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SHORT COMMUNICATION

In vitro and *in vivo* survivality of *gdhA* derivative *Pasteurella multocida* B:2

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Aims: This study was carried out to determine the *in vitro* and *in vivo* survival pattern and period for the newly created *gdhA* derivative *Pasteurella multocida* B:2.
Methodology and results: For the *in vitro* study, the *gdhA* derivative *Pasteurella multocida* B:2 was cultured in BHI and stored at 4 °C and at room temperature (27 °C). This was followed by determination of the colony-forming unit (CFU)/ mL at weekly intervals. When kept at 4 °C, the bacterial concentration (CFU/mL) showed insignificant ($p > 0.05$) weekly reduction until week 3. By week 4, the concentration was less than 10^2 CFU/mL. In the *in vivo* study, the derivative was injected intraperitoneal into a group of mice and compared with the group injected intraperitoneal with wild-type *P. multocida* B:2 and another control group injected with PBS. It was found that the *gdhA* derivative *P. multocida* B:2 remained intact for a period of 8 h, lost its kanamycin casette after the 8-h period but remained avirulent throughout the 96 h study period when none of the mice died. On the other hand, all mice that were exposed to the wild-type *P. multocida* B:2 were dead within 24 h post-injection.
Conclusion, significance and impact study: The *gdhA* derivative *P. multocida* B:2 seemed to survive better when kept at 4 °C and survived in the host for at least 4 days without returning the virulency. These are important since this strain can be used as live vaccine in animals with minimal possibility of returning back to virulent strain while the live vaccine can be stored at 4 °C for at least 4 weeks.

Keywords: Haemorrhagic septicemia, live vaccine of *gdhA* derivative *Pasteurella multocida* B:2, Survival

INTRODUCTION

Pasteurella multocida B:2 is the causative agent of haemorrhagic septicemia (HS) of cattle and buffalo (Rafidah *et al.*, 2012) resulting in high mortality and great economic loss to the farmers (Wijewardana, 1992). The *gdhA* derivative *Pasteurella multocida* B:2 has recently been developed and was found to be suitable for preparation of live vaccine against haemorrhagic septicaemia (Othman, 2007) enhancing immunity (Chau, 2009) and vaccination coverage in field buffalo, and provided protection for 12 months post-vaccination (Rafidah *et al.*, 2012). With the excellent experimental and field vaccination results, the use of *gdhA* derivative as live vaccine is of concern without a thorough safety study. A study on the *in vitro* and *in vivo* survivality of the *gdhA* derivative *P. multocida* B:2 was undertaken to determine the *in vitro* and *in vivo* survival pattern and period. Therefore, the aim of this study was to determine the in vitro survivality of the *gdhA* derivative *P. multocida* B:2 that were stored at 4 °C and at room temperature and to determine the *in vivo* rate of survivality following inoculation into mouse model.

MATERIALS AND METHODS

In vitro study

To study the *in vitro* survivality, both wild-type and *gdhA* derivative *P. multocida* B:2 were inoculated into 10 mL of BHI broth. Following incubation, 1.25 mL of each culture was transferred into 50 mL of broth medium, incubated in a 150 rpm shaking incubator at 37 °C (Shah *et al.*, 2008). After overnight and 46 h incubation, respectively, each culture was divided into two portions; one was kept at 4 °C while the other was kept at room temperature (27 °C). Culture samples were collected at weekly interval for determination of the bacterial concentration using the

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plate counting technique (Alcamo, 1997). Approximately 30–300 colonies were counted to determine the viability colony-forming unit. The bacterial concentration was expressed as colony forming unit (CFU/mL). The experiment was terminated once the bacterial concentration was $<10^2$ CFU/mL.

In vivo study

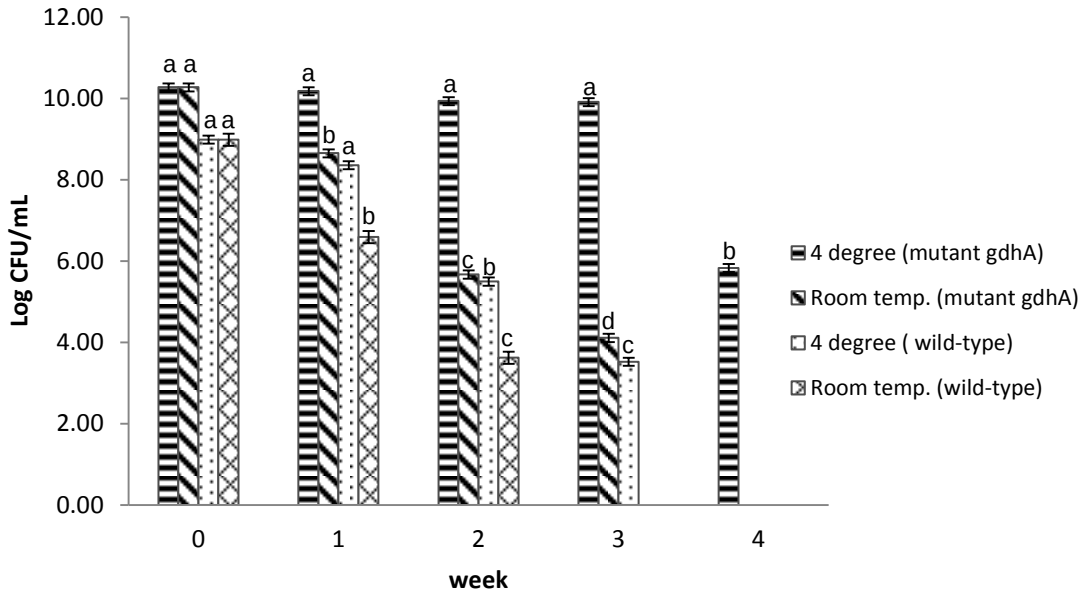
One hundred and eight healthy white mice were selected for the trials. All mice were divided into three groups and kept in three cages. Each group consisted of 36 mice. Group 1 (n= 36) was designated as the unexposed group and was injected intraperitoneally with 0.5 mL of sterile phosphate buffered saline (PBS) pH 7.4. Mice of group 2 (n= 36) were similarly injected with 0.5 mL of the inoculum containing 106 CFU/ mL of wild-type *P. multocida* B:2 while mice of group 3 (n= 36) were similarly injected with 0.5 mL of the inoculum containing 10^6 CFU/mL of the *gdhA* derivative *P. multocida* B:2. All mice were observed for signs of disease or mortality at 8 h intervals post-injection for a period of 96 h, when three mice from each group were killed.

Post-mortem examinations were carried out on all dead mice. Samples of heart, lung and liver were collected aseptically before direct smears were prepared on glass slide. The organ samples were also subcultured onto blood agar with and without antibiotics (Othman, 2007). The agar plates were incubated at 37 °C overnight or for 46 hours for wild-type and *gdhA* derivative, respectively. Suspected cultures of *P. multocida* B:2 (Table 1) were confirmed by polymerase chain reaction (PCR) of Othman (2007). The PCR produced the 1.108 kb band for the wild-type *P. multocida* B:2 and the 2.4 kb band for the *gdhA* derivative *P. multocida* B:2.

RESULTS

In vitro survival

Figure 1 shows the in vitro survival pattern and period for the *gdhA* derivative *P. multocida* B:2. The organism remained viable at 4 °C until the end of the 4 week study period, which was significantly ($p< 0.05$) better than survival for 3 weeks at room temperature. Similarly, the concentrations of the *gdhA* derivative *P. multocida* B:2 kept at 4 °C showed insignificant ($p> 0.05$) weekly reduction for 3 weeks before significantly ($p< 0.05$) reduced at week 4. When kept at room temperature, the *gdhA* derivative showed gradual but significant ($p< 0.05$) weekly reduction in the concentrations until no organism was detected in week 4. In contrast, the wild-type kept at 4 °C showed insignificant (p> 0.05) reduction for the first 1 week before started to show significant ($p< 0.05$) weekly reductions until week 3. When kept at room temperature, the wild-type showed significantly ($p< 0.05$) weekly reduction in the concentrations until week 2 (Figure 1).



Note: [a,b,c] Different letters signify significant differences at "Prob>F" less than 0.05

Figure 1: *In vitro* survival period and pattern for the *gdhA* derivative *Pasteurella multocida* B:2 compared with the wild-type *Pasteurella multocida* B:2 kept at different storage temperatures.

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In vivo survival

Table 1 summarises the *in vivo* survival of *gdhA* derivative *P. multocida* B:2 in mice. The wild-type P. multocida B:2 was highly virulent for mice, killing all mice of group 2 within 24 h post-infection (Table 1). It was successfully re-isolated from the heart, lung and liver of all dead mice of group 2.

Direct smears from the organs of mice of group 3 that were infected with the *gdhA* derivative *P. multocida* B:2 revealed the presence of the organism as early as 8 h post-inoculation (Table 1). The *gdhA* derivative *P. multocida* B:2 was subsequently isolated from the heart, lung and liver, and persisted in the organs until 64 h post-inoculation. The *gdhA* bacterium, however, was absent

Table 1: Rate of successful re-isolation of the *gdhA* derivative and wild-type *Pasteurella multocida* B:2 from different organs of mice following intraperitoneal inoculation.

	Hour post-inoculation											
Group	8	16	24	32	40	48	56	64	72	80	88	96
Group 1 (Control):												
Heart	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
Lungs	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
Liver	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
Mortality	0	0	0	0	0	0	0	0	0	0	0	0
Group 2 (Wild-type *P. mutocida* B:2)												
Heart	3/3	3/3	3/3	-	-	-	-	-	-	-	-	-
Lungs	3/3	3/3	3/3	-	-	-	-	-	-	-	-	-
Liver	3/3	3/3	3/3	-	-	-	-	-	-	-	-	-
Mortality	0	10	17									
Group 3 (*gdhA* derivative *P. multocida* B:2)												
Heart	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	1/3	1/3	1/3	0/3
Lungs	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	1/3	0/3	0/3	0/3
Liver	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	2/3	0/3	0/3	0.3
Mortality	0	0	0	0	0	0	0	0	0	0	0	0

from the liver of mice at 72 h, from the lungs at 80 h and from the heart at 96 h post-inoculation. Following PCR, only isolates from mice that were killed at 8 h post-inoculation revealed the 2.4 kb band typical for the *gdhA* derivative *P. multocida* B:2. Isolations between 16 and 80 h post-inoculation revealed the 1.108 kb band of wild-type *P. multocida* B:2. Nevertheless, all inoculated mice of group 3 survived the infection (Table 1).

DISCUSSISON

In general, the gdhA derivative *P. multocida* B:2 showed longer *in vitro* survival period than the wild-type *P multocida* B:2. Similarly, keeping at 4 °C retained the higher concentrations for a much longer period than keeping at room temperature. The *in vitro* survivality and safety of the master seed are extremely important in attempts to produce live vaccines for animals (OIE, 2012). Results of this study revealed that the gdhA derivative *P. multocida* B:2 should be kept at 4 °C to maintain the bacterial concentrations a period of at least 3 weeks. Similar observations were reported for other live veterinary vaccines (Milstien *et al.*, 2006; Ramakrishnan *et al.*, 2007).

Other than in vitro survival, it is important that the live vaccine stays in the host for some time to stimulate immune response. At the same time, the vaccine strain should not stay long in the host to minimize possibility of revert back to virulency. This study revealed that the *gdhA* derivative remained in the host and avirulent for at least 4 days. However, Othman (2007) reported 14-day in vivo survival of the *gdhA* derivative in mice. Therefore, 4 to 14 days in vivo survival of the *gdhA* mutant strain

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provides ample time for the mucosa to be stimulated (Rafidah *et al.,* 2011).

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