantly ($p<0.05$) higher than Group 3 but insignificant ($p>0.05$) than Group 2. Similar pattern was observed for death rate of macrophages. Therefore, this study revealed that although the attachment, phagocytosis and intracellular killing rates of *P. multocida* B:2 were similar compared to other bacterial species used in this study, it seemed that more neutrophils and macrophages were dead following infection by *P. multocida* B:2 compared to *E. coli* and *M. haemolytica* A:2.

Then, the endothelial cells that were obtained from the aorta of buffaloes were prepared as monolayer cell cultures. The cultures were divided into 3 groups. Group 1 was inoculated with 10^7 cfu/ml of whole cell *P. multocida* B:2, Group 2 with LPS that was extracted earlier from 10^7 cfu/ml of wild-type *P. multocida* B:2 while Group 3 with sterile cell culture medium with neither *P. multocida* B:2 nor LPS. The assessments on the cellular changes were done using transmission electron microscope at different points of time; at 0, 6, 12, 18, 24, 36 and 48 hours post-incubation. Groups exposed to whole cell *P.multocida* B:2 and LPS demonstrated moderate to severe changes, characteristic of acute cellular injury leading to cell lysis. The ultrastructural changes were consistent with necrotic

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changes that increased in the severity with time of incubation. There were no significant differences (p>0.05) between the two infected groups in the first 18 h post-inoculation before the severity of lesions became significant (p<0.05) thereafter. All infected groups showed significantly (p<0.05) more severe changes compared to control Group 3 from 6 h post-inoculation onwards. The severity scores by 48 h post-inoculation reached peak with score 3 for whole cell treated BAEC and score 2.8 for LPS treated BAEC. However, this study revealed that although both whole cells and LPS endotoxin showed similar moderate to severe alterations of cellular damage, it seemed that *P. multocida* B:2 whole cells were more potent in causing much severe damage than LPS alone.

In conclusion, the *in-vitro* attachment and distribution of wild type *P. multocida* B:2 on the cells of respiratory and urinary tracts of buffalo were found to increase over time. They were comparable with septicaemic bacteria of *E. coli* and the respiratory bacteria of *M. haemolytica* A:2. Furthermore, there was positive correlation between the attachment on the surface and the distribution. Thus, this findings affirm the pathogenic role during bacterial colonization, which allows the bacterium to exert its pathogenic and immunogenic effects on the host leading to acute septicaemia. Nevertheless, *P. multocida* B:2 was found to have high ability to cause death of neutrophils and macrophages allowing more survival of bacterial cells with less phagocytic cells to prevent infection. Moreover, introduction of whole cell and LPS endotoxin of *P. multocida* B:2 leads to endothelial cells damage thus, proposed the possible mechanism of translocation of *P. multocida* B:2 in acute HS to allow successful translocation from the blood vessels into the tissue and vice versa.

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Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PERBANDINGAN PATOGENESIS SECARA *In-Vitro* OLEH JANGKITAN *Pasteurella multocida* B:2 TERHADAP KERBAU

Oleh

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Septisemia Hemoraj adalah penyakit septisemik yang disebabkan oleh *Pasteurella multocida* serotype B:2. Penyakit ini menjadi wabak dengan menyebabkan kematian yang tinggi dikalangan lembu dan kerbau. Organisma akan memasuki perumah (*host*) melalui pernafasan atau oral kerana *P. multocida* B:2 telah berjaya dipencilkan dari kedua-dua bahagian iaitu nasofarinks dan usus dari lembu dan kerbau yang sudah mati. Selain itu, saluran kencing juga memainkan peranan penting dalam pembentukan dan transmisi HS. Oleh itu, memahami interaksi di antara *P. multocida* B:2 dan sel-sel perumah pada titik masuk dan perkumuhuan, dan kemudian sel-sel endotelium yang membawa kepada kerosakan dan kemusnahan yang teruk adalah penting. Seterusnya, setelah *P. multocida* B:2 masuk ke dalam peredaran darah, keupayaan *P. multocida* B:2 untuk hidup terhadap sel fagosit dalam mengatasi perumah adalah kritikal. Terdapat beberapa kajian yang melibatkan patogenesis *in-vitro* tetapi tidak ada yang dilakukan dengan menggunakan sel kerbau, organ atau tisu. Memandangkan kerbau lebih mudah terjangkit dengan HS, kajian ini menggunakan kerbau untuk penilaian lampiran dan taburan *P. multocida* B:2 ke sel saluran pernafasan dan saluran kencing, ketahanan *P. multocida* B:2 sel fagosit dan tahap kerosakan sel endotelium yang disebabkan oleh *P. multocida* B:2. Kajian ini melibatkan pengenalan endotoksin dan keseluruhan sel (*whole cell*) *P. multocida* B:2. Untuk mencapai objektif ini, eksplan dari saluran pernafasan dan saluran kencing telah disediakan dan dijangkitkan (*challenged*) dengan *P. multocida* B:2, neutrofil dan makrofaj telah dituai (*harvest*) dan di kulturkan sebelum jangkitan dilakukan manakala endotelium monolapisan telah dituai dan turut dijangkitkan. Pelekatan (*attachment*) dan taburan (*distribution*), ketahanan terhadap sel fagosit, dan pemusnahan sel endotelium ditentukan menggunakan sistem penskoran.

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Tiga ekor kerbau yang sihat tanpa sejarah vaksinasi terhadap septisemia hemoraj dikorbankan sebelum eksplan dari paru-paru dan pundi kencing disediakan. Eksplan kemudian dijangkitkan dengan 10^9 cfu/ml *P. multocida* B:2 hidup. Pada masa yang sama, bakteria dari saluran pernafasan yang diketahui iaitu, *Mannhemia haemolytica* A:2 dan bakteria septisemia, *Escherichia coli* digunakan sebagai perbandingan kerana HS adalah penyakit septisemia dan saluran pernafasan adalah kawasan yang disukai oleh *P. multocida* B:2. Eksplan telah dituai dalam tempoh 2 jam sehingga 12 jam sebelum kadar skor lekatan ditentukan dengan menggunakan mikroskopi pengimbasan elektron manakala taburan ditentukan dengan menggunakan pewarnaan *immunoperoxidase*. Semua strain bakteria menunjukkan susunan dan corak taburan yang serupa. *Pasteurella multocida* B:2 dan *M. haemolytica* A:2 menunjukkan signifikasi ($p<0.05$) peningkatan pelekatan dan taburan dengan masa untuk mencapai kemuncak pada 8-10 jam dan 12 jam pasca inokulasi. Selain itu, *E. coli* menunjukan signifikasi ($p<0.05$) peningkatan pelekatan dan taburan dengan masa untuk mencapai kemuncak pada 12 h pasca-inokulasi. Terdapat signifikasi ($p<0.05$) korelasi antara kadar pelekatan dan taburan oleh semua bakteria. Secara amnya, pelekatan dan taburan oleh *P. multocida* B:2 pada paru-paru dan pundi kencing kerbau adalah lebih baik berbanding bakteria di saluran pernafasan yang diketahui, *M. haemolytica* A2, dan sebaik bakteria septisemia, *E. coli.* Oleh itu, *P. multocida* B:2, faktanya, adalah bakteria septisemia yang mempunyai potensi yang tinggi untuk menyebabkan penyakit septisemia.

Neutrofil dan monosit dituai dari kerbau yang sihat untuk menilai keberkesanan fagositosis *in-vitro* dan keupayaan membunuh bakteria. Neutrofil disediakan melalui kaedah kultur sel manakala monosit dibiarkan matang sehingga menjadi makrofaj sebelum disiapkan sebagai kultur sel. Sel-sel ini dibahagikan kepada 3 kumpulan. Kumpulan 1 di inokulasi dengan *P. multocida* B: 2, Kumpulan 2 dengan *E. coli* manakala Kumpulan 3 dengan *M. haemolytica* A: 2. Inokulasi dilakukan menggunakan 10^7 cfu/ml bakterium. Sel-sel yang telah di inokulasi telah dituai pada 0, 30, 60 dan 120 minit pasca pendedahan dan kadar sel fagositik, kadar kematian dan sel fagositik yang mati telah ditentukan. Kedua-dua fagositosis dan kadar kematian semua bakteria meningkat dari masa ke semasa. Fagositosis melibatkan antara 71% dan 73% neutrofil dan antara 60% dan 64% daripada makrofaj pada 120 minit. Kematian bakteria yang berjaya ditunjukkan antara 76% dan 79% daripada neutrofil dan antara 70% dan 74% daripada makrofaj pada 120 minit. Kadar kematian neutrofil adalah antara 67% dalam Kumpulan 3 dan 88% dalam Kumpulan 1 pada 120 minit, dengan signifikan ($p<0.05$) lebih tinggi daripada Kumpulan 3 tetapi tidak signifikan ($p>0.05$) berbanding Kumpulan 2. Corak serupa diperhatikan untuk kadar kematian makrofaj. Oleh itu, kajian ini menunjukan bahawa walaupun pelekatan, fagositosis dan kadar kematian intrasel oleh *P. multocida* B:2 adalah sama berbanding sepsis bakteria yang lain yang digunakan dalam kajian ini, lebih neutrofil dan makrofaj mati selepas di infeksi dengan *P. multocida* B:2 berbanding *E. coli* dan *M. haemolytica* A:2.

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Kemudian, sel-sel endotelium yang diperoleh dari aorta kerbau telah disediakan sebagai monolapisan kultur sel. Sel kultur dibahagikan kepada 3 kumpulan. Kumpulan 1 di inokulasi dengan 10^7 cfu/ml keseluruhan sel *P. multocida* B: 2, Kumpulan 2 dengan ekstrak LPS dalam bentuk cecair, telah diekstrak lebih awal dari 10^7 cfu/ml *P. multocida* jenis liar (*wild-type*) B: 2 manakala Kumpulan 3 dengan media kultur sel yang steril tanpa *P. multocida* B: 2 atau LPS. Taksiran mengenai perubahan sel telah dilakukan menggunakan mikroskopi pengimbasan elektron pada masa yang berlainan; iaitu pada 0, 6, 12, 18, 24, 36 dan 48 jam pasca inkubasi. Kumpulan yang terdedah kepada kepada sel seluruh (*whole cell*) *P.multocida* B:2 dan LPS menunjukan perubahan yang sederhana hingga parah (*severe*), ciri-ciri kecederaan sel akut yang menjurus kepada sel lisis (*lysis*). Perubahan ultrastruktur adalah konsisten dengan perubahan peningkatan nekrotic dengan keparahan dan masa inkubasi. Tidak ada perbezaan yang signifikan ($p>0.05$) di antara kedua-dua kumpulan yang dijangkiti dalam tempoh 18 h selepas inokulasi pertama sebelum lesi (*lesion*) yang ketara menjadi signifikan ($p<0.05$) selepas itu. Semua kumpulan yang dijangkiti menunjukkan peningkatan yang signifikan ($p<0.05$) berbanding dengan Kumpulan kawalan 3 dari 6 jam pasca inokulasi. Skor keparahan (*severity*) pada 48 h pasca inokulasi mencapai puncak dengan skor 3 untuk keseluruhan sel dirawat dengan BAEC dan skor 2.8 untuk LPS yang dirawat dengan BAEC. Walau bagaimanapun, kajian ini mendedahkan bahawa walaupun kedua-dua sel-sel dan endotoksin LPS menunjukkan perubahan yang sama dengan kerosakan selular yang sederhana dan parah, sel keseluruhan *P. multocida* B:2 lebih poten dalam menyebabkan kerosakan teruk berbanding LPS sahaja

Kesimpulannya, pelekatan *in-vitro* dan taburan *P. multocida* B:2 jenis liar yang terdapat pada sel-sel pernafasan dan saluran kencing kerbau didapati meningkat dari semasa ke semasa. Selain itu, terdapat korelasi positif antara pelekatan pada permukaan dan taburan. Oleh itu, penemuan ini mengesahkan peranan patogen semasa kolonisasi bakteria, yang membolehkan bakteria memberi kesan patogenik dan imunogenik kepada perumah yang membawa kepada septisemia akut. Walau bagaimanapun, *P. multocida* B:2 didapati mempunyai keupayaan tinggi untuk menyebabkan kematian neutrofil dan makrofaj kerbau yang membolehkan lebih banyak sel bakteria hidup dengan sel kurang fagositik untuk mencegah jangkitan. Lebih-lebih lagi, pengenalan terhadap keseluruhan sel dan endotoksin sel LPS *P. multocida* B:2 membawa kepada kerosakan sel endotelium, seterusnya, mencadangkan kemungkinan mekanisma translokasi *P. multocida* B: 2 dalam Septisemia Hemoraj akut untuk membolehkan translokasi dari pembuluh darah ke tisu berjaya atau sebaliknya.

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LIST OF ABBREVIATIONS

µg	microgram
µl	microliter
µm	micrometer
^{0}C	degree Celcius
ANOVA	analysis of variance
BAECs	buffalo aortic endothelial cells
BHI	brain-heart infusion
BSA	bovine serum albumin
cfu	colony forming unit
CO_2	carbon dioxide
cm	centimetre
DAB	3,3'-Diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DMEM	dulbecco's modified eagle's medium
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EtOH	ethanol or ethyl alcohol
g	gram
h	hour
HBSS	hank's balanced salt solution
HS	haemorrhagic septicaemia
IF	immunofluorescent
IgG	immunoglobulin G
IL	interleukin
IP	immunoperoxidase

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LPS	lipopolysaccharide
LSD	least significant difference
M	molar
min	minute
ml	mililiter
nm	nanometre
OMP	outer membrane protein
PA	paraformaldehyde
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PBST	phosphate-buffered saline with tween 20
PCR	polymerase chain reaction
pH	potential hydrogen
RER	rough endoplasmic reticulum
rpm	revolution per minute
RPMI	roswell park memorial institute
s	second
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate – polyacrylamide gel electrophoresis
SEM	scanning electron microscope
SPSS	Statistical Packages for the Social Sciences
TBE	tris-boric acid-EDTA
TEM	transmission electron microscopy
TNF-α	tumor necrosis factor-α
W	watt
xg	times gravity

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CHAPTER 1

INTRODUCTION

Haemorrhagic septicaemia (HS) is one of the important diseases of cattle and buffaloes in Asia, leading to high morbidity and mortality. It is also reported in Africa and certain countries in the Middle East and Southern Europe (Benkirane and De Alwis, 2002). The disease is associated with a specific serotype of *Pasteurella multocida* (Kuranasree, 2016) and is particularly important in Southeast Asian countries including Malaysia (Thomas, 1972; Saharee *et al*., 1993; Kahn and Line, 2005; Jesse *et al*., 2013a; Chung *et al*., 2015). Buffaloes are more susceptible than cattle and the disease is usually observed more frequently following poor husbandry, particularly in countries with disease surveillance that are not well developed (Annas *et al*., 2014; Moustafa *et al.,* 2017). A study in Malaysia also revealed that the mortality rate was higher among buffaloes compared to cattle despite the fact that the buffalo population in Malaysia was half that of cattle (Rafidah *et al*., 2010).

Pasteurella multocida is a Gram-negative coccobacillus bacterium. Using a combination of capsular and somatic typings, *P. multocida* serotype B:2 known as the Asian serotype and E:2 known as the African serotype by Carter-Heddlestone system, which correspond to 6B and 6E by Namioka-Carter system are the specific serotypes that HS in ruminants (Carter, 1963; Heddleston *et al*., 1972). Following exposure, the agent enters susceptible animals by inhalation or oral route leading to septicaemia (Khin *et al*., 2010; Abubakar *et al*., 2013) and localization in various organs (Annas *et al.,* 2015a). Occasionally, following exposure, the animals would survive and became carriers harbouring *P. multocida* B:2 even though most animals would succumb to peracute or acute HS (De Alwis, 1995). If the animal survived the initial infection and became carier, it would exhibit minimal clinical signs that are easily overlooked, such as transient pyrexia and mild depression (De Alwis, 1999). However, the peracute or acute HS are characterised by a short clinical course (Biswas *et al*., 2004; Zamri-Saad and Shafarin, 2007). The clinical signs include severe depression, pyrexia, submandibular oedema, dsypnoea, recumbency and death (Horadagoda *et al*., 2002; Zamri-Saad and Shafarin, 2007; Abubakar and Zamri-Saad, 2011).

Recently, the urinary tract was reported to play significant role in the development and transmission of the disease following detection of *P. multocida* B:2 in the tract (Annas *et al*., 2014, 2015b). The outcome of infection depends on the interaction between the virulent organism and the host, particularly on the ability of the organism to attach or adhere to the host cells. Therefore, bacterial adhesion is crucial in colonization and eventually in disease development (Nuriqmaliza *et al.,* 2017). The interaction between the bacterium and the target cells, known as adherence enables colonization to occur, which allows the

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bacterium to exert its pathogenic and immunogenic effects on the host (Nuriqmaliza *et al.,* 2017).

As the first line of defense, both neutrophils and macrophages play important role in phagocytosis and killing of invading organisms. So far, the role of macrophages in adhesion and phagocytosis of *P. multocida*, and subsequent bactericidal activity has been studied in turkeys (Harmon *et al*., 1991; Harmon *et al*., 1992; Pruimboom *et al*., 1996), chicken (Poermadjaja *et al*., 2000), mouse (Shah *et al*., 1996) and bovine (Maheswaran and theis, 1979). Previous studies have reported that *in-vitro* interaction of leukotoxin from *Mannhaemia haemolytica* and lipopolyscharide (LPS) endotoxin of *P. multocida* with bovine leucocytes resulted in extensive cell death including apoptosis and necrosis (Stevens and Czuprynski, 1996; Sun *et al*., 2000; Periasamy *et al*., 2018]. Furthermore, data from *in-vivo* studies suggest that endotoxin from *P. multocida* could mediate severe inflammatory changes and tissue pathology during pasteurellosis (Praveena *et al*., 2010, 2014; Annas *et al*., 2015a). However, there is limited studies on how phagocytic cells (neutrophils and macrophages) respond to *in-vitro* infection by live *P. multocida*. Although, *P. multocida* could replicates exponentially in extracelullar milieu at infection site, the phagocytic and intracellular killing abilities of innate immune cells are not known clearly.

The critical feature of HS disease is the rapid spread of the infecting bacterium from the respiratory tract to the blood and lymph that leads to fatal septicaemia within 16-72 h (Annas *et al*., 2015b). To pass into the bloodstream, the bacterium migrates through the epithelial layer into the pulmonary interstitium leading to septicaemia (Sarah *et al*., 2012). Subsequently, understanding the interaction of *P. multocida* B:2 with the host endothelial cells that leads to severe damage and destruction, is the mechanism that enables *P. multocida* B:2 to translocate from blood vessels into organs and vice versa leading to toxaemia and later septicaemia (Annas *et al*., 2014). *In-vitro* study revealed that *P. multocida* B:2 has the ability to adhere and invade bovine aortic endothelial cells (Galdiero *et al*., 2001) while Sarah *et al*. (2012) showed that *P. multocida* B:2 could be found to be internalised in embryonic bovine lung (EBL) cells.

It is extremely important to understand the development of septicaemia in HS, particularly the entrance point of *P. multocida* B:2 into the blood circulation. So far, the recognition of respiratory, gastrointestinal and urinary tracts as points of entry were made following *in-vivo* study using both cattle and buffalo. There were few studies involving *in-vitro* pathogenesis but none was done using buffalo's cells, organs or tissues. Since buffalo is more susceptible to HS, a study using buffalo would be beneficial in evaluating the rate of attachment and the severity of endothelial cells infected by *P. multocida* B:2. Nevertheless, different strains of *P. multocida* isolated from different hosts with different diseases are known to have different degree of invasiveness (Galdiero *et al*., 2001). Considering this paucity of information and high susceptibility of buffaloes to *P. multocida* B:2, several understandings need to be developed to better understand the

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pathogenesis of HS in buffalo. Therefore, the main goal of the study was to investigate the *in-vitro* interaction of wild-type *P. multocida* B:2 with the buffalo cells or tissues. Since HS is a septicaemic disease and the respiratory tract is the preferred sites for *P. multocida* B:2 (Annas *et al*., 2014), hence *Escherichia coli*, a known septicaemic organism and *Mannhaemia haemolytica* A:2, a known respiratory organism were used as comparison. In order to achieve this eventual goal, the research hypotheses and objectives have been structured as follows :

1.1 Null Hypotheses

1. *In-vitro* attachment and distribution of wild type *P. multocida* B:2 on the cells of respiratory and urinary tracts of buffaloes do not increase over time and not comparable with the septicaemic bacterium, *E. coli* and the respiratory bacterium, *M. haemolytica* A:2.
2. The *in-vitro* rates of phagocytosis and intracelullar killing of *P. multocida* B:2 by phagocytic cells of buffaloes do not increase over time and not comparable to the septicaemic bacterium, *E. coli* and the respiratory bacterium, *M. haemolytica* A:2.
3. There is no difference in the severity of damages to the endothelial cell monolayers following introduction of the whole cell and endotoxin of wild-type *P. multocida* B:2.

1.2 Alternative Hypotheses

1. *In-vitro* attachment and distribution of wild type *P. multocida* B:2 on the cells of respiratory and urinary tracts of buffaloes increase over time and are comparable with the septicaemic bacterium, *E. coli* and the respiratory bacterium, *M. haemolytica* A:2.
2. The *in-vitro* rates of phagocytosis and intracelullar killing of *P. multocida* B:2 by phagocytic cells of buffaloes increase over time and are comparable with the septicaemic bacterium, *E. coli* and the respiratory bacterium, *M. haemolytica* A:2.
3. The endotoxin of *P. multocida* B:2 causes more severe damage to the endothelial cell monolayers than the whole cell of *P. multocida* B:2.

1.3 Objectives of the study

1. To determine and to compare the *in-vitro* attachment and the extend of distribution of wild-type *Pasteurella multocida* B:2 with the septicaemic bacterium *E. coli* and the respiratory bacterium *M. haemoltytica* A:2 on the cells of respiratory and urinary tracts of buffaloes

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2. To evaluate and to compare the extend of *in-vitro* phagocytosis and intracellular killing of wild-type *P. multocida* B:2 with the septicaemic bacterium *E. coli* and the respiratory bacterium *M. haemoltytica* A:2 by the phagocytic cells obtained from buffaloes.
3. To compare the severity of *in-vitro* damages on the endothelial cell monolayers following introduction of the whole cell and endotoxin of wild-type *P. multocida* B:2.

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