Subcutaneous Vaccination with Attenuated *Salmonella enterica* Serovar Choleraesuis C500 Expressing Recombinant Filamentous Hemagglutinin and Pertactin Antigens Protects Mice against Fatal Infections with both *S. enterica* Serovar Choleraesuis and *Bordetella bronchiseptica*†

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*Salmonella enterica* serovar Choleraesuis strain C500 is a live, attenuated vaccine that has been used in China for over 40 years to prevent piglet paratyphoid. We compared the protective efficacies of subcutaneous (s.c.) and oral vaccination of BALB/c mice with C500 expressing the recombinant filamentous hemagglutinin type I domain and pertactin region 2 domain antigen (rF1P2) of *Bordetella bronchiseptica*. Protective efficacy against both *S. enterica* serovar Choleraesuis infection in an oral fatal challenge model and *B. bronchiseptica* infection in a model of fatal acute pneumonia was evaluated. Both the s.c. and oral vaccines conferred complete protection against fatal infection with the virulent parent *S. enterica* serovar Choleraesuis strain (C78-1). All 20 mice vaccinated s.c. survived intranasal challenge with four times the 50% lethal dose of virulent *B. bronchiseptica* (HH0809) compared with 4 of 20 vector-treated controls and 1 of 18 phosphate-buffered salinetreated controls that survived, but no significant protection against HH0809 was observed in orally vaccinated animals. Both the s.c. and oral vaccines elicited rF1P2-specific serum immunoglobulin G (IgG) and IgA antibodies. However, lung homogenates from s.c. vaccinated animals had detectably high levels of rF1P2-specific IgG and IgA; a much lower level of rF1P2-specific IgG was detected in samples from orally vaccinated mice, and the latter showed no evidence of local IgA. Furthermore, a more abundant and longer persistence of vaccine organisms was observed in the lungs of mice immunized s.c. than in those of mice immunized orally. Our results suggest that s.c. rather than oral vaccination is more efficacious in protecting mice from fatal challenge with *B. bronchiseptica*.

*Bordetella bronchiseptica* is an etiological agent of atrophic rhinitis and bronchopneumonia in young pigs. Although the primary disease is important, more significant is the fact that this bacterium predisposes pigs to colonization and disease with other viral and bacterial pathogens (6). *B. bronchiseptica* is also a contributory agent in the porcine respiratory disease complex, a multifactorial disease state that is increasingly problematic for swine producers (2). However, vaccine efficacy is reported to be low, and atrophic rhinitis remains an important disease problem in grower/finisher pigs (1, 34).

Several studies demonstrated that pertactin-specific active or passive immunization against *B. bronchiseptica* protects against mortality and disease in mice and pigs (19, 24, 26). The pertactin protein has two repeated regions, regions 1 and 2; region 2 is identified as being an immunodominant protective epitope (4). The filamentous hemagglutinin (FHA) of *B. pertussis* is defined as being an important attachment factor and protective immunogen (28, 36), with two main immunodominant regions, identified as type I and type II domains (8, 20). In addition, the individual type I domain of FHA induced an immune response that protected BALB/c mice against intranasal (i.n.) infection by the clearance of *Bordetella pertussis* from the lung (18). Because protein structure and immunological analyses suggest that the FHA proteins from *B. pertussis* and *B. bronchiseptica* are similar and have a common set of immunogenic epitopes (21, 27, 30), we hypothesized that a truncation of the FHA of *B. bronchiseptica* that includes the immunodominant type I domain may serve as a protective antigen against porcine bordetellosis.

Over the last decade, the use of recombinant attenuated *Salmonella* vaccine strains for heterologous antigen delivery has increased considerably. A range of strategies has been developed to allow the controlled and stable delivery of antigens and improved immunogenicity where required. The evaluation of different routes of immunization is an important way to modulate immune responses according to clinical requirements. The oral route of antigen delivery is the most common and most frequently explored among the mucosal immunization routes and stimulates both systemic and mucosal immune responses (32, 33). In addition, other immunization routes have been extensively explored in mice, including nasal, rectal, vaginal, and intraperitoneal administration (for examples, see references 7 and 14). However, there have been few previous studies of systemic immunity following subcutaneous (s.c.) vaccination based on this principle.

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Strains

**E. coli**
- DH5α
- BL21(DE3)
- X7213
- X6097

**S. enterica serovar Choleraesuis**
- C500
- C78-1
- C501
- C501(pYA-F1P2)

**B. bronchiseptica**
- HH0809

Plasmids

- pBluescript
- pRE112
- pET28a(+)
- pET-F1P2
- pYA3493
- pYA-F1P2

DNA fragments

- F1
- P2

### MATERIALS AND METHODS

**Bacterial strains, plasmids, media, and growth conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* and *S. enterica* serovar Choleraesuis cultures were grown at 37°C in Luria-Bertani (LB) broth or on LB agar (3). When required, antibiotics were added to culture media at the following concentrations: ampicillin at 100 μg/ml, kanamycin at 50 μg/ml, and chloramphenicol at 30 μg/ml; *de*-α-Diaminopimelic acid (DAP) was added (50 μg/ml) for the growth of Asd- strains (25). LB agar containing 5% sucrose was used for sacB gene-based counterselection in allelic exchange experiments (12). *B. bronchiseptica* HH0809 was grown on Bordet-Gengou agar (Difco, Detroit, MI) supplemented with 15% (vol/vol) defibrinated sheep blood at 37°C.

**Expression of rF1P2 in *E. coli*** DNA manipulations were carried out as described previously by Sambrook et al. (31). Transformation of *E. coli* and *Salmonella* was performed by electroporation (Bio-Rad, Hercules, CA). The PCR conditions were as follows: denaturation at 95°C for 30 s, primer annealing at 56°C for 30 s; polymerization at 72°C for 30 s, and a final extension step at 72°C for 10 min. The 465-bp fragment specifying the important immunodominant type I domain (F1) of the FHA gene was PCR amplified from the genome of *B. bronchiseptica* HH0809 using a pair of primers (N-terminal primer 5'-TTTAAG AATTCCTGACTGCCCTGGACAAT-3' and C-terminal primer 5'-TTTAAATG CGACTCGAGATCGCCGAAA-3'). The N-terminal primer contains an EcoRI site (underlined), and the C-terminal primer contains a SalI site (underlined). The 465-bp amplified fragment, digested with restriction enzymes EcoRI and SalI, was then cloned into the EcoRI and SalI sites of vector pET-28a, resulting in pET-F1. Similarly, the 300-bp fragment specifying the main immunodominant region II (P2) of the pertactin gene was amplified using a pair of primers (N-terminal primer 5'-TTAAATG CGACTCGAGATCGCCGAAA-3' and C-terminal primer 5'-TTTAAATG CGACTCGAGATCGCCGAAA-3') and cloned into the SalI and HindIII sites (underlined), respectively, of pET-F1, resulting in pET-FIP2, which harbored a 765-bp fragment of *B. bronchiseptica* HH0809. In-frame cloning of pET-FIP2 was confirmed by nucleotide sequencing using ABI Prism fluorescent Big Dye terminators according to the manufacturer's instructions (PE Biosystems, Norwalk, CT). *E. coli* DH5α was transformed with pET-FIP2 by electroporation (24) and then grown in LB medium containing 50 μg/ml of ampicillin for 4 h at 37°C. The cell pellet was harvested by centrifugation and incubated in TBS (10 mM Tris-HCl, pH 7.5, 250 mM NaCl) with 0.5% Triton X-100 and 0.5% SDS for 1 h at 4°C. The cells were then sonicated and centrifuged to remove cell debris. The supernatant was used for SDS-PAGE analysis.

**Expression of rF1P2 in *B. bronchiseptica*** The recombinant plasmid pET-FIP2 was transformed into *B. bronchiseptica* HH0809 by electroporation. The transformants were selected on LB agar containing 50 μg/ml of ampicillin and 50 μg/ml of kanamycin. The resulting strain was grown in LB medium containing 50 μg/ml of ampicillin and 50 μg/ml of kanamycin at 37°C. The cell pellet was harvested by centrifugation and incubated in TBS (10 mM Tris-HCl, pH 7.5, 250 mM NaCl) with 0.5% Triton X-100 and 0.5% SDS for 1 h at 4°C. The cells were then sonicated and centrifuged to remove cell debris. The supernatant was used for SDS-PAGE analysis.

**Affinity purification of rF1P2** The supernatant from the culture of *B. bronchiseptica* expressing rF1P2 was applied to a Ni-NTA agarose column (Quiagen) and washed with TBS containing 0.5% Triton X-100 and 0.5% SDS. The bound protein was eluted with TBS containing 500 mM imidazole. The eluted protein was then dialyzed against TBS and subjected to SDS-PAGE analysis.

**Antigenicity and immunogenicity of rF1P2** The antigenicity and immunogenicity of rF1P2 were assessed by ELISA and Western blotting. The recombinant protein was used as a coating antigen for ELISA, and the serum from the vaccinated mice was used as the primary antibody. The antigenicity and immunogenicity were determined by comparing the absorbance of the experimental group with the control group.

**Protection against experimental infections** The recombinant protein was administered intranasally to BALB/c mice. The mice were challenged with *B. bronchiseptica* at 37°C. The survival rate of the mice was determined at 7 days postchallenge.

**Safety and efficacy of the recombinant vaccine** The safety and efficacy of the recombinant vaccine were assessed by evaluating the side effects and protection against experimental infections. The side effects were determined by monitoring the general health and behavior of the mice. The protection against experimental infections was determined by measuring the survival rate of the mice after challenge with *B. bronchiseptica*.

**Conclusions** The results of this study showed that the recombinant protein rF1P2 is an effective and safe vaccine against *B. bronchiseptica* infections. The recombinant protein was able to induce a strong immune response in mice and provide complete protection against experimental infections. The safety and efficacy of the recombinant vaccine were demonstrated by the lack of side effects and the high survival rate of the vaccinated mice.

### TABLE 1. Strains, plasmids, and DNA fragments used in this study

<table>
<thead>
<tr>
<th>Strain, plasmid, or DNA fragment</th>
<th>Relevant characteristics</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli</strong> DH5α</td>
<td>supE44 ΔlacU169 (φ80lacΔM15) hisD17 recA1 endA1 gyrA96 thi-1 relA1</td>
<td>Takara</td>
</tr>
<tr>
<td>BL21(DE3)</td>
<td>F ompT hsdSB (rB-mB-lB) DE3 is a λ derivative carrying lacIq and T7 RNA polymerase genes under pLacUV5 control</td>
<td>Takara</td>
</tr>
<tr>
<td>X7213</td>
<td>thi-1 thr-1 leuB6 hisA21 lacY1 glnV44 ΔasdB4 recA1 RP4 2-Tc:Mu [λpir] Km'</td>
<td>9</td>
</tr>
<tr>
<td>X6097</td>
<td>F' ara Δ(pro-lac) rpsL ΔasdB4 Δ [l2f-2::Tn10] thi φ80lacΔM15</td>
<td>25</td>
</tr>
<tr>
<td><strong>S. enterica serovar Choleraesuis</strong> C500</td>
<td>Live vaccine attenuated from C78-1 by chemical methods; used to prevent piglet paratyphoid in China; serotype 6,7,C:1,5</td>
<td>CIVDC</td>
</tr>
<tr>
<td>C78-1</td>
<td>ΔasdB derivative of C500</td>
<td>CIVDC</td>
</tr>
<tr>
<td>C501</td>
<td>C501 harboring pYA-F1P2</td>
<td>This work</td>
</tr>
<tr>
<td>C501(pYA-F1P2)</td>
<td>Wild-type, virulent strain</td>
<td>This work</td>
</tr>
<tr>
<td><strong>B. bronchiseptica</strong> HH0809</td>
<td>pBluescript SK(+)</td>
<td>Phagemid cloning vector, ori CodE1 orF1(+)+ bla lacZα</td>
</tr>
<tr>
<td>pRE112</td>
<td>oriT oriV ΔasdB Cm' sacB; counterselectable suicide plasmid</td>
<td>22</td>
</tr>
<tr>
<td>pET28a(+)</td>
<td>IPTG-inducible expression vector; Km'</td>
<td>Nowagen</td>
</tr>
<tr>
<td>pET-F1P2</td>
<td>pET28a(+)- derivative expressing rF1P2 with an N-terminal His6 tag; Km'</td>
<td>This work</td>
</tr>
<tr>
<td>pYA3493</td>
<td>Asd' vector; pBR322 orI; derivative β-lactamase signal sequence-based periplasmic secretion plasmid</td>
<td>17</td>
</tr>
<tr>
<td>pYA-F1P2</td>
<td>765-bp DNA encoding the type I region of FHA and R2 repeat domain of pertactin in pYA3493</td>
<td>This work</td>
</tr>
<tr>
<td><strong>DNA fragments</strong></td>
<td>465-bp fragment specifying the important immunodominant type I domain at the carboxy terminus of the FHA gene containing the most reactive epitopes</td>
<td>This work</td>
</tr>
<tr>
<td>F1</td>
<td>300-bp fragment specifying the repeated region II domain of the pertactin gene encoding an immunodominant protective epitope</td>
<td>This work</td>
</tr>
</tbody>
</table>

* CIVDC, China Institute of Veterinary Drug Control (Beijing, China).
mice were fed 200 orogastric gavage with a 12-gauge ball-tipped gavage needle. Ten minutes later, vector C501(pYA3493), recombinant vaccine C501(pYA-F1P2), or the parent C500 vaccine strain (2.1 × 10^6 CFU in 200 μl PBS). After 14 days, all animals were boosted with the same dose of the appropriate vaccine.

Serum, lung, and gut mucus samples were collected on days 0, 28, and 56. Five animals from all groups before vaccination and five animals from each group after vaccination were anesthetized with an intraperitoneal injection containing xylazine (0.5 mg) and ketamine (2.5 mg) and then bled thoroughly by removing the eyeball. Additional blood samples were collected from the retro-orbital sinuses of mice in groups of five for monitoring the kinetics of serum antibody responses on days 14 and 42. Serum was collected by centrifugation at 1,700 × g for 10 min and then stored at −80°C until use. To collect gut mucus, mice were sacrificed, and the gut was removed between just distal to the stomach and just proximal to the anus. The mucus was scraped from the luminal surface and suspended in 1 ml PBS. The lung was removed aseptically and homogenized in 2 ml PBS. After centrifugation at 12,000 × g for 5 min at 4°C, supernatant fluids from gut mucus and lung homogenates were collected, and samples were analyzed immediately using an indirect enzyme-linked immunosorbent assay (ELISA).

For in vivo localization of bacteria, groups of four mice were sacrificed on days 2, 8, and 14 after the initial immunization. Lungs, spleens, and Peyer’s patches were removed aseptically. The tissues were homogenized in sterile PBS and plated onto MacConkey agar (Tianjie, Hangzhou, China) plates with or without 1% maltose to examine the distribution and persistence of the recombinant vaccine.

Intranasal infection with B. bronchiseptica. A highly virulent strain of B. bronchiseptica, HH0809, originally isolated by our laboratory from a pig suffering from atrophic rhinitis, was used for the challenge of mice on day 30 after the initial immunization. HH0809 cells were grown on Bordet-Gengou agar for 48 h as described above. Bacteria were resuspended and diluted in 1% Casamino Acids and then serially diluted to provide challenge inoculum dilutions. For respiratory infection, 20 μl of the bacterial suspension containing approximately four times the 50% lethal dose (LD₅₀) of virulent strain HH0809 (5.2 × 10^8 CFU) was deposited into each nostril of mice that had been anesthetized with an intraperitoneal injection containing xylazine (0.25 mg) and ketamine (1.25 mg). Morbidity and mortality were observed for 30 days after the challenge.

ELISA for Salmonella and rF1P2. An ELISA was used to assay antibodies to whole Salmonella cells or to rF1P2 in samples of serum, lung homogenate, and intestinal mucus from individual mice. Each sample well of polystyrene 96-well flat-bottomed microtiter plates (Kangji Ltd., China) was coated with 100 ng of purified rF1P2 diluted in 100 μl 0.1 M carbonate buffer (pH 9.6). For anti-Salmonella antibody titration, S. enterica serovar Choleraesuis C500 cells were grown overnight, harvested by centrifugation, and resuspended in PBS at 3 × 10^11 CFU/ml. Bacteria were heat killed for 10 min at 80°C and stored at −80°C. Each sample well was coated with 100 μl of this suspension diluted 100-fold in carbonate buffer. The coated plates were incubated at 37°C for 1 h, followed by an overnight incubation at 4°C. Free binding sites were blocked with a blocking buffer (PBS, 0.1% Tween 20, and 5% skim milk). Samples of serum, lung homogenate, or gut mucus were added to each well and incubated at 37°C for 30 min. After three washes, plates were treated with biotinylated goat anti-mouse immunoglobulin G (IgG) (Southern Biotechnology Inc., Birmingham, AL) for sera and lung homogenates, or IgA for all samples, at 37°C for approximately 10 min; the catalytic reaction was stopped by adding 50 μl 1% SDS. The optical density was read at 630 nm using an ELISA reader.

Statistics. All analyses were performed by use of SAS system software. Salmonella- and rF1P2-specific IgG and IgA titers and numbers of vaccine organisms recovered in murine tissues in log_{10} units were compared by a Student’s t test. For survival studies, data were analyzed by Fisher’s exact test. In these two tests, a P value of <0.05 was considered to be significant.

RESULTS

Characterization of combinatorial S. enterica serovar Choleraesuis C500 vaccine expressing rF1P2. S. enterica serovar Choleraesuis C500 asd deletion mutant C501 lost the ability to synthesize DAP and was unable to grow on medium without DAP. Its ability was restored when C501 harbored plasmid pYA3493 or pYA-F1P2, resulting in C501(pYA3493) and C501(pYA-F1P2). The mean generation times of recombinant C500(pYA3493), recombinant vaccine C501(pYA-F1P2), or the parent C500 vaccine strain (2.1 × 10^6 CFU in 200 μl PBS). After 14 days, all animals were boosted with the same dose of the appropriate vaccine.
S. enterica serovar Choleraesuis C501(pYA-F1P2), the vector control C501(pYA3493), and the parent avirulent C500 vaccine strain in Luria broth were 30.7, 28.1, and 27.9 min, respectively. The fermentation patterns of the various strains on different carbohydrates, and the levels of production of H$_2$S, were similar. The O and H antigens of C501(pYA3493) and C501(pYA-F1P2) were 6:H1C1.5, identical to the parent strain C500. Recombinant strain C501(pYA-F1P2) expressed the rF1P2 protein at an approximate molecular mass of 30 kDa, consistent with the calculated size of rF1P2 (Fig. 1). Analysis of Coomassie bright-stained SDS-polyacrylamide gels showed that the amount of the rF1P2 protein accounted for up to approximately 1.1% of the total C501(pYA-F1P2) protein; approximately 71.4% of rF1P2 was located in the cell lysates, and 28.6% was located in the culture supernatants. To examine the stability of plasmids pYA3493 and pYA-F1P2 in C501 in vitro, C501 cells containing pYA3493 and pYA-F1P2 were cultured with a daily passage of 1:1,000 dilutions for five consecutive days in LB broth containing DAP. Cells obtained from the last-day culture expressed amounts of the 30-kDa rF1P2 that were similar to those from the first day (data not shown), whereas there were no survivors in a group of 10 PBS controls. The LD$_{50}$ after s.c. immunization with C501(pYA-F1P2) was $10^{1.1}$ (equal to that obtained when animals were immunized with C500), and the LD$_{50}$ were $10^{1.6}$ and $10^{1.7}$ for oral immunization with C501(pYA-F1P2) and C500, respectively. These results indicate that s.c. immunization with C501(pYA-F1P2) or C500 is avirulent for mice and can provide complete protection from S. enterica serovar Choleraesuis infection.

**Antibody responses to rF1P2 in sera.** The IgG and IgA responses in sera were assessed following two doses of vaccine (Fig. 2). The kinetics of the rF1P2-specific serum IgG and IgA antibody responses of vaccine-inoculated mice were monitored and compared with those of PBS- and vector-treated mice (data not shown). Primary immunization with C501(pYA-F1P2) given s.c. induced significant levels of serum anti-rF1P2 IgG and IgA within 2 weeks. Serum samples taken 2 weeks after booster immunization showed a further increase in the levels of rF1P2-specific IgG and IgA; the highest total IgG and IgA titers were observed 4 and 2 weeks after booster immunization. A similar trend was observed in orally vaccinated mice, except that the highest IgG and IgA titers were observed 6 and 4 weeks after booster immunization. It should be noted that s.c. immunization with C501(pYA-F1P2) elicited lower levels of serum IgA (Fig. 2B) but induced significantly higher ($P < 0.01$) serum IgG antibody levels than oral immunization with titers of 3,584 and 14,336 on days 28 and 56 (Fig. 2A).

**B. bronchiseptica wild-type challenge.** In the survival study, mice were inoculated with vector and PBS, regardless of the route of immunization, died between days 2 and 7, with the peak of mortality on day 4, after challenge with highly virulent B. bronchiseptica HH0809. Complete protection (20/20) over a 30-day observation period was seen in mice that had received the recombinant vaccine by the s.c. route; the survival rates were much lower in mice that received either PBS (1/18) or vector (4/20) alone (Table 2). In addition, no obvious signs of disease were observed in the mice immunized s.c. with C501(pYA-F1P2) during the entire experimental period. However, i.n. challenge of orally vaccinated mice with HH0809 (5.2 $\times$ 10$^{6}$ CFU) failed to produce a significant difference in survival rates between vaccine-treated mice and PBS- and vector-treated controls (Table 2), even though these mice showed a significant increase in survival as a function of time postinfection compared with the vector- or PBS-treated controls (data not shown). Altogether, these results suggest that vaccine strain C501(pYA-F1P2) provides complete protection against challenge with virulent wild-type B. bronchiseptica HH0809 and is more efficacious when delivered by the s.c. route.

**Antibody responses to rF1P2 in lung.** Having produced evidence indicating that s.c. rather than oral immunization provides complete protection in the i.n. challenge model, we...
wanted to determine whether a difference in the antibody profiles existed at the site of infection after either vaccination. To accomplish this, lung homogenates were obtained from mice after s.c. and oral inoculation. In mice that received the s.c. vaccine, high levels of total IgG and IgA were observed in lung homogenates, whereas only low-level reactivity for total IgG was seen in orally vaccinated animals, with no evidence of rF1P2-specific IgA (titer, \(10^{10}\)) (Fig. 2C and D). Interestingly, the IgA level in lung homogenates was significantly higher \((P < 0.01)\) than that in sera 4 and 8 weeks following s.c. immunization (Fig. 2B and D). These results suggest that local lymphoid tissues in lung may be a source of protective antibodies, in addition to transudation from the circulation. The lack of IgA in lung homogenates from orally vaccinated mice was unexpected, as this antibody was observed in serum samples from these animals. These results suggest that not only IgG but also IgA in lung tissue may play an important role in mediating protection in s.c. vaccinated mice.

In vivo distribution of vaccine organisms. The presence of live C501(pYA-F1P2) vaccine organisms was then observed in lungs, spleen, and Peyer’s patches following both s.c. and oral delivery (Table 3). In these two groups, similar numbers of organisms were isolated from the Peyer’s patches of mice after inoculation. In contrast, the numbers of vaccine colonies isolated from spleens of mice inoculated s.c. were significantly higher than in those of mice inoculated orally on days 2, 8, and 14 following immunization. Most importantly, much larger numbers of vaccine colonies were isolated from the lungs of s.c. immunized mice on day 2, with a significant increase on day 8 and then a rapid decline on day 14. However, only smaller numbers of vaccine colonies were detected on day 2, and no organisms were detected in lungs of mice immunized orally on days 8 and 14, suggesting the temporary persistence of vaccine organisms in murine lungs. Interestingly, very similar numbers of organisms were isolated from the lungs, spleen, and Peyer’s

### TABLE 2. Effectiveness of s.c. or oral immunization with recombinant S. enterica serovar Choleraesuis vaccine strain C501(pYA-F1P2) in protecting BALB/c mice against i.n. challenge with wild-type B. bronchiseptica HH0809

<table>
<thead>
<tr>
<th>Strain (genotype) or control</th>
<th>Inoculation route</th>
<th>Immunization dose (CFU)</th>
<th>Challenge dose (CFU)</th>
<th>No. of survivors/total no. of mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>C501(pYA3493) s.c.</td>
<td>2.1 (\times) 10^6</td>
<td>5.2 (\times) 10^6</td>
<td>4/20</td>
<td></td>
</tr>
<tr>
<td>C501(pYA-F1P2) s.c.</td>
<td>2.1 (\times) 10^6</td>
<td>5.2 (\times) 10^6</td>
<td>20/20</td>
<td></td>
</tr>
<tr>
<td>PBS s.c.</td>
<td>200 (\mu)l</td>
<td>5.2 (\times) 10^6</td>
<td>1/8</td>
<td></td>
</tr>
<tr>
<td>C501(pYA3493) Oral</td>
<td>2.1 (\times) 10^10</td>
<td>5.2 (\times) 10^6</td>
<td>4/18</td>
<td></td>
</tr>
<tr>
<td>C501(pYA-F1P2) Oral</td>
<td>2.1 (\times) 10^10</td>
<td>5.2 (\times) 10^6</td>
<td>4/20</td>
<td></td>
</tr>
<tr>
<td>PBS Oral</td>
<td>200 (\mu)l</td>
<td>5.2 (\times) 10^6</td>
<td>3/18</td>
<td></td>
</tr>
</tbody>
</table>

a Mice were s.c. or orally immunized twice at 2-week intervals with the indicated vaccine strains or with PBS and challenged 30 days after the primary immunization with wild-type B. bronchiseptica HH0809. Morbidity and mortality observations were recorded daily for 30 days postchallenge.

b A total of 5.2 \(\times\) 10^6 CFU represents about four times the LD_50 of HH0809 in nonimmunized BALB/c mice.

c \(P < 0.0001\) by Fisher’s exact test for vector versus vaccine, PBS versus vaccine, and s.c. versus oral vaccination.

d Value in microliters.
patches in s.c. immunized mice at day 2 or 8 after immunization (Table 3). Parent strain C500 localized in the same tissues, and no statistical differences were observed in the counts of the live bacteria alone compared to bacteria carrying plasmid pYA-F1P2 (data not shown).

**DISCUSSION**

*S. enterica* serovar Choleraesuis strain C500 is an avirulent vaccine strain attenuated by chemical methods, which is highly immunogenic and safe and has been used widely to prevent piglet paratyphoid in China for over 40 years (10, 15, 23). In this study, all mice immunized s.c. with C500 or C501(pYA-F1P2) survived, and no signs of disease were observed in the immunized mice during the entire experimental period. Either s.c. or oral vaccination in BALB/c mice induced immune responses to both *Salmonella* and rF1P2 and provided effective protection against fatal challenge with a virulent *S. enterica* serovar Choleraesuis strain, C78-1. These results indicate that s.c. vaccination with C500 or C500 with an Asd+ plasmid is avirulent for mice. Furthermore, rF1P2-specific immunity did not interfere with immunity against *Salmonella* itself. Previous works on the protective efficacy of FHA and pertactin revealed that a single antigen or an immunodominant protective domain alone may provide sufficient protective efficacy against *Bordetella* challenge if effective immunity can be induced via vaccination (18, 19, 24, 28). In this study, the secretion of the rF1P2 antigen expressed in *Salmonella* was confirmed, which may augment immune responses by facilitating the adequate exposure of rF1P2 antigen to antigen-presenting cells for processing (17).

Although routes of delivery of antigens expressed in recombinant *Salmonella* strains have been extensively explored in mice (32, 33), there were few previous studies demonstrating that s.c. vaccination produced effective immune responses and protection based on this principle. In this study, the results from immunization experiments demonstrated that s.c., but not oral, vaccination with this strain provided complete protection against i.n. challenge with *B. bronchiseptica*, which is supported by much higher anti-rF1P2 IgG and IgA levels detected in lungs of mice following s.c. but not oral inoculation. These findings suggest that protection against i.n. infection correlates with the local systemic responses in murine lungs elicited by s.c. vaccination. These findings also indicate that the degree of activation of gut-associated lymphoid tissue by oral vaccination is insufficient for antibody-secreting B cells to localize to the respiratory lymphoid tissue based on this principle, even though mice immunized orally showed an increase in survival as a function of time postinfection (data not shown). The sources of anti-rF1P2 IgG and IgA antibodies in the respiratory tract after s.c. immunization have not yet been directly determined; however, we suspect that local lymphoid tissues may be a source of the protective antibodies rather than transudation from the circulation alone. This interpretation is based on the findings that oral vaccine promoted a potential total anti-rF1P2 IgA response in serum but not in lung and that s.c. immunization induced significantly higher anti-rF1P2 IgA antibody levels in lung homogenates than in sera ($P < 0.01$).

We then performed kinetic bacterial distribution assays of murine tissues after inoculation. The current finding that the vaccine organisms persisted more abundantly and longer in the lungs and spleen of s.c. inoculated mice suggests that, for some reason, the vaccine organisms might reach these murine tissues more effectively following s.c. inoculation than following oral inoculation. This persistence of vaccine organisms in the lungs is in agreement with the greater immunogenicity of the s.c. immunization. In contrast, orally inoculated mice had only a short persistence of vaccine organisms, which might be not sufficient to stimulate antibody responses in the lungs, and this likely accounted partially for the poor antibody responses against the heterologous antigen in this local tissue. The stronger persistence of vaccine organisms in the Peyer’s patches may result from the environment, which is less hostile than that in either the lungs or the spleen of mice based on tissue specificity (13). Simultaneously, large numbers of organisms were isolated from the lungs, spleen, and Peyer’s patches of mice following s.c. inoculation, suggesting that the s.c. inoculum may establish a reservoir in the lymph nodes, where *Salmonella* readily spreads throughout the body via the lymph stream and becomes systemic (5, 35), finally reaching the lungs and other tissues for the generation of systemic and local immune responses.

In conclusion, we have shown that s.c. vaccination with recombinant attenuated *Salmonella* vaccine strain C500 is a more suitable immunization route than oral immunization for the induction of protective immune responses against fatal infections with both *S. enterica* serovar Choleraesuis and *B. bronchiseptica* in this model. It is likely that this *Salmonella* expression and delivery system could be easily adapted to develop multivalent recombinant *Salmonella* vaccines against other infectious agents. Further work is needed to determine the potential of the vaccine in pigs before comprehensive evaluation and practical application are done. In addition, it would be interesting to compare the protective efficacy of s.c. vaccination

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**TABLE 3. Persistence of recombinant *S. enterica* serovar Choleraesuis vaccine strain C501(pYA-F1P2) in deep organs of BALB/c mice**

<table>
<thead>
<tr>
<th>Route</th>
<th>Lungs on day:</th>
<th>Spleen on day:</th>
<th>Peyer's patches on day:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
<td>8</td>
<td>14</td>
</tr>
<tr>
<td>s.c.</td>
<td>2.7*</td>
<td>3.4</td>
<td>1.4</td>
</tr>
<tr>
<td>Oral</td>
<td>1.6</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

a Mice were inoculated orally or s.c. with a single dose of $2.1 \times 10^9$ or $2.1 \times 10^5$ CFU C501(pYA-F1P2) organisms. Data are averages of values for four mice, and ND represents no detectable organisms. *, $P < 0.01$ by Student’s t test between the s.c. and orally inoculated groups.

b One mouse had low counts of vaccine organisms, and the other three mice had cleared all organisms.
with recombinant *Salmonella* vaccine strain C500 to that of i.n. vaccination.

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