SHORT COMMUNICATION

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

Siti Oslan Hazwani1, Mohd Zamri Saad2, Mohamad Rosfarizan2, Bejo Siti-Khairani3

1Research Centre for Ruminant Diseases, Faculty of Veterinary Medicine, Universiti Putra Malaysia 43400 Serdang, Malaysia.
2Department of Bioprocess Technology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia 43400 Serdang, Malaysia.
3Department of Veterinary Pathology & Microbiology, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 Serdang, Malaysia.

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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 cassette 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 survivability 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 survivability 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 survivability following inoculation into mouse model.

MATERIALS AND METHODS

In vitro study

To study the in vitro survivability, 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
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 <10⁶ 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 10⁶ 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 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).
**In vivo survival**

Table 1 summarises the in vivo survival of gdhA derivative *Pasteurella 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.

<table>
<thead>
<tr>
<th>Group</th>
<th>Hour post-inoculation</th>
<th>8</th>
<th>16</th>
<th>24</th>
<th>32</th>
<th>40</th>
<th>48</th>
<th>56</th>
<th>64</th>
<th>72</th>
<th>80</th>
<th>88</th>
<th>96</th>
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</thead>
<tbody>
<tr>
<td>Group 1 (Control):</td>
<td>Heart</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
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<td></td>
<td>Lungs</td>
<td>0/3</td>
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<tr>
<td></td>
<td>Liver</td>
<td>0/3</td>
<td>0/3</td>
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<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
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<tr>
<td></td>
<td>Mortality</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Group 2 (Wild-type <em>P. multocida</em> B:2)</td>
<td>Heart</td>
<td>3/3</td>
<td>3/3</td>
<td>3/3</td>
<td>-</td>
<td>-</td>
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<tr>
<td></td>
<td>Lungs</td>
<td>3/3</td>
<td>3/3</td>
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<td>-</td>
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<tr>
<td></td>
<td>Liver</td>
<td>3/3</td>
<td>3/3</td>
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<tr>
<td></td>
<td>Mortality</td>
<td>0</td>
<td>10</td>
<td>17</td>
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<td>Group 3 (gdhA derivative <em>P. multocida</em> B:2)</td>
<td>Heart</td>
<td>3/3</td>
<td>3/3</td>
<td>3/3</td>
<td>3/3</td>
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<tr>
<td></td>
<td>Lungs</td>
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</tr>
<tr>
<td></td>
<td>Liver</td>
<td>3/3</td>
<td>3/3</td>
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<tr>
<td></td>
<td>Mortality</td>
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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 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).

**DISCUSSION**

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

REFERENCES


