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Immunologic Effect of Attenuated *Salmonella enterica* Serovar Choleraesuis C501 Expressing Recombinant *Mycoplasma hyopneumoniae* P97R1 Adhesin and NrdF Antigens in Mice

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Abstract

The immunogenicity of the P97 adhesin repeat region R1 (P97R1) and the ribonucleotide reductase R2 subunit (NrdF) antigens of *Mycoplasma hyopneumoniae* were evaluated as vaccines in mice. Mice were immunized orally or injected intramuscularly (IM) with attenuated *Salmonella enterica* serovar Choleraesuis strain C501 harboring a prokaryotic expression vector encoding P97R1 and NrdF (pYA97R1N). Local and systemic immune responses were analyzed in vaccinated mice. The results showed that P97R1N-specific serum IgG and IgA antibodies were detected in serum and lung samples. P97R1-specific serum IgG and IgA antibodies were also detected in serum, but in lung sample only P97R1-specific IgG response was induced. ELISA assays demonstrated that IFN- γ and IL-4 production was induced in cultural supernatants from splenocytes stimulated with specific antigens *in vitro*. *M. hyopneumoniae* whole cell-specific antibody was induced in vaccinated mice and a good immunogenic effect by this vaccine was indicated. Importantly, animals vaccinated by IM had higher levels of P97R1N-specific IgG and IgA than those vaccinated by oral route. Furthermore, mice immunized with the C501 vaccine elicited significantly higher levels of P97R1N-specific IgA and IgG in lungs and serum ($P < 0.05$), and IFN- γ and IL-4, than those who received the live *M. hyopneumoniae* vaccine. Taken together, these results suggest that IM vaccination with the C501 vaccine might provide an effective method of inducing an immune response to *M. hyopneumoniae*.

Keywords: *M. hyopneumoniae*; Splenocytes; Serovar choleraesuis

Introduction

Mycoplasma hyopneumoniae is the causative agent of porcine enzootic pneumonia (PEP) that reduces growth rate [1]. Colonization also predisposes the host to more severe infections from secondary pathogens [2]. Vaccines of whole-cell and subunit *M. hyopneumoniae* have only a partial protective effect against primary or secondary infection [3-5]. Additionally, the cost of producing the vaccines is very high. Therefore development of an improved vaccine is desirable.

The ribonucleotide reductase R2 subunit (NrdF) gene fragment of *M. hyopneumoniae* has a protective effect on *M. hyopneumoniae* infection: it induces NrdF-specific lung IgA in mice [6], and significantly reduced lung lesion scores in pigs [7].

The adhesin P97 is a potential antigen of *M. hyopneumoniae*, enabling the bacteria to adhere to respiratory ciliated epithelial cells [8]. A repeat region of P97 (P97R1) has been shown to include both cilium- and antibody-binding sites [9,10] and is reported to function independently from other P97 regions [11].

P97R1 induces P97R1-specific serum IgG but not IgA in both pigs and mice [12,13]. Studies have demonstrated that a combined NrdF and P97 vaccine (pYA97R1N) can provide a partial protection in vaccinated animals [7,14,15].

Recent studies showed that attenuated *Salmonella*-derived vaccines induce both humoral and cell-mediated immunity responses [16-18]. Particularly, *S. enterica* serovar Choleraesuis strain C500 is highly immunogenic and safe, and has been widely used in China for over 40 years to prevent piglet paratyphoid [19,20]. In the present work, we combined two *M. hyopneumoniae* antigens, P97R1 and NrdF (P97R1N), into *S. enterica* serovar Choleraesuis C501 [21]. Therefore, a recombinant attenuated *S. enterica* vaccine was evaluated by oral and intramuscular (IM) routes in a mouse

model. The immunogenicity of *S. enterica* strain C501 expressing P97R1N protein was also investigated.

Materials and Methods

Bacterial strains

M. hyopneumoniae HNYT wild type strain isolated from tissues on primary porcine lung (Laboratory stock). Attenuated *S. enterica* serovar Choleraesuis strain C501 was constructed with the help of our colleagues [21]. *Escherichia coli* X7213 and JM105 were obtained from the Agricultural Microbiology Laboratory, Huazhong Agricultural University, China.

Growth conditions and isolation of genomic DNA of *M. hyopneumoniae*

M. hyopneumoniae was grown in Friis modified mycoplasma broth (FMFB) as described previously [22]. The culture was grown at 37°C at 5% CO₂ until acidity was observed. Genomic DNA was extracted using a Quantum Prep AquaPure Genomic DNA Kit (Bio-Rad).

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Construction of recombinant plasmids

P97 and NrdF genes were based on the published sequences (GenBank accession no. NC_006360). To construct the pYAP97R1N plasmid, the P97R1 fragment was obtained by PCR from strain HNYE genomic DNA using the N-terminal primer 5'-AAAGAATTCGAAGGTAAAAGAGAAGA-3', and the C-terminal primer 5'-TAAGGATCCTGTAAGTGAAAAGCCA-3'. The N-terminal primer contains an *EcoRI* site (underlined), and the C-terminal primer contains a *BamHI* site (underlined). The 330-bp amplified fragment, digested with restriction enzymes *EcoRI* and *BamHI*, was then cloned into the *EcoRI* and *BamHI* sites of expression vector pYA3493 [23], resulting in pYAP97R1. Similarly, the 288-bp fragment specifying the NrdF of *M. hyopneumoniae* genome was amplified using the N-terminal primer 5'-CAGGATCCCCAAGAGGAGATCTATTATATAA-3', and the C-terminal primer 5'-AAAAAGCTTAACTCCCAATCTTCATCT-3' and cloned into the *BamHI* and *HindIII* site (underlined), respectively, of pYA-P97R1, resulting in pYAP97R1N which harbored a 618-bp fragment of *M. hyopneumoniae* HNYE. The N-terminal primer contained linker sequences (double underlined). To induce expression of P97R1N in *E. coli*, the P97R1 and NrdF fragments were excised from pYAP97R1N using *EcoRI* and *HindIII* enzymes and then ligated into the eukaryotic expression vector pGEX-KG [24] resulting in pGEX-KG-P97R1N. Similarly, the P97R1 fragments was amplified using the N-terminal primer 5'-AAAGGATCCGAAGGTAAAAGAGAAGA-3', and the C-terminal primer 5'-TAATGTAAGTGAAAAGCCA-3' and cloned into *BamHI* and *HindIII* site (underlined), respectively, of pGEX-KG, resulting in pGEX-KG-P97R1. The resultant plasmids, pYA-P97R1N, pGEX-KG-97R1N and pGEX-KG-P97R1, were purified using a QIAprep Spin Miniprep kit (BioFlux) and sequenced.

Expression of P97R1 and P97R1N in *E. coli*

Recombinant plasmid pGEX-KG-P97R1 and pGEX-KG-P97R1N were transformed into *E. coli* JM105 expression competent cells by heat shock following a standard method [25]. The *E. coli* JM105 harboring pGEX-KG-97R1N was induced by 1 mM IPTG at mid-exponential phase and expressed a large amount of glutathione S-transferase (GST)-tagged P97R1N in its cytoplasm. P97R1 and P97R1N were purified by an affinity purification process with glutathione-Sepharose 4B columns (GE). Its expression was confirmed by immunoblot performed as previously described [26], using anti-GST-HRP antibody in the Western blot.

Expression of pYAP97R1N in *S. enterica* serovar *choleraesuis* strain C501

The plasmids pYAP97R1N were transformed into *E. coli* X7213 by heat shock following a standard method [25]. Purified plasmids were subsequently electroporated into the *S. enterica* serovar *Choleraesuis* strain C501 using a 2 mm cuvette at 200 p, 25 μ F and 2.0 Kv (Electro Cell Manipulator ECM600). P97R1N expression was analyzed by SDS-PAGE using a 12% gel and Bio-Rad Mini Protean II Cell Apparatus according to the manufacturer's instructions. Western blot analysis to detect the expressed P97R1N was carried out using a positive serum sample obtained from a pig which had been tested for *M. hyopneumoniae* antibody using an ELISA kit (IDEXX Labs).

Animal experiments

Groups of 6-week-old BALB/c mice (ten mice per group) were immunized IM or orally with the recombinant vaccine C501 (pYAP97R1N) (4×10^8 c.f.u. in 200 μ L PBS) on day 0 and day 14, respectively. Mice were also treated with live *M. hyopneumoniae* vaccine

168 (1×10^6 c.f.u. in 200 μ L PBS) (Tianbang Bio-Industry, China) or 200 μ L of PBS, for positive and negative controls, respectively. Blood samples were collected on days 14, 28 and 42 for serological tests. On day 42, lung samples and splenocytes were collected. The lung homogenate was prepared as previously described [21].

Antibody tests

An ELISA was used to assay antibodies to whole *M. hyopneumoniae* or P97R1N in samples of serum and lung homogenate from individual mice. The antibodies against P97R1N protein were measured using a P97R1N-based ELISA we developed and the *M. hyopneumoniae*. Antibody Test Kit was obtained from R&D. Similarly, we constructed a P97R1-based ELISA method to assay P97R1-specific antibodies. Each sample well of polystyrene 96-well flat-bottomed microtiter plates (Kangjia) was coated with 20 μ g/mL of purified P97R1N or P97R1 in 0.1 M carbonate buffer (pH 9.6). 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% BSA). Samples of serum and lung homogenate were added to each well and incubated at 37°C for 30 min. After four washes, plates were treated with biotinylated goat anti-mouse IgG (Southern Biotechnology) for sera and lung homogenates, at 37°C for 30 min, followed by six washes. The substrate solution containing 3,3',5,5'-tetramethylbenzidine (TMB) and H_2O_2 (50 μ L) was then added to each well and incubated at room temperature in the dark for approximately 10 min; the catalytic reaction was stopped by adding 50 μ L 1% SDS. The optical density was read at 630 nm using an ELISA reader.

Analysis of IFN- γ and IL-4

The spleens of mice from each vaccine group were aseptically removed and pooled into 10 mL Dulbecco's Modified Eagle Medium (DMEM, Gibco) containing 100 U/mL⁻¹ penicillin-streptomycin on ice. Splenocytes were isolated using a cell constrainer (Becton Dickinson) and centrifuged at 800 g for 10 min at room temperature. The pellet was resuspended in 10 mL of 150 mmol NH_4Cl for 10 min on ice. The cells were then washed with DMEM and centrifuged. The washed cell pellet was resuspended in 10 mL DMEM containing 100 U/mL⁻¹ penicillin-streptomycin and 10% fetal bovine serum, and then diluted to 4×10^6 cells mL⁻¹. Two milliliters were added to each well of a 12-well plate. Mouse splenocytes were stimulated *in vitro* with 4 μ g/mL⁻¹ of purified P97R1N. The plate was then incubated in a 37°C incubator in the presence of 5% CO₂ for 72 h. The splenocyte culture supernatants were collected and stored at -20°C, or -80°C long-term. Mouse cytokine ELISA kits (R&D) were used according to the manufacturer's instructions. IL-4 and IFN- γ productions were measured as key T-helper1 (Th1) and T-helper 2 (Th2) response, respectively.

Statistical analysis

Values for antibody titers and cytokine levels were compared by one-way analysis of variance (ANOVA) using the computing software Statistical Package for the Social Sciences (SPSS). Results were considered significant at probability (*P*-values) 0.05.

Results

Expression of P97R1N and P97R1 in *E. coli*

Western blot result showed that the expressions of P97R1 and P97R1N in *E. coli* JM105 were induced with or without IPTG in Luria-Bertani (LB) broth culture at mid-exponential phase. The P97R1 and P97R1N fusion protein were detected by anti-GST-HRP antibody by

Western blot. The size of P97R1 was estimated to be about 41 kDa and P97R1N 50 kDa, including the 26 kDa GST tag from the vector (Figure 1).

Expression of P97R1N in *S. enterica* serovar *Choleraesuis* C501

S. enterica serovar *Choleraesuis* C501 was constructed by a method described in our previous work [21]. The recombinant *S. enterica* serovar *Choleraesuis* C501 had a normal growth rate and expressed the P97R1N protein with an approximate molecular mass of 25 kDa, consistent with the calculated size of P97R1N (Figures 2a and 2b). To examine the stability of plasmids pYAP97R1N in C501 *in vitro*, C501 cells containing pYAP97R1N were cultured with a daily passage of 1:1000 dilutions for five consecutive days in LB broth. Cells obtained from the last-day culture expressed amounts of the 25 kDa P97R1N that were similar to those from the first day (Figure 2c), suggesting the stable expression of P97R1N without rearrangements.

Serum antibody titers to P97R1, P97R1N, and *M. hyopneumoniae* whole-cell

The kinetics of the responses of vaccinated mice were monitored for P97R1- and P97R1N-specific serum IgG and IgA, and the *M. hyopneumoniae* whole-cell specific antibody. These responses were compared with those of PBS- and live- *M. hyopneumoniae* 168 vaccine-treated mice. The kinetics of the responses of vaccinated mice to P97R1-specific serum IgG and IgA were analyzed. A low level of P97R1-specific IgA response was observed in all groups vaccinated with the C501 (pYAP97R1N) or live 168 vaccine IM and orally (Figure 3a).

However, only the IM immunization with the C501 group induced a significantly higher specific-P97R1 IgG antibody response than that of the live 168 vaccine group on days 42 ($P<0.05$; Figure 3b). When recombinant P97R1N protein was used in the ELISA assay, there was a low level of serum anti-P97R1N IgG and IgA antibody induced by primary immunization with C501 (pYAP97R1N) or vaccine strain 168 given IM on day 14. However after booster immunization, serum samples showed a tremendous increase in the levels of P97R1N-specific IgG and IgA. A similar trend but only half the levels of IgA were observed in orally vaccinated mice on days 28 and 42. It was noted that IM immunization with the C501 induced a significantly higher level of serum IgA antibody than oral immunization ($P<0.05$), with titers of 133, 6826, and 1066 vs. 106, 3840, and 426 on days 14, 28 and 42, respectively (Figure 3c). Similarly, IM immunization with the C501 (pYAP97R1N) induced a significantly higher level ($P<0.05$) of serum

IgG than oral immunization, with titers of 533, 1706, and 8533 vs. 66, 106, and 213, on days 14, 28, and 42, respectively (Figure 3d). The IM and oral immunization with the C501 (pYA97R1N) groups induced a significantly higher specific-P97R1N antibody response than with live 168 vaccine on days 14, 28, and 42 ($P<0.05$; Figures 3c and 3d). Sera from all the groups, apart from the PBS group, showed serum antibody responses against *M. hyopneumoniae* whole-cell lysate (Table 1). The IM and oral immunization with the C501 groups induced higher anti-*M. hyopneumoniae* antibody responses than with live 168 vaccine on days 14 and 42, and they were significantly higher than the control PBS group ($P<0.05$).

Antibody responses to P97R1N and P97R1 in lung homogenates

All immunized mice survived, and no signs of disease in the

Groups	<i>M. hyopneumoniae</i> antibody ng/mL	
Control (PBS)	28.50 ± 1.72	33.86 ± 2.41
C501 (pYA-P97R1N) i.m.	194.64 ± 4.47 ^a	218.02 ± 12.74 ^a
C501 (pYA-P97R1N) orally	165.65 ± 5.51 ^a	214.12 ± 7.92 ^a
168 strain i.m.	204.62 ± 7.57 ^a	181.72 ± 8.26 ^a
168 strain orally	94.76 ± 3.1 ^a	122.77 ± 9.64 ^a

Serum samples were collected on day 14 and day 42 to determine the *M. hyopneumoniae* whole cell-specific antibody titers for primary and secondary immune responses, respectively.

^aDifferent superscript indicates a significantly different from each other at the same day ($p < 0.05$ as calculated by student's *t* test).

Table 1: Antibody 1 responses to *M. hyopneumoniae* in vaccinated mice.

Figure 2: Expression of P97R1N in *S. enterica* serovar *Choleraesuis* C501. Vaccine strain C501 (P97R1N) and vector control strain C501 (pYA3493) were cultured in LB broth at 37°C. Total cells and concentrated culture supernatants were subjected to SDS-PAGE analysis, and P97R1N was detected by immunoblotting with anti-*M. hyopneumoniae* antibody. (a) Coomassie brilliant blue-stained gel analysis. M, protein ladder; lane 1, total cell extracts of C501 (pYA97R1N); lane 2, concentrated supernatants of C501 (pYA97R1N); lane 3, C501 (pYA3493); (b) Immunoblot analysis. M, protein ladder; lane 1, total cell extracts of C501 (P97R1N); lane 2, concentrated supernatants of C501 (pYA97R1N); lane 3, C501 (pYA3493). (c) Analysis of P97R1N stable expression from the recombinant strain C501 (pYAP97R1N) by SDS-PAGE. Lane 1-9, second passage to ten passage from C501 (pYAP97R1N), lane 10, C501 (pYA3493).

Figure 3: Humoral immune responses in vaccinated mice. Mice were immunized IM or orally with C501 (pYA97R1N), live *M. hyopneumoniae* 168 vaccine, or PBS. Booster immunization was performed 2 weeks later. Serum samples were collected on day 14 (for primary response), and days 28 and 42 (for secondary response) to determine (a) the P97R1-specific IgA (b) the P97R1-specific IgG (c) the P97R1N-specific IgA (d) IgG antibody titers. All data represent the 2 mean $\pm$ SD. Data with different letters (a–d) indicate significant differences between groups ($p < 0.05$). * $p < 0.05$. ** $p < 0.01$.

Figure 4: Local immune responses in lungs of vaccinated mice. Lung homogenates were tested by ELISA for (a) IgA antibodies and (b) IgG antibodies against P97R1N. Data with different letters (a–d) indicate significant differences between groups ($p < 0.05$).

immunized mice were observed during the entire experimental period. The P97R1N-specific IgA and IgG antibody responses of vaccine-inoculated mice were assessed 6 weeks following the initial immunization and compared with those of PBS- and live- *M. hyopneumoniae* 168 vaccine mice (Figures 4a and 4b). Immunization with the C501 (pYAP97R1N) IM injection elicited higher IgA (titer, 8533) and IgG (titer, 1066) response, while oral immunization, elicited less IgA (titer, 1066) and IgG (titer, 26) response in lungs. In contrast, immunization with live *M. hyopneumoniae* 168 vaccine elicited minimal levels of IgA (66 and 133) and IgG (26 and 16) by IM and oral routes, respectively.

The C501 (pYAP97R1N) induced significantly higher titers of the P97R1N-specific IgG and IgA than live *M. hyopneumoniae* 168 vaccine in local immunity ($P < 0.05$). However, when measuring the P97R1-specific antibody responses, only minimal level of IgG was induced in both groups with IM route (Figure 5).

Measurement of cytokine production by ELISA

Splenocytes isolated from pooled spleens from each group of five

mice were stimulated with the P97R1N fusion protein and cultured in duplicate *in vitro* for 72 h. The cultural supernatants were removed and used for determination of cytokine production using mouse cytokine ELISA. Both the C501 (pYAP97R1N) and vaccine strain 168 groups produced high levels of P97R1N-induced IFN- γ and IL-4 (Table 2) when compared with the PBS control group. This demonstrated that both immunization C501 (pYA97R1N) and live *M. hyopneumoniae* 168 vaccine induced cell-mediated and humoral responses, although this result was not statistically significant for any of these groups.

Discussion

Vaccination of attenuated, live recombinant vaccine with a bacterial expression system is a promising method for antigen delivery. *S. enterica* serovar *Choleraesuis* strain C500 is an avirulent vaccine strain and has been used widely to prevent piglet paratyphoid in China for over 40 years [19,20]. Recently, strain C500 was used as a delivery system for foreign antigens and for the development of a safe and protective live bacterial vaccine vector [21]. In this study, we investigated the immunogenicity of the recombinant C501 (pYAP97R1N) vaccine

Groups	IL-4 (pg/ml)	IFN- γ (pg/ml)
PBS	56.1 $\pm$ 4.8	60.6 $\pm$ 0.8
C501 (pYAP97R1N) i.m.	161.2 $\pm$ 6.8	147.0 $\pm$ 11.6
C501 (pYAP97R1N) orally	172.4 $\pm$ 0.5	94.1 $\pm$ 8.3
168 strain i.m.	119.9 $\pm$ 2.4	126.1 $\pm$ 2.0
168 strain orally	67.8 $\pm$ 4.1	123.5 $\pm$ 5.0

Splenocytes were isolated 42 days after primary immunization and stimulated with purified P97R1N proteins for 72 h to determine IFN- γ and IL-4 production in the culture supernatants by ELISA.

Table 2: Cytokine induction by 3 P97R1N proteins in the splenocyte culture.

expressing P97R1 and NrdF genes of *M. hyopneumoniae*. All mice immunized with live *M. hyopneumoniae* 168 or C501 vaccine survived during the entire experimental period. Immunization with the C501 (pYAP97R1N) elicited higher IgA and IgG responses in lungs by i.m. rather than oral route and the C501 induced significantly higher titers of the P97R1N-specific IgG and IgA than live *M. hyopneumoniae* 168 vaccine. Our results were similar to Zhao's group that live avirulent *Salmonella choleraesuis* C501 (pYA-F1P2) induced a better specific-IgA and IgG by subcutaneous (s.c.) rather than oral vaccination [21]. Although the vector pYA3493 could lead to secretion of the recombinant P97R1N protein into the periplasm of *S. enterica* as previously reported [21], our experimental results indicated that most of the recombinant P97R1N protein was found in the intracellular space of *S. enterica*. The disease process of PEP is initiated by adherence of *M. hyopneumoniae* to the cilia of swine respiratory epithelium through an interaction involving P97, a surface-associated protein, and cilia-specific receptors. We analyzed the antigenicity of P97R1 using DNA star bioinformatics software. The P97R1 antigen is hydrophobic and may not be secreted into the periplasmic space [9]. This may explain why the P97R1 vaccine induces only P97R1-specific serum IgG but not IgA in both pigs and mice [12,13]. It has been conceptually challenging to produce an immunogenic and protective vaccine based on vaccination by the i.m. route, because there were few previous studies on the systemic immunity that results from i.m. vaccination based on this principal. Here we show that i.m., but not oral, immunization with a live avirulent *Salmonella choleraesuis* C501 strain expressing P97R1NrdF antigen produce an immunogenic in mice. Mice vaccinated with C501, orally or IM, induced measurable P97R1N-specific IgA, IgG antibodies and *M. hyopneumoniae* whole-cell-specific antibody as compared with the control PBS groups. P97 adhesin has been known for the adherence of *M. hyopneumoniae* to respiratory ciliated epithelial cells in swine [8]. A repeat region of P97 called R1 has been identified as containing both cilium- and antibody binding sites [9,10]. NrdF gene fragments of *M. hyopneumoniae* have been cloned as an experimental vaccine

for porcine enzootic pneumonia [6] and the NrdF vaccine induces significant NrdF-specific lung IgA in mice and reduced lung lesion scores in pigs [7,27]. Studies have reported that multiple antigens confer better protection than a single antigen [28-30]. We showed that the C501 vaccine expressing two model antigens (P97R1 and NrdF) from *M. hyopneumoniae* had a better immunogenicity in mice as suggested by a recent study [31]. The present results showed that mice immunized IM and orally with the C501 or live 168 vaccines generated a significant level of P97R1-specific serum and lung IgG, but not P97R1-specific IgA. However, both P97R1N-specific IgG and IgA were significantly induced in the sera or lungs of mice vaccinated with this C501. Previous studies used *S. typhimurium aroA* to express single Adh [13] and NrdF [7,27,32] antigens. Mice immunized orally with *S. typhimurium aroA* strain SL3261 expressing the *M. hyopneumoniae* NrdF fragment induced a significant NrdF-specific IgA in the lungs [27]. IgA blocks *M. hyopneumoniae* attachment to the mucosal surface, and therefore NrdF is believed to play a key role in protection [33,34]. However, orally immunized mice with *aroA*-attenuated *S. typhimurium* strain expressing NrdF, P97R1, or *nrdF-adh* genes failed to elicit an IgA serologic immune response [13,31,32,35] developed a system in which the C-terminal region of the P97 adhesin of *M. hyopneumoniae* (regions R1 and R2) is expressed and exposed on the surface of an attenuated strain of *Erysipelothrix rhusiopathiae* YS-1. However, this vaccine did not produce significant levels of anti-R1 IgA in the respiratory tract [36] constructed the R1 region of the P97 adhesin of *M. hyopneumoniae* (R1) fused to the B subunit of the heat-labile enterotoxin of *Escherichia coli* (LTB), and it was able to induce specific P97R1-IgA antibody. The attenuated *S. enterica* Serovar typhimurium *aroA* SL3261 expressed a recombinant *M. hyopneumoniae* NrdF fragment fused to β -galactosidase and was able to elicit significant NrdF-specific IgA antibody in lung [27]. However, this NrdF vaccine cannot induce P97R1-specific IgA. Both immunization with the C501 and live *M. hyopneumoniae* 168 vaccine elicited *M. hyopneumoniae* whole-cell antigen-specific responses, and the C501 groups (IM and oral) elicited higher P97R1N-specific IgA and IgG responses than the live 168 vaccine groups ($P < 0.05$). When routes of vaccination were compared, the IM injection with C501 induced higher than oral inoculation for serum IgG anti-P97R1N antibody, and lungs IgA antibody. Similarly, it induced significant higher serum IgG anti-P97R1 antibody than oral inoculation. This observation is supported indirectly by a previous study [21] in which mice vaccinated subcutaneously with *S. enterica* serovar *Choleraesuis* strain C500 induced higher levels IgA in lungs than oral vaccination.

A significant level of IFN- γ was induced in splenocytes from immunized mice stimulated with the purified P97R1N protein. This was in agreement with IFN- γ production from splenocytes induced by NrdF or P97R1 [32]. Splenocytes from immunizing mice with C501 or live 168 vaccines produced higher levels of IFN- γ and IL-4 than control PBS groups when stimulated with P97R1N, indicating that both vaccines induced humoral immune and cell-mediated immunities. Furthermore, the immunized mice with C501 (pYAP97R1N) elicited higher levels of IFN- γ and IL-4 than live *M. hyopneumoniae* 168 vaccination by IM.

Studies have shown the importance of cellular and humoral immune responses induced by vaccination [37-39] or experimental infection [40]. Antibodies induced locally in various animal species with *M. pneumoniae* provide a protection against these diseases [41-43]. Cellular and humoral immune responses can reduce the lesion of pigs inoculated with *M. hyopneumoniae* vaccine [1,39].

In conclusion, these results indicated that IM vaccination with

recombinant attenuated *S. enterica* vaccine strain C501 is more suitable than oral immunization for eliciting immune responses. The C501 expressing P97R1N antigen induced P97R1N-specific IgA and Th1 and Th2 responses by IM route. Therefore, the C501 delivery system may be an attractive approach for prevention of infectious diseases such as PEP, in which Th1 and Th2 responses play important roles in disease protection [44-48].

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