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Field efficacy of a combined vaccine supplemented with recombinant *Pasteurella multocida* toxin subunits against atrophic rhinitis

Mi Lan Kang[1], Seung Won Shin[1], Nabin Rayamahji[1], Yeon Soo Seo[1], Su In Lee[1], Won Hyung Lee[2], Han Sang Yoo[1,*]

[1]*College of Veterinary Medicine, BK21 for Veterinary Science and KRF Zoonotic Disease Priority Research Institute, Seoul National University, Seoul 151-742, Korea*
[2]*XP Bio Inc, Anseong 456-914, Korea*
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Abstract : We have investigated efficiency of a recombinant subunit *Pasteurella multocida* toxin (PMT) that was mixed with a vaccine consisted of inactivated whole cells of *Bordetella bronchiseptica*, *P. multocida* (types A and D). For verification of the efficacy of the vaccine, all experimental pigs (suckling piglets, sow and gilts) in the three farms were vaccinated. Antibody titers against *B. bronchiseptica* and *P. multocida* type A of the vaccinated pigs by microplate agglutination were significantly higher than those of the control pigs ($p < 0.05$). Similar patterns were observed in the analysis of anti- PMT neutralizing antibody by serum neutralizing method using Vero cell ($p < 0.05$). Anti- *P. multocida* type D antibody titer of the vaccinated sows and gilts by ELISA showed significant differences with those of the non-vaccinated pigs ($p < 0.05$). Although antibody titers increased, it was unable to find out the difference in the clinical signs between the vaccinated and non-vaccinated pigs. However, the increase in body weight of the vaccinated piglets was observed in comparison with the non-vaccinated piglets on a farm. At slaughtering of the pigs, pathological lesions in the turbinate bones of the vaccinated pigs were significantly lower than those of the non-vaccinated pigs ($p < 0.001$). These results suggested that efficacy of the vaccine in pigs demonstrated to protect against atrophic rhinitis in Korea.

Keywords : atrophic rhinitis, field trial, *Pasteurella multocida* toxin, recombinant subunit vaccine

Introduction

Infection with virulent *B. bronchiseptica* strain is a common risk factor in the establishment of toxin-producing strains of *P. multocida* types A and D in the nasal cavity of pigs leading to the disease, atrophic rhinitis (AR) [7, 13, 15]. The more severe form of AR is attributed to colonization in nasal cavity of pigs by toxigenic *P. multocida* and is known as progressive atrophic rhinitis (PAR) [16]. Many capsular type D and occasionally type A strains of *P. multocida* produce a toxin (PMT), which is a major virulence factor to develop turbinate atrophy in pigs [3]. PAR triggers significant global economic loss in the swine industry due to reduced growth rates and feed conversion efficiency because of developing a number of characteristic lesions including turbinate bone hypoplasia, facial distortion and nasal hemorrhage [14]. However, controlling PAR in the swine industry remains problem to be solved until today.

A recombinant PMT as a vaccine candidate was shown to induce protective response against challenge with a lethal dose of PMT in mice [11] and efficient immunoprotective properties against a challenge with *B. bronchiseptica* and toxigenic *P. multocida* in vaccinated pigs [9]. Also, a previous study demonstrated that three recombinant subunit PMT (rsPMT), characterizing the N-terminal, middle, and C-terminal segments of PMT could induce specific and protective immune responses in pigs [8].

*Corresponding author: Han Sang Yoo
College of Veterinary Medicine, Seoul National University, Seoul 151-742, Korea
[Tel: +82-2-880-1263, Fax: +82-2-874-2738, E-mail address: yoohs@snu.ac.kr]

Considering the proposed synergistic role of *B. bronchiseptica* and *P. multocida* in PAR, the common treatment of *B. bronchiseptica* and *P. multocida* toxoid/ bacterins to control AR is warranted. Therefore, combined vaccine of the bacterins with rsPMT would represent a novel approach to control atrophic rhinitis in pigs. Based on the situations, this study was designed to evaluate the rsPMT vaccine with inactivated whole cells of *B. bronchiseptica*, *P. multocida* (types A and D) under field condition in typical Korea farms.

Materials and Methods

Pigs and bacterial strains

The field trial was carried out at three different scales of typical farms without any vaccination history against AR in Korea that hold about 4,300, 2,000 and 800 pigs, respectively. YLD cross breed pigs were used in this experiment. There were no outbreaks of PAR in those farms. The experiment was carried out with 3 groups of pigs (1-3 weeks old piglets, sows and gilts). Breeding equipment and environment in the farms were described on Table 1. Swine *B. bronchiseptica* type 1 (Bb-1) and *P. multocida* types A (Pm-A) and D (Pm-D) isolates were from National Institute for Animal Health, Council of Agriculture in Taiwan. *E. coli* BL21 transformed with Tox1, Tox2 and Tox7 of rsPMT [8] was from Graduate Institute of Veterinary Pathology, National Chung Hsing University in Taiwan.

Vaccine preparation

The vaccine, BayoVac swine atrophic rhinitis recombinant toxin and inactivated combined bacterin (AR + rsPMT), was manufactured by Kaohsiung Biological Product (Taiwan) and distributed by Bayer Taiwan. Characteristics and generation of PMT in the experimental vaccine were previously described by Liao et al. [8]. The vaccine contained *B. bronchiseptica* bacterin, *P. multocida* types A and D bacterins, rsPMT

Table 1. Breeding and raising environment of experimental farms

Items	Classification	Farm A	Farm B	Farm C
Pig inventory	No. of boar	4	1	17
	No. of sow	940	125	455
	No. of lactating piglet	1,028	200	537
	No. of weaned piglet	2,300	420	990
Ventilation method	Farrowing unit	Windowless, Upper inlet, Exhaust fan	Windowless, Upper inlet, Exhaust fan	Natural ventilation
	Nursery unit	Windowless, Upper inlet, Exhaust fan	Windowless, Upper inlet, Exhaust fan	Mechanical ventilation
	Grower unit	Winch curtain, Exhaust fan	Natural ventilation	Natural ventilation
	Finisher unit	Winch curtain, Exhaust fan	Natural ventilation	Natural ventilation
Waste management	Farrowing unit	Slurry system	Slurry system	Slurry system
	Nursery unit	Slurry system	Slurry system	Slurry system
	Grower unit	Sawdust	Slurry system	Slurry system
	Finisher unit	Sawdust	Slurry system	Slurry system
Feeding type in each stage	Pregnant sow	Wet feeding	Wet feeding	Wet feeding
	Lactating piglet	Dry feeding	Dry feeding	Dry feeding
	Weaned piglet	Dry feeding	Dry feeding	Dry feeding
	Grower	Dry feeding	Dry feeding	Dry feeding
	Finisher	Dry feeding	Dry feeding	Dry feeding
Vaccination	Atrophic rhinitis	Non- vaccinated	Non- vaccinated	Non- vaccinated
Biosecurity	Entrance disinfection equipment	have	have	have
	Foot dipping	have	have	have
	Isolation unit	have	have	have

and aluminum hydroxide gel as an adjuvant. One dose of the vaccine was composed of 1×10^9 CFU of *B. bronchiseptica*, *P. multocida* types A and D each bacterins, 2,000 μg of *P. multocida* toxin containing 50 μg of rsPMT and 19.8 mg of aluminum hydroxide gel.

Vaccination of pigs

The experiment was carried out with 3 groups of pigs in each farm. The piglets, sows and gilts in vaccinated groups consisted of 30, 5 and 5 pigs, respectively. The pigs in control groups consisted of same number of pigs in vaccinated groups. The piglets were immunized by intramuscular injection with 1 ml of the vaccine at 1 week old and 2 ml of the vaccine at 3 weeks old. The gilts were vaccinated by intramuscular injection with 2 ml at 1 month after copulation and 2 ml at 3 weeks before parturition. The sows were vaccinated one time by intramuscular injection with 2 ml at 3 weeks before parturition. Control pigs with same age in each group were raised without any administration. Serum was collected from a jugular vein of the pigs at two week intervals during the experimental period to determine specific antibody response.

Microplate agglutination test

The value of serum anti- *B. bronchiseptica* and *P. multocida* type A antibody titers was determined by microplate agglutination test (MAT) using the standard procedure [2] with minor modifications. *B. bronchiseptica* and *P. multocida* type A strains were isolated from Korea. The bacterial cells were cultured in tryptic soy broth (Difco, USA) at 37°C for 24 h under shaking condition. The bacteria were harvested, washed and resuspended with phosphate-buffered saline (PBS, pH 7.4). Serum samples were heat-inactivated by the incubation at 56°C for 30 min and then two-fold serially diluted in PBS from 1 : 10 to 1 : 1,280. The quantity of 50 μl each serum diluents was added to the wells of a U-shaped microplate followed by the addition of antigens (2 μg/well). The plate was incubated at 37°C for 2 h and kept at 4°C for overnight. Positive and negative sera were included as controls. The titer was expressed as reciprocal of the number indicating the highest dilution that showed agglutination.

ELISA

The value of anti- *P. multocida* type D antibody titer was determined by ELISA kit (EB44-09; Anigen, Korea). ELISA was performed by the manufacturer's procedure.

Serum neutralization assay

Neutralizing antibody titers were determined by the ability of antibody to inhibit PMT-induced cytopathic effect in Vero cells [10] with some modifications of the previous report [8]. Serum was serially diluted with DMEM supplemented with 3% FBS (Gibco BRL, USA), 200 UI/ml of penicillin G and 200 μg/ml streptomycin sulfate. The serially diluted sera were transferred to a 96-well cell culture plate (50 μl/well) followed by the addition of an equal volume of DMEM containing a four-fold minimal toxic dose (MTD) of authentic PMT [8]. The plate was incubated at 37°C for 1 h, and 100 μl of a Vero cell suspension containing 2×10^4 cells per well was then added to the plate. The plate was incubated at 37°C for another 5-7 days. The endpoint-titer was expressed as the highest dilution of immune serum that could neutralize a one-fold MTD of PMT.

Body weight and clinical signs

The body weight was measured in the piglets of both vaccinated and non-vaccinated groups for 6 weeks after the vaccination. Clinical signs were observed in the experimental animals, piglets, sows and gilts in every week for 6 weeks after the vaccination. Clinical signs were determined by the presence or absence of survival, decrease in movement and feed conversion and typical symptoms of AR such as nasal discharge, eye discharge, pyrexia, anorexia, atrophy, sneeze, pink eye, hypokinesia and dyspnea, erythema, rhinorrhagia, necrosis and redness of injected site, etc.

Turbinate score

At slaughtering of the experimental pigs, heads of the pigs were collected and all snouts were sectioned transversely at the first premolar tooth region. Turbinate lesions of the rostral face were subjectively scored through gross examination by a group of collaborative investigators. Each snout was visually scored from the method of previous report [5]. Left and right turbinate atrophy and deviation of the nasal septum were each graded separately on a scale of 0 (normal) to 3 (complete atrophy). Normal turbinate was received a grade of 0. Slight, but obvious, atrophy was graded as 1, moderate atrophy of not less than half the turbinates, especially the dorsal and ventral scrolls was graded as 2, and

severe atrophy of the dorsal and ventral scrolls was graded as 3. No, slight, moderate and severe deviation of the septum were graded from 0 to 3, respectively. The three scores from each snout were then added together and divided by 3 to determine final visual scores for each pig, ranging from 0 to 3. Group means were calculated.

Statistical analysis

Statistical analysis was performed using the computer-based MS-Excel 2004 program. All results were expressed as the mean ± SD. Statistical differences between the groups were analyzed with two-tailed, non-paired Student's *t* test assuming unequal variance. Differences were considered significant if probability values of $p < 0.05$ were obtained.

Results

Anti- *B. bronchiseptica* antibody titer

Anti- *B. bronchiseptica* antibody titer was detected by MAT using *B. bronchiseptica* antigens. The titers against *B. bronchiseptica* in serum of the vaccinated piglets significantly increased from 4 weeks post vaccination. The titers of all farms at 6 weeks after the vaccination were significantly higher than those of the control piglets ($p < 0.01$) (Fig. 1). High antibody titers against *B. bronchiseptica* in the sows and gilts were detected in 2 weeks after the vaccination and maintained more than six weeks even though the antibody titers in each farm were not same. The vaccinated sows and gilts in all farms showed significantly higher agglutination titers than those of the non-vaccinated pigs at 6 weeks post immunization ($p < 0.05$) (Fig. 1).

Anti- *P. multocida* type A antibody titer

Anti- *P. multocida* type A antibody titer was detected by MAT using *P. multocida* type A antigens. The antibody titers against *P. multocida* type A of the vaccinated piglets in the farms A and B were significantly higher than those of the control piglets after the vaccination ($p < 0.05$) (Fig. 2). Anti- *P. multocida* type A antibody titers in the sows and gilts showed similar pattern to those of *B. bronchiseptica* in most farms. In serum samples of the vaccinated sows and gilts at 6 weeks post vaccination, *P. multocida* type A specific titers were significantly higher than those of the control groups ($p < 0.05$) (Fig. 2).

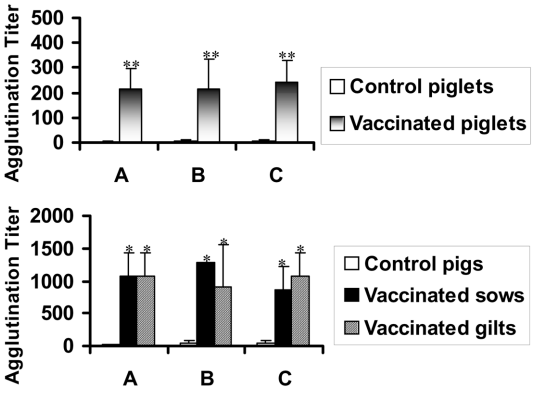

Fig. 1. Anti- *B. bronchiseptica* antibody titers in serum of the experimental pigs at 6 week post immunization by MAT. A, B and C indicate the antibody titers in farms A, B and C, respectively. Significant differences were expressed as $^{**}p < 0.01$ and $^{*}p < 0.05$.

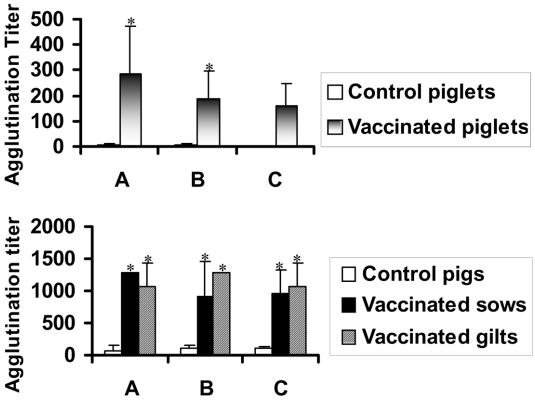

Fig. 2. Anti- *P. multocida* type A antibody titers in serum of the experimental pigs at 6 week post immunization by MAT. A, B and C indicate the antibody titers in farms A, B and C, respectively. Significant difference between control and vaccinated pigs was expressed as $^{*}p < 0.05$.

Anti- *P. multocida* type D antibody titer

The antibody titer against *P. multocida* type D was determined by ELISA. Most of the vaccinated piglets showed increase of the antibody titer with time after the vaccination. However, there were no significant differences between the vaccinated and control piglets in all farms (data not shown). In the results of pregnant pigs, the most of the vaccinated sows and gilts showed significantly higher antibody titers against *P. multocida* type D than those of the control pigs even though the patterns of increase were different (Fig. 3). In case of the farm A, the control pigs showed increase of

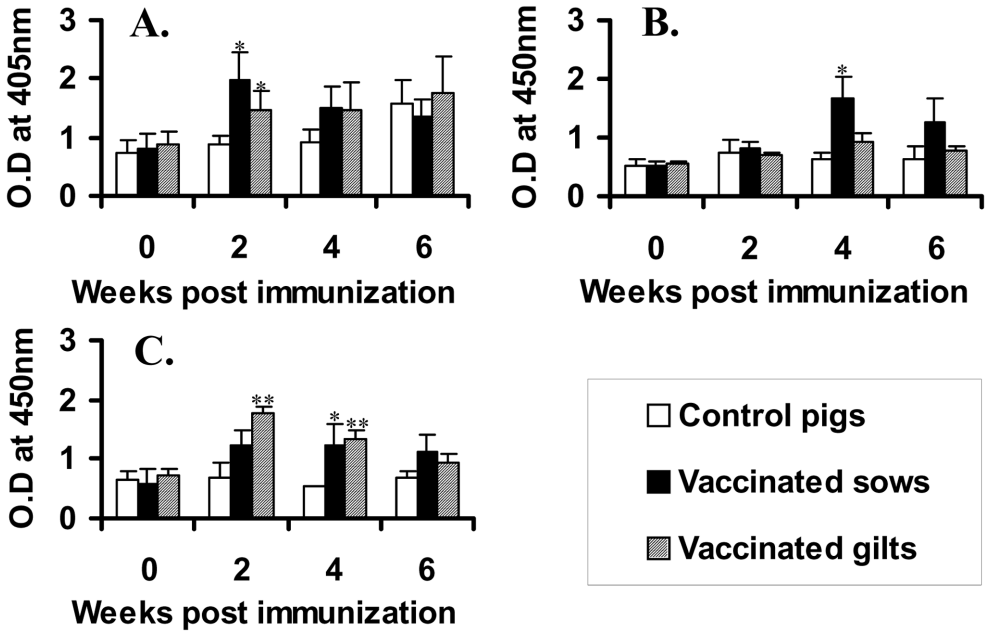

Fig. 3. Anti- *P. multocida* type D antibody titers by ELISA. A, B and C indicate the antibody titers in the farms a, b and c, respectively. Significant differences between the control and vaccinated pigs were expressed as $^{**}p < 0.01$ and $^{*}p < 0.05$.

antibody response against *P. multocida* type D after 4 week. The increase seems to be due to natural infection of the bacterin since there was no vaccination history.

PMT- specific neutralizing antibody titer

The PMT- specific neutralizing antibody titer was detected as its inhibitory ability to the cytopathic effects induced by PMT in Vero cells. At 6 weeks after the vaccination, PMT- specific neutralizing antibody titer ($\geq 1:64$) was significantly higher in the vaccinated piglets of the farm A than that of the non-vaccinated piglets ($p < 0.05$) (Fig. 4). However, the vaccinated piglets in other farms showed moderate increase of neutralizing antibody titer ($\geq 1:32$) against PMT even though there was not significance. The vaccinated sows in the farm A could generate the highest PMT-specific neutralizing antibody titers after 6 weeks (1 : 128). The titers were significantly higher than those of the control pigs ($p < 0.05$) (Fig. 4). The vaccinated sows in the farm B and C showed high levels of PMT- specific neutralizing antibody titer ($\geq 1:64$) even though the value was lower than that of the vaccinated sows in the farm A. In the farm C, the neutralizing antibody titers in both of the vaccinated sows and gilts were significantly higher than those of the non-vaccinated pregnant pigs ($p < 0.05$) (Fig. 4).

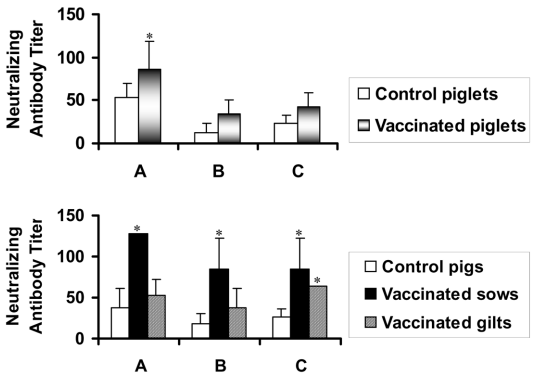

Fig. 4. *P. multocida* toxin-specific serum neutralizing antibody titers at 6 week post immunization. The SN-titer was expressed as the end-point dilution of serum that could inhibit the cytotoxicity of four fold MTD of authentic PMT on Vero cells. A, B and C indicate the neutralizing antibody titer in farms A, B and C, respectively. Significant difference between the control and vaccinated pigs was expressed as $^{*}p < 0.05$.

Body weight and clinical signs

The significant increase in body weight of the vaccinated piglets was not observed in all farms even though there was increase in one farm (Fig. 5). Also,

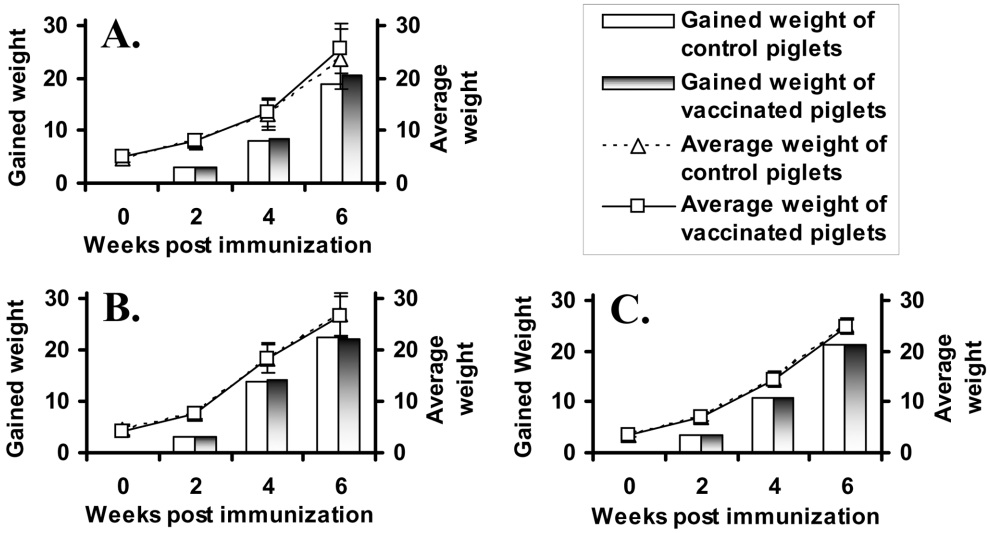

Fig. 5. Body weight of the experimental piglets. Gained weight was calculated as difference with weight of the beginning. Average weight measured through all experimental piglets (n = 30). A, B and C indicate the farms A, B and C, respectively.

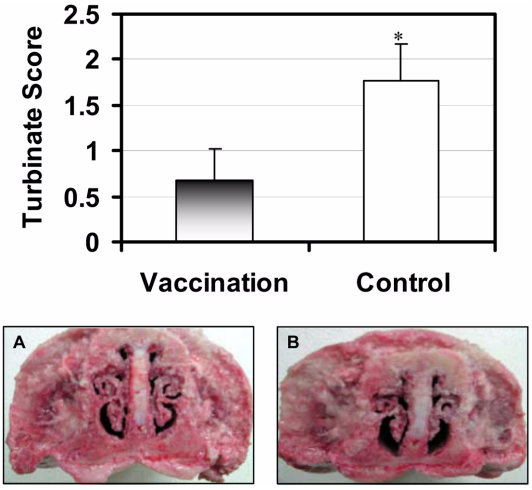

Fig. 6. Gross pathological scores of turbinate in the experimental pigs. The grade scores of gross morphological changes in turbinate bones at slaughtering were estimated according to the grade from 0 (normal) to 3 (complete atrophy). Atrophy of left and right turbinates and deviation of the nasal septum were each graded separately. The graph indicates mean of the three scores in turbinate bone of each pig. Significant difference between control and vaccinated groups was expressed as $^{*}p < 0.001$ (n = 3). The photographs are cross sections of turbinate bone tissues in (A) vaccinated pig and (B) control pig.

it was very difficult to distinguish the difference in the degree of clinical signs regardless of the vaccination.

Turbinate scores

In the scoring of gross pathological lesions in nasal turbinate produced low turbinate atrophy scores ranging from 0 to 1 in the vaccinated pigs. The average score of turbinate atrophy in the vaccinated group was 0.67 ± 0.34. In contrast, the non-vaccinated pigs showed mild to severe turbinate atrophy with average scores of 1.78 ± 0.34, which was significantly severed from the vaccinated pigs ($p < 0.001$) (Fig. 6). Some pigs in the control group showed severe atrophy of turbinate, especially dorsal, ventral scrolls and deviation of the septum (Fig. 6B).

Discussion

Toxigenic strains of *P. multocida* and virulent strains of *B. bronchiseptica*, alone, or combined with environmental factors are the primary causes of AR [1, 3, 6, 7, 13, 15]. Especially, toxigenic *P. multocida* has been accepted as an essential but not sufficient factor for the development of PAR but virulent strains of *B. bronchiseptica* can cause mild and subclinical AR. Chanter *et al.* [1] suggested that the toxin of *B. bronchiseptica* responsible for dermonecrosis may enhance the growth of toxigenic strains of *P. multocida* on the nasal turbinate mucosa. Considering the proposed synergistic role of *B. bronchiseptica* and *P. multocida* in PAR, the common treatment of *B.*

bronchiseptica and *P. multocida* toxoid/bacterins to control AR is warranted. In this study, we investigated the efficacy of a combined vaccine consisting of three fragments of rsPMT and inactivated bacterins of *B. bronchiseptica* and *P. multocida* (types A and D) in field condition of Korea.

Recent studies have been investigated several types of vaccine preparations to prevent or control PAR. In general, a genetically modified nontoxigenic PMT [17] and a purified formalin-inactivated PMT toxoid [4] have been demonstrated an effect for the control PAR. However, PMT constitutes less than 0.6% of total cellular proteins of *P. multocida*, making it necessary to culture a large scale of bacteria to obtain sufficient antigen for the use of commercial scale. Therefore, a nontoxic but immunogenic derivative of PMT may be an attractive alternative strategy for large quantity of PMT production. The expression efficiencies of the three kinds of rsPMT derivatives in *E. coli* and the effective immunogenicity for inducing PMT- specific immune responses were demonstrated [8].

For evaluating the immunogenicity of the combined vaccine in the field condition, the specific immune responses elicited by the vaccinated pigs were analyzed by MAT, ELISA and serum neutralization assay. Antibody titer against bacterins and PMT- specific neutralizing antibody titer generally increased by the vaccination even though there was not significant depending on the farm. The difference in the farms might be due to different conditions such as health, nutrition, and breeding environment, etc. Although vaccination with the rsPMT can induce serum neutralizing antibodies, other vaccine components of toxigenic strains of *B. bronchiseptica* and *P. multocida* should be considered. The bacterial capsular antigens including lipopolysaccharide, polysaccharide, or pili may play roles in bacterial colonization on turbinate mucosa [12]. Uchida C *et al.* [18] have demonstrated that *P. multocida* cell-free antigens could induce strong resistance to an intratracheal challenge with toxigenic *P. multocida* in mice. Our results showed that significant *B. bronchiseptica* and *P. multocida* specific antibodies were induced by intramuscular injection of the combined vaccine (Figs 1-3). Most of the vaccinated pigs in all farms showed significant increase of specific antibodies by the vaccination. Also, the vaccinated pigs exhibited lower levels of turbinate conchal atrophy than those of the non-vaccinated pigs (Fig. 6) ($p < 0.001$). Therefore, these results suggested that the combined vaccine used in this study could induce significant specific immune responses in the field. Based on the results of the body weight increase, clinical evaluation of clinical signs and turbinate atrophy, the vaccine we evaluated the efficacy in the field will be useful to prevent AR.

Acknowledgments

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References

1. **Chanter N, Magyar T, Rutter JM.** Interactions between *Bordetella bronchiseptica* and toxigenic *Pasteurella multocida* in atrophic rhinitis of pigs. Res Vet Sci 1989, **47**, 48-53.
2. **Cole JR Jr, Sulzer CR, Pursell AR.** Improved microtechnique for the leptospiral microscopic agglutination test. Appl Microbiol 1973, **25**, 976-980.
3. **Foged NT.** *Pasteurella multocida* toxin. The characterization of the toxin and its significance in the diagnosis and prevention of progressive atrophic rhinitis in pigs. APMIS Suppl 1992, **25**, 1-56.
4. **Foged NT, Nielsen JP, Jorsal SE.** Protection against progressive atrophic rhinitis by vaccination with *Pasteurella multocida* toxin purified by monoclonal antibodies. Vet Rec 1989, **125**, 7-11.
5. **Gatlin CL, Jordan WH, Shryock TR, Smith W**C. The quantitation of turbinate atrophy in pigs to measure the severity of induced atrophic rhinitis. Can J Vet Res 1996, **60**, 121-126.
6. **Hamilton TD, Roe JM, Webster AJ.** Synergistic role of gaseous ammonia in etiology of *Pasteurella multocida*-induced atrophic rhinitis in swine. J Clin Microbiol 1996, **34**, 2185-2190.
7. **Jacques M, Parent N, Foiry B.** Adherence of *Bordetella bronchiseptica* and *Pasteurella multocida* to porcine nasal and tracheal epithelial cells. Can J Vet Res 1988, **52**, 283-285.
8. **Liao CM, Huang C, Hsuan SL, Chen ZW, Lee WC, Liu CI, Winton JR, Chien MS.** Immunogenicity and efficacy of three recombinant subunit *Pasteurella multocida* toxin vaccines against progressive atrophic rhinitis in pigs. Vaccine 2006, **24**, 27-35.

9. **Nielsen JP, Foged NT, Sorensen V, Barfod K, Bording A, Petersen SK.** Vaccination against progressive atrophic rhinitis with a recombinant *Pasteurella multocida* toxin derivative. Can J Vet Res 1991, **55**, 128-138.
10. **Pennings AM, Storm PK.** A test in Vero cell monolayer for toxin production by strains of *Pasteurella multocida* isolated from pigs suspected of having atrophic rhinitis. Vet Microbiol 1984, **9**, 503-508.
11. **Petersen SK, Foged NT, Bording A, Nielsen JP, Riemann HK, Frandsen PL.** Recombinant derivatives of *Pasteurella multocida* toxin: candidates for a vaccine against progressive atrophic rhinitis. Infect Immun 1991, **59**, 1387-1393.
12. **Pijoan C, Trigo F.** Bacterial adhesion to mucosal surfaces with special reference to *Pasteurella multocida* isolates from atrophic rhinitis. Can J Vet Res 1990, **54** (Suppl), S16-21.
13. **Rhodes MB, New CW Jr, Baker PK, Hogg A, Underdahl NR.** *Bordetella bronchiseptica* and toxigenic type D *Pasteurella multocida* as agents of severe atrophic rhinitis of swine. Vet Microbiol 1987, **13**, 179-187.
14. **Rutter JM.** Atrophic rhinitis in swine. Adv Vet Sci Comp Med 1985, **29**, 239-279.
15. **Rutter JM, Taylor RJ, Crighton WG, Robertson IB, Benson JA.** Epidemiological study of *Pasteurella multocida* and *Bordetella bronchiseptica* in atrophic rhinitis. Vet Rec 1984, **115**, 615-619.
16. **Schöss P.** Diagnosis and control of progressive atrophic rhinitis. ALTEX 1994, **11**, 55-58.
17. **To H, Someno S, Nagai S.** Development of a genetically modified nontoxigenic *Pasteurella multocida* toxin as a candidate for use in vaccines against progressive atrophic rhinitis in pigs. Am J Vet Res 2005, **66**, 113-118.
18. **Uchida C, Kimura Y, Kubota S, Sasaki O.** Protective effect of *Pasteurella multocida* cell-free antigen and toxoid against challenge with toxigenic strains of *Pasteurella multocida* in mice. J Vet Med Sci 2003, **65**, 737-740.