

## Effective methods for the production of immunoglobulin Y using immunogens of *Bordetella bronchiseptica*, *Pasteurella multocida* and *Actinobacillus pleuropneumoniae*

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### ABSTRACT

Swine respiratory diseases induce severe economic losses in the swine industry worldwide. Several methods have been developed and applied to control these diseases. However, there are still problems of disease control in the swine industry. Recently, egg yolk antibodies have been found to offer several advantages for disease control in animals and humans.

In a previous study (24), antibodies to several causative pathogens of swine respiratory diseases were developed. However, several problems remained, especially in terms of reduced laying rates. Therefore, experimental vaccines were reformulated with various bacterial antigens of the swine respiratory diseases. After immunizing hens with the antigens, antibody profiles and other effects including laying rates were investigated and compared to those of the previous study.

Profiles of antibody titers were very similar with those of the previous study. However, side effects, such as depression, weakness, reduction of laying rates and mortality, were dramatically lowered and laying rates were increased in hens injected with certain experimental vaccines. In particular, laying rates of hens injected with vaccines against atrophic rhinitis were increased up to 84% by injecting a vaccine composed of only the DNTs of *B. bronchiseptica* and *P. multocida* D:4. Efficacies of the vaccines against swine pneumonic pasteurellosis and pleuropneumonia were very similar with those of the previous study. These results suggest that new vaccines could be effective in the production of egg yolk antibodies against the causative agents of swine

respiratory diseases.

**Key words ;** IgY, Swine, Respiratory Diseases.

### Introduction

Although several attempts such as vaccination and use of antibiotics have been made to control infectious diseases in swine industry, problems, such as appearance of antibiotics resistant bacteria, shock due to vaccination remain, especially in terms of the respiratory diseases (2, 32). During the last decade, passive immunization with mammal antibodies has been considered as an alternative mean of controlling infectious diseases. However, the method has associated problems in terms of cost and productivity. Recently, the utilization of immunoglobulin Y (IgY) from eggs of chickens which were immunized against certain pathogens to mammals has been the focus of attention in immunotherapy and immunodiagnosis, since the birds can actively transfer immunoglobulin G (IgG) to the egg yolks of their offsprings in the same levels of concentration as in mammals (3, 4, 14, 16, 27, 30). The use of chicken antibody for large scale production of antibodies to mammalian pathogens offers several advantages such as; animal welfare, low costs in production and safety for dangerous regaining of pathogenicity of mammalian pathogens in birds as the unusual host (3, 12, 16, 31, 32). The efficacy of IgY has been proven in several applications for the treatment and prevention of fatal enteric colibacillosis in neonatal piglets (15, 29) and calves (13), viral diarrhea in infants (10), dental caries (11), canine parvovirus (21), and snake venom (1).

Swine respiratory diseases such as atrophic rhinitis (AR), pneumonic pasteurellosis, and swine pleuropneumonia are the most important swine diseases, both economically and clinically (20, 23).

AR, caused by toxigenic *Pasteurella multocida* alone or in combination with *Bordetella bronchiseptica*, is characterized by hypoplasia of the nasal turbinate bones in young swine under 6 weeks old (23). Toxigenic *P. multocida* and *B. bronchiseptica* are widespread and are of global economic

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significance to swine industry. *P. multocida* type A is the etiologic agent of swine pneumonic pasteurellosis as well as an opportunistic pathogen (16). Recently, outer membrane proteins (OMPs) of the bacterium were proposed to be associated with the *in vivo* antigen (6, 7, 18, 22, 34). Moreover, it has been suggested that the *in vivo* expression of the cross-protective antigen could be induced by the low concentration of iron present *in vivo* (18, 22, 34). *Actinobacillus pleuropneumoniae* is the causal agent of porcine contagious pleuropneumonia, and is one of the most important causes of economic loss in the industry, due to its high morbidity and mortality (8, 9).

Previous studies (24, 25, 26) have reported upon the production of IgYs against the causative agents of AR, swine pneumonic pasteurellosis and porcine contagious pleuropneumonia. Moreover, the specificities and protectivities of these IgYs were confirmed by Western blot and challenge test in mice, respectively. However, some problems were experienced, such as depression, weakness, a reduction in the laying rate, and increased mortality, especially in hens injected with atrophic rhinitis causative agents. Therefore, vaccine components were devised and applied to hens in an attempt to solve these problems and improve IgY production in the present study.

## Materials and Methods

### Bacterial strains

The bacterial strains used in this study were *B. bronchiseptica* and *P. multocida* D:4 as the causative agents of AR, *P. multocida* A:3 for pneumonic pasteurellosis and *A. pleuropneumoniae* serotypes 2 and 5 for porcine contagious pleuropneumonia. These bacterial strains were isolated and identified from field samples, as described in a previous study (24).

### Preparation of immunogens

Immunogens such as bacterin, OMPs, and dermonecrotxin (DNT) used in this study were prepared as described in a previous study by Shin et al. (24).

### Preparation of live cells of *P. multocida*

The bacteria were grown in tryptic soy broth (TSB) (Difco Co., Detroit, MI, USA) at 37°C for 18 hrs with shaking. After incubation, the bacteria were harvested by centrifugation at 8,000 rpm for 30 min, washed with phosphate buffered saline (PBS, pH 7.0), and resuspended in PBS. The number of live cells was determined using the standard plate count method and adjusted to  $1.0 \times 10^5$  CFU/ml.

### Preparation and analysis of iron-restricted outer membrane proteins (IROMPs) from *P. multocida* A:3

IROMPs were extracted by a method described by Choi et al. (5) with minor modifications. Briefly, cells were cultured in brain heart infusion (BHI) (Difco Co.) with 100 mM 2,2-dipyridyl (Sigma Co., St. Louis, Mo, USA) at 37°C for 18

hrs with shaking. The bacteria were harvested, washed twice and resuspended in 10 mM HEPES buffer (pH 7.4) and finally sonicated with an ultrasonic homogenizer (Bahdelin Co., Germany) for 30 seconds. After centrifugation of the sonicated bacterial culture broth at 8,000 rpm for 30 min, the supernatant was centrifuged at 20,000 rpm for 1 hr at 40°C and the pellet was resuspended in 2 ml of 2% sodium lauryl sarcosinate detergent in 10 mM HEPES buffer (pH 7.4) and incubated at 22°C for 1 hr. Sarkocyl detergent-insoluble OMP was harvested by centrifugation at 20,000 rpm for 1 hr at 4°C, washed, suspended in distilled water and then stored at 20°C. To analyze the prepared IROMP, SDS-PAGE analysis was carried out, as described by Marandi and Mittal (18). Briefly, the IROMP was denatured in sample buffer by incubation at 100°C for 5 min. The 20 ml of the sample were applied in 12% polyacrylamide gel at 80 V for 2 hr. Protein bands were visualized by staining the gel with 0.1%(w/v) Coomassie brilliant blue R-250.

### Preparation and immunization of experimental vaccines in hens

Experimental vaccines were made using antigens prepared as described above. Vaccine formulations are shown in Table 1. Twenty-week-old white laying hens purchased from a commercial farm were used for immunization. The chickens were raised in the ordinary outside condition from April to October, 2000. Birds were allocated into 9 experimental groups based on the antigen involved and the antigen concentration, as shown in Table 1. Oil adjuvant ISA75 (Seppic, Com) was used as an adjuvant based on the results of a previous study (24). Primary immunization was conducted by injecting 1.0 ml of the vaccine into the pectoral muscle. The second and third vaccination was carried out 2 and 4 weeks after the first vaccination. The last immunization was performed at 14 weeks after the first inoculation.

### Extraction of egg yolk antibody

Eggs were collected from immunized hens in one-week intervals after the first inoculation. Egg yolk antibodies were extracted from the eggs as described by previous studies (24, 26). Briefly, egg yolk was separated from the egg white and homogenized after mixing with an equal volume of PBS. The homogenized egg yolk was then mixed with two volumes of chloroform and incubated for 2 hrs at room temperature. After this incubation, the supernatant was collected. To compare the antibody titers of egg yolks, the sera of hens were also collected at 2 weeks intervals after the first inoculation.

### Immunological assay

Antibody titers in egg yolks and sera were measured by ELISA. OMPs and DNT from *B. bronchiseptica* and *P. multocida* D:4, OMPs from *A. pleuropneumoniae* serotypes 2 and 5 and IROMPs from *P. multocida* A:3 were used as

**Table 1.** Experimental groups designed in this study

Groups	Target Diseases	Bacterial species	Antigens	No. of Chickens
1	Atrophic rhinitis	<i>B. bronchiseptica</i> <i>P. multocida</i> D:4	Whole cells ( $1.0 \times 10^9$ cells/ml)	7
2	Atrophic rhinitis	<i>B. bronchiseptica</i> <i>P. multocida</i> D:4	DNT (150 $\mu$ g/head)	7
3	Atrophic rhinitis	<i>B. bronchiseptica</i> <i>P. multocida</i> D:4	First ; whole cells <sup>1</sup> Second ; DNT <sup>2</sup> Third ; mixture of whole cells and DNT	7
4	Atrophic rhinitis	<i>B. bronchiseptica</i> <i>P. multocida</i> D:4	Whole cells ( $1.0 \times 10^9$ cells/ml), DNT (100 $\mu$ g/head)	7
5	Pneumonic pasteurellosis	<i>P. multocida</i> A:3	First ; live cells <sup>3</sup> Second ; killed cells <sup>4</sup>	7
6	Pneumonic pasteurellosis	<i>P. multocida</i> A:3	Whole cells ( $1.0 \times 10^9$ cells/ml) IROMP (150 $\mu$ g/head)	7
7	Porcine Pleuropneumonia	<i>A. pleuropneumonia</i> serotypes 2 and 5	Whole cells ( $1.0 \times 10^9$ cells/ml) OMP (150 $\mu$ g/head)	7
8	Pneumonic pasteurellosis and Porcine Pleuropneumonia	<i>P. multocida</i> A:3 <i>A. pleuropneumonia</i> serotypes 2 and 5	Whole cells ( $1.0 \times 10^9$ cells/ml) IROMP (150 $\mu$ g/head) OMP (150 $\mu$ g/head)	7
9	Control	PBS		7

<sup>1</sup> Whole cells ;  $1.0 \times 10^9$  cells/ml, <sup>2</sup> DNT ; 150 mg/head,

<sup>3</sup> Live cells ( $1.0 \times 10^4$  cells/ml), <sup>4</sup>Killed cells ;  $1.0 \times 10^9$  cells/ml

coating antigens for ELISA. Egg yolk and serum from each bird were pooled and used for measurement of antibody titers. Ninety-six well microplates were coated with the antigens diluted in coating buffer by overnight incubation at 4°C. After the incubation, the plates were washed with 0.05% Tween 20-PBS (T-PBS) buffer three times, blocked with PBS containing 1% BSA, and washed with T-PBS buffer three times after incubation at 37°C for 1 hr. Egg yolk antibodies were incubated with appropriate antigens at 37°C for 1 hr after diluting with PBS at the appropriate concentration. After washing out the unreacted antibodies with T-PBS three times, rabbit anti-chicken IgG conjugated with horseradish peroxidase (Cappel Co.) was applied to each well of the plate and incubated at 37°C for 1 hr. Substrate, 2,2-azino-bis (3-ethylbenz-thiazoline-6- sulfonic

acid) (Sigma Co.) was added into each well of the plate. Optical densities at 405 nm were measured with a microplate ELISA reader after stopping of the reaction with 2 M HCl solution.

### Laying rates

The laying rate in each experimental group was calculated by dividing the total number of eggs by accumulated number of hens during days of laying.

## Results

### Immune responses to *B. bronchiseptica*

*B. bronchiseptica* was injected using four different regimens as shown in Table 1. Antibody profiles against

bacteria in sera and egg yolks were analyzed by ELISA using OMP and DNT of *B. bronchiseptica* as antigens. Generally, antibody titers peaked at 4 and 6 weeks in sera and egg yolks, respectively, after the first injection (Fig. 1 and 2). The titers of the vaccinated groups were three times higher than those of the control group. Antibody profiles against OMP and DNT of *B. bronchiseptica* were similar (Fig. 1 and 2). Early immune responses in the egg yolks of hens inoculated with DNT at the first inoculation (groups 2 and 4) were more remarkable than those in hens inoculated with only bacterin (groups 1 and 3) (Fig. 1 and 2). However, the response rapidly increased by the second inoculation with DNT (group 3 in Fig. 1 and 2). Group 2 showed the highest antibody titers against antigens, and this was also more stably maintained in the experimental animals immunized against AR. Serum responses were also very similar to those shown in egg yolks even though the responses in sera occurred sooner (data not shown).

#### Immune responses to *P. multocida* D:4

Generally, the immune response profiles against DNT and OMP of *P. multocida* D:4 were very similar to those against DNT and OMP of *B. bronchiseptica* except for a sudden decrease of antibody titers against DNT in egg yolks from 10-12 weeks after the first inoculation (Fig. 3 and 4).

#### Immune responses to *P. multocida* A:3

Immune response profiles against *P. multocida* A:3 in egg yolks and the sera of hens immunized with *P. multocida* A:3 alone and *P. multocida* A:3 combined with *A. pleuropneumoniae* were very similar in patterns in the analysis of antibody titer by ELISA using IROMP and OMP of *P. multocida* A:3 (Fig. 5 and 6). However, the response in group 5 was lower than in the other groups in case of using IROMP as a coating antigen (Fig. 5). Earliest immune response in egg yolks was observed in group 6 (Fig. 5). There was no difference in the antibody titers regardless of combining *P. multocida* A:3 with *A. pleuropneumoniae* serotypes 2 and 5 (Fig. 6).

#### Immune responses to *A. pleuropneumoniae* serotypes 2 and 5

Immune responses to *A. pleuropneumoniae* serotypes 2 and 5 in egg yolks and sera steadily increased, and were maintained for 14 weeks (Fig. 7 and 8). Antibody titers against *A. pleuropneumoniae* serotypes 2 and 5 were increased by combination with *P. multocida* 3A (group 8) in sera (data not shown).

#### Laying and survival rates

The laying and survival rates of hens inoculated with *B. bronchiseptica* and *P. multocida* D:4, as the causative agents of AR, varied depending on the nature of bacterial antigens (Fig. 9). The laying rate in the group 2, in which all birds survived, was the highest. Laying in some hens

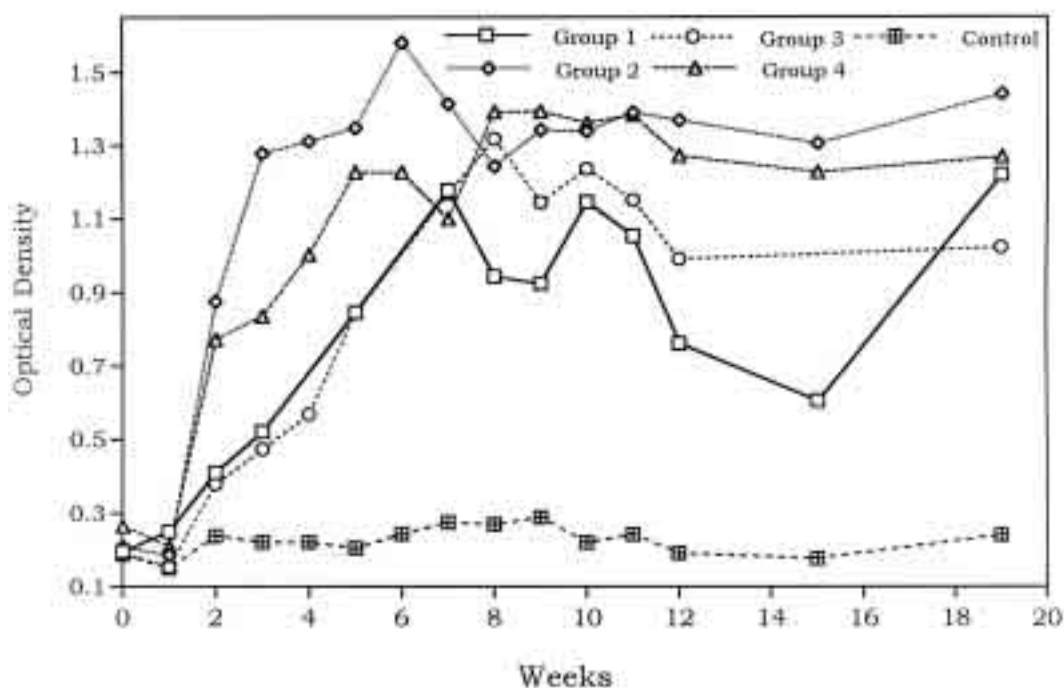
stopped temporally after the first inoculation and then gradually recovered.

## Discussion

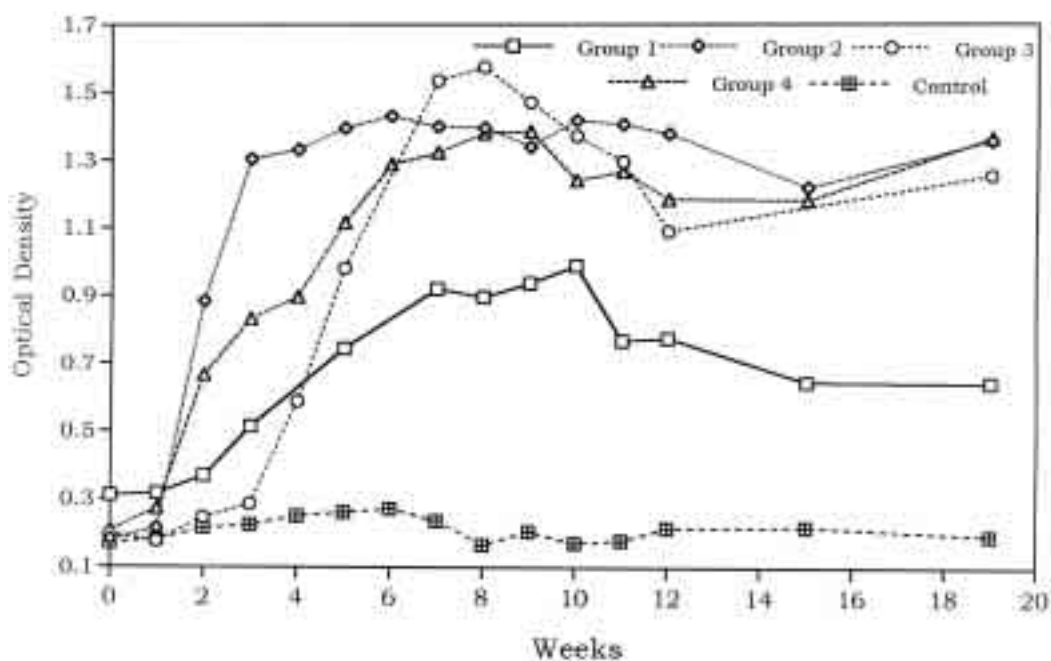
*A. pleuropneumoniae*, *P. multocida* and *B. bronchiseptica* are the most important infectious pathogens in the porcine respiratory tract. Although several attempts have been made to control these agents in swine industry worldwide, the diseases caused by these bacteria remain problematic and induce severe economic losses in infected swineherds worldwide (2, 32). Recently, egg yolk antibodies were introduced in an effort to control swine diseases such as gastrointestinal diseases (17, 30). To develop egg yolk antibodies to control swine respiratory disease, immune responses against these agents in the egg yolks and sera of hens were examined for specificity and protectivity in mice (24, 25, 26). However, problems were encountered in terms of laying and survival rates, particularly with respect to the causative agents of AR. In this study, we tried to solve the problem by reformulating experimental vaccines. For this purpose, 4 different groups were designed based on the vaccine components of the causative agents of AR, *B. bronchiseptica* and *P. multocida* 4:D, and animals were immunized using different methods (Table 1). Results from the 4 groups suggest that the LPS of the killed whole cells of *P. multocida* 4:D was the most important factor that could influence laying rate. The results from groups 5 and 6 immunized with *P. multocida* A:3 also support the above suggestion. These findings are in agreement with previous reports, which indicate that the toxicity of *P. multocida* A:3 in hens, as indicated by a reduction of egg production and increases with mortality. Survival rates of experimental groups (1 to 4) injected with the causative agents of AR suggested that crude DNT could influence on the death rate by LPS and/or capsular polysaccharide contamination. Results from analysis of laying rate and antibody titers indicate that immunization with only DNT was most effective in terms of survival and laying rates for the production of egg yolk antibodies against *B. bronchiseptica*. However, bacterin was most effective in the case of *P. multocida* 4:D.

Antibody titers reached a peak at 6-8 weeks in egg yolk, with 2 weeks in advance of titers in serum after the first inoculation, though differences were observed in the groups. This result agrees with those of previous reports (3, 4, 12, 27, 28, 33). Titers in egg yolk dropped significantly around 12 weeks after the first inoculation. However, it was found that the titer could increase again by re-inoculation 14 weeks after first injection. This finding indicated that egg yolk antibody can be continuously produced by re-inoculating immunogens when the antibody titers in the sera begins to decline.

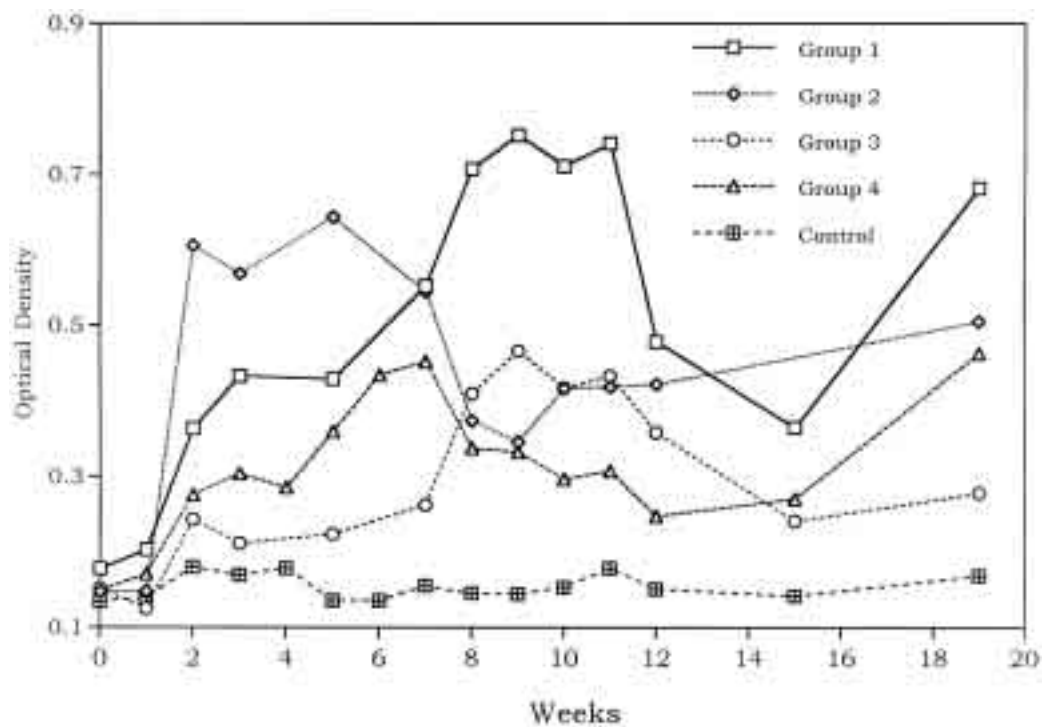
To produce egg yolk antibody against *P. multocida* A:3, live bacteria and IROMP were used to prepare experimental



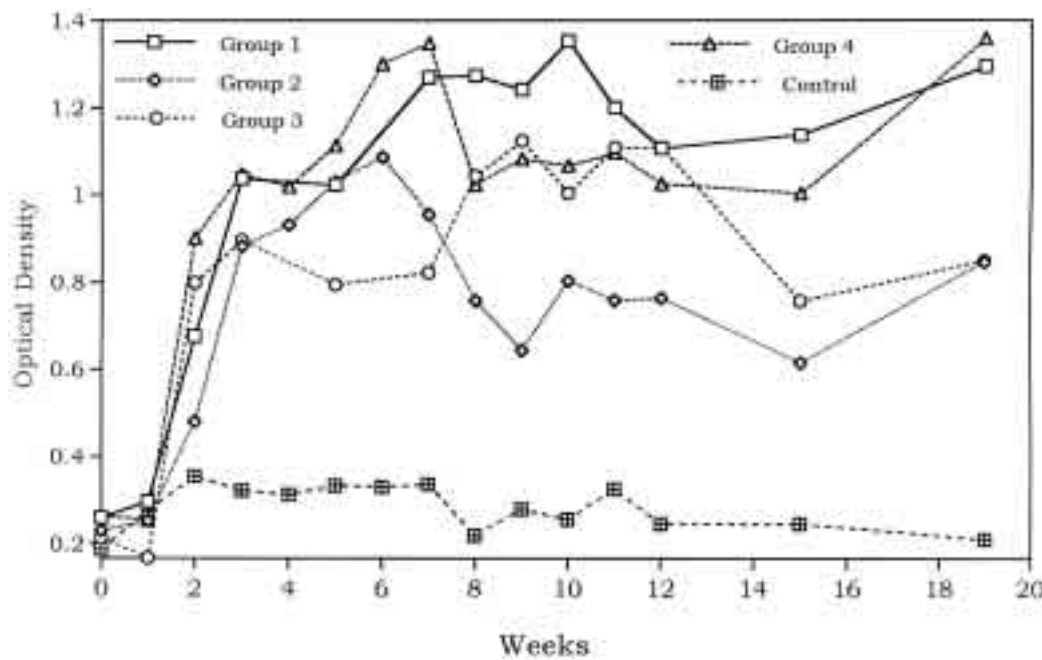
**Fig. 1.** Antibody titers against *Bordetella bronchiseptica* DNT in the egg yolks of hens immunized with various antigens of *B. bronchiseptica* and *P. multocida* D:4, as shown in Table 1.



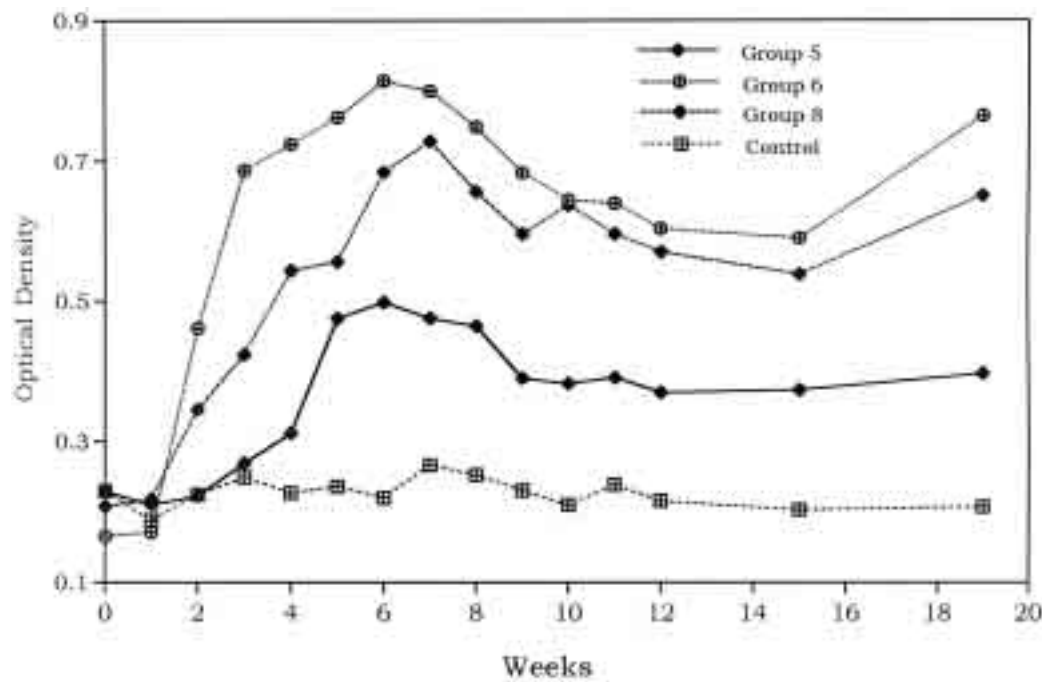
**Fig. 2.** Antibody titers against *Bordetella bronchiseptica* OMP in the egg yolks of hens immunized with various antigens of *B. bronchiseptica* and *P. multocida* D:4, as shown in Table 1.



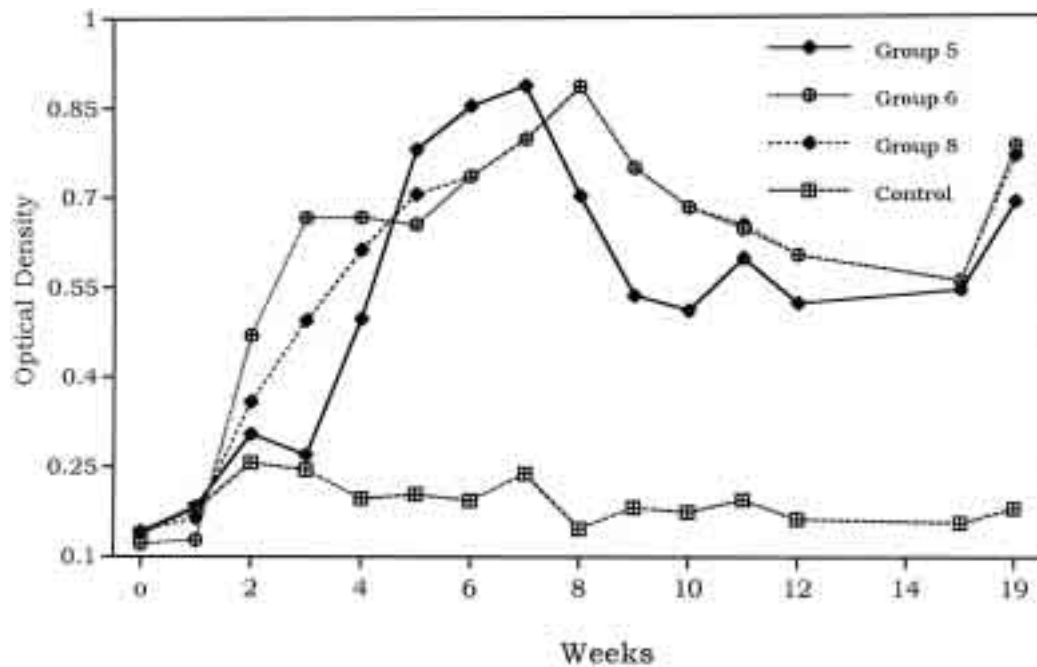
**Fig. 3.** Antibody titers against *Pasteurella multocida* D:4 DNT in the egg yolks of hens immunized with various antigens of *B. bronchiseptica* and *P. multocida* D:4, as shown in Table 1.



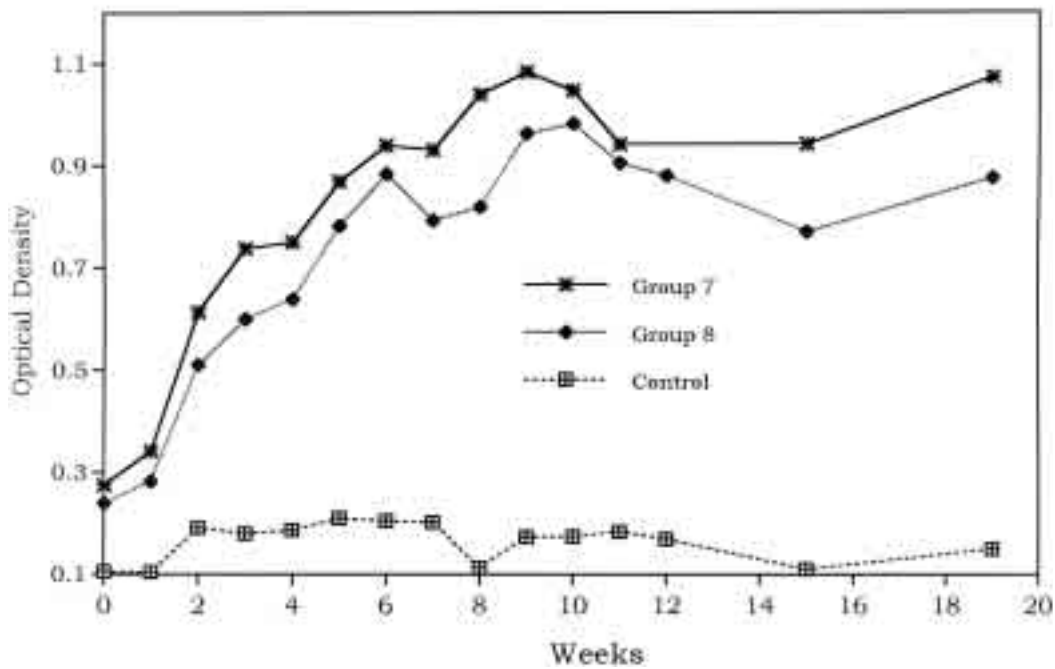
**Fig. 4.** Antibody titers against *Pasteurella multocida* D:4 OMP in the egg yolks of hens immunized with various antigens of *B. bronchiseptica* and *P. multocida* D:4, as shown in Table 1.



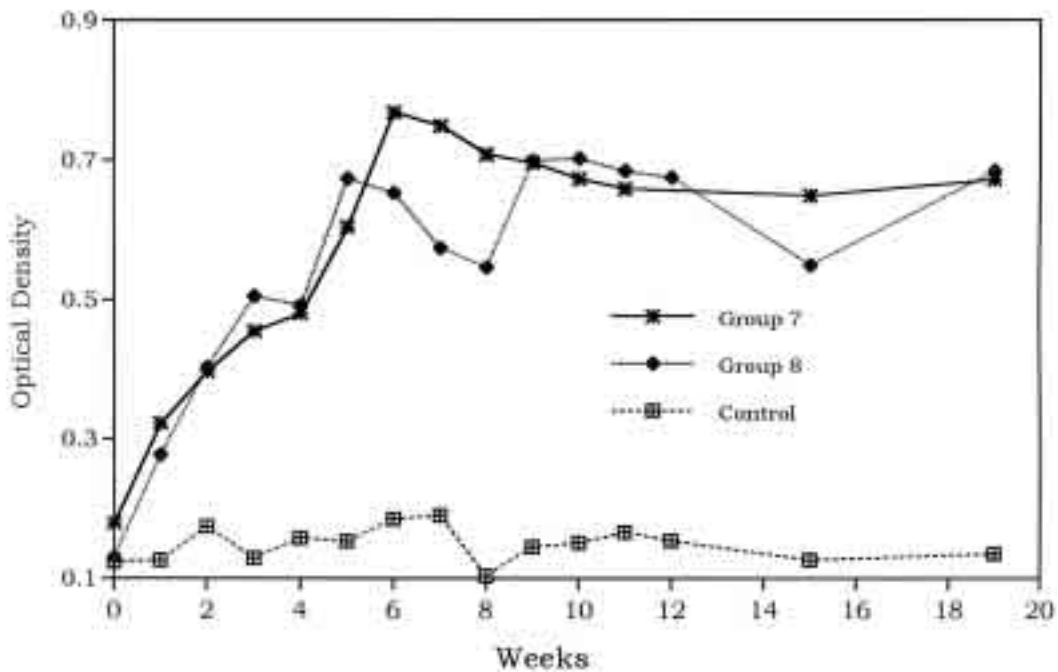
**Fig. 5.** Antibody titers against *Pasteurella multocida* A:3 IROMP in the egg yolks of hens immunized with antigens of *P. multocida* A:3 or *P. multocida* A:3 combined with *Actinobacillus pleuropneumoniae* serotypes 2 and 5, as shown in Table 1.



**Fig. 6.** Antibody titers against *Pasteurella multocida* A:3 OMP in the egg yolk of hens immunized with antigens of *P. multocida* A:3 or *P. multocida* A:3 combined with *Actinobacillus pleuropneumoniae* serotypes 2 and 5, as shown in Table 1.

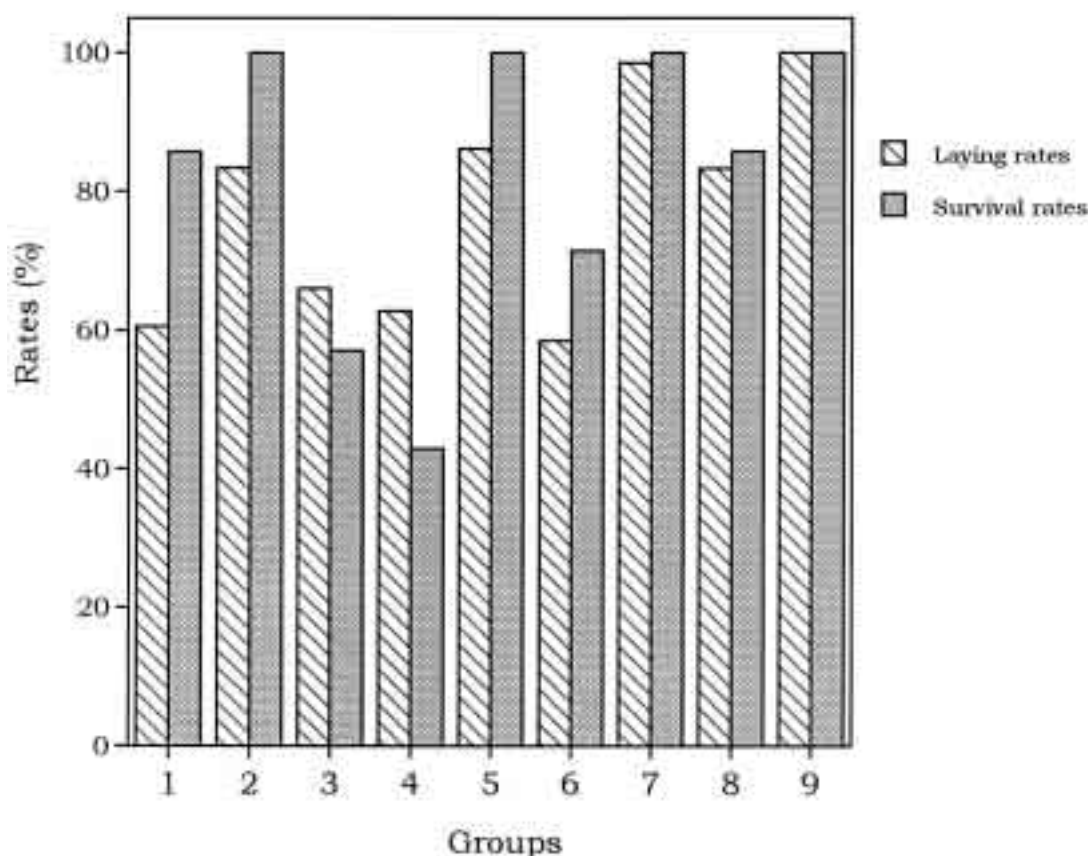


**Fig. 7.** Antibody titers against *Actinobacillus pleuropneumoniae* serotype 2 OMP in the egg yolk of hens immunized with antigens of *A. pleuropneumoniae* serotypes 2 and 5 or *A. pleuropneumoniae* combined with *P. multocida* A:3, as shown in Table 1.



**Fig. 8.** Antibody titers against *Actinobacillus pleuropneumoniae* serotype 5 OMP in the egg yolk of hens immunized with antigens of *A. pleuropneumoniae* serotypes 2 and 5 or *A. pleuropneumoniae* combined with *P. multocida* A:3, as shown in Table 1.





**Fig. 9.** Laying rates and survival rates of hens immunized with various combination of antigens of *B. bronchiseptica*, *P. multocida* D:4, *P. multocida* A:3 and *A. pleuropneumoniae* serotypes 2 and 5.

vaccines since bacterin alone could not induce full protection and cross-reactive immunity. However, live bacteria were insufficient to induce antibody, especially for early immune response in comparison with other groups. This phenomenon might be due to the inoculation of a low dose of bacteria (Table 1). IROMP is one of a number of novel surface antigens that were found to be expressed only under iron-restricted condition or *in vivo* status (5, 19, 34). In addition, the protein can induce protective immunity against both homologous and heterologous serotypes of *P. multocida* A:3. The induction of immune response against IROMP was remarkable. Therefore, IROMP of 74, 87, and 99 kDa could be one of the most effective antigens for producing egg yolk antibodies against *P. multocida* A:3 and the antibody was increased further by a fourth booster injection. *A. pleuropneumoniae* was proved as the most effective antigen for antibody production with and had associated high survival and laying rates. In addition, the bacteria could relieve the sensitivity for LPS and capsular substance of *P. multocida* A:3.

In conclusion, IgY production, hen survival and laying rates were closely related with the components of the immunizing vaccine. Moreover, protection against disease might be closely related with the antigens. Future study

should be undertaken to determine subunit antigens with effective immunogenicity but with low toxicity.

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