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RESEARCH ARTICLE

In Vitro Attachment and Distribution of *Pasteurella multocida* B:2 in the Lung and Urinary Bladder of Buffaloes

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A B S T R A C T

Haemorrhagic septicaemia is caused by *Pasteurella multocida* B:2 that leads to septicaemia following adherence and colonization of the respiratory tract. However, recent studies revealed the possible involvement of urinary tract in the pathogenesis of haemorrhagic septicaemia. This study is conducted to determine the *in vitro* adherence and distribution of *P. multocida* B:2 in the lungs and urinary bladder of buffaloes. Three buffalo calves with no history of vaccination against haemorrhagic septicaemia were killed before 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 respiratory tract bacterium, *Mannhemia haemolytica* A:2 and a septicaemic *E. coli* were used as comparison. The explants were harvested at 2-h intervals until 12 hours before the rate of adherence was determined using scanning electron microscopy while the distribution was determined using immunoperoxidase staining. All bacterial strains showed similar adherence and distribution patterns. *Pasteurella multocida* B:2 showed significantly ($P<0.05$) increased rates of adherence and distribution with time to reach peak at 8-10 h and 12 h post-inoculation, respectively. There were significant ($P<0.05$) correlation between the rate of adherence and distribution. In general, *M. haemolytica* A:2 remained least septicaemic bacterium with relatively low rates of adherence and distribution while *E. coli* was the most septicaemic with highest rates of adherence and distribution.

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INTRODUCTION

Haemorrhagic septicaemia 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 (Jesse *et al*., 2013; Chung *et al.*, 2015). Buffaloes are more susceptible than cattle and the disease is usually observed more frequently following poor husbandry conditions in countries with disease surveillance that is not well developed (Annas *et al*., 2014; Moustafa *et al.*, 2017). Similarly, in Malaysia, 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*., 2012).

Following exposure, the aetiological agent enters susceptible animals by inhalation or oral route leading to septicaemia (Khin *et al*., 2010; Abubakar *et al*., 2012) and localization in various organs (Annas *et al.*, 2015a). 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; Annas *et al.*, 2015b).

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

the adherence of *P. multocida* B:2 to the host cells is important, particularly at the point of entry that leads to severe damage and destruction.

The aims of this study were to investigate the rate of *in-vitro* attachment and the distribution of *P. multocida* B:2 on the cells of respiratory and urinary tracts of buffaloes.

MATERIALS AND METHODS

Three healthy buffalo calves were selected and ensured of free from *P. multocida* B:2 by bacterial isolation and polymerase chain reaction (Zamri-Saad *et al*., 2006). The buffaloes were kept in individual pens and fed cut grass at the rate of 4 kg/animal/day and supplemented feed (pellet) at 400 g/animal/day.

Wild type *P. multocida* B:2 (strain PmTB) used in this study was isolated earlier from an outbreak of haemorrhagic septicaemia (Zamri-Saad *et al*., 2006), *M. haemolytica* A:2 was from infected lungs of a goat while *E. coli* was from a calf that died of septicaemia. They were cultured on blood agar at 37ºC for 24 h, then in brain-heart infusion (BHI) broth at 37ºC with shaking at 150 rpm for 18h. The bacterial concentrations were determined using serial dilution method to produce an infective dose of $1.0x10^9$, $1.2x10^9$ and $1.1x10^9$ colony forming unit (cfu) per ml of live *P. multocida* B:2, *M. haemolytica* A:2 and *E. coli*, respectively.

At the start of the experiment, all buffaloes were killed before the lungs and urinary bladder were collected and placed in Dulbeco's minimum essential medium (DMEM) supplemented with antibiotic-antimycotic (10,000 units/ml Penicillin G Sodium, streptomycin 10,000 πg/ml and 25 πg/ml amphotericin B) solution before being prepared as explants (Al-Haddawi *et al*., 2000).

The explants of each animal were then divided into three groups. Explants of Group 1 were added with 1 ml of the inoculum containing live *P. multocida* B:2, Group 2 was similarly added with live *E. coli* while Group 3 with live *M. haemolytica* A:2 before they were incubated at 37ºC in 5% CO_2. At 2-h intervals until 12h post-inoculation, 3 explants from each group were harvested and processed for immunoperoxidase staining and scanning electron microscopic (SEM) examinations.

For SEM, the explants were fixed in 4% glutaraldehyde buffered in 0.1M sodium cacodylate buffer for 4 h at 4ºC and processed for viewing (JEOL IT 100, Japan). The intensity of colonization by the respective bacterium was determined according to Al-Haddawi *et al*. (2000). The colonization was scored as follows: 0=no bacterial attachment; 1=mild colonization with few bacteria scattered on the explants; 2=moderate colonization with some bacterial aggregations on the explants and 3=severe colonization with numerous bacteria covering more than half of the explants. Fifteen random microscopic fields at 2500X magnification of each of the triplicate explant samples were examined. The results were expressed as the mean score with standard deviation.

The tissue sections were dewaxed for immunoperoxidase staining according to Abubakar *et al*. (2012). Immunoperoxidase scoring was done in relation to the extent of the distribution of *P. multocida* B:2, *M. haemolytica* A:2 and *E. coli* antigens (Abubakar *et al.*, 2012). The scoring criteria were 0 for no observable bacterial distribution, 1 for focal distribution, 2 for multifocal distribution and 3 for diffuse distribution of bacteria. Fifteen random microscopic fields at 400x magnification of each of the triplicate explant samples were examined. The results were expressed as mean scoring with standard deviation.

All the data were analysed using One-Way ANOVA and LSD multiple comparison. The data were considered significant at $P<0.05$. Spearman's rho correlation test was used to determine the correlation between the bacterial colonization and distribution. The value of correlation coefficient was used to determine the strength of correlation, based on categorisation by Dancey and Reidy (2004). Briefly, value 0 indicated no relation, 0.1 to 0.3 indicated weak positive correlation, 0.4 to 0.6 indicated moderate positive correlation, 0.7 to 0.9 indicated strong positive correlation, and 1 indicated perfect correlation.

RESULTS

***In vitro* attachment onto the lung:** All exposed explants of Groups 1, 2 and 3 showed bacterial attachment and colonization as early as 2 h post-inoculation (Fig. 1). There was significant ($P<0.05$) increasing attachment patterns of all bacteria with time post-inoculation (Fig. 2). Both Groups 1 and 3 showed peak attachment rate at 8 h post-inoculation while Group 2 at 12 h post-inoculation. The rate of attachment for Groups 1 and 3 was significantly ($P<0.05$) decreased at 12 h post-inoculation compared to Group 2. At 4, 6 and 8 h post-inoculation, Group 1 showed insignificantly ($P>0.05$) higher rate of attachment than Group 2 with scoring of 1.27±0.45, 1.87±0.35 and 2.8±0.41, respectively (Fig. 2), while Group 3 showed significantly ($P<0.05$) lower rate of attachment compared with Groups 1 and 2. At 10 hours post-inoculation, the attachment rates of Groups 1 and 3 started to decrease with scoring of 2.60±0.50 and 2.27±0.59, respectively while Group 2 showed non-significant ($P>0.05$) increasing scoring (2.60±0.50 and 2.87±0.35) pattern at 10 and 12 h post-inoculation, respectively.

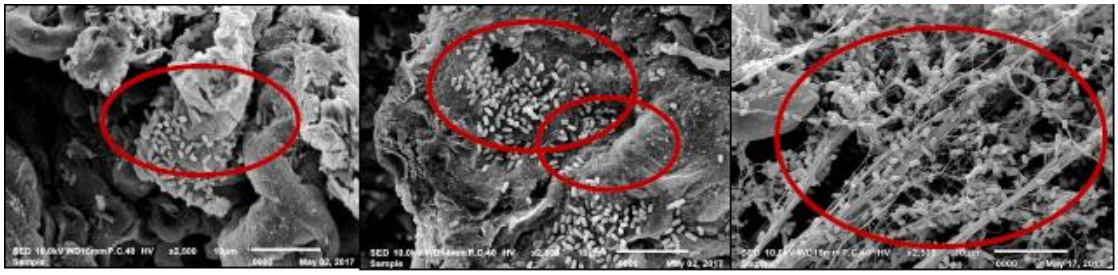

Fig. 1: Scanning electron micrographs showing mild (left), moderate (middle) and severe (right) rates of attachment of *Pasteurella multocida* B:2 onto the lung explants of buffalo calves.

***In vitro* attachment onto the urinary bladder:** The Fig. 3 reveals the attachment patterns onto the explants of urinary bladder. There was significant (P<0.05) increasing attachment for Groups 1 and 3 that reached peak at 10h post-inoculation (score 2.20±0.56 and 2.07±0.45, respectively) before no significant (P>0.05) decreasing pattern was noted. On the other hand, Group 2 showed increasing attachment pattern until it reached peak at 12h post-inoculation with score 2.80±0.41 (Fig. 3). The rate of attachment of *P. multocida* B:2 onto urinary bladder increased significantly (P<0.05) from mild score to severe score between 4 and 8 h post-inoculation.

***In vitro* distribution onto lungs:** The Fig. 4 shows the bacterial distribution pattern on the lung explants. The immunoperoxidase staining reaction was observed at 2 h post-inoculation (Fig. 5) and increased steadily with time. The three bacterial species did not show significant (P>0.05) differences in the distribution pattern, especially during the first 6 h post-inoculation. Focal distribution started at 2 h post-inoculation and significantly (P<0.05) increased to reach peak at 12 h post-inoculation with score 2.93±0.25, 2.93±0.25 and 2.40±0.50, respectively. Generally, *M. haemolytica* A:2 showed insignificantly (P>0.05) lower rate of distribution on the lung explants throughout the study period (Fig. 4).

***In vitro* distribution on the urinary bladder:** The Fig. 6 shows the bacterial distribution pattern on the explants of urinary bladder. There was no attachment of any bacteria to the explants at 0 hour. However mild distribution observed at 2 h post-inoculation. There was generally an increasing pattern of distribution of all the bacteria with time of exposure. There was significant (P<0.05) increasing rates of distribution in Group 1 between 0, 2, 4, 6 hours post-inoculation before remained insignificant (P>0.05) after 8 hours. Similar distribution pattern was observed in Group 2 and Group 3, when the rate of distribution showed significant (P<0.05) increasing pattern in the first 6 hours post-inoculation before became insignificant (P>0.05) high thereafter.

Correlation between rates of attachment and distribution: As showed in Table 1, there were significantly (P<0.05) positive correlations between the rate of attachment and the distribution of each bacterial strain within the lung and bladder explants. Particularly strong positive correlations (0.7 to 0.90) were observed in the *P. multocida* B:2 and *M. haemolytica* A:2, while perfect correlation was observed for *E. coli*.

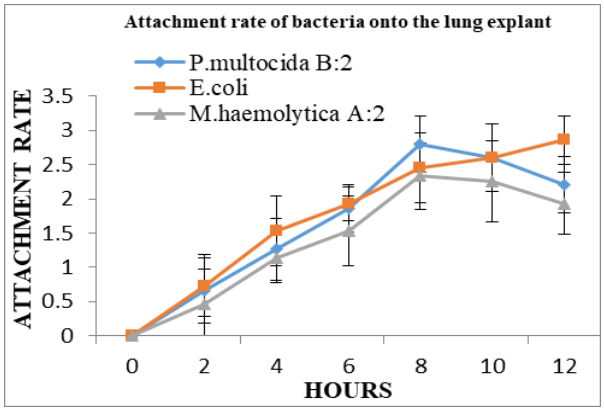

Fig. 2: Attachment patterns of the different types of bacteria onto the lung explants. Both *Pasteurella multocida* B:2 and *Mannhemia haemolytica* A:2 reached peak at 8h post-inoculation while *E. coli* at 12h post-inoculation.

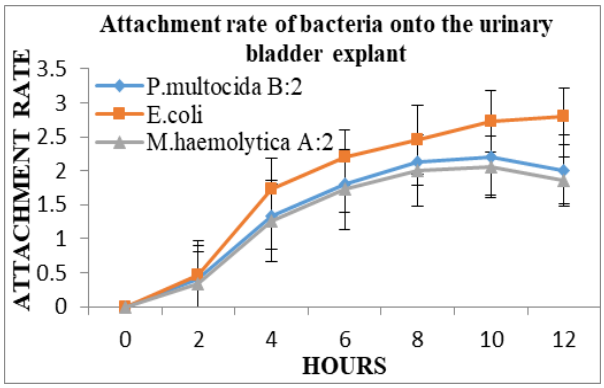

Fig. 3: Attachment patterns of the different types of bacteria onto the urinary bladder explants. Both *Pasteurella multocida* B:2 and *Mannhemia haemolytica* A:2 reached peak at 10h post-inoculation while *E. coli* at 12h post-inoculation.

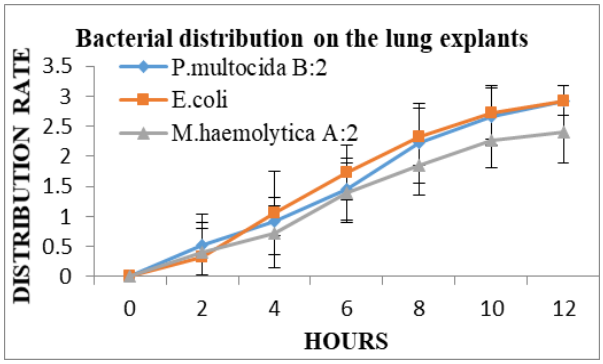

Fig. 4: Distribution pattern of the different types of bacteria on the lung explants. The three bacterial species show focal distribution at 2 h post-inoculation but increased to reach peak at 12 h post-inoculation.

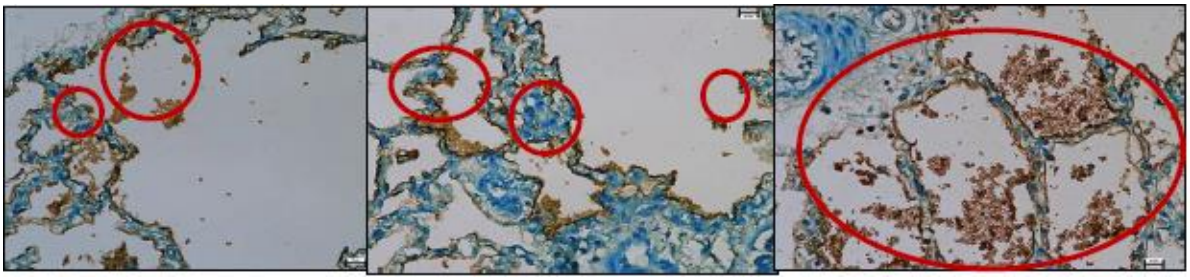

Fig. 5: Micrographs of the lung explants that were exposed to *Pasteurella multocida* B:2 showing mild (left), moderate (middle) and severe (right) distributions of the organism. IP, bar = 10nm.

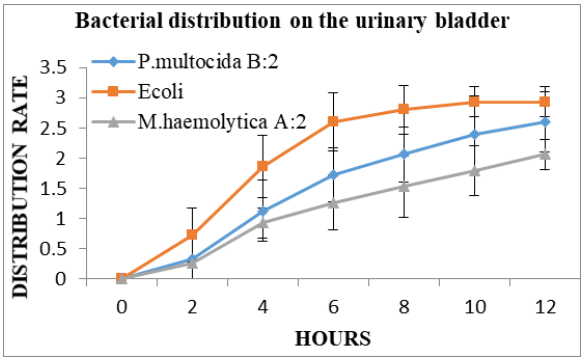

Fig. 6: Distribution pattern of the different types of bacteria on the urinary bladder explants. The three bacterial species show mild distribution at 2 h post-inoculation but increased with time of exposure.

Table 1: Correlation between the rate of attachment onto the surface of the explants and the immunoperoxidase distribution scorings for each bacterial species. There are strong correlations between the rate of attachment and the distribution of all bacterial species used in this study but the strongest was observed for *Escherichia coli*

Bacteria coefficient	Organ	Correlation	P-value
Pasteurella multocida B:2	Lungs	0.857*	0.014
	Bladder	0.893*	0.007
Escherichia coli	Lungs	1*	0.000
	Bladder	0.99*	0.000
Mannhemia haemolytica A:2	Lungs	0.857*	0.014
	Bladder	0.893*	0.007

*P<0.05.

DISCUSSION

This study evaluates the rates of attachment and distribution of *P. multocida* B:2 onto the cells of lungs and urinary bladder of buffaloes. It was compared with the known septicaemia organism of *E. coli* and respiratory organism of *M. haemolytica* A:2.

It was found that *P. multocida* B:2 tends to attach to the lung as early as 2 hours post-inoculation with a mild colonization. In natural infection, colonisation and proliferation of *P. multocida* started in the nasal mucosa before it is inhaled and deposited in the lungs leading to multifocal points of infective sites (Gonzales and Maheswaran, 1993; Singh *et al.*, 2017). This study revealed that the rate of attachment increased to reach peak at 10 hours post-inoculation. Consequently, the rate of distribution within the lung explants also increased over the time to reach peak at 12 hours post-inoculation. There was strong positive correlation between the rate of attachment of *P. multocida* B:2 and the rate of distribution in the lungs. These patterns of attachment and distribution of *P. multocida* B:2 are in agreement with an earlier report that infected buffalo calves showed signs of dullness, depression, anorexia, dyspnea, serous nasal discharge, pyrexia and recumbency, followed by death between 6 and 12 hours post-infection (Annas *et al*., 2014).

This study describes for the first time the attachment and distribution rates of *P. multocida* B:2 in the urinary bladder of buffaloes. Similarly, *P. multocida* B:2 showed increasing rates of attachment and distribution in the urinary bladder with time to reach peak at 10 hours post-inoculation. Septicaemia develops rapidly following entrance of the causative agent leading to peracute disease, which is characteristic in natural outbreaks of HS (Annas *et al*., 2015a). This is due to the wide distribution of the bacterium in the various organs (Sarah *et al*., 2012; Singh *et al*., 2017). The possible involvement of urinary tract in transmission of HS was first reported by Annas *et al*. (2014) following detection and isolation of high concentrations of *P. multocida* B:2 from the kidney, urine and the urinary bladder. These findings are in agreement with the present study, which demonstrates that *P. multocida* B:2 was distributed into the lumen of urinary bladder and showed increasing intensity of distribution with time.

Although all bacteria used in this study showed similar attachment and distribution patterns, it seemed that *M. haemolytica* A:2 is the least septicaemic organism when it showed least rates of attachment and distribution while *E. coli* is the most septicaemic organism with highest rates of attachment and distribution.

Conclusions: *In-vitro* attachment and distribution rates of wild type *P. multocida* B:2 on the cells of respiratory and urinary tracts of buffalo were found to increase over time. There was positive correlation between the attachment rate observed on the surface of each explants and the distribution rate within the explants leading to septicaemia.

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Authors contribution: YP involved in planning of the study, sample processing, data analysis and drafting of manuscript. MNAR assisted in processing of sample and data analysis while AS helped in data analysis and review of the manuscript. MZS involved in study design, data analysis and final review of the manuscript.

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