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 DNIs of *B. bronchiseptica* and *P. multocida* D4. Efficacies of the vaccines against swine pneumatic 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 offspring 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 usual 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), pneumatic 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
significance to swine industry. *P. multocida* type A is the etiologic agent of swine pneumatic 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 pneumatic pasteurellosis and porcine contagious pleuropneumonia. Moreover, the specificities and protectiveities 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 pneumatic 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 dermonecrotoksin (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 X 10^6 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 (Bheledlin 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. Sarkoetyl 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 (Sepprinc. 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