Evaluation of the Antibody Response in Pigs Vaccinated against *Streptococcus suis* Capsular Type 2 using a Double-antibody Sandwich Enzyme-linked Immunosorbent Assay

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ABSTRACT

A double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) was standardized for the detection of specific antibodies following vaccination with *Streptococcus suis* capsular type 2 bacterins. No statistically significant increase of antibody titers was detected in vaccinated piglets compared to the non-vaccinated control group, even if a minority of piglets demonstrated an important postvaccinal response. Three of four vaccinated sows showed a low antibody response to vaccine and specific immunity was detected in piglets of only one litter of these three sows. Passive protection studies showed that none of the sera from vaccinated piglets were protective for mice whereas serum obtained from hyperimmunized pigs gave protection.

RÉSUMÉ

Une épreuve ELISA a été développée pour la détection d'anticorps spécifiques chez des porcs vaccinés au moyen d'une bactérine contenant *Streptococcus suis* sérotype 2. Aucune différence significative n'a pu être mise en évidence entre les titres détectés chez les porcelets vaccinés et ceux retrouvés chez les sujets contrôle et ce, même si quelques porcelets ont démontré une bonne réponse post-vaccinale. Trois des quatre truies vaccinées n'ont produit qu'une faible réponse sérologique envers le vaccin et des anticorps correspondant n'ont pu être détectés que chez les porcelets d'une seule des portées. Des études de protection effectuées au moyen d'un modèle d'infection expérimentale chez la souris, ont démontré une absence de protection par le sérum des porcs vaccinés, alors qu'un sérum hyperimmun, préparé chez des porcs, s'est avéré protecteur.

INTRODUCTION

During the last decade, the swine industry has directed major efforts towards the production of minimal disease herds. Using standard procedures such as antibiotic therapy, medicated early weaning, or the culling of infected animals, several pig producers have succeeded in eliminating infectious agents responsible for diseases such as pleuropneumonia, atrophic rhinitis and swine dysentery. Nonetheless, standard control measures (antibiotic therapy, antibiotic prophylaxis or vaccination) give unsatisfactory results against a less specific agent, *Streptococcus suis*, which can be found in the nasopharynx of a majority of pigs (1–5). There are currently 29 capsular types of *S. suis* (6–8) and capsular type 2 is the most prevalent in diseased pigs (9–13).

Economic losses due to *S. suis* are important and there is a need to explain why classic control measures give equivocal results. Because of the absence of a reliable serological method, knowledge is almost nonexistent about the level of infection in different types of operations, the maternal immunity, the immune response and the level of protection induced in sows and/or in piglets following vaccination with a *S. suis* bacterin. Some authors have evaluated serological methods, such as a mixed reversed passive antiglobulin hemagglutination test, an indirect hemagglutination test, a direct and indirect bactericidal test, a phagocytic test with pig neutrophils and an indirect enzyme-linked immunosorbent assay (ELISA) to detect antibodies against *S. suis* capsular type 2 (4,14–17). However, they concluded that these methods lacked specificity.

The aim of this study was to standardize a double-antibody sandwich ELISA for the detection of antibodies against *S. suis* capsular type 2. This technique was then used to study the kinetics of the immune response induced in piglets by vaccination with a *S. suis* bacterin and the transfer of maternal immunity from vaccinated sows to their piglets. An additional objective was to use serum from vaccinated piglets to assess its protective capacity in mice against a virulent strain of *S. suis* capsular type 2.

MATERIALS AND METHODS

BACTERIAL STRAINS

This study was carried out using reference strains of *S. suis* capsular types 1, 2, 3, 4 and 22 (S428, R735, 4961, 6407, 88-1861 respectively), a capsulated virulent serotype 2 strain, 89-1591 (18,19), and a less capsulated

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avirulent mutant from that strain, M-42 (19). A *Streptococcus bovis* strain (79-1582) and an *Enterococcus faecalis* strain (ATCC 19433) were also used for specificity studies.

PRODUCTION OF HYPERIMMUNE SERA

New Zealand white rabbits weighing approximately 2.5 kg were immunized against the five *S. suis* reference strains and against the *S. bovis* and *E. faecalis* strains. Two 500 kg Holstein cows were immunized against *S. suis* capsular type 2 reference strain and two six-week-old piglets were immunized against *S. suis* capsular type 2 strain 89-1591. Animals were housed and fed according to the guidelines issued by the Canadian Council on Animal Care. Prior to immunization, sera from all animals were negative by slide agglutination test for the presence of anti-*S. suis* antibodies. Inoculations were carried out with whole-cell formalinized antigens, prepared as previously described (20). Rabbits, pigs and cows were inoculated with increasing doses of the antigen adjusted to an optical density (OD) of 1.0 at 540 nm and injected intravenously twice weekly during a minimum of four weeks or until a satisfactory titer (1:16 or more) was obtained by the slide agglutination test, against the homologous antigen. The first week, the cows also received two intramuscular inoculations of the antigen mixed with incomplete Freund's adjuvant. Sera from five normal rabbits and from two germ-free piglets were also collected. All sera were heat-inactivated for 30 min at 56°C and stored at −20°C until utilization.

PURIFICATION OF IMMUNOGLOBULIN G (IgG) FRACTION

The IgG fraction from bovine antisera was purified by affinity chromatography on a protein A-superose column coupled to a fast protein liquid chromatography system (Fine Chemical, Uppsala, Sweden), as described (21).

ELISA PROCEDURE

Each well of polystyrene 96-well microtiter plates (Immunomodule U-16, Life Technologies, Burlington, Ontario) was first coated with anti-*S. suis* capsular type 2 bovine IgGs optimally diluted in 50 µL 0.1 M carbonate buffer (pH 9.6). Plates were incubated overnight at 4°C and washed for three periods of 3 min in phosphate-buffered saline containing 0.05% polysorbate Tween-20 (PBS-T20). A volume of 50 µL of inactivated antigen, diluted to an OD of 0.1 (540 nm) in PBS-T20, which corresponded to a concentration of 10^7 CFU/mL, was added to each well. The antigen used for the ELISA was a whole-cell suspension of the capsular type 2 reference strain prepared by the same procedure as the one used for immunizations. Plates were incubated for 30 min at room temperature (22–23°C) and washed as mentioned above.

Rabbit or pig sera to be tested were diluted serially in PBS-T20 and added in 50 µL amounts to appropriate wells. Negative controls (PBS-T20 for studies on rabbit sera and axenic pig serum for studies on pig sera) and positive controls (anti-*S. suis* capsular type 2 hyperimmune rabbit and pig sera) were added to each plate. After incubation for 30 min at 22–23°C, plates were washed and 50 µL of a commercial horseradish peroxidase conjugated goat antirabbit IgG heavy and light chains (Prince Laboratories Inc., Toronto, Ontario) or antipig (Jackson Immunoresearch Laboratories, West Grove, Pennsylvania), diluted in PBS-T20, was added. After 15 min of incubation at 22–23°C, plates were washed and a volume of 100 µL of 0.4 mM 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) and 0.5 M H_2O_2 in 0.05 M citrate buffer (pH 4.0) was added to each well. Absorbance was measured at 414 nm on a kinetic microplate reader (Molecular Devices Corp., Menlo Park, California) after a 30 min incubation period. All sera were tested in duplicates in the same plate and the mean absorbance of each dilution was calculated. Optimal dilutions of coating antibodies, antigen and peroxidase conjugated antirabbit or antipig were determined by separate checkerboard titrations in preliminary studies.

Results were reported as titers which were defined as the reciprocal of the lowest serum dilution having an optical density equal to at least the mean optical density of four wells containing negative controls plus three standard deviations, calculated for each plate.

Interplate and intraplate variations were controlled as follows: positive and negative controls were previously tested in ten different plates on different days and the mean control values were calculated. For subsequent tests, a plate was rejected if the variation of the controls was higher than 20%. A plate was also rejected if the intraplate variation of the controls was higher than 15%.

Specificity studies were carried out using the hyperimmune rabbit sera raised against the *S. suis* reference strains (excluding capsular type 2) and against the *S. bovis* and the *E. faecalis* strains.

PIG VACCINATIONS

Vaccines — Two different bacterins were used. One was produced with a virulent strain of *S. suis* capsular type 2 isolated from a pig, strain 89-1591 (vaccine A), and the other with an avirulent less capsulated mutant (M-42) of strain 89-1591 (vaccine B). The latter was used to evaluate if a less capsulated strain could induce the same amount of antibodies as a highly capsulated strain. Both vaccines were tested in piglets while only vaccine A was administered to sows. Piglets were vaccinated at four weeks of age and received a booster two weeks later and sows were vaccinated five and three weeks before parturition, as recommended by the manufacturer.

Animals — Two groups of animals were used: vaccinated piglets from nonvaccinated sows, and vaccinated sows with their respective litter. The first group consisted of the litters from eight nonvaccinated sows; four litters were immunized with vaccine A (group A) and four with vaccine B (group B). In each litter, six piglets were selected at random to be vaccinated. For the second group, four pregnant sows were vaccinated with vaccine A. Five to six piglets from each litter were selected at random and included in the study. A total of ten nonvaccinated piglets from those litters from nonvaccinated sows were included in the study as controls (C). All the animals in the study originated from the same swine operation.

TABLE I. Titers obtained with seven different rabbit antisera and a normal rabbit serum, as tested by ELISA against *Streptococcus suis* capsular type 2 antigen

Serum	Titer[a]
S. suis capsular type 2	200,000
S. suis capsular type 1	960
S. suis capsular type 3	1280
S. suis capsular type 4	1920
S. suis capsular type 22	1920
Streptococcus bovis	400
Enterococcus faecalis	1920
Normal	420

[a]Titer was defined as the reciprocal of the lowest dilution having an OD equal to at least the mean OD of four wells containing negative control plus three SD, calculated for each plate

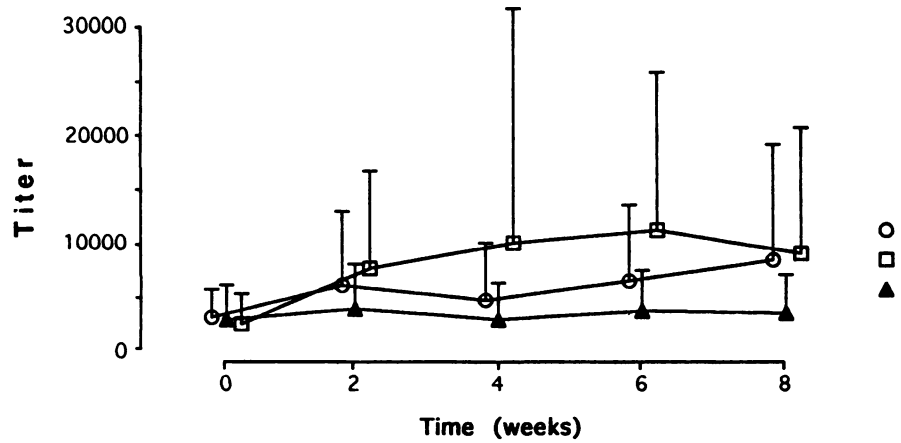

Fig. 1. Antibody response of piglets to a *Streptococcus suis* capsular type 2 vaccine, measured by ELISA. Results are shown as mean titers with one standard deviation in vaccinated piglets compared to the control group (C). Two different vaccines were tested, one derived from the virulent strain 89-1591, tested in group A, and one derived from the avirulent strain M-42, tested in group B.

Collection of sera — All animals were tested before vaccination. For vaccinated piglets, blood was collected every two weeks during two months. Sows were bled before vaccination and two weeks after they delivered. Piglets from vaccinated sows were bled three times at the age of two, four and six weeks.

STATISTICAL ANALYSIS

Differences in titers among groups A, B and C were evaluated at baseline using one-factor ANOVA (Superanova, Abacus, California). The effects of time and the interactions between time and groups were evaluated with a one-factor ANOVA for repeated measures, using an adjusted univariate approach. A probability level of less than 0.05% was considered significant for all tests used.

MOUSE PROTECTION TEST

Sera from vaccinated piglets were pooled on the basis of their antibody titers in order to have three groups of sera with different antibody titers for both vaccines, 89-1591 and M-42. As shown in Table II, we prepared for each vaccine a pool of high titer (pools 1 and 4), medium titer (pools 2 and 5) and low titer sera (pools 3 and 6). Pool 7 was composed of non-vaccinated control piglets sera. Only sera from eight and ten-week-old piglets were used and each pool contained sera from at least three piglets. Hyperimmune anti-*S. suis* capsular type 2 pig antiserum was tested undiluted and diluted 1:4 and 1:10 to obtain titers comparable to those of pools of high titers, and 1:20. The titer of each pool was determined by ELISA.

The mouse protection test was carried out using a previously described mouse-model (18). *Streptococcus suis* capsular type 2 strain 89-1591 was grown in Todd-Hewitt broth with 10% normal bovine serum (THS) in a waterbath at 37°C, with agitation until the culture reached an OD of 0.1 at 540 nm (2–3 h). Groups of five four-week-old mice (strain CF1) were inoculated intraperitoneally with 0.5 mL of the broth culture mixed with 0.5 mL of axenic pig serum (negative control), 0.5 mL from the different pools described earlier or 0.5 mL of hyperimmune pig antiserum (non-diluted and diluted). The exact number of viable cells was counted with the pour plate method using blood-agar plates incubated for 18 h at 37°C. It was estimated that the mice received about 10^7 bacteria, which corresponded to the minimal lethal dose as measured previously (18). Death or presence of clinical signs such as meningitis was monitored at 24 h intervals over the next six days. The test was done in duplicates for each serum. Sterile THS mixed with axenic serum was also inoculated into one group of mice as control.

RESULTS

SPECIFICITY STUDIES ON RABBIT SERA

Specificity studies are shown in Table I. It appeared that the titer obtained with the homologous antiserum was 200,000 whereas titers obtained with sera raised against capsular types 4 and 22 and *E. faecalis* were 2000. Titers of antisera against *S. suis* capsular type 1 and *S. bovis* were 960 and 400 respectively and were considered negligible.

DETECTION OF ANTI-*S. SUIS* SEROTYPE 2 ANTIBODIES IN PIG SERA

A titer of 100,000 was obtained with the anti-*S. suis* capsular type 2 hyperimmune pig sera.

It appeared that before vaccination (day 0), there was no difference in titers among groups A, B and the control group (Fig. 1). Means of titers obtained from the piglets vaccinated with strain 89-1591 (group A) ranged from 3300 (day 0) to 8600 (week 8), the latter being the maximum value. Animals vaccinated with strain M-42 (group B) had similar titers; in this case, the mean ranged from 3000 to 9100, with a maximum of 11,200 (week 6). Means of titers for control animals (group C) were constantly between 3100 and 4100 throughout the testing period. There was no statistically significant change in the antibody response throughout the period of time following vaccination. However, a wide variety of serological responses were observed among animals. A minority of vaccinated piglets developed an antibody response against *S. suis* capsular type 2. Four vaccinated piglets out of 24 immunized with the 89-1591 strain and five out of 24 piglets immunized with the M-42 strain, developed titers

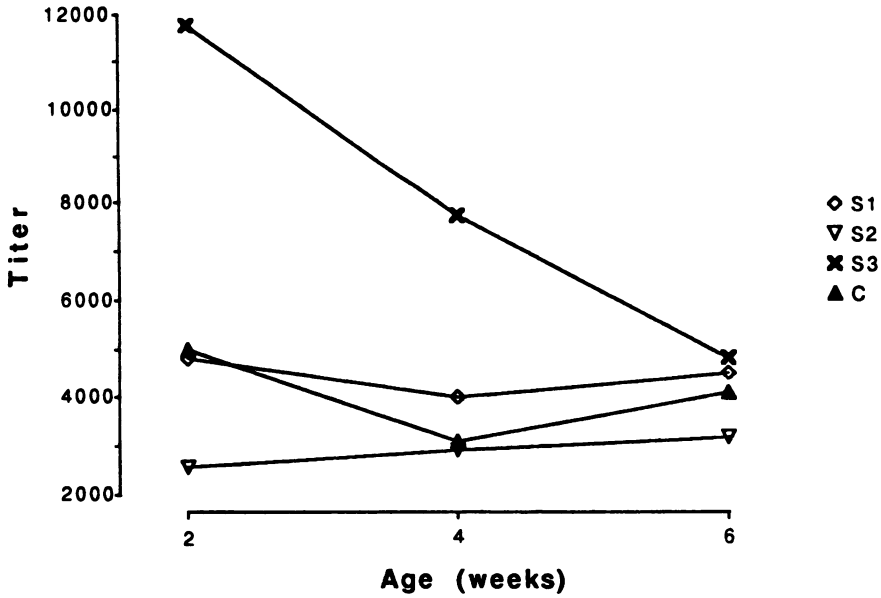

Fig. 2. Antibody titers of piglets from sows vaccinated with strain 89-1591. Results are shown as means of titers for three litters from three different sows (S1, S2 and S3), compared to the means of titers of piglets from nonvaccinated sows (C).

of 25,600 or more. Although similar results were obtained with both vaccines, the production of antibodies seemed to be more homogeneous in group A (vaccine 89-1591) than in group B (vaccine M-42). For example, two weeks after the second dose of vaccine, titers ranged from 800 to 51,200 with a standard deviation (SD) of 5000 for group A and from 800 to 102,400, with a SD over 21,000 for group B. Standard deviations were in general lower in group A (Fig. 1).

Of the four sows (S1, S2, S3 and S4) vaccinated with strain 89-1591, only one (S4) did not develop an antibody response to the vaccine; thus, its litter was not included in the study designed to evaluate the passive immunity in piglets from those sows. For the three other sows, the rise of antibody titer was at most one dilution. Titers increased from 3200 to 6400 for S1 and S2, and from 6400 to 12,800 for S3. Piglets from S1 and S2 had titers ranging from 1600 to 6400, and at two weeks of age, the mean titers were comparable to the mean titers of the control group (Fig. 2). At two weeks of age, titers of piglets from S3 were 6400 for three of them, 12,800 for the other two, and 25,600 for one piglet. The mean of those titers (11,730) was about the same as the titer of the sow. At six weeks of age the mean of titers was close to the mean of titers of the control group (C).

PROTECTION STUDIES

The seven pools of sera from vaccinated piglets and hyperimmune serum were used for the mouse protection test against *S. suis* capsular type 2 strain 89-1591. As shown in Table II, a 100% protection was obtained with the undiluted hyperimmune serum. The protection rate was 80% when that serum was diluted to a titer of 25,600 or more, but protection decreased under this value. It is noteworthy that at a given antibody titer as measured by ELISA, a much higher level of protection was observed with dilutions of the hyperimmune serum than with sera from vaccinated piglets. Hyperimmune serum diluted 1:4, and pool 4, for which the titer was 25,600, gave a level of protection of 80% and 0% respectively. The hyperimmune serum diluted 1:10 protected 30% of the mice whereas no protection was obtained with the pool 1, the titer for both sera being 12,800. No protection was observed with any of the pools from vaccinated piglets. All mice inoculated with antigen and axenic serum died. Inoculation of sterile THS mixed with axenic serum did not affect any of the mice.

DISCUSSION

Polysaccharides adsorb poorly to polystyrene plates commonly used in ELISA procedures because of their hydrophilic character (22). Preliminary studies indicated that it was difficult to obtain reliable results with an indirect ELISA carried out with live or formalinized antigen for the coating of plates. Indeed, after using an indirect ELISA for a serological study on pigs, authors expressed doubts about the specificity of the technique (4). Nonetheless, other authors did obtain satisfactory results with an ELISA using purified polysaccharides from *S. suis* serotype 2 for coating (23). In fact, the problem of adsorption of polysaccharides to polystyrene can be avoided by precoating the plates with specific immunoglobulins as performed in the sandwich ELISA.

Specificity of the ELISA depends in large part on the quality of the antigen used. For this reason, specific purified haptens are commonly used, instead of whole-bacteria preparations. For example, purified polysaccharides have been used in ELISA for the detection of antibodies against *Streptococcus pneumoniae* and groups A, B, C and G streptococci (24–28). In the present study, a whole-cell preparation has been used because of the lack of knowledge about specific immunogenic components of *S. suis*. At this point, it is not known if major antibodies are directed against the capsular polysaccharide. The importance of anticapsular antibodies is well established in the protection against many bacterial species such as *S. pneumoniae*, group B streptococci, *Neisseria meningitidis* and *Haemophilus influenzae* type b (29); yet, this importance has not been clearly demonstrated for *S. suis*. Some authors have suggested that protection would involve antibodies against cell-wall proteins (30,31).

The present study has shown that although common epitopes seemed to be present in different streptococcal species, a specific antibody response against *S. suis* capsular type 2 could be measured by a sandwich ELISA. However, the use of the sandwich ELISA for other purposes such as diagnosis or detection of chronically infected carriers will need additional refinements to obtain even more specificity. Thus, the development of type or species specific haptens and monoclonal antibodies raised against

TABLE II. Antibody titers and mouse protection results obtained with sera from axenic and hyperimmunized piglets, piglets vaccinated against strain 89-1591 (pools 1, 2 and 3), piglets vaccinated against strain M-42 (pools 4, 5 and 6) and nonvaccinated piglets (pool 7)

Serum	Titer	% of protected mice
Axenic	0	0
Hyperimmune[a] (undiluted)	100,000	100
Hyperimmune (diluted 1:4)	25,600	80
Hyperimmune (diluted 1:10)	12,800	30
Hyperimmune (diluted 1:20)	6400	10
Pool 1	12,800	0
Pool 2	6400	0
Pool 3	3200	0
Pool 4	25,600	0
Pool 5	6400	0
Pool 6	3200	0
Pool 7	1600	0

[a]Formalinized killed cells, injected intravenously twice weekly

those antigens will be necessary. Indeed, in the ELISA used in this study, cross-reactivity of the antigen with other antibodies in pig serum, and background caused by other reagents of the ELISA, could generate nonspecific reactions. Titers noted in piglet sera before vaccination and in control animals were, in general, between 800 and 6400. It is difficult to know if those titers reflect a nonspecific reaction or a specific immunological response due to the previous presence of *S. suis* in the swine operation.

Results obtained following hyperimmunization of swine are in accordance with previous data which indicated that repeated intravenous injections of formalinized antigens could induce antibody production against *S. suis* capsular type 2 (32). Using the bactericidal test, these authors also detected a humoral response against *S. suis* in swine injected twice intramuscularly with formalinized antigen, but not in animals receiving only one injection. In another study, opsonizing antibodies against *S. suis* have been detected in most animals immunized twice with the purified capsular polysaccharide, when combined with incomplete Freund's adjuvant (33). However, in the present study, vaccination of piglets against *S. suis* capsular type 2 had no statistically significant effect on specific antibody titers. Similarly, Clifton-Hadley *et al* (4) have demonstrated that within a group of seven-week-old piglets experimentally infected with live bacteria, many of them showed no increase in their antibody titers. Furthermore, the humoral response of some infected piglets was similar to that of some healthy carrier animals. In the present study, only a few piglets demonstrated an important postvaccinal increase in antibodies. These results did not allow the demonstration of a clear difference between the two vaccines. Standard deviation values indicated that the response against the more capsulated strain was most homogeneous. Contrary to that of the parent strain, the capsular material of M-42 mutant strain was thin, irregular and unstable (19). Variations in the capsular composition may spontaneously occur, and this could explain the broad range of humoral responses observed.

In the second part of this study, maternal immunity induced by vaccine A was studied. The aim of the study was to determine if maternal antibodies against *S. suis* capsular type 2 were transferred to the piglets. Specific immunity was detected in piglets of only one of the three litters originating from sows which had produced antibodies against this microorganism. These results suggest that maternal antibodies can be protective in piglets only if their titer has reached a certain level. Although the number of sows used in the present investigation was small, the fact that sows responded poorly or not at all to vaccination may explain, in part, the unsatisfactory results of vaccination noted in some swine operations. Many other factors could be involved in the development of an adequate immunological response against *S. suis*, among which are age, immunological status of animals, sanitary status of the operation, contacts with healthy carriers, different *S. suis* serotypes, vaccine preparation, and schedule of vaccination.

Passive protection studies indicated that serum from pigs hyperimmunized intravenously with formalin-killed whole-cell bacteria protect mice against a subsequent challenge with the homologous strain. Authors have shown that protection observed in pigs following repeated intravenous injections with live virulent cultures of *S. suis* capsular type 2 was transferred passively to other pigs by inoculation of sera from protected pigs (34). To our knowledge, there are no published data about correlation between antibody titers and protection level for *S. suis*. In our study, a very good protection was obtained with hyperimmune sera with titers of 25,600 or more. Moreover, no protection was obtained with sera from piglets inoculated with a commercial killed vaccine, even if antibody titers of those sera were similar to that of hyperimmune serum. Preliminary results showed that the difference in protection obtained with both groups of sera seems not to be related to the presence of antibodies against proteins.

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