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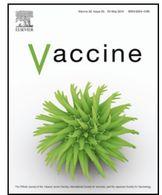

Generation of an attenuated *Salmonella*-delivery strains expressing adhesin and toxin antigens for progressive atrophic rhinitis, and evaluation of its immune responses in a murine model

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ABSTRACT

An expression/secretion plasmid containing genes encoding the FimA, CP39, PtfA, ToxA and F1P2 antigens associated with porcine pneumonic pasteurellosis and progressive atrophic rhinitis (PAR) was constructed and harbored in an attenuated *Salmonella* Typhimurium, which was used as the vaccine candidate. The immune responses induced by this delivery strain were investigated in a murine model. Each antigen secreted from the delivery strain was confirmed by Western blot analysis. Thirty BALB/c mice were divided equally into two groups; group A were intranasally inoculated with the mixture of the five delivery strains, and group B were inoculated with sterile PBS. In group A, all antigen-specific serum IgG were significantly increased compared to those of group B from the 2nd week post-inoculation (WPI) till the 8th WPI. All antigen-specific mucosal IgA in group A were also significantly greater than those of group B. In addition, the significant splenic lymphocyte proliferative responses, the elevations of $CD3^+CD4^+$, $CD3^+CD8^+$ and B-cell populations, and the induction of IFN-γ expression in group A were observed. In conclusion, the mixture of five delivery strains expressing specific antigen for these diseases was found to be capable of inducing significant humoral and cellular immune responses.

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1. Introduction

Pneumonic pasteurellosis, caused by both toxigenic and non-toxigenic strains of *Pasteurella multocida* serotypes A and D, which is responsible for significant losses in the swine industry [1]. In addition, progressive atrophic rhinitis (PAR) is an important upper respiratory disease is caused by *Bordetella bronchiseptica* and *P. multocida* serotype D, which is characterized by the shortening and twisting of the snout, dark tear staining, and pneumonia in swine [2,3] Potential virulence factors of *P. multocida* and *B. bronchiseptica*, which are involved in bacterial attachment and internalization, include hemagglutinating molecules, capsule, fimbriae, and toxin [4,5]. These virulence factors can be targeted for the prevention of *P. multocida* and *B. bronchiseptica* infection [6]. The *fimA* gene encodes a subunit of fimbriae, fimbrilin (FimA) of *P. multocida* strains, which abundantly expressed bacterial surface protein [7]. This protein has been shown to be a potent target for host immunity [8]. Cp39 is a capsule-associated adhesin, and a cross-protective antigen among *P. multocida* strains [9,10]. Its properties were found to be identical to OmpH, which stimulates significant protection against heterologous challenge in chickens [10]. The PtfA, one among colonization factors of *P. multocida* strains, showed variation in their potency as a vaccine based on the ability of this fimbrial antigen to cause a heterologous, and capsule- and serotype-independent protection [11]. In addition, *P. multocida* toxin (PMT), a monomeric 146 kDa protein encoded by the *toxA* gene, is produced by *P. multocida* serotype A and D strains [12,13]. A nontoxic but immunogenic PMT derivative, such as the N-terminal truncated fragment of PMT may be advantageous in the development of efficient vaccines against PAR [13,14]. An adhesin protein, F1P2 of *B. bronchiseptica*, which consists of the immunodominant protective type I domain (F1) of filamentous hemagglutinin and the immunogenic region II domain (P2) of pertactin may serve as a protective antigen against porcine bordetellosis in pigs [15,16].

Live vaccine vehicles can powerfully induce both mucosal and systemic immune responses against pathogenic microorganisms [17]. In recently, many protection studies using delivery systems to express the immunogenic antigens of *P. multocida* [18–20] or *B. bronchiseptica* [15,16] have been performed to prevent *P. multocida* infections or *B. bronchiseptica* infections. In particular, live,

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attenuated *Salmonella* strains have been manipulated to express a wide range of antigens from bacterial, parasitic, and viral sources [17,21]. *Salmonella* Typhimurium colonizes the gastrointestinal system, and the respiratory system [22]. The intranasal inoculation of mice with the *Salmonella* systems was particularly efficient at inducing antibody responses both in serum and respiratory tracts [23,24]. A balanced-lethal host-vector system based on the essential bacterial gene for aspartate β-semialdehyde dehydrogenase (*asd*) has been used to specify recombinant antigens from Asd + plasmids that are retained in vivo in *Salmonella* strains with the *asd* gene deleted [25]. The objective of this study was to construct a live *Salmonella* delivery system expressing FimA, CP39, PtfA ToxA and F1P2 antigens associated with pneumonic pasteurellosis and PAR for young pigs in a murine model. Immune responses induced via intranasal inoculation with this delivery system were examined using a murine model. Mice inoculated with the delivery system not only showed robust systemic and mucosal immune responses, but also demonstrated powerful cellular immune responses.

2. Materials and methods

2.1. Experimental animals

Five-week-old, 30 female BALB/c mice were equally divided into two groups. Mice were maintained in 12 h light/dark cycles and allowed free access to a standard rodent diet and water. The animal experiments mentioned in this study were conducted under ethics approval (CBU 2011-0017) from the Chonbuk National University Animal Ethics Committee in accordance with the guidelines of the Korean Council on Animal Care.

2.2. Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. Wild-type *P. multocida* type A, JOL976, isolated from a piglet was used to amplify gene encoding FimA adhesin antigen. In addition, JOL977, *P. multocida* type D isolated from a piglet, was used to amplify genes encoding CP39 and PtfA adhesin antigens, and the ToxA toxin antigen. *B. bronchiseptica* isolate from a piglet, JOL978, was used to amplify the gene encoding the F1P2 adhesin antigen (Table 1). The JOL977 was inoculated in mice and subsequently isolated from internal organs. In this way, the strain was passaged three times to increase the virulence of JOL977. After three passages, this strain was renamed as JOL1080 and was used as the virulent challenge strain. These strains were kindly supplied by the National Veterinary Research and Quarantine Service, Korea. The attenuated *Salmonella* Typhimurium ($\Delta lon \Delta cpxR \Delta asd$) mutant strain, JOL912 [26], and the pBP244 plasmid [27] were used as a host and a vector for the delivery of individual antigens.

2.3. Cloning of individual recombinant fimbrial antigens

CP39, FimA, PtfA, ToxA and F1P2 proteins were purified from JOL1136, JOL1234, JOL1214, JOL1135 and JOL991, respectively, (Table 1) for enzyme-linked immunosorbent assay (ELISA), and splenocyte stimulation in flow cytometry and quantitative real-time polymerase chain reaction (PCR). Briefly, genes for CP39, FimA, PtfA, ToxA and F1P2 proteins were amplified by PCR using specific primer sets (Table 2). The PCR fragments of each gene were digested with restriction enzymes, and were subsequently cloned into pQE31 or pET28a. These plasmids were then transformed into *Escherichia coli* TOP10 or *E. coli* BL21 in order to create JOL1136, JOL1234, JOL1214, JOL1135 and JOL991 strains. The recombinant CP39, FimA, PtfA, ToxA and F1P2 antigens were prepared from JOL1136, JOL1234, JOL1214, JOL1135 and JOL991, respectively, using an affinity purification process with nickel-nitrilotriacetic acid-agarose (Qiagen, Valencia, CA, USA). The identities of the purified antigens were confirmed via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). All purified antigens were stored at −70 °C until use.

Table 1
Bacterial strains and plasmids used in this study.

Strain/plasmid	Description	Source
Strains		
E. coli		
Top10	F-mcrA (mrr-hsdRMS-mcrBC) 80lacZM15 lacX74 recA1 ara139 (ara-leu)7697 galUgalKrpsL (Str^R)endA1nupG	Lab stock
BL21(DE3)pLysS	F^-, *ompT*, *hsd*S_B($r_B{}^-$, $m_B{}^-$), *dcm*, *gal*, λ(DE3), pLysS, Cm^r	Lab stock
JOL1136	BL21 with pET-CP39	This study
JOL1234	BL21 with pET-FimA	This study
JOL1214	Top10 with pQE-PtfA	This study
JOL1135	BL21 with pET-ToxA	This study
JOL991	BL21 with pET-F1P2	This study
Salmonella Typhimurium		
JOL401	*Salmonella* Typhimurium wild type	Lab stock
JOL911	*Salmonella* Typhimurium JOL401derivave $\Delta lon \Delta cpxR$	Lab stock
JOL912	*Salmonella* Typhimurium JOL911 derivative Δasd	Lab stock
JOL932	JOL912 with pBP244	This study
JOL1240	JOL912 with pBP244-CP39	This study
JOL1251	JOL912 with pBP244-FimA	This study
JOL1247	JOL912 with pBP244-PtfA	This study
JOL1244	JOL912 with pBP244-ToxA	This study
JOL1074	JOL912 with pBP244-F1P2	This study
P. multocida		
JOL976	*Pasteurella multocida* sero type A wild type (PmA037)	Lab stock
JOL977	*P. multocida* (PDNT)sero type D wild type	Lab stock
B. bronchiseptica		
JOL978	*Bordetella bronchiseptica* wild type	Lab stock
Plasmids		
pQE31	IPTG-inducible expression vector; Am^r	Qiagen
pET28a	IPTG-inducible expression vector; Km^r	Novagen
pBP244	pYA3493 derivative containing *lepB*, *secA* and *secB* genes	Lab stock

2.4. Purification of fimbria-specific antisera

Preparation of specific antibodies against the individual adhesin or toxin antigens was carried out via subcutaneous injection of an emulsion containing approximately 250 μg of each purified recombinant antigen in 1 ml of sterile phosphate buffered saline (PBS) and 1 ml of complete Freund adjuvant (Sigma-Aldrich, St. Louis, MO, USA) into New Zealand white rabbits. Two boosters with the same antigen quantity in incomplete Freund adjuvant (Sigma-Aldrich, St. Louis, MO, USA) were administered at 14 and 28 days post-prime immunization. Blood was collected for the preparation of antisera at 14 days after the final injection. The each antigen-specific antibody was stored at −70 °C until use.

2.5. Preparation of Salmonella delivery strain formulation

The delivery strain formulations were prepared as previously described with a slight modification [27]. The genes for CP39, FimA, PtfA and F1P2 adhesin, and ToxA toxin antigen were prepared by digestion from JOL1136, JOL1234, JOL1214, JOL1135 and JOL991 strains, respectively. And then each gene was inserted in pBP244 for

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