Booster vaccination with safe, modified, live-attenuated mutants of *Brucella abortus* strain RB51 vaccine confers protective immunity against virulent strains of *B. abortus* and *Brucella canis* in BALB/c mice

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*Brucella abortus* attenuated strain RB51 vaccine (RB51) is widely used in prevention of bovine brucellosis. Although vaccination with this strain has been shown to be effective in conferring protection against bovine brucellosis, RB51 has several drawbacks, including residual virulence for animals and humans. Therefore, a safe and efficacious vaccine is needed to overcome these disadvantages. In this study, we constructed several gene deletion mutants (*DcydC, DcydD* and *DpurD* single mutants, and *DcydC*DcydD and *DcydC*DpurD double mutants) of RB51 with the aim of increasing the safety of the possible use of these mutants as vaccine candidates. The RB51*DcydC, RB51*DcydD, RB51*DpurD, RB51*DcydC*DcydD and RB51*DcydC*DpurD mutants exhibited significant attenuation of virulence when assayed in murine macrophages *in vitro* or in BALB/c mice. A single intraperitoneal immunization with RB51*DcydC, RB51*DcydD, RB51*DpurD, RB51*DcydC*DcydD and RB51*DcydC*DpurD mutants was rapidly cleared from mice within 3 weeks, whereas the RB51*DpurD mutant and RB51 were detectable in spleens until 4 and 7 weeks, respectively. Vaccination with a single dose of RB51 mutants induced lower protective immunity in mice than did parental RB51. However, a booster dose of these mutants provided significant levels of protection in mice against challenge with either the virulent homologous *B. abortus* strain 2308 or the heterologous *Brucella canis* strain 26. In addition, these mutants were found to induce a mixed but T-helper-1-biased humoral and cellular immune response in immunized mice. These data suggest that immunization with a booster dose of attenuated RB51 mutants provides an attractive strategy to protect against either bovine or canine brucellosis.

**INTRODUCTION**

*Brucella* spp. are Gram-negative, facultative and intracellular bacteria that cause a zoonotic disease that is distributed almost worldwide. This disease is a major cause of abortions and infertility in numerous animal species (Godfroid *et al.*, 2005; Seleem *et al.*, 2010). In humans, infection with several *Brucella* spp. has been reported, and the most pathogenic and invasive species for humans are *Brucella melitensis* and *Brucella abortus*, followed in descending order by *Brucella suis* and *Brucella canis* (Godfroid *et al.*, 2005; Lucero *et al.*, 2010; Seleem *et al.*, 2010). In Korea, outbreaks of bovine and canine brucellosis, mainly caused by *B. abortus* and *B. canis*, have been reported in cattle and dogs, respectively (Bae & Lee 2009; Yoon *et al.*, 2010). *B. abortus* and *B. canis* infect not only their preferred hosts, but also other domestic or wild animal species, e.g. *B. abortus* infection in elk, dog and Chinese water deer or *B. canis* infection in cattle (Baek *et al.*, 2003, 2012; Her *et al.*, 2010; Kang *et al.*, 2011; Truong *et al.*, 2011) have been reported. Of note, from 2002 to 2010, 651 human brucellosis cases were also reported to be associated with the livestock industry in Korea (Jang *et al.*, 2011; Park *et al.*, 2005).

As a result of the economic and global public health risks, extensive efforts have been made to control and prevent brucellosis in animals through eradication programs (Corbel, 2006; Yoon *et al.*, 2010). In addition to the implementation of a ‘test-and-slaughter’ strategy,
vaccination is a critical tool for control of bovine brucellosis (Avila-Calderón et al., 2013). In the last few decades, live-attenuated vaccines such as *B. abortus* strains S19 and RB51 have been developed and widely used against bovine brucellosis (Avila-Calderón et al., 2013; Schurig et al., 2002). The strain RB51 vaccine was first reported in 1991 as a spontaneous mutant of *B. abortus* strain 2308 that developed an attenuated phenotype after repeated *in vitro* passage in the presence of rifampicin (Schurig et al., 2002). Numerous efficacy studies of this vaccine conducted in experimental mice and in cattle have demonstrated that the vaccinated animals, particularly cattle, are effectively protected from exposure to WT *B. abortus* vaccine conducted in experimental mice and in cattle have demonstrated that the vaccinated animals, particularly cattle, are effectively protected from exposure to WT bacteria (Schurig et al., 2002; Wang & Wu, 2013). The protective efficacy of RB51 is variable and depends on a range of factors, including the age of the vaccinated animal and the dose and route of the vaccination. Although RB51 typically exhibits low virulence in cattle, the vaccine may cause abortions when administered to pregnant animals. It was also found that after vaccination of cattle with RB51, the bacteria possibly survived for extended periods and were excreted in milk or vaginal exudate (Arellano-Reynoso et al., 2004). In addition, the potential for persistent local and systemic adverse events associated with accidental human exposure to RB51 has also been reported (Ashford et al., 2004).

*B. abortus* is known to survive and replicate in nutrient- or oxygen-limited environments inside hosts. Therefore, *B. abortus* mutants lacking the genes encoding cytochrome terminal oxidases or required for the biosynthesis of an essential nutrient are unlikely to survive in that harsh environment. Using transposon mutagenesis, previous work in our laboratory has identified the *B. abortus* genes *cydC* (encoding the ATP-binding/permease protein) and *purD* (encoding phosphoribosylamine–glycine ligase) as being required for intracellular survival and virulence *in vitro* and *in vivo* (Truong et al., 2014, 2015). BALB/c mice immunized with the *cydC*::Tn5 and *purD*::Tn5 transposon mutants were protected against challenge with virulent *B. abortus* strain 544. Although transposon mutants are not appropriate for use as a vaccine, these genes may merit consideration as ideal targets for future vaccine development. In the present study, in an attempt to diminish the virulence of the strain RB51 vaccine, we constructed unmarked single (*AcydC, AcydD* and *ApurD*) and double (*AcydCAcydD* and *ΔcydDpurD*) gene deletion mutants of RB51, and evaluated their safety and their induction of protective immunity in BALB/c mice. Deletion in RB51 of *cydC*, *cydD* or *purD*, or both *cydC* and *cydD* or *purD*, has several positive effects, including significantly reduced survival or persistence of bacteria in cultured macrophages and in BALB/c mice, and a diminished inflammatory response associated with vaccination. These effects lead to the increased safety of the vaccine strain, because high doses of RB51 alone elicit splenomegaly, an undesirable side effect of vaccination (Stevens et al., 1995; Wang & Wu, 2013). In addition, we also investigated the capacity of RB51 mutants to elicit *Brucella*-specific immune responses, and to protect the mice against challenge with virulent homologous *B. abortus* strain 2308 and heterologous *B. canis* strain HID90026.

**METHODS**

**Bacterial strains.** Bacterial strains used in this study (Table 1) included *Escherichia coli* DH5α, *B. abortus* strain RB51 vaccine, *B. abortus* strain 2308 (S2308) and *B. canis* strain HID90026 (*B. canis* S26). The virulent *B. canis* S26 was isolated from a dog by our laboratory in 2010. All *Brucella* strains and mutants were routinely grown in Tryptic Soy Broth (Difco) or on Tryptic Soy Agar (TSA) at 37 °C in 5% CO₂. *E. coli* DH5α was cultivated in Luria–Bertani (Difco) broth or agar. Electrocompetent cells of RB51 and mutants were prepared as described previously (McQuiston et al., 1995).

**Construction of the unmarked RB51 single- and double-deletion mutants.** To construct the plasmids for deleting *cydC*, *cydD*, *purD*?
and purD, we designed two sets of primers to amplify from RB51 DNA fragments in the upstream and downstream regions of each gene of interest. The sequences of each primer pair are listed in Table 2. Using previously published methods (Kahl-McDonagh & Ficht, 2006; Wang et al., 2013), the respective plasmids pEX18ΔcydC, pEX18ΔcydD and pEX18ΔpurD were created by a two-round PCR amplification, restricted digestion and ligation, and then introduced into RB51 and the RB51ΔcydC mutant via electroporation. Transformants were selected in the presence of 100 μg carbencicillin ml⁻¹ for the first screening and 5 % sucrose for the second screening. The deletions of cydD (D5F/D6R), cydC (C5F/C6R) and purD (P5D/P6D) in RB51 or RB51ΔcydC were then verified by PCR amplification and DNA sequencing analysis, and the resulting mutants referred to as RB51ΔcydD, RB51ΔcydC, RB51ΔpurD, RB51ΔcydCAcydD and RB51ΔcydCAcydDpurD. Genetic complementation strains corresponding to each single mutant were also constructed and characterization of these mutants was performed as described previously (Truong et al., 2014, 2015).

Intracellular growth of RB51 mutants in murine macrophages. Preparation of and the intracellular bacterial growth assays within RAW 264.7 cells (RAW cells) and bone marrow-derived macrophages (BMDMs) from BALB/c mice were performed as previously described (Trant et al., 2010; Truong et al., 2015). Briefly, the RAW cells and BMDMs were seeded at 2 × 10⁵ cells per well in complete medium [CM; RPMI 1640 (GenDepot), 10 mM HEPES and 10 % FBS] into 10 cm² tissue culture plates, and infected with RB51 and its isogenic mutants at m.o.i. 100. The plates were centrifuged at 250 g for 5 min and then incubated at 37 °C for 30 min. After washing twice with PBS, the cells were incubated for 1 h in the presence of 50 μg gentamicin ml⁻¹ (Sigma) to kill extracellular bacteria. The cells were then replaced by CM containing 10 μg gentamicin ml⁻¹, and incubated at 37 °C for 0, 6, 24 and 48 h. At different times post-infection (p.i.), the infected cells were washed three times with PBS and lysed with 0.1 % Triton X-100 in sterile double-distilled water. Tenfold serial dilutions of the lysates were plated on TSA plates to determine the c.f.u. count. These assays were performed in triplicate and repeated twice.

Clearance of RB51 mutants in mice. Female 7-week-old BALB/c mice were purchased from ORIENTS and kept in individually ventilated cage rack systems in the Biosafety Level 2+ facilities of Kangwon National University. All animal care and experimental procedures were approved by Kangwon University Institutional Animal Care and Use Committee (permit KW-150729-2). Groups of mice were inoculated intraperitoneally with a single dose of 3.9–4.8 × 10⁹ c.f.u. RB51 or RB51 mutants in 0.2 ml PBS. Three weeks after the first inoculation, a booster inoculation of 3.5–4.6 × 10⁹ c.f.u. RB51 mutants was given. At 1, 2, 3, 4, 5, 6, 7 and 9 weeks after the single inoculation or 4 weeks after the booster inoculation, five mice from each group were euthanized, and spleens were aseptically removed, weighed and homogenized in PBS. Tenfold serial dilutions of spleen homogenates were plated on TSA plates and incubated for 3–5 days at 37 °C. The c.f.u. counts were determined and results presented as the mean ± SD for each group.

Evaluation of protective efficacy of RB51 mutants in mice. For the single immunization, groups of mice were vaccinated intraperitoneally with a single dose of 3.9–4.8 × 10⁹ c.f.u. RB51 and RB51 mutants in 0.2 ml PBS. For the booster immunization, groups of mice were boosted with 3.5–4.6 × 10⁹ c.f.u. RB51 mutants 3 weeks after the first vaccination. Control groups were injected with PBS only. At 7 weeks post-vaccination, all groups were challenged intraperitoneally with 1.7 and 2.8 × 10⁸ c.f.u. per mouse of virulent B. abortus S2308 and B. canis S26, respectively. At 2 weeks post-challenge, five mice per group were sacrificed, and the spleens were collected and weighed. The numbers of c.f.u. recovered from spleens were determined as described above. The degree of protection was expressed as the mean ± SD c.f.u. of S2308 or B. canis S26 for each mouse group obtained after challenge. Mean log c.f.u. reduction or log units of protection (U) were obtained by subtracting the mean log c.f.u. for the vaccinated group from the mean log c.f.u. for the PBS control group.

Evaluation of serum antibody response. Mice were bled from the orbital sinus during the immunization schedule and sera were stored at −70 °C until tested. The IgG and IgG subclass antibodies against whole-cell antigens (WCAs) of RB51 and B. canis S26 were measured by indirect ELISA as described previously (Arenas-Gamboa et al., 2008). Briefly, ELISA plates were coated with 25 μg per well WCAs of each strain in carbonate buffer overnight at 4 °C. The plates were then washed with PBS-T (PBS containing 0.05 % Tween 20) and blocked with blocking buffer (1 % BSA in PBS). Subsequently, triplicate samples of 1 : 200 diluted serum in blocking buffer were added and incubated at 37 °C for 1 h. After three PBS-T washes, goat anti-mouse IgG, IgG1 and IgG2a horseradish peroxidase conjugate ( Fitzgerald) at a dilution of 1 : 2000 was added and incubated at 37 °C for 1 h. After extensive washing, o-phenylenediamine dihydrochloride peroxidase substrate (Sigma) was added and incubated for 20 min, and then the A450 was measured with an ELISA reader (Bio-Rad).

Cytokine detection in spleen cell culture supernatants. Splenocytes from the mice were prepared as previously described (Kahl-McDonagh & Ficht, 2006). At 7 weeks post-vaccination, individual spleens from five mice per group were aseptically removed. Single-cell suspensions were obtained by passing the spleens through a 40 μm mesh cell strainer (SPL Lifesciences). The cells were then pelleted by centrifugation at 1000 g for 10 min and then treated for 5 min with ACK erythrocyte lysis buffer (150 mM NH₄Cl, 1 mM KHCO₃,

Table 2. Primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’→3’) (restriction enzyme used)*</th>
</tr>
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<tbody>
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<td>C1F</td>
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</tr>
<tr>
<td>C2R</td>
<td>GCATTACAAGGATCCGCCGATAGGTTAGCTAGT</td>
</tr>
<tr>
<td>C3F</td>
<td>CCTCTAGGGATCCGTCGTAAGTGAACATTCTTCATTCC</td>
</tr>
<tr>
<td>BamHI</td>
<td></td>
</tr>
<tr>
<td>C4R</td>
<td>GCCTTAGGCTGTGCTCTCGCAAGAGA (XbaI)</td>
</tr>
<tr>
<td>C5F</td>
<td>AACGGTTCATAGTGGCA</td>
</tr>
<tr>
<td>C6R</td>
<td>CGAGCATGATGAACGCTG</td>
</tr>
<tr>
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<td>GGAATTCAATGGCAACCTTCTCATCG (EcORI)</td>
</tr>
<tr>
<td>D2R</td>
<td>TGTTTACAGGGATCCGTTGTCGTCGCAAGAGA (BamHI)</td>
</tr>
<tr>
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<td></td>
</tr>
<tr>
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</tr>
<tr>
<td>GATCC (BamHI)</td>
<td></td>
</tr>
<tr>
<td>D4R</td>
<td>GCCTTAGAAGCCAGTCAACGGATTCCATG (XbaI)</td>
</tr>
<tr>
<td>D5F</td>
<td>CTGCCATACGAGCTTGCTTGTGTCGCAAGAGA (BamHI)</td>
</tr>
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<td>D6R</td>
<td>CGAGGTCCTGAGGACATTCTTCATCG (EcORI)</td>
</tr>
<tr>
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</tr>
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<td>PD6R</td>
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*Bold indicates restriction enzyme site.
Statistical analysis. The data from assays for survival of RB51 and its isogenic mutants in murine macrophages and BALB/c mice and from efficacy studies were expressed as the mean ± SD log c.f.u. for each group. Statistical comparison between means was performed using ANOVA. The significance of differences between the experimental groups was analysed by paired Student’s t-test and ANOVA followed by either Dunnett’s post-test (comparison with control group) or Tukey’s post-test (comparison between several groups). P < 0.05 was considered to indicate significance.

RESULTS

Intracellular survival of RB51 mutants in murine macrophages

To examine the intracellular survival of bacteria in vitro, RAW cells and BMDMs were infected with RB51 and its isogenic RB51ΔcydC, RB51ΔcydD, RB51ΔpurD and RB51ΔcydCΔpurD mutants. As shown in Fig. 1, RB51 mutants displayed a similar rate of internalization as the parental RB51 as measured immediately after infection, indicating that absence of cydC, cydD or purD does not affect internalization. However, RB51 mutants showed an almost 1 log reduction in c.f.u. compared with RB51 at 6 h p.i., in both RAW cells and BMDMs (Fig. 1a, b, P < 0.001), suggesting that RB51 mutants were more susceptible to macrophage killing than parental RB51. At 24 and 48 h p.i., RB51 mutants showed a significantly reduced rate of survival within RAW cells and BMDMs compared with parental RB51 (P < 0.001): RB51 mutants exhibited a 2–4 log reduction in the number of c.f.u. at 48 h p.i. in both cell types. These results suggested that RB51 mutants are more attenuated in murine macrophages than RB51, and indicated that the deletions of cydC, cydD and purD significantly affect the ability of RB51 to survive within both cell types.

Safety of RB51 mutants in mice

To assess the attenuation of RB51 mutants, BALB/c mice were inoculated intraperitoneally with a high dose (10^8 c.f.u.) of RB51 or its isogenic mutants and the numbers of viable organisms in the spleens were determined at different time points after inoculation. As shown in Fig. 2(a), at 1 and 2 weeks post-inoculation, significantly fewer bacteria were recovered from the spleens of mice inoculated with a single dose of RB51 mutants than from those of mice inoculated with the parental RB51. Recovery of RB51 mutants was reduced by ~2–3 log compared with that of the parental RB51 (P < 0.001). By 3 weeks post-inoculation, when the RB51ΔcydC, RB51ΔcydD, RB51ΔcydCΔcydD and RB51ΔcydCΔpurD mutants had been completely cleared from spleens of mice, RB51 and the RB51ΔpurD mutant persisted in spleens at 3.8 and 1.3 log c.f.u., respectively (Fig. 2a). No viable organisms were detected at 4 weeks post-inoculation in spleens of mice inoculated with RB51ΔpurD, whereas RB51 was completely cleared by 7 weeks post-inoculation. The significantly reduced persistence of RB51 mutants in mice correlated with reduced spleen weights compared with those in mice inoculated with RB51 (Fig. 2b). In fact,
enlarged spleens were observed only in RB51-inoculated mice, but not in mice inoculated with RB51 mutants, implying that there was significantly reduced splenomegaly and inflammatory response in mice inoculated with RB51 mutants (Fig. 2b). These results demonstrated that RB51 mutants were highly attenuated in mice.

When a second inoculation of RB51ΔcydC, ΔcydD, ΔpurD, ΔcydCAcydD or ΔcydCAPurD mutants was given in mice 3 weeks after the first inoculation, no mice in the experimental groups showed evidence of splenomegaly, even though they were re-inoculated with high doses (3.5–4.6 × 10^8 c.f.u. per mouse) of RB51 mutants (data not shown). Moreover, these mutants were completely cleared from spleens by 4 weeks after the second inoculation (data not shown). The enhanced clearance and the lack of splenomegaly associated with either single or booster inoculation suggested that from a safety perspective RB51 mutants are attractive as live vaccine candidates.

**Efficacy against *B. abortus* challenge of single and booster doses of RB51 mutants**

The above demonstration that RB51 mutants are highly attenuated and have limited persistence in vitro and in mice suggested that the rapid clearance of RB51 mutants may enhance their safety as vaccine strains, but provide possibly reduced levels of protection compared with RB51. To test this assumption, the groups of vaccinated mice were challenged at 7 weeks post-vaccination with the virulent *B. abortus* S2308. The mean log c.f.u. values of the challenge strain in spleens of mice are presented in Table 3. At 2 weeks post-challenge, there was a significant reduction (P<0.001) in bacterial load in the spleens from mice vaccinated with a single dose of RB51, RB51ΔcydC, RB51ΔcydD, RB51ΔpurD, RB51ΔcydCAPurD or RB51ΔcydCAcydD mutants relative to those in the PBS-vaccinated mice, with 1.35, 0.83, 0.91, 1.08, 0.76 or 0.72 log reductions, respectively, in c.f.u. in the spleens. Vaccination with a single dose of RB51 resulted in better protection against challenge with virulent S2308 than did a single dose of RB51 mutants.

The improved benefit of diminished splenomegaly and respective safety post-vaccination exhibited by RB51 mutants provided us with an opportunity to evaluate the use of a booster dose of RB51 mutants in mice. To determine whether a booster dose of RB51 mutants improved protection against a challenge infection, vaccinated mice were challenged with the virulent homologous S2308 at 4 weeks after a second vaccination. As shown in Table 3, booster vaccination of mice with RB51 ΔcydC, RB51ΔcydD, RB51ΔpurD, RB51ΔcydCAPurD or RB51ΔcydCAcydD mutants offered a 1.81, 1.86, 1.94, 1.74 or 1.68 log reduction in bacterial burden in the spleens relative to that in the PBS control group (P<0.001). Mice vaccinated with a booster dose of RB51 mutants showed enhanced protection against S2308 challenge compared with those given a single dose of either RB51 mutants or RB51 (Table 3). These results indicate that the use of booster vaccination with RB51 mutants could provide better protective efficacy against challenge than single vaccination with parental RB51 or RB51 mutants.

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**Fig. 2.** Clearance of RB51 and RB51 mutants in BALB/c mice. Groups of mice were inoculated intraperitoneally with a single dose of ~4–5 × 10^8 c.f.u. RB51, or RB51ΔcydC, RB51ΔcydD, RB51ΔpurD, RB51ΔcydCAcydD or RB51ΔcydCAPurD mutants. At the indicated time points, the spleens of inoculated mice were aseptically removed, and assessed for (a) levels of c.f.u. of strain RB51 and its mutants, and (b) spleen weight changes during infection. Data are presented as mean ± SD spleenic c.f.u. and weights. **P<0.001 indicates the log numbers of c.f.u. or spleen weights are significantly different from those of PBS-inoculated mice. *P<0.05 indicates a significant difference in c.f.u. levels between mutants.**
To evaluate the immune response in mice vaccinated with RB51 mutants, we assessed the antibody responses in serum and cytokine responses in splenocytes from vaccinated mice at 7 weeks post-vaccination. The antibody levels were assessed by ELISA to reflect the humoral immune response against WCAs of either RB51 or B. canis strains. The levels of relevant specific antibodies in the sera of the immunized mice at 7 weeks post-vaccination are shown in Fig. 3. Mice immunized with a single dose of RB51 mutants were capable of producing significantly higher levels of specific IgG antibodies against WCAs of RB51 than PBS-immunized mice (P<0.001) (Fig. 3a). As expected, mice immunized with two doses of RB51 mutants developed significantly higher levels of IgG specific for the WCAs of RB51 than did those mice immunized with a single dose of RB51 mutants (P<0.05). In addition, assays of IgG1 and IgG2a antibody isotypes revealed that both were present at significantly higher levels in mice given a booster dose of RB51 mutants than in mice immunized with a single dose of RB51 mutants (P<0.05) or in mice inoculated with PBS (P<0.001) (Fig. 3b, c). Similarly, when sera from mice immunized with either a single dose of RB51 or two doses of RB51 mutants were examined for antibody responses against WCAs from B. canis S26, high levels of IgG, IgG1 and IgG2a antibodies to WCAs of B. canis S26 were detected at 7 weeks post-vaccination (Fig. 3). Notably, the antibody responses to WCAs from B. canis S26 were slightly higher than those to WCAs from RB51 (Fig. 3).

### Efficacy against heterologous B. canis challenge of a booster dose of RB51 mutants

Strain RB51 vaccine has been shown to be protective in mice and dogs against challenge with the virulent rough strains of Brucella ovis and B. canis, respectively (Hur & Baek, 2010; Jiménez de Bagüés et al., 1994). Therefore, to determine whether a booster dose of highly attenuated RB51 mutants would be protective against rough strains of Brucella, vaccinated mice were challenged at 7 weeks post-vaccination with the rough, virulent, heterologous B. canis S26. As shown in Table 3, booster vaccination of mice with RB51 mutants conferred significant levels of protection at 2 weeks post-challenge. RB51ΔpurD mutant provided the best protection, defined as a 2.20 log decrease in bacterial load compared with the PBS control group (P<0.001), followed by RB51ΔcydC at 2.11 log, RB51ΔcydD at 2.03 log, RB51ΔcydCΔcydD at 1.88 log and RB51ΔcydCΔpurD at 1.83 log (P<0.001). Notably, booster vaccination with RB51 mutants provided better protection than single vaccination with parental RB51 (1.56 U). These results suggested that a booster dose of RB51 mutants could provide effective protection in mice against challenge with heterologous virulent B. canis.

### Humoral and cell-mediated immune response induced by RB51 mutants

To evaluate the immune response in mice vaccinated with either single or booster vaccination with RB51 mutants, we

### Table 3. Protection against B. abortus S2308 and B. canis S26 provided to BALB/c mice by vaccination with RB51 and its isogenic mutants

<table>
<thead>
<tr>
<th>Group (n=5)</th>
<th>No. of vaccinations</th>
<th>Log c.f.u. B. abortus S2308 in spleen*</th>
<th>Log units (U) of protection</th>
<th>Log c.f.u. B. canis S26 in spleen*</th>
<th>Log units (U) of protection</th>
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<tr>
<td>PBS</td>
<td>1</td>
<td>6.28 ± 5.97</td>
<td>1.35†</td>
<td>6.22 ± 6.03</td>
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<tr>
<td>RB51</td>
<td>1</td>
<td>4.93 ± 4.69</td>
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<tr>
<td>RB51ΔcydC</td>
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<td>4.02 ± 4.11</td>
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<tr>
<td>RB51ΔcydCΔcydD</td>
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<td>5.52 ± 5.27</td>
<td>1.94†</td>
<td>4.34 ± 4.39</td>
<td>1.88†</td>
</tr>
<tr>
<td>IVK15ΔcydCΔpurD</td>
<td>2</td>
<td>5.56 ± 5.36</td>
<td>1.68†</td>
<td>4.39 ± 4.35</td>
<td>1.83†</td>
</tr>
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</table>

* Mice were vaccinated once with RB51, and once or twice at an interval of 3 weeks with RB51 mutants. Seven weeks after the first vaccination, all groups of mice were challenged intraperitoneally with the virulent strains of B. abortus S2308 (1.7 × 10<sup>5</sup> c.f.u. per mouse) and B. canis S26 (2.8 × 10<sup>5</sup> c.f.u. per mouse), and mean ± SD splenic c.f.u. was counted at 2 weeks post-challenge.
† Significant difference between the vaccinated groups and the PBS control group (P<0.001).
‡ Significant difference between the mouse groups immunized with two doses of the RB51ΔpurD mutant and a single dose of parental RB51 (P<0.05).
Fig. 3. Anti-Brucella antibodies in sera from mice vaccinated with either a single dose or two doses of RB51 mutants or a single dose of RB51. At 7 weeks post-vaccination, the serum samples were collected from mice vaccinated with either a single dose or two doses of RB51 mutants or a single dose of RB51. (a) IgG, (b) IgG1 and (c) IgG2a antibody levels against Brucella spp.
To evaluate the cellular immune response associated with vaccination with RB51 and its mutants, capture ELISAs were used to detect the levels of IFN-γ and IL-10 in supernatants of splenocytes from immunized mice at 7 weeks post-vaccination stimulated with heat-killed RB51 or B. canis S26, concanavalin A or medium. The results shown in Fig. 4 clearly demonstrate that there were no differences in the levels of IFN-γ and IL-10 in supernatants of splenocytes from immunized mice stimulated with either heat-killed RB51 or heat-killed B. canis S26. Splenocytes from mice vaccinated with RB51 or RB51 mutants produced significantly higher amounts of IFN-γ and IL-10 than those from non-vaccinated mice (P<0.001). The IFN-γ concentration was significantly higher in supernatants of splenocytes from mice vaccinated with a booster dose of RB51 mutants than in those from mice vaccinated with a single dose of RB51 mutants (P<0.05). However, there was no significant increase in levels of IL-10 in any of vaccinated groups. As expected, splenocytes from all mice that were stimulated with concanavalin A produced significant amounts of IFN-γ and IL-10, whereas no cytokine production was induced by medium stimulation (data not shown).

**DISCUSSION**

In the campaign to eradicate bovine brucellosis, the extensive use of the strain RB51 vaccine has played an important role in reducing the incidence of the disease in cattle in many countries. Although the use of RB51 has virtually eliminated interference with the serological diagnosis of field infections, several drawbacks are associated with this vaccine strain, including its variable efficacy and its ability to induce human infections and sometimes abortions in pregnant animals (Moriyón et al., 2004; Wang & Wu, 2013). In dogs, oral or intravenous inoculation with RB51 did not result in bacterial shedding in urine or faeces or abortion in pregnant dogs. However, it caused infection of the oropharyngeal lymph nodes, liver, spleen and placenta, and infected canine placental membranes or fluids may be a source of infection for other animals and humans (Palmer & Cheville, 1997). Therefore, questions have arisen concerning vaccine safety, which poses a significant challenge for *Brucella* vaccine development. For this reason, the development of new, safer or improved vaccines is still a matter of great interest.

Our previous studies using transposon mutagenesis have identified *cydC* and *purD* as being required for intramacrophage survival and virulence of *Brucella* in the purine- or oxygen-limited environments inside hosts (Truong et al., 2014, 2015). In particular, *cydC*::Tn5 and *purD*::Tn5 mutants were found to be attenuated for virulence and survival in both RAW and HeLa cells and in BALB/c mice, and protected the mice against challenge with virulent *B. abortus* strain 544. These data indicate that *cydC* and *purD* can be used as the ideal candidate genes for the generation of new or improved vaccine strains (Truong et al., 2014, 2015). In the present study, in an attempt to enhance the safety of RB51, *cydC*, *cydD* (ATP-binding protein) and *purD* were therefore selected for deletion to further attenuate strain RB51 vaccine.

The resulting RB51Δ*cydC*, RB51Δ*cydD*, RB51Δ*purD*, RB51Δ*cydD*Δ*cydD* and RB51Δ*cydD*Δ*purD* mutants were examined for survival and attenuation in RAW cells and BMDMs. As shown in this study, the deletion of *cydC*, *cydD* and *purD* did not affect bacterial internalization, but had a marked effect on the survival of RB51 in RAW cells and BMDMs. Notably, RB51 mutants were more attenuated in these murine macrophages than the parental RB51. This observation was consistent with our previous finding that a transposon insertion in *cydC* of *B. abortus* crippled the ability of *Brucella* to survive inside macrophages by diminishing its resistance to host defence mechanisms (Truong et al., 2014). It is not surprising that a single deletion of *cydD* or deletion of both *cydC* and *cydD* in RB51 had the same attenuated phenotype *in vitro* as did the *cydC* mutant, because both genes encode an ATP-binding cassette (ABC)-type transporter that is required for the activity of cytochrome *bd* and *c* oxidases, similar to that seen in *E. coli* (Goldman et al., 1996; Poole et al., 1994). In addition, the deletion of *purD* renders the RB51Δ*cydC* mutant severely attenuated. In this case, the attenuation of RB51Δ*cydD*Δ*purD* is based on two altered behaviours: (1) the phenotype observed in the RB51Δ*cydC* mutant and (2) the purine auxotrophy caused by deletion of *purD*, which has previously been demonstrated to be required for *de novo* purine biosynthesis and survival of *Brucella* in macrophages (Truong et al., 2015).

In BALB/c mice, we found that RB51 mutants were cleared faster than the parental RB51. Notably, the RB51Δ*cydC*, RB51Δ*cydD*, RB51Δ*cydD*Δ*cydD* and RB51Δ*cydD*Δ*purD* mutants were rapidly cleared from spleens of mice by 3 weeks post-inoculation, whereas the RB51Δ*purD* mutant and its parent RB51 were detectable in spleens up to 4 and 7 weeks post-inoculation, respectively. Moreover, no splenomegaly was observed in mice after intraperitoneal inoculation of RB51 mutants in mice, compared with the splenomegaly induced by the parental RB51 (Fig. 2b). The results presented here are consistent with our previous findings (Truong et al., 2014, 2015) and the work of other investigators (Alcantara et al., 2004; Yang et al., 2010) demonstrating the important roles of purine biosynthesis (*purD*, *purE*) and ABC-type...
transporter (cydC) in the virulence of \textit{B. abortus} in mice. These results suggest that the rapid clearance of RB51 mutants from mice may provide a measure of safety for the host species. These properties make these novel mutant strains attractive candidates as safe vaccines.

To investigate whether RB51 mutants induce protection, the protective efficacy of these mutants was assessed in mice. The protection study showed that a single dose of RB51 and its RB51\textDelta\textit{cydC}, RB51\textDelta\textit{cydD}, RB51\textDelta\textit{purD}, RB51\textDelta\textit{cydC}\textDelta\textit{cydD} and RB51\textDelta\textit{cydC}\textDelta\textit{purD} mutants could

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\caption{Cytokine production by stimulated splenocytes from mice vaccinated with either a single dose or two doses of RB51 mutants or a single dose of RB51. At 7 weeks post-vaccination, mice were euthanized and splenocytes were harvested and stimulated with heat-killed (HK) RB51 and HK \textit{B. canis} S26 or with concanavalin A or medium alone as controls. IFN-\gamma (a) and IL-10 (b) production after 72 h of stimulation was detected using ELISA. Cytokine production is expressed as mean \pm SD cytokine concentration for each group of mice. Significance: * (P<0.001) indicates significant difference between vaccinated and non-vaccinated mice; \beta (P<0.05) indicates significant difference between mutant-vaccinated and RB51-vaccinated mice; \alpha (P<0.05) indicates significant difference between single vaccination and booster vaccination with RB51 mutants.}
\end{figure}
protect the mice against homologous *B. abortus* S2308 challenge, but vaccine efficacy was reduced compared with that induced by RB51 in the mice vaccinated with a single dose of RB51 mutants. The lower protection levels observed in RB51 mutant-vaccinated mice probably resulted from the reduced persistence and lower numbers of bacteria following vaccination with mutants than with parental RB51, which was induced by the additional deletion of *cydC*, *cydD* and *purD* in RB51 or *cydD* and *purD* in RB51Δ*cydC*. When a booster dose of attenuated RB51 mutants was administered 3 weeks after the first vaccination, these mutants were also rapidly cleared from mice without causing a marked inflammatory response. Therefore, we next investigated whether prime–boost vaccination with highly attenuated RB51 mutants could enhance their protective efficacy in mice. As shown here, booster vaccination with RB51 mutants was significantly more effective than single vaccination with either RB51 or RB51 mutants. Mice vaccinated with two doses of RB51Δ*cydC*, RB51Δ*cydD*, RB51Δ*purD*, RB51Δ*cydCAcydD* or RB51Δ*cydCApurD* mutants produced significantly higher log units of protection against challenge with virulent *B. abortus* S2308 than mice vaccinated with a single dose of these mutants. To the best of our knowledge, this trial represents the first time that the use of booster vaccination with RB51 mutants can increase protection against *B. abortus* infection in mice. Also, the use of this strategy is in agreement with previous studies that have shown that RB51 booster vaccination is an effective vaccination strategy for enhancing protection against *B. abortus* infection in [19]. Water buffalo and cattle (Caporale et al., 2010; Leal-Hernández et al., 2005; Olsen & Johnson, 2012).

In terms of protection, vaccination with RB51 has been shown previously to provide effective protection against infections caused by *B. abortus*, *B. melitensis* and *B. ovis* in mice (Jiménez de Bagüés et al., 1994; Moriyón et al., 2004) or *B. canis* in dogs (Hur & Baek, 2010). Therefore, it is to be expected that attenuated mutants of RB51 would also be the best choice for a vaccine targeting *B. canis* infection. As described herein, a single vaccination with RB51 or a booster vaccination with RB51 mutants not only provided protection against challenge with the homologous *B. abortus* S2308, but also conferred high levels of protection in mice against challenge with the heterologous *B. canis* S26. Remarkably, vaccination with RB51 or its isogenic RB51 mutants afforded even better protection against *B. canis* challenge than against *B. abortus* challenge. In dogs, brucellosis has been shown to be caused mainly by *B. canis* and occasionally by *B. abortus* (Baek et al., 2003, 2012; Forbes, 1990; Wanke, 2004); however, there is no vaccine available at the current time that protects dogs against these bacterial strains. Therefore, highly attenuated mutants such as RB51 mutants could be ideal safe vaccine candidates to protect against canine brucellosis caused by *B. abortus* and *B. canis*.

To ascertain the protective mechanisms induced by vaccination with RB51 and its mutants, serum antibody responses and the cytokines produced by splenocytes of vaccinated mice were also investigated. The data showed that mice immunized with a single dose of highly attenuated RB51 mutants produced significantly higher levels of specific IgG, IgG1 and IgG2a antibodies and IFN-γ compared with PBS-immunized mice; however, these levels were lower than those from mice immunized with parental RB51. This can be explained by the rapid clearance of RB51 mutants, because these mutants do not persist long enough in the host to elicit a strong immune response. As expected, further parenteral boosting with RB51 mutants administered 3 weeks after the first dose was observed to induce a significant enhancement of antibody and cellular immune responses, as shown by higher levels of IgG, IgG1 and IgG2a antibodies and IFN-γ compared with those produced by a single dose of RB51 mutants. Furthermore, the presence in the serum of vaccinated mice of predominantly IgG2a rather than IgG1 antibodies specific for RB51 antigens indicates the preferential development of a T-helper 1 (Th1)-type immune response. This is further corroborated by the elevated levels of IFN-γ compared with IL-10. As protective immunity induced by RB51 vaccination against smooth *Brucella* strains generally depends on the generation of a Th1-type cellular immune response characterized by secretion of IFN-IFN-γ from antigen-specific T cells (Pasquale et al., 2001; Stevens et al., 1995), we therefore speculate that the high level of IFN-γ induced by a booster dose of RB51 mutants may offer an explanation for the better protection provided by these mutants against homologous *B. abortus* S2308 challenge.

As RB51 has the same rough phenotype as the experimental *B. canis* strain used in this study, several factors can be invoked to explain the high level of protective immunity to *B. canis* in BALB/c mice. First, it is likely that the antibodies induced by RB51 and its mutants, probably directed against the outer membrane proteins of *B. canis*, could also protect against rough *B. canis* infection. In fact, when the WCAs from *B. canis* were used for testing the humoral immune response induced by RB51 and its mutants, high levels of specific IgG antibodies were observed in immunized mice. These antibody responses were similar to those determined by assays using WCAs from RB51. This observation suggests that immunization with RB51 and its mutants offered cross-reactive immunity to *B. canis* antigens, and that this antibody response may have contributed to protection against challenge with *B. canis*. Second, we showed here that the protective properties of RB51 and its mutants may be the result of their ability to induce a strong Th1-type cellular immune response, as shown by the high levels of serum IgG2a antibodies and IFN-γ secretion by splenocytes from RB51- and RB51 mutant-immunized mice in response to exposure to *B. canis* whole cells – an immune response that is considered to play a crucial role in protection against *Brucella* infection (Baldwin & Parent, 2002).

In conclusion, to the best of our knowledge, this is the first study to investigate the protective immunity against *B. canis* infection conferred by vaccination of mice with...
RB51 or its isogenic mutants. Although a single dose of genetically modified, live-attenuated mutants of RB51 may not be an ideal vaccine candidate for use in animals, our results demonstrate that the use of two doses of RB51 mutants is superior to a single dose of RB51 or RB51 mutants, not only in inducing a specific antibody response, but also in eliciting specific cytokine secretion, resulting in significant protection of mice against challenge with virulent strains of *B. abortus* and *B. canis*. The enhanced safety of RB51 mutants and the promising efficacies achieved by booster vaccination with these mutants suggest that RB51 mutants, such as RB51ΔcydD and RB51ΔpurD, which provide the best protective immunity in mice, could be suitable as live-attenuated booster vaccine candidates against brucellosis caused by *B. abortus* and *B. canis*, and deserve further investigation in domestic animals such as cattle or dogs.

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