# Protective Effect of NaOH-Extracted *Erysipelothrix rhusiopathiae* Vaccine in Pigs

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ABSTRACT. A vaccine was prepared from a NaOH-extracted antigen of the Kyoto strain (serovar 2) of *Erysipelothrix rhusiopathiae (E. rhusiopathiae)* with an oil adjuvant, and was injected twice at 3-week intervals into SPF pigs and conventional pigs with maternal antibodies. After the second vaccination, IgG-GA titers of immunized SPF pigs were more than 256-fold at 3 weeks, and immunized pigs with maternal antibodies were 64-fold at 7 weeks. The pig with maternal antibodies vaccinated once with live vaccine had less than 4-fold titers. The ELISA antibody titers which were measured by using the NaOH-extracted antigen showed similar transition to the IgG-GA antibody titers. All immunized pigs and nonvaccinated control pigs were challenged with the strains Fujisawa (serovar 1a) or Saitama-1 (serovar 2). After challenge exposure, all pigs immunized with the NaOH-extracted vaccine showed no clinical signs and survived, and the pig immunized with the live vaccine had a local rhomboidal lesion at the site of the injection. Nonvaccinated pigs developed typical symptoms of *E. rhusiopathiae* infection and one of them died. After the autopsy, the challenge strains were not recovered from the main organs except tonsils of the pigs immunized with the NaOH-extracted vaccine. These results indicated that the NaOH-extracted vaccine induces a protective effect in pigs with maternal antibodies as well as in SPF pigs negative for such antibodies, and that 67–64, 62–60 kDa proteins in the NaOH-extracted antigen play an important role in protecting against *E. rhusiopathiae* infection.
— KEY WORDS: *Erysipelothrix rhusiopathiae*, NaOH-extracted antigen, vaccine.

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*Erysipelothrix rhusiopathiae (E. rhusiopathiae)* is a Gram-positive bacillus and the causative agent of swine erysipelas. The infection induces septicemia, endocarditis and death in pigs, therefore causing great economic loss to the pig industry. In Japan a live vaccine prepared from a acriflavin-fast attenuated strain of Koganei 65–0.15 [9] of *E. rhusiopathiae* has been used widely to control the disease. This vaccine can induce protection against *E. rhusiopathiae* infection in most pigs, but the extent of the protective effect depends on the susceptibility of the pigs to vaccination. The most important agent which prevents the effect is maternal antibodies from the parent stock. In contrast, the live vaccine sometimes causes strong reactions such as urticaria, depression, pyrexia and systemic eruption, in highly susceptible pigs such as SPF pigs, because of excessive multiplication. To solve these problems with the live vaccine, it is necessary to develop a new inactivated vaccine.

There have been many attempts to develop an inactivated *E. rhusiopathiae* vaccine. Groschup *et al.* [2] reported that 10 mM NaOH extracts of *E. rhusiopathiae* were protective in mice. Kobayashi *et al.* [3] reported that culture filtrate and alkaline-extracted antigens from cells of the attenuated strain Koganei of *E. rhusiopathiae* induced protective immunity in mice. Sawada *et al.* [7] and Sawada and Takahashi [8] demonstrated that pigs vaccinated with culture filtrate prepared from the Koganei strain of *E. rhusiopathiae* showed signs of strong protection against challenge exposure with a virulent strain of *E. rhusiopathiae*. But there have not been reports on the protective effect of the NaOH-extracted antigen in pigs. In this study, we produced an inactivated vaccine prepared from the NaOH-extracted antigen with oil adjuvant. This vaccine was also examined for its safety and protective effect in SPF pigs, and antibody response to pigs with maternal antibodies.

## MATERIALS AND METHODS

*Bacterial strains*: The Kyoto strain (serovar 2) of *E. rhusiopathiae* isolated from a pig affected with chronic erysipelas was used for preparation of the NaOH-extracted antigen. Virulent strains of Fujisawa (serovar 1a) and Saitama-1 (serovar 2) were used for challenge exposure. The Marienfelde strain was used for the growth agglutination (GA) test. These strains were obtained from the National Veterinary Assay Laboratory, Kokubunji, Tokyo, Japan.

*Animals*: Six approximately 30-day-old SPF pigs (secondary, LW: Landrace × White Yorkshire), and four age-matched conventional pigs (LWD: LW × Duroc) were used. None of the SPF pigs had IgG-GA antibodies to E. rhusiopathiae. The conventional pigs had 16- to 32-fold IgG-GA antibody titers.

*Preparation of NaOH-extracted vaccine*: The Kyoto strain of *E. rhusiopathiae* was cultured for 24 hr at 37°C in trypticase soy broth (TSB, BECTON DICKINSON, U.S.A.) containing 0.1% Tween 80 and 2% horse serum. After incubation, the bacteria ($8.2 \times 10^8$ CFU/m*l*) were collected by centrifugation at 10,000 × for 20 min and washed with 0.01 M phosphate buffered saline (PBS) pH 7.2. The surface components of the bacteria were extracted by gentle stirring in 0.01 N NaOH solution, which was about 1/100 of the culture volume, for 18 hr at 4°C. After extraction the suspensions were centrifuged at 10,000 × g for 20 min, and

the supernatants were filtrated with 0.22 $\mu$m membrane to remove the small remaining bacteria. The volume of the filtrated supernatants was adjusted to 1/100 or 1/20 (total protein concentrations of 980 $\mu$g/m*l* and 196 $\mu$g/m*l*) of the culture volume with PBS. The supernatant was emulsified with the oil adjuvant ISA-25 (SEPPIC, Cometies/Pharmacy Division, Paris) at a ratio of 3:1, and was used as the NaOH-extracted vaccine.

*Live vaccine*: Commercial lyophilized live vaccine (Kyoto Biken Laboratories, Japan) prepared from acriflavine-fast attenuated *E. rhusiopathiae* Koganei 65–0.15 (serovar 1a) strain was used. The reconstituted vaccine contained approximately $1.0 \times 10^8$ CFU/m*l* of *E. rhusiopathiae*.

*Vaccination and challenge exposure*: Six SPF pigs were randomly divided into 2 groups of 3 animals each. Two pigs in each group (Groups A and B) were immunized intramuscularly with 3 m*l* of the NaOH-extracted vaccine containing 2,205 $\mu$g/dose antigen twice at 3-week intervals. Two of 4 conventional pigs were vaccinated intramuscularly with 1 m*l* of the NaOH-extracted vaccine containing 147 $\mu$g/dose antigen twice at 3-week intervals, one was vaccinated subcutaneously once with 1 m*l* of the live vaccine and the remaining one animal was not vaccinated as a control (Group C). The pigs in Group A were challenged intradermally in the flank with 0.1 m*l* of the Fujisawa strain ($5.7 \times 10^6$ CFU) and those in Group B were challenged with the Saitama-1 strain ($3.4 \times 10^7$ CFU) 3 weeks after the second immunization. The pigs in Group C were challenged intradermally in the flank with 0.1 m*l* of the Fujisawa strain ($6.5 \times 10^7$ CFU) at 7 or 10 weeks after the last immunization. The clinical responses were observed and the rectal temperature was measured for a week after each immunization and for 3 weeks after the challenge exposure. After the vaccination, serum samples were collected from all pigs every week.

*Autopsy and isolation of E. rhusiopathiae*: At 3 weeks after the challenge exposure, all surviving pigs were sacrificed to observe the injection scar of the NaOH-extracted vaccine and to recover *E. rhusiopathiae* from main organs (liver, spleen, kidneys, heart, lung, lymphonoidi mesenterici), tonsils and synovial fluid. Isolation of the bacteria from the each sample, and serotyping of bacteria identified as *E. rhusiopathiae* were performed by the method of Takahashi *et al.* [14].

*IgG-growth agglutination (GA) test*: The growth agglutination (GA) test was performed by a modified method of Sawada *et al.* [6]. Briefly, 2-fold dilutions of serum samples treated with 2-Mercaptethanol were prepared with TSB containing 0.1% Tween 80 in test tubes. One *ml* of each serum dilution was mixed with 0.025 m*l* of TSB culture of the Marienfelde strain (serovar 1a) and the mixture was incubated at 37°C for 18 to 24 hr. After incubation, the titers were expressed as the reciprocal of the highest dilution of serum that showed signs of agglutination.

*Measurement of ELISA titer*: The serum antibody titers to the NaOH-extracted antigen of immunized pigs were measured by ELISA. The NaOH-extracted antigen (0.61 $\mu$g/m*l* in carbonate/bicarbonate buffer) was loaded into microtiter plates. After overnight incubation at 4°C, the plates were washed with PBS containing 0.05% Tween 20 (T-PBS), 1:100 diluted pig serum was added and they were incubated for 1 hr at 37°C. The plates were washed with T-PBS, 1:10,000 diluted horseradish peroxdase-conjugated anti-swine IgG was added and they were incubated for 1 hr at 37°C. After washing, O-phenylene diamine (SIGMA: U.S.A.) substrate was applied and the plates were incubated for 1 hr at 37°C. The reaction was stopped after 30 min with 1M $H_2SO_4$ and the O.D. read at 490 nm.

*SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Immunoblotting analysis*: SDS-PAGE of NaOH-extracted antigen was performed by the method of Laemmli [5]. Five $\mu$*l* of marker protein and 10 $\mu$*l* of the NaOH-extracted antigen containing 196 $\mu$g/m*l* were separated in 12% polyacrylamide slab gels. After SDS-PAGE, the separated proteins were transferred to Immobilon membranes (Millipore, Bedford, MA., U.S.A.) by means of a transblotting apparatus, according to the method of Towbin *et al.* [16]. The strips containing marker protein and the NaOH-extracted antigen were stained with amid black 10B. The membranes were treated with 1: 500 diluted serum collected from pre- and post- challenge SPF pigs immunized with the NaOH-extracted vaccine, and convalescent control animals. After washing, the membranes were incubated with 1:1,000 diluted horseradish peroxdase-conjugated anti-swine IgG (CAPPEL: U.S.A.). The color on the membranes was developed with 3-3'-diaminobenzidine and 0.02% $H_2O_2$.

## RESULTS

*Clinical signs in pigs after vaccination*: After immunization, none of the SPF or conventional pigs vaccinated with the NaOH-extracted vaccines showed any clinical signs, such as depression, pyrexia or urticarial lesions. The pigs immunized with the live vaccine also did not showed clinical signs and a red patch at the site of the injection.

*Effect of vaccination in protecting pigs against challenge with virulent strain*: After the challenge exposure with the virulent Fujisawa or Saitama-1 strain, all nonvaccinated control pigs in each group showed typical clinical responses of *E. rhusiopathiae* infection: i.e. depression, anorexia, pyrexia (above 41°C), lameness due to arthritis, and systemic urticarial lesions. The urticarial lesions, which were observed as purplish red square regions of anthema, appeared on the 2nd or 3rd day after the challenge exposure. One control pig in each of Groups A and B, showed the disappearance of systemic urticarial lesions and finally recovered, and one control animal in Group C died 6 days after challenge with the Fujisawa strain. The pig injected with the live vaccine did not show any clinical response and survived, but a local rhomboidal lesion developed at the site of intradermal exposure. This lesion was observed for about a week and then gradually disappeared. In contrast, all the pigs immunized with the NaOH-extracted vaccine in each

Table 1. Clinical responses of pigs after challenge with virulent strains

| Group[a)] | Pig No. | Immunogen[b)] | Challenge strain (serovar) | Responses after challenge | | | Mortality |
|---|---|---|---|---|---|---|---|
| | | | | Pyrexia[c)] | Arthritis | Erythema[d)] | |
| A | 251 | NaOH-extracted vaccine | | – | – | – | – |
| | 252 | NaOH-extracted vaccine | Fujisawa(1a) | – | – | – | – |
| | 401 | Control | | ++ | + | ++ | – |
| B | 191 | NaOH-extracted vaccine | | – | – | – | – |
| | 192 | NaOH-extracted vaccine | Saitama-1(2) | – | – | – | – |
| | 542 | Control | | ++ | + | ++ | – |
| C | 351 | NaOH-extracted vaccine | | – | – | – | – |
| | 352 | NaOH-extracted vaccine | Fujisawa(1a) | – | – | – | – |
| | 986 | Live vaccine | | – | – | + | – |
| | 81 | Control | | ++ | + | ++ | + |

a) Group A and B are SPF pigs, Group C are conventional pigs. b) Total protein concentration of the NaOH-extracted antigen: Group A and B are 980 mg/ml, Group C are 196 mg/ml. c) Temperature is expressed as –: <40°C, +: 40~41°C, ++: >41°C. d) Development of lesion at the site of injection is expressed as –: no response, +: local, ++: systemic skin lesions.

Table 2. Isolation of challenge strains of *E. rhusiopathiae* from pigs

| Group | Pig No. | Liver | Spleen | Kidney | Heart | Lung | Lymphonoidi mesenterici | Tonsil | Synovial fluid |
|---|---|---|---|---|---|---|---|---|---|
| A | 251 | – | – | – | – | – | – | + | – |
| | 252 | – | – | – | – | – | – | + | – |
| | 401 | – | – | – | – | – | – | + | + |
| B | 191 | – | – | – | – | – | – | + | – |
| | 192 | – | – | – | – | – | – | + | – |
| | 542 | – | – | – | – | – | – | + | + |
| C | 351 | – | – | – | – | – | – | + | – |
| | 352 | – | – | – | – | – | – | + | – |
| | 986 | – | – | – | – | – | – | + | – |
| | 81 | + | + | + | + | + | + | + | + |

+: *E. rhusiopathiae* which were same serotype with challenge strain recovered from each samples. –: *E. rhusiopathiae* were not recovered.

group survived and showed no sign of either a clinical response or local lesion at the site of the injection after the challenge exposure (Table 1).

*Observation of injection scars of the NaOH-extracted vaccine and isolation of E. rhusiopathiae*: At autopsy no injection scar was observed in any of the pigs immunized with the NaOH-extracted vaccine. No *E. rhusiopathiae* were isolated from main organs of any of the surviving pigs after the challenge exposure but, the live bacteria which were of same serotype as the challenge strain were recovered from the tonsils of all the pigs and the synovial fluid of the pigs showed signs of arthritis. In contrast, *E. rhusiopathiae* were isolated from all organs of the dead pig after the challenge exposure (Table 2).

*IgG-GA antibody titers*: In Groups A and B, all serum samples collected from SPF pigs had less than a 4-fold IgG-GA antibody titer before the first immunization. The IgG-GA antibody titers of pigs immunized with the NaOH-extracted vaccine were 64- to 128-fold after the first immunization, and these increased to 256- to 512-fold after the second immunization. At 3 weeks after the challenge exposure with the virulent strain of Fujisawa or Saitama-1, the IgG-GA antibody titers of the immunized pigs were equivalent to those before the challenge, or decreased slightly, but those of the nonvaccinated pigs were increased 256- to 512-fold. In Group C, all the samples from conventional pigs had 16- to 32-fold IgG-GA antibody titers before immunization. These pigs immunized once with the NaOH-extracted vaccine showed a decrease in the IgG-GA antibody titer to 8-fold after the first immunization, followed by increases of 64- to 128-fold after the second immunization. The IgG-GA antibody titer of pigs injected with the live vaccine or not vaccinated decreased to less than 4-fold titer before challenge exposure. At 3 weeks after challenge exposure with the Fujisawa strain, the antibody titers of all samples of these pigs had increased to

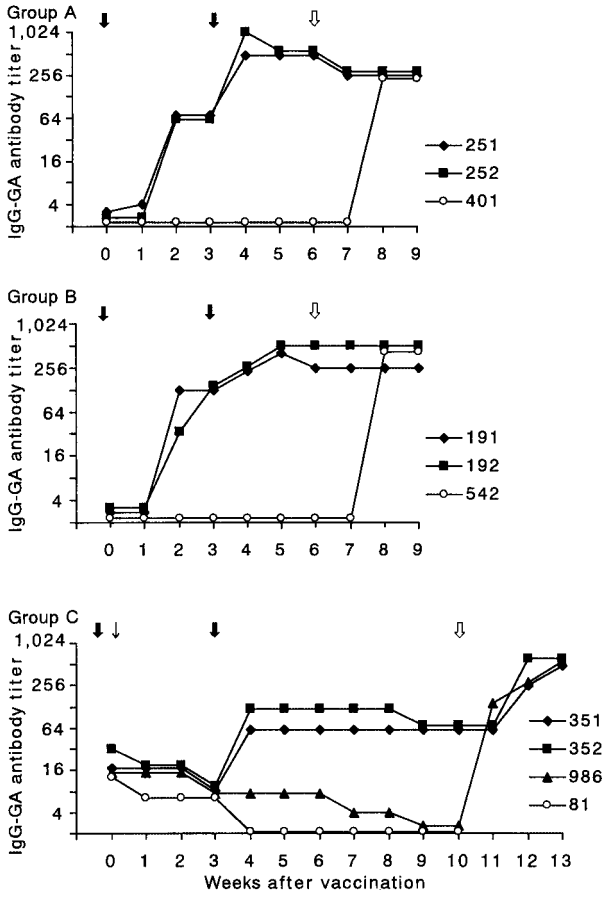


Fig. 1. IgG-GA antibody titers of vaccinated pigs. ⬇ Immunization with the NaOH-extracted vaccine, ↓ Immunization with the live vaccine, ⇩ Challenge exposure with the Fujisawa strain (Group A,C), with the Saitama-1 strain (Group B). No. 251, 252, 191, 192, 351, 352: Pigs immunized with the NaOH-extracted vaccine. No. 986: Pig immunized with the live vaccine. No. 401, 542, 81: Control pigs.

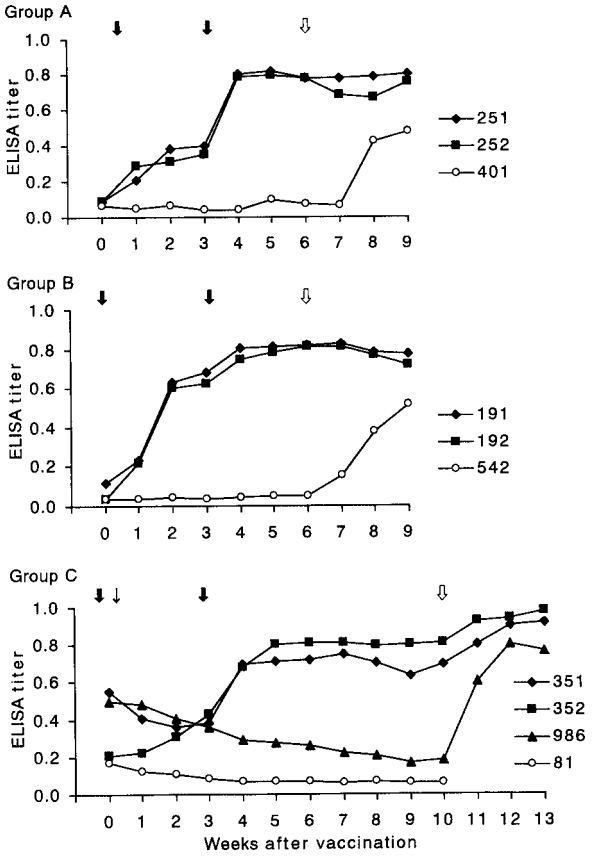


Fig. 2. ELISA titers of vaccinated pigs. ⬇ Immunization with the NaOH-extracted vaccine, ↓ Immunization with the live vaccine, ⇩ Challenge exposure with the Fujisawa strain (Group A,C), with the Saitama-1 strain (Group B). No. 251, 252, 191, 192, 351, 352: Pigs immunized with the NaOH-extracted vaccine. No. 986: Pig immunized with the live vaccine. No. 401, 542, 81: Control pigs.

512-fold (Fig. 1).

*ELISA antibody response*: In Groups A and B, all serum samples of SPF pigs showed about a 0.8 ELISA titer after the second immunization with the NaOH-extracted vaccine. At 3 weeks after the challenge exposure, the sera of immunized pigs had a 0.7 to 0.9, and those of the nonvaccinated pigs increased from a 0.5 to a 0.6 ELISA titer. In Group C, all the samples from conventional pigs had a 0.1 to 0.6 ELISA titer before the immunization. After the second immunization with the NaOH-extracted vaccine, the samples increased from a 0.6 to a 0.8 ELISA titer. Those of the pig injected with the live vaccine and nonvaccinated pig gradually decreased to less than 0.2 ELISA titer. At 3 weeks after challenge exposure, all samples from these pigs had 0.7 to 1.0 ELISA titers (Fig. 2).

*Immunoblotting analysis*: Immunoblotting analysis was performed with the NaOH-extracted antigens prepared from the Kyoto strain and sera of SPF pigs immunized with the NaOH-extracted vaccine or sera of nonvaccinated pigs. In the reactions with the sera of the immunized pigs before challenge exposure, two protein bands of 67–64 and 62–60 kDa were observed, and no reactions were found in the sera of nonvaccinated pigs. All the samples from the immunized pigs after challenge exposure with the Fujisawa or Saitama-1 strain showed the same bands. The 67–64 kDa protein band was mainly and the 62–60 kDa protein band reacted weakly with convalescent sera of the nonvaccinated pigs challenged with the virulent strains (Figs. 3, 4).

## DISCUSSION

The live vaccine prepared from the attenuated strain of Koganei 65-0.15 (serovar 1a) has been used for the prevention of swine erysipelas. Takahashi *et al.* [13] reported that the live vaccine induced protection in immunized pigs against challenge with virulent strains of various serovars. The live vaccine can be effective for the control of swine erysipelas, but the extent of the effect depends on maternal antibodies in pigs that inhibit antibody production following vaccination in the field. We

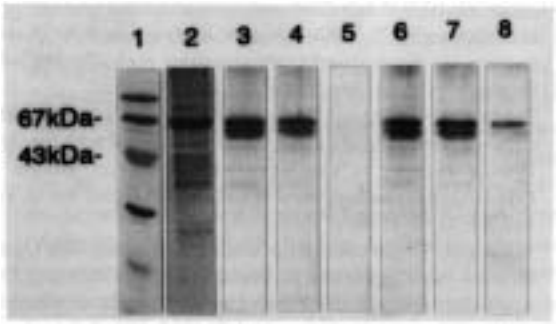


Fig. 3. Immunoblotting analysis of the NaOH-extracted antigen with sera of SPF pigs. Lane 1: Marker protein. Lane 2: NaOH-extracted antigen. Lane 3~5: Before challenge exposure. (3: No. 251. 4: No. 252. 5: No. 401.) Lane 6~8: After challenge exposure. (6: No.251. 7: No.252. 8: No.401.)

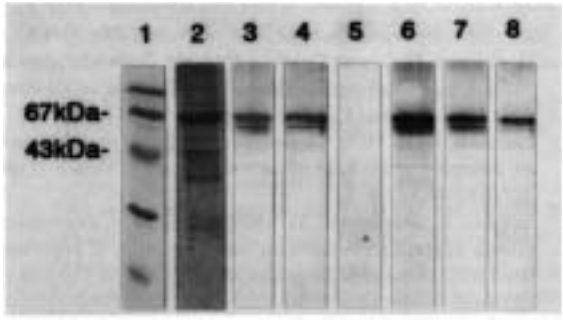


Fig. 4. Immunoblotting analysis of the NaOH-extracted antigen with sera of SPF pigs. Lane 1: Marker protein. Lane 2: NaOH-extracted antigen. Lane 3~5: Before challenge exposure. (3: No.191. 4: No. 192. 5: No. 542.) Lane 6~8: After challenge exposure. (6: No. 191. 7: No. 192. 8: No. 542.)

investigated the protective activity of the bacterin and inactivated vaccine prepared from culture filtrate and NaOH-extracted antigen in mice. The NaOH-extracted vaccine had a highly reproducible protective effect in these vaccines (data not shown). The present study was performed to investigate the protective effect of NaOH-extracted vaccine prepared from the Kyoto strain (serovar 2) in pigs.

A number of *E. rhusiopathiae* have been isolated from naturally affected pigs showing the typical clinical signs of erysipelas, septicemia, arthritis, urticaria and endcarditis. Generally, most of these isolates belong to serovar 1 (subtypes 1a and 1b) and 2. Takahashi *et al.* [10, 12] reported that about 80% of isolates from slaughtered pigs affected with erysipelas were of serovar 1 or 2 in Japan. Wood and Harrington [20] also reported a similar result in the United States. These reports suggested that protection against challenge with serovar 1 and 2 strains would be significant for the control of swine erysipelas. We investigated the protective effect in the SPF pigs immunized with the NaOH-extracted vaccine against challenge with the virulent strains of serovar 1a or serovar 2. None of the immunized pigs showed any clinical signs, but the challenge strains were recovered from their tonsils. Takahashi *et al.* [11] reported that the rate of isolation of serovar 2 was about 31% and that of serovar 1 was 1.6% in healthy slaughtered pigs, and in addition, that the carrier pigs may become a persistent source. Wood demonstrated cross protection in swine immunized with erysipelas adsorbate bacterin against challenge exposure with various serotypes strains [19], but the results of the recovery of the challenge bacteria are not described in this report. It is necessary to investigate whether it is possible for the pigs immunized with the NaOH-extracted vaccine to discharge live bacteria.

The NaOH-extracted vaccine induced a high IgG-GA and ELISA antibody titer in not only SPF pigs but also in conventional pigs possessing maternal antibodies. It seems that the transition of the IgG-GA antibody titers was closely related with that of the ELISA antibody titers measured by using the NaOH-extracted antigen. Pigs immunized with the NaOH-extracted vaccine did not even develop local rhomboidal lesions, but these were observed in the conventional pigs injected with live vaccine, after challenge exposure. Pigs immunized with oil adjuvanten NaOH-extracted vaccine had higher antibody titers than pigs immunized with aluminum phosphate gel adjuvant vaccine (data not shown). It was thought that a high antibody level induced by the NaOH-extracted vaccine inhibited the development of local lesions.

Many investigators have studied the protective antigens of *E. rhusiopathiae* [1, 15, 17, 18]. Lachmann and Deicher [4] reported that major proteins with molecular weights of 78, 72, 68 and 48 kDa were obtained in EDTA, CHAPS, TritonX, and SDS extracts of *E. rhusiopathiae* T28. Groschup *et al.* [2] found that a 66–64 kDa antigen prepared by extraction of *E. rhusiopathia*e with 10 mM NaOH had a protective effect in mice. It is therefore considered that one of the protective antigens was the 66–64 kDa protein released from the cell surface of *E. rhusiopathiae*. Similar results were obtained in our experiments; SDS-PAGE and immunoblotting analysis showed that the NaOH-extracted antigen mainly contained 67–64 and 62–60 kDa proteins, but the major immunologically reactive band in convalescent pigs sera was the 67–64 kDa band. We consider the 67–64 kDa protein in NaOH-extracted antigen is related to protection. It is necessary to determine the role of the 67–64 and 62–60 kDa proteins in protection. Furthermore, it is necessary for the safety of the NaOH-extracted vaccine, the relation between immunoreaction and antigen density, and the protective mechanism to be studied further.

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