

IMMUNOGLOBULIN G RESPONSE OF STREPTOCOCCUS SUIS BACTERIN - VACCINATED PIGS

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Thirty SPF pigs known to be free of *Streptococcus suis* infection were randomly selected from four litters at 10 to 12 days of age and allocated to 20 vaccinates and 10 controls. Each of the vaccinates was injected intramuscularly with a 1 ml dose of a *S. suis* bacterin at 10 - 12 days of age, followed by a 2.0 ml dose at 26 days of age. All of the animals were challenged with *S. suis* 16 days following the second vaccination. The vaccinates were protected and the controls showed clinical signs and necropsy lesions. Western blot analysis identified the special proteins between 35 and 50 kD that were recognized by all sera from pigs vaccinated twice and also by sera from pigs surviving challenge. These 35 - 50 kD proteins were not recognized by sera from prevaccinates and negative controls. An indirect ELISA using 35 - 50 kD proteins for coating plates was standardized for the detection of specific antibodies following vaccination and challenge.

Streptococcus suis has become one of the major pig infections in today's modern pig operations using medicated early weaning (MEW), segregated early weaning (SEW) or any other high health technologies to eliminate most of the pathogens from pig herds (9). The reason is that pigs are colonized with *S. suis* during parturition or between 5 and 10 days of age, which is far earlier than the actual weaning day in the above early weaning systems. Colonized piglets will transmit *S. suis* into other nursery pigs and cause outbreak of clinical disease as maternal antibody declines (1). For this reason, to vaccinate pigs with a *S. suis* bacterin at an appropriate age could be one method of keeping this disease under control. Attempts to control this disease with vaccines have been made by several researchers (2,4,6). However, in the absence of a reliable serological method to detect the antibody response, it is difficult to evaluate the immune response and the level of protection induced in vaccinated pigs.

The aim of this study was to identify certain bacterial protein fractions that can only be detected by sera from vaccinated pigs which survived virulent challenge, and to develop an ELISA to measure specific IgG levels against these proteins in vaccinated or challenged pigs.

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MATERIALS AND METHODS

Test Bacterin: Streptococcus Suis Bacterin, MVP Serial LS 101, is a killed bacterin adjuvanted with Emulsigen (an oil-in-water adjuvant).

Challenge Strain: *S. suis*, Serotype 2 (MVP Reference Strain 7837).

Efficacy Test and Serum Collection: Thirty *S. suis* free SPF pigs from four litters were randomly assigned to a test group (20 pigs) and a control group (10 pigs) when they were 10 - 12 days old. Each of the 20 pigs from the test group was injected intramuscularly with a 1 ml dose of test bacterin at 10 and 11 days of age, followed by a 2.0 ml dose at 31 and 32 days of age. At the same time as the test group, each of the ten control pigs was injected with similar volumes of a sham vaccine which contained only the growth medium plus adjuvant.

All 30 pigs were challenged with a 1.0 ml intravenous dose containing 10^7 CFU of virulent *S. suis* 16 days after the second injection. Clinical signs were recorded daily.

Serum samples were taken prior to each vaccination and prior to challenge. Serum samples were also taken at 10 days after challenge or just prior to death by euthanasia.

The challenged pigs were temperatured and observed daily for clinical signs of *S. suis* infection. Observations were made for seven to 10 days following challenge.

Clinical signs such as dyspnea, lameness (joint swelling), meningitis and depression (anorexia, etc.) were evaluated daily on a 3 point scale as follows:

<u>Score/Day</u>	<u>Degree of Dysfunction</u>
0	Normal
1	Mild
2	Moderate
3	Severe

Necropsy observations and histopathology results were scored in a similar fashion (normal, mild, moderate, severe) on a scale of 0 to 3.

Fever was scored daily as follows:

<u>Score/Day</u>	<u>Temperature Range</u>
0	< 103.0°F
1	103.0 to 103.9°F
2	104.0 to 104.9°F
3	=> 105.0°F

Death attributed to *S. suis* infection received a score of 15.

Production of Hyperimmune Pig Serum: Prior to immunization, a young pig was bled and its serum was tested by slide agglutination to confirm absence of *S. suis* antibodies. The sero-negative pig was immunized with two doses of MVP Streptococcus Suis Bacterin (Serial LS 101, above), followed by weekly intravenous injections of 10^7 CFU of washed, formalin-inactivated *S. suis* for 3 months. The hyperimmune sera was aliquoted and stored at -20°C . Non-reactive sera from 10 SPF pigs were also stored at -20°C for use as a negative control.

SDS - PAGE and Western blotting: Ten (10) ml of the formalin-killed *S. suis* whole culture was centrifuged at 1000 G for 10 minutes at room temperature. The culture was lysed by resuspending the pellet in 1 ml of cold (4°C) Lysis Buffer (0.1M Tris pH 8.0, 0.8 mg EDTA, 1 mg lysozyme) for 5 minutes. Approximately 240 ul of the lysed whole cells were mixed with 120 ul of gel loading buffer (DTT: 0.11 gm, 20% SDS: 0.71 ml, 1M Tris pH 6.8: 0.57 ml, glycerol: 1.07 ml, 1% Bromophenol Blue: 4 ul, H_2O : qs. 7 ml) and heated to 100°C for 1 minute. The SDS-PAGE (7.5%) gels were prepared and 20 ul of the heated sample was loaded on each well of 2 gels and 10 ul of the molecular weight marker was also loaded on the outside well of each gel. After electrophoresis and electroblotting, blots were probed with pig serum.

ELISA Procedure (Using Special Fractions of *S. suis* as Antigen): A formalin inactivated culture of *S. suis* was concentrated 10 fold and an SDS-PAGE was performed as described above. After electrophoresis, the gel was removed and placed in a staining box for silver staining. As soon as the bands started to appear, the stain reaction was stopped by adding 5% citrate solution. With a scalpel, the gel band of interest was excised (the area between 35 and 50 kD) and fragmented using a tissue grinder. One (1.0) ml of extraction buffer (0.5% SDS in 125 mM Tris-HCl, pH 6.8) was added and the suspension was incubated overnight at 5°C .

The suspension was centrifuged at 1000 G for 10 minutes and the supernatant was transferred to a fresh centrifuge tube containing 1 ml of cold precipitation buffer (90% acetone, 5% acetic acid, and 5% trimethylamine). The tube was put on ice for 30 minutes and then centrifuged at 5000 G at 5°C for 10 minutes. The supernatant was removed and the pellet was resuspended with an appropriate amount of PBS (pH 7.2) to make an antigen solution containing 0.125 mg protein per ml. Each well of the 96-well microtiter plates (Dynatech Immulon-2) was coated with 50ul (6.25 ug/well) of the diluted antigen. The plates were air dried overnight at room temperature. Plates were then filled with 50 ul per well of a blocking agent (1% BSA in PBS pH 7.2) and incubated at room temperature for 30 minutes. After washing 3 times with 150 ul per well of PBS-Tween 20, the antigen-coated microplates were incubated at room temperature for 90 minutes with 50 ul of diluted test serum. Hyperimmune pig serum was used as a positive control and serum from non-exposed pigs was used as negative control. Each plate contained positive and negative control samples diluted in the same manner as test serum samples. Serial dilutions of test sera were made from 1:400 to 1:12,800. After another three washes with PBS-Tween 20, 50 ul of alkaline phosphatase conjugated rabbit anti-porcine IgG (Sigma Chemical Co.) diluted 1:3000 was added to each well. Plates were incubated at room temperature for 90 minutes. After three washes with PBS-Tween, 100 ul of

alkaline phosphatase substrate solution (Sigma Chemical Co.) was added to each well. The plates were allowed to react at room temperature until the OD 405 of the negative control was between 0.20 and 0.30. Then 50 ul of 5N NaOH was added to stop the color reaction. The plates were read at 405 nm. Results were reported as titers which were defined as the reciprocal of the lowest serum dilution having a ratio (OD test / OD negative control) of 1.5 or larger.

ELISA Procedure (Using Washed Whole Cells of *S. suis* as Antigen): One (1.0) ml of a whole culture containing 10^7 CFU per ml of *S. suis* was added to 9 ml of sterile PBS and mixed well by vortex. After centrifugation at 500 G for 10 minutes, the pellet was saved and resuspended in 10 ml PBS. More PBS was added to adjust the cell suspension to an OD of 0.10 at 405 nm. A 1:100 dilution of this cell suspension was made in PBS and used to coat each well of a 96-well microtiter plate with 100 ul of cell suspension. The plate was centrifuged at 1000 G for 20 minutes. The supernatant was removed and the plate was allowed to air dry before the ELISA was started. The ELISA procedure was the same as described above.

ELISA Procedure (Using Heat Extracted Antigens of *S. suis* as Antigen): The confluent growth from overnight cultures of *S. suis* serotype 2 was grown on a sheep blood agar plate and was harvested with sterile 0.85% NaCl solution and heated at 121°C for 2 hours. Cells were pelleted by centrifugation and the supernatant was adjusted to 6.25 ug of protein per ml with PBS. Fifty (50) ul of the diluted antigen extract was used to coat each well of a 96-well plate and air dried. The ELISA procedure was the same as described above.

Serological study of baby pigs from vaccinated and nonvaccinated sows using ELISA: A total of 16 baby pigs, 8 from a vaccinated sow and 8 from a nonvaccinated sow, were randomly selected from their siblings for *S. suis* serological study after receiving colostrum by nursing. All of the 16 baby pigs were bled at the age of 2, 5, 8 and 11 days old. The sows were bled at parturition. The antibody titer profiles of each of the 16 pigs and 2 sows were determined by using the ELISA procedure described above.

RESULTS

Efficacy Test: The vaccinates were protected from a virulent challenge with 1×10^7 CFU of *S. suis* serotype 2 while the controls showed clinical signs and necropsy lesions. The mean cumulative scores of clinical signs, necropsy lesions, histopathology and mortality was 3.9 for vaccinates and 51.0 for the controls ($P < 0.05$, see Table 1).

Western blots: Western blot analysis of the cellular proteins from homologous *S. suis* serotype 2 with pig sera obtained from prevaccinates and vaccinates identified protein bands between 35 and 50 kD which were recognized by all sera from pigs vaccinated twice and also by sera from pigs surviving virulent challenge. These bands were not recognized by sera from pre-vaccinates and negative controls (Fig. 1, 2). A band of 59 kD was frequently recognized by most of the pig sera including pre-vaccinates, vaccinates, and survivors after challenge. (Fig. 2).

Development of ELISA: Serial dilutions of pig sera from negative control pigs, *S. suis* bacterin-vaccinated pigs, and *S. suis*-hyperimmune pigs were assayed using 3 different antigens of *S. suis*, serotype 2 as described above. At a serum dilution of 1:800, absorbance values from *S. suis*-vaccinated and *S. suis*-negative pigs were widely separated when 96-well microtiter plates were coated either with 35 - 50 kD proteins of *S. suis* serotype 2. (Fig. 3) or with heat extracted antigens of *S. suis* serotype 2 (Fig. 4). Absorbance values from *S. suis*-vaccinated and *S. suis*-negative pigs were quite similar to each other when plates were coated with washed whole cells of *S. suis* serotype 2 (Fig. 5).

ELISA procedures using either 35 - 50 kD proteins of *S. suis* serotype 2 or heat-extract antigens of *S. suis* serotype 2 for coating plates were compared in their detections of antibody response in the same group of pigs, before and after immunization, or before and after virulent challenge. Comparison was also made between the vaccinated and non-vaccinated groups of pigs. (Table 2, Table 3). At a serum dilution of 1:800, background readings (absorbance value from *S. suis*-negative pigs) were higher when heat extract antigens were used as coated antigens, and low when special fractions (35 - 50 kD proteins) were used as coated antigens. IgG antibody titers increased from 0 (prevaccinate) to 800 (after vaccination) and 3200 (post challenge) when 35 - 50 kD proteins of *S. suis* serotype 2 were used as antigens.

Sensitivity and specificity: Sensitivity of ELISA using 35 - 50 kD proteins of *S. suis* serotype 2 as coated antigens was established with sera from 64 pigs which were obtained from infected pig herds showing clinical signs of *S. suis* infection and at least one positive culture for *S. suis* serotype 2. Sera from 2 pigs had a S/N (signal to noise) ratio below the 1.5 cutoff value, giving a sensitivity of 97%. Specificity of the ELISA was also determined with sera from another group of 64 pigs, which were obtained from SPF pig herds and conventional pig herds showing no clinical signs and no positive culture for *S. suis* serotype 2. Sera from 6 *S. suis* serotype 2-negative pigs had a S/N ratio above the 1.5 cutoff value, giving a specificity of 91%. (Table 4).

Serological study of baby pigs: Antibody titer of the unvaccinated sow was 0 while the titer of the vaccinated sow was 1600. Piglets from the vaccinated sow had titers of 800 at 2 days, 5 days, and 8 days, and 0 at 11 days and 15 days. Piglets from the unvaccinated sow had titers of 0 at 2 days, 5 days, 8 days, 11 days and 15 days.

(Insert photograph for Figure 1 here)

Fig. 1. Western blot of lysed *S. suis*, serotype 2, probed with negative pig serum (lanes 1,2), pre vaccinated pig serum (lanes 4,5), and vaccinated pig serum (lanes 7,8). Lanes 3 and 6 are low range molecular weight markers (106 kD, 50 kD, 35 kD, 28 kD and 20 kD). Lane 9 is a high range marker (205 kD, 117 kD, 80 kD and 50 kD).

(Insert photograph for Figure 2 here)

Fig. 2. Western blot of lysed *S. suis*, serotype 2, probed with control pig serum (lane 1), post challenge control pig (lane 3), pre vaccinated pig (lane 6), and vaccinated pigs after challenge (lane 8). Lane 2 is high range marker and lane 9 is low range marker.

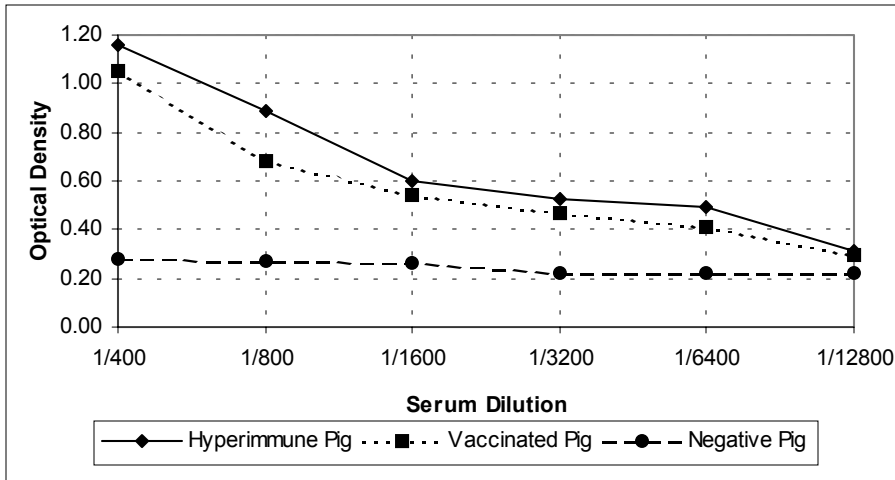


Fig. 3. Serial dilutions of pig sera from *S. Suis* Bacterin-vaccinated pigs, and *S. suis* hyperimmune pig were compared to those from *S. suis*-negative pig by ELISA using special fractions of *S. suis* as antigens.

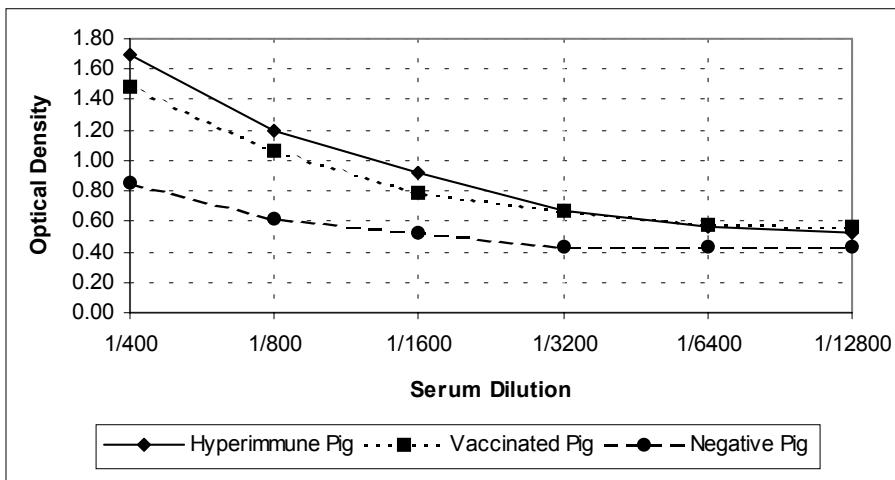


Fig. 4. Serial dilutions of pig sera from *S. Suis* Bacterin-vaccinated pigs, and *S. suis* hyperimmune pig were compared to those from *S. suis*-negative pig by ELISA using heat-extracted antigens of *S. suis*.

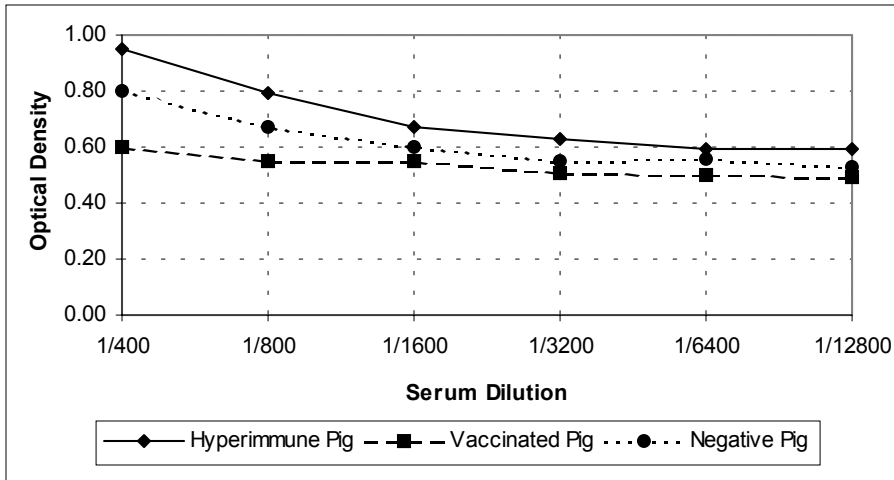


Fig. 5. Serial dilutions of pig sera from *S. Suis* Bacterin-vaccinated pigs, and *S. suis* hyperimmune pig were compared to those from *S. suis*-negative pig by ELISA using washed whole cells of *S. suis*.

Table 1. Scores of clinical signs, necropsy lesions, histopathology and mortality after challenge of both vaccinates and nonvaccinated pigs with virulent *S. suis* serotype 2.

Test Animals	Clinical Signs	Necropsy Lesions	Histopathology Lesions	Cumulative Score
Vaccinates				
Pig # 1	5	1	0	6
2	2	0	0	2
3	3	0	0	3
4	4	0	0	4
5	0	0	0	0
6	1	0	1	2
7	6	0	2	8
8	4	0	1	5
9	7	0	2	9
10	6	0	0	6
11	0	0	0	0
12	0	0	0	0
13	5	0	0	5
14	2	0	0	2
15	3	2	0	5
16	1	0	0	1
17	0	0	0	0
18	7	0	0	7
19	11	0	0	11
20	2	0	0	2
		Mean cumulative score:		3.9
Controls:				
Pig # 21	41	3	0	44
22	64	3	2	39
23	69	3	2	74
24	28	0	0	28
25	33	2	0	35
26	52	3	0	55
27	59	3	0	62
28	51	1	2	54
29	36	2	0	38
30	50	1	0	51
		Mean Cumulative Score:		51

Table 2. Absorbance and Antibody Titers Obtained with Pooled Sera from *S. Suis* Bacterin-Vaccinated and Non-Vaccinated Pigs by ELISA Using a Specific Fraction (35 - 50kD) of *S. suis* serotype 2 as Antigens

Group	Dilution of Pig Serum										Antibody Titer
	1:800		1:1600		1:3200		1:6400		1:12,800		
	OD	S/N	OD	S/N	OD	S/N	OD	S/N	OD	S/N	
<u>Vaccinates</u>											
Pre-vaccination	0.29	1.0	0.26	0.9	0.20	1.0	0.27	1.0	0.26	0.9	0
After 1st Vaccination	0.49	1.8	0.33	1.2	0.32	1.1	0.29	1.0	0.26	0.9	800
After 2nd Vaccination	0.57	2.0	0.39	1.4	0.34	1.2	0.29	1.0	0.26	0.9	800
Post-Challenge	1.14	4.1	0.75	2.7	0.53	1.9	0.35	1.3	0.29	1.0	3200
<u>Controls</u>											
Pre-challenge	0.27	1.0	0.30	1.1	0.25	0.9	0.23	0.8	0.22	0.8	0
Post-Challenge	1.42	5.1	0.80	2.9	0.53	1.9	0.48	1.7	0.40	1.4	6400
Hyperimmune Pig	0.96	3.4	0.54	1.9	0.44	1.6	0.35	1.3	0.31	1.1	3200
Negative Pig	0.28		0.27		0.26		0.22		0.27		0

Table 3. Absorbance and Antibody Titers Obtained with Pooled Sera from *S. suis* Bacterin-Vaccinated and Non-Vaccinated Pigs by ELISA Using Heat-Extract Antigens of *S. suis* serotype 2 as Antigens

Group	Dilution of Pig Serum										Antibody Titer
	1:800		1:1600		1:3200		1:6400		1:12,800		
	OD	S/N	OD	S/N	OD	S/N	OD	S/N	OD	S/N	
<u>Vaccinates</u>											
Pre-vaccination	0.36	0.6	0.36	0.6	0.40	0.7	0.41	0.7	0.42	0.7	0
After 1st Vaccination	0.70	1.1	0.58	0.9	0.53	0.9	0.48	0.8	0.47	0.8	0
After 2nd Vaccination	1.06	1.7	0.79	1.3	0.67	1.1	0.58	0.9	0.57	0.9	800
Post-Challenge	0.94	1.5	0.79	1.3	0.65	1.0	0.55	0.9	0.54	0.9	800
<u>Controls</u>											
Pre-challenge	0.62	1.0	0.47	0.8	0.42	0.7	0.42	0.7	0.45	0.7	0
Post-Challenge	0.99	1.6	0.78	1.3	0.63	1.0	0.55	0.9	0.55	1.9	800
Hyperimmune Pig	1.19	1.9	0.92	1.5	0.67	1.1	0.56	0.9	0.53	0.9	1600
Negative Pig	0.62		0.52		0.43		0.43		0.45		0

Table 4. Sensitivity and Specificity of the ELISA using 35 - 50 kD proteins of *S. suis* serotype 2 as Coated Antigens in Detecting Antibodies to *S. suis* serotype 2 in Pigs.

ELISA Result	Number of Pigs with Indicated <i>S. suis</i> Antibody Test Result		Total
	Positive	Negative	
Positive	62	6	68
Negative	2	58	60
Total	64	64	

Table 5. Antibody Titers Obtained with Pooled Sera from Baby Pigs at 2 - 15 Days of Age by ELISA Using 35-50 kD Proteins of *S. suis* as Antigens

Age of Pig (Days)	Titers of Pooled Sera from 8 Baby Pigs Farrowed by Unvaccinated Sow	Titers of Pooled Sera from 8 Baby Pigs Farrowed by Vaccinated Sow
2	0	800
5	0	800
8	0	800
11	0	0
15	0	0
Unvaccinated Sow	0	NA
Vaccinated Sow	NA	1600

DISCUSSION

Previous experiments have shown that IgG and IgM directed against surface components of *S. suis* serotype 2 are important in protection of pigs that had been inoculated with live and killed cultures of *S. suis* serotype 2 (2,5). It has also been reported that rabbit IgG generated against such cell surface components could passively protect mice against the challenge with reference *S. suis* serotype 2 strain (7,10). While one report indicated that mice injected with cell proteins of 33, 128 and 136 kD were protected against challenge with the homologous *S. suis* strain (10), another paper indicated rabbit serum against 78 and 94 kD proteins protected mice against challenge (7). However, no experiment has been done to identify such bacterial cell surface components by sera from pigs surviving virulent challenge.

In this experiment, we found that 35 - 50 kD proteins were recognized by all sera from pigs vaccinated twice with *S. Suis* Bacterin and also by sera from pigs surviving virulent challenge. These 35 - 50 kD proteins were not recognized by sera from prevaccinates and negative controls. These data suggest that these 35 - 50 kD proteins of *S. suis* serotype 2 may play a role in stimulating protective antibodies in pigs. In a separate experiment, we also found that such 35 - 50 kD proteins could be identified by sera either from mice immunized with a subunit vaccine containing 35 - 50 kD proteins or from mice immunized with a *S. suis* serotype 2 bacterin using Western blot analysis and an indirect ELISA (8). This further confirms the usefulness of mice for studying *S. suis* infection in pigs as described before by other authors (3,11). This may indicate the feasibility of developing a standardized in-vivo serological test to compare the antibody titers induced by two or more *S. suis* bacterins. Further experiments using mouse as an animal model for pigs will be needed.

Test results in this experiment indicated that it was difficult to get reliable results with an indirect ELISA using formalinized whole culture of *S. suis* serotype 2 for coating of plates. This is consistent with a previous report (2).

In order to confirm a strong correlation between protection of pigs vaccinated twice with *S. suis* bacterin and the presence of antibody titers of 800 or higher against the

specific proteins (35 - 50 kD) of *S. suis* serotype 2 an indirect ELISA using 35 - 50 kD proteins for coating of plates was developed. The present study has shown an acceptable sensitivity and specificity of the ELISA, using 35 - 50 kD proteins of *S. suis* for coating plates. In the present study, a heat extract antigen of a whole cell preparation of *S. suis* serotype 2 was also used to develop an indirect ELISA. Test results showed that the ELISA using 35 - 50 kD proteins for coating of plates gave lower background readings than that using heat extract antigens for coating of plates.

Due to the present lack of knowledge about the specific immunogenic components of *S. suis* serotype 2, it is still premature to adopt such an ELISA for routine *S. suis* serology study. However, data from the present study did show that a specific antibody response against *S. suis* could be measured by an indirect ELISA. Further use of such ELISA for determining the protection levels in pigs after immunization will need further studies.

It is known that pigs are colonized with *S. suis* before 15 days of age and that such colonization is largely responsible for *S. suis* disease (9). Therefore, it may be necessary to immunize sows with *S. suis* bacterin to provide maternal protection for young pigs on farms adopting the SEW system. However, there are no reported data about the level of maternal antibody titers in young pigs shortly after birth and before 15 days of age. In this experiment we found the titer of antibodies against 33 - 50 kD proteins of *S. suis* serotype 2 was 0 for an unvaccinated sow and its offspring, 1600 for a vaccinated sow when pigs were farrowed, 800 when pigs from a vaccinated sow were 2 days, 5 days and 8 days of age, and 0 when pigs from a vaccinated sow were 11 days and 15 days of age. In order to study the maternal protection of young pigs on farms using a SEW system, further experiments are needed.

ACKNOWLEDGMENT

We thank J. McGonigle for critical reading of the manuscript and B. Brotherton for assistance with the preparation of the manuscript.

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Immunoglobulin G Response of Streptococcus Suis Bacterin-Vaccinated Pigs

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ABSTRACT

Thirty (30) specific pathogen free pigs known to be free of *Streptococcus suis* infection were randomly selected from four litters at 10 to 12 days of age and allocated to 20 vaccinates and 10 controls. Each of the vaccinates was injected intramuscularly with a 0.85 ml dose of a *S. suis* bacterin at 10 or 11 days of age, followed by a 1.7 ml dose three weeks later at 31 or 32 days of age. All of the animals were challenged with *S. suis* 16 days following the second vaccination. The vaccinates were protected and the controls showed clinical signs and necropsy lesions. An indirect enzyme linked immunosorbant assay using 35 to 50-kDa proteins from *S. suis* for coating plates was standardized for the detection of specific antibodies following vaccination and challenge. Western blot analysis identified the special proteins between 35 and 50 kDa that were recognized by all sera from pigs vaccinated twice and also by sera from pigs surviving challenge. These 35 to 50-kDa proteins were not recognized by sera from prevaccinates and negative controls.

INTRODUCTION

Streptococcus suis has become one of the major pig infections in today's modern pig operations using medicated early weaning (MEW), segregated early weaning (SEW) or any other high health technologies to eliminate most of the pathogens from pig herds (9). The reason is that pigs are colonized with *S. suis* during parturition or between 5 and 10 days of age, which is far earlier than the actual weaning day in the above early weaning systems. Colonized piglets will transmit *S. suis* into other nursery pigs and cause outbreak of clinical disease as maternal antibody declines (1). For this reason, to vaccinate pigs with a *S. suis* bacterin at an appropriate age could be one method of keeping this disease under control. Attempts to control this disease with vaccines have been made by several researchers (2,4,6). However, in the absence of a reliable serological method to detect the antibody response, it is difficult to evaluate the immune response and the level of protection induced in vaccinated pigs.

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Test Bacterin: Streptococcus Suis Bacterin, Serial LS-101, MVP Laboratories, Inc., is a killed bacterin adjuvanted with Emulsigen (an oil-in-water adjuvant) and aluminum hydroxide.

Challenge Strain: *S. suis*, Serotype 2 (MVP Reference Strain 7837).

Efficacy Test and Serum Collection: Thirty (30) *S. suis* free specific pathogen free (SPF) pigs from four litters were randomly assigned to a test group (20 pigs) and a control group (10 pigs) when they were 10-11 days old. Each of the 20 pigs from the test group was injected intramuscularly with a 0.85 ml dose of test bacterin at 10 or 11 days of age, followed by a 1.7 ml dose three weeks later at 31 or 32 days of age. At the same time as the test group, each of the ten control pigs was injected with similar volumes of a sham vaccine which contained only the growth medium plus adjuvant.

All 30 pigs were challenged with a 1.0 ml intravenous dose containing 10^7 CFU of virulent *S. suis* 16 days after the second injection. Clinical signs were recorded daily.

Serum samples were taken prior to each vaccination and prior to challenge. Serum samples were also taken at 10 days after challenge or just prior to death by euthanasia.

The challenged pigs were temperatured and observed daily for clinical signs of *S. suis* infection. Observations were made for seven to 10 days following challenge.

Clinical signs such as dyspnea, lameness (joint swelling), meningitis and depression (anorexia, etc.) were evaluated daily on a 3 point scale as follows:

Score/Day	Degree of Dysfunction
0	Normal
1	Mild
2	Moderate
3	Severe

Necropsy observations and histopathology results were scored in a similar fashion (normal, mild, moderate, severe) on a scale of 0 to 3.

Fever was scored daily as follows:

Score/Day	Body Temperature Range
0	< 103.0°F
1	103.0 to 103.9°F
2	104.0 to 104.9°F
3	=> 105.0°F

Death attributed to *S. suis* infection received a score of 15.

The cumulative scores of vaccinated animals and control animals were compared using the one-tailed Student's t test.

Production of Hyperimmune Pig Serum: Prior to immunization, a young pig was bled and its serum was tested by slide agglutination to confirm absence of *S. suis* antibodies. The sero-negative pig was immunized with two doses of Streptococcus Suis Bacterin Serial LS-101, followed by weekly intravenous injections of 10^7 CFU of washed, formalin-inactivated *S. suis* for 3 months. The hyperimmune serum was aliquoted and stored at -20°C. Non-reactive sera from 10 SPF pigs were also stored at -20°C for use as a negative control.

SDS-PAGE and Western blotting: Ten (10) ml of the formalin-killed *S. suis* whole culture was centrifuged at 1000 X g for 10 minutes at room temperature. The culture was lysed by resuspending the pellet in 1 ml of cold (4°C) lysis buffer (0.1M Tris pH 8.0, 0.8 mg EDTA, 1 mg lysozyme) for 5 minutes. Approximately 240 ul of the lysed whole cells were mixed with 120 ul of gel loading buffer (DTT: 0.11 gm, 20% sodium dodecyl sulfate (SDS): 0.71 ml, 1M Tris pH 6.8: 0.57 ml, glycerol: 1.07 ml, 1% Bromophenol Blue: 4 ul, H₂O: qs. 7 ml) and heated to 100°C for 1 minute. The SDS-polyacrylamide gel electrophoresis (PAGE) 7.5% gels were prepared and 20 ul of the heated sample was loaded on each well of 2 gels. Ten (10) ul of the molecular weight marker was also loaded on the outside well of each gel. After electrophoresis and electroblotting, blots were probed with pig serum.

ELISA Procedure Using Special Fractions of *S. suis* as Antigen: A formalin inactivated culture of *S. suis* was concentrated 10 fold and an SDS-PAGE was performed as described above. After electrophoresis, the gel was removed and placed in a staining box for silver staining. As soon as the bands started to appear, the stain reaction was stopped by adding 5% citrate solution. With a scalpel, the gel band of interest was excised (the area between 35 and 50 kDa) and fragmented using a tissue grinder. One (1.0) ml of extraction buffer (0.5% SDS in 125 mM Tris-HCl, pH 6.8) was added and the suspension was incubated overnight at 5°C.

The suspension was centrifuged at 1000 X g for 10 minutes and the supernatant was transferred to a fresh centrifuge tube containing 1 ml of cold precipitation buffer (90% acetone, 5% acetic acid, and 5% trimethylamine). The tube was put on ice for 30 minutes and then centrifuged at 5000 X g at 5°C for 10 minutes. The supernatant was removed and the pellet was resuspended with an appropriate amount of phosphate buffered saline (PBS), pH 7.2, to make an antigen solution containing 0.125 mg protein per ml. Each well of the 96-well microtiter plates

(Dynatech Immulon-2) was coated with 50 μ l (6.25 μ g/well) of the diluted antigen. The plates were air dried overnight at room temperature. The plates were then filled with 50 μ l per well of a blocking agent (1% bovine serum albumen [BSA] in PBS pH 7.2) and incubated at room temperature for 30 minutes. After washing 3 times with 150 μ l per well of PBS-Tween 20, the antigen-coated plates were incubated at room temperature for 90 minutes with 50 μ l of diluted test serum. Hyperimmune pig serum was used as a positive control and serum from non-exposed pigs was used as negative control. Each plate contained positive and negative control samples diluted in the same manner as test serum samples. Serial dilutions of test sera were made from 1:400 to 1:12,800. After another three washes with PBS-Tween 20, 50 μ l of alkaline phosphatase conjugated rabbit anti-porcine IgG (Sigma Chemical Co.) diluted 1:3000 was added to each well. Plates were incubated at room temperature for 90 minutes. After three washes with PBS-Tween, 100 μ l of alkaline phosphatase substrate solution (Sigma Chemical Co.) was added to each well. The plates were allowed to react at room temperature until the optical density (OD) of the negative control was between 0.20 and 0.30 at 405 nm. Then 50 μ l of 5N NaOH was added to stop the color reaction. Results were reported as titers which were defined as the reciprocal of the lowest serum dilution having a ratio (OD test / OD negative control) of 1.5 or larger.

ELISA Procedure Using Washed Whole Cells of *S. suis* as Antigen: One (1.0) ml of a whole culture containing 10^7 CFU per ml of *S. suis* was added to 9 ml of sterile PBS and mixed well by vortexing. After centrifugation at 1000 X g for 10 minutes, the pellet was saved and resuspended in 10 ml PBS. More PBS was added to adjust the cell suspension to an OD of 0.10 at 405 nm. A 1:100 dilution of this cell suspension was made in PBS. Each well of a 96-well microtiter plate was coated with 100 μ l of the cell suspension. The plate was centrifuged at 1000 X g for 20 minutes. The supernatant was removed and the plate was allowed to air dry before the ELISA was started. The ELISA procedure was the same as described above.

ELISA Procedure Using Heat Extracted Antigens of *S. suis* as Antigen: The confluent growth from overnight cultures of *S. suis* serotype 2 was grown on a sheep blood agar plate and was harvested with sterile 0.85% NaCl solution and heated at 121°C for 2 hours. Cells were pelleted by centrifugation and the supernatant was adjusted to 6.25 μ g of protein per ml with PBS. Fifty (50) μ l of the diluted antigen extract was used to coat each well of a 96-well plate and air dried. The ELISA procedure was the same as described above.

Serological study of baby pigs from vaccinated and nonvaccinated sows using ELISA: A total of 16 baby pigs, 8 from a vaccinated sow and 8 from a nonvaccinated sow, were randomly selected from their siblings for a *S. suis* serological study after receiving colostrum by nursing. All of the 16 baby pigs were bled at 2, 5, 8 and 11 days of age. The sows were bled at parturition. The antibody titer profiles of each of the 16 pigs and 2 sows were determined by using the ELISA procedure described above.

RESULTS

Efficacy Test: The vaccinates were protected from a virulent challenge with 1×10^7 CFU of *S. suis* serotype 2 while the controls showed clinical signs and necropsy lesions. The mean cumulative scores of clinical signs, necropsy lesions, histopathology and mortality was 3.9 for vaccinates and 51.0 for the controls (P <0.05, Table 1).

Western blots: Western blot analysis of the cellular proteins from homologous *S. suis* serotype 2 with pig sera obtained from prevaccinates and vaccinates identified protein bands between 35 and 50 kDa which were recognized by all sera from pigs vaccinated twice and also by sera from pigs surviving virulent challenge. These bands were not recognized by sera from prevaccinates and negative controls (Fig. 1, 2). A band of 59 kDa was frequently recognized by most of the pig sera including pre-vaccinates, vaccinates, and survivors after challenge (Fig. 2).

Development of ELISA: Serial dilutions of pig sera from negative control pigs, *S. suis* bacterin-vaccinated pigs, and *S. suis*-hyperimmune pigs were assayed using 3 different antigens of *S. suis* serotype 2 as described above. At a serum dilution of 1:800, absorbance values from *S.*

suis-vaccinated and *S. suis*-negative pigs were widely separated when 96-well microtiter plates were coated either with 35 to 50 kDa proteins of *S. suis* serotype 2 (Fig. 3) or with heat extracted antigens of *S. suis* serotype 2 (Fig. 4). Absorbance values from *S. suis*-vaccinated and *S. suis*-negative pigs were quite similar to each other when plates were coated with washed whole cells of *S. suis* serotype 2 (Fig. 5).

ELISA procedures using either 35 to 50 kDa proteins of *S. suis* serotype 2 or heat-extract antigens of *S. suis* serotype 2 for coating plates were compared in their detections of antibody response in the same group of pigs, before and after immunization, or before and after virulent challenge. Comparison was also made between the vaccinated and non-vaccinated groups of pigs (Table 2, Table 3). At a serum dilution of 1:800, background readings (absorbance value from *S. suis*-negative pigs) were higher when heat extract antigens were used as coated antigens, and low when special fractions (35 to 50 kDa proteins) were used as coated antigens. IgG antibody titers increased from 0 (prevaccination) to 800 (postvaccination) and 3200 (post challenge) when 35 to 50 kDa proteins of *S. suis* serotype 2 were used as antigens.

Sensitivity and specificity: Sensitivity of ELISA using 35 to 50 kDa proteins of *S. suis* serotype 2 as coated antigens was established with sera from 64 pigs which were obtained from infected herds showing clinical signs of *S. suis* infection and at least one positive culture for *S. suis* serotype 2. Sera from 2 of the 64 pigs had a S/N (signal to noise) ratio below the 1.5 cutoff value, giving a sensitivity of 97%. Specificity of the ELISA was also determined with sera from another group of 64 pigs, which were obtained from SPF pig herds and conventional pig herds showing no clinical signs and no positive culture for *S. suis* serotype 2. Sera from 6 of this group of 64 *S. suis* serotype 2-negative pigs had a S/N ratio above the 1.5 cutoff value, giving a specificity of 91%.

Serological study of baby pigs: Antibody titer of the unvaccinated sow was 0 while the titer of the vaccinated sow was 1600. Piglets from the vaccinated sow had titers of 800 at 2 days, 5 days, and 8 days, and 0 at 11 days and 15 days. Piglets from the unvaccinated sow had titers of 0 at 2 days, 5 days, 8 days, 11 days and 15 days (Table 4)

DISCUSSION

Previous experiments have shown that IgG and immunoglobulin M directed against surface components of *S. suis* serotype 2 are important in protection of pigs that had been inoculated with live and killed cultures of *S. suis* serotype 2 (2,5). It has also been reported that rabbit IgG generated against such cell surface components could passively protect mice against the challenge with reference *S. suis* serotype 2 strain (7,10). While one report indicates that mice injected with cell proteins of 33, 128 and 136 kDa were protected against challenge with the homologous *S. suis* strain (10), another paper indicates rabbit serum against 78 and 94 kDa proteins can protect mice against challenge (7). However, no experiment has been done to identify such bacterial cell surface components by sera from pigs surviving virulent challenge.

In this experiment, we found that 35 to 50 kDa proteins were recognized by all sera from pigs vaccinated twice with Streptococcus Suis Bacterin and also by sera from pigs surviving virulent challenge. These 35 to 50 kDa proteins were not recognized by sera from prevaccinates and negative controls. These data suggest that the 35 to 50 kDa proteins of *S. suis* serotype 2 may play a role in stimulating protective antibodies in pigs. In a separate experiment, we also found that such 35 to 50 kDa proteins could be identified by sera either from mice immunized with a subunit vaccine containing 35 to 50 kDa proteins or from mice immunized with a *S. suis* serotype 2 bacterin using Western blot analysis and an indirect ELISA (8). This further confirms the usefulness of mice for studying *S. suis* infection in pigs as described before by other authors

(3,11). This may indicate the feasibility of developing a standardized in-vivo serological test to compare the antibody titers induced by two or more *S. suis* bacterins. Further experiments using mice as an animal model for pigs will be needed.

Test results in this experiment indicate that it is difficult to get reliable results with an indirect ELISA using formalinized whole culture of *S. suis* serotype 2 for coating of plates. This is consistent with a previous report (2).

In order to confirm a strong correlation between protection of pigs vaccinated twice with *S. suis* bacterin and the presence of antibody titers of 800 or higher against the specific proteins (35 to 50 kDa) of *S. suis* serotype 2, an indirect ELISA using 35 to 50 kDa proteins for coating of plates was developed. The present study has shown an acceptable sensitivity and specificity of the ELISA, using 35 to 50 kDa proteins of *S. suis* for coating plates. In the present study, a heat extract antigen of a whole cell preparation of *S. suis* serotype 2 was also used to develop an indirect ELISA. Test results showed that the ELISA using 35 to 50 kDa proteins for coating of plates gave lower background readings than that using heat extract antigens for coating of plates.

Due to the present lack of knowledge about the specific immunogenic components of *S. suis* serotype 2, it is still premature to adopt such an ELISA for routine *S. suis* serology study. However, data from the present study show that a specific antibody response against *S. suis* can be measured by an indirect ELISA. Further use of such ELISA for determining the protection levels in pigs after immunization will need further studies.

It is known that pigs are colonized with *S. suis* before 15 days of age and that such colonization is largely responsible for *S. suis* disease (9). Therefore, it may be necessary to immunize sows with *S. suis* bacterin to provide maternal protection for young pigs on farms adopting the SEW system. However, there are no reported data about the level of maternal antibody titers in young pigs shortly after birth and before 15 days of age. In this experiment we found the titer of antibodies against 33-50 kDa proteins of *S. suis* serotype 2 was 0 for an unvaccinated sow and its offspring, 1600 for a vaccinated sow when pigs were farrowed, 800 when pigs from a vaccinated sow were 2 days, 5 days and 8 days of age, and 0 when pigs from a vaccinated sow were 11 days and 15 days of age. In order to study the maternal protection of young pigs on farms using a SEW system, further experiments are needed.

ACKNOWLEDGMENTS

We thank J. McGonigle for critical reading of the manuscript and B. Brotherton for assistance with the preparation of the manuscript.

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FIG. 1. Western blot of lysed *S. suis* serotype 2 probed with negative pig serum (lanes 1,2), pre vaccinated pig serum (lanes 4,5), and vaccinated pig serum (lanes 7,8). Lanes 3 and 6 are low range molecular weight markers (106 kDa, 80 kDa, 50 kDa, 35 kDa, 28 kDa and 20 kDa). Lane 9 is a high range marker (205 kDa, 117 kDa, 80 kDa and 50 kDa).

FIG. 2. Western blot of lysed *S. suis* serotype 2 probed with serum from unvaccinated control pig (lane 1), post challenge control pig (lane 3), pre vaccinated pig (lane 4), twice vaccinated pig (lane 6), and vaccinated pigs after challenge (lane 8). Lane 2 is high range marker and lane 9 is low range marker.

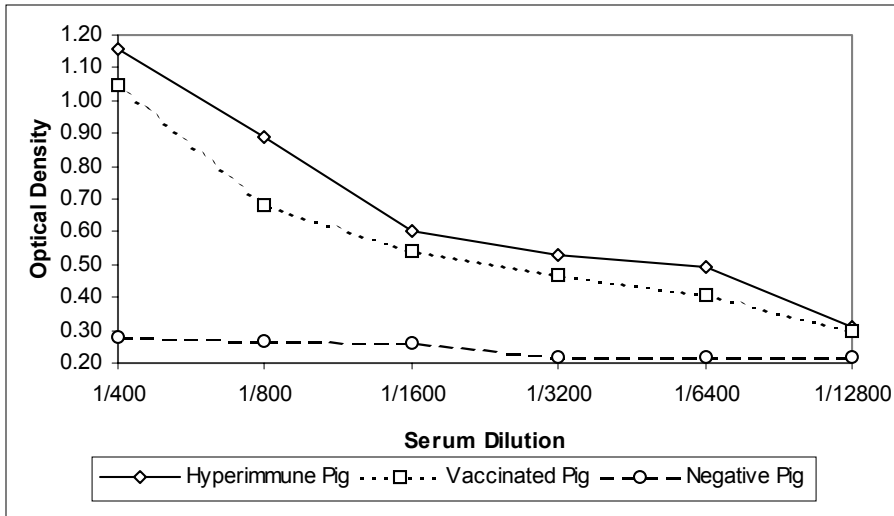


FIG. 3. Serial dilutions of pig sera from *S. suis* Bacterin-vaccinated pigs, and *S. suis* hyperimmune pig were compared to those from *S. suis*-negative pig by ELISA using special fractions of *S. suis* as antigens.

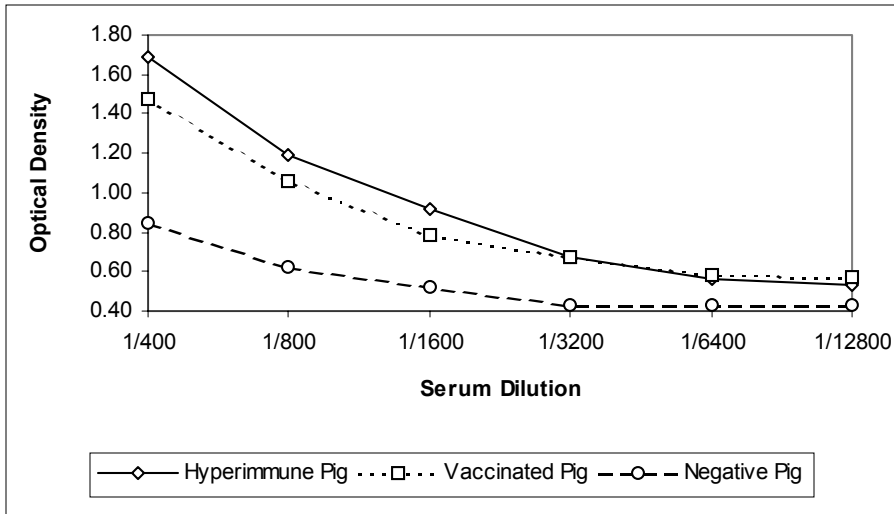


FIG. 4. Serial dilutions of pig sera from *S. suis* Bacterin-vaccinated pigs, and *S. suis* hyperimmune pig were compared to those from *S. suis*-negative pig by ELISA using heat-extracted antigens of *S. suis*.

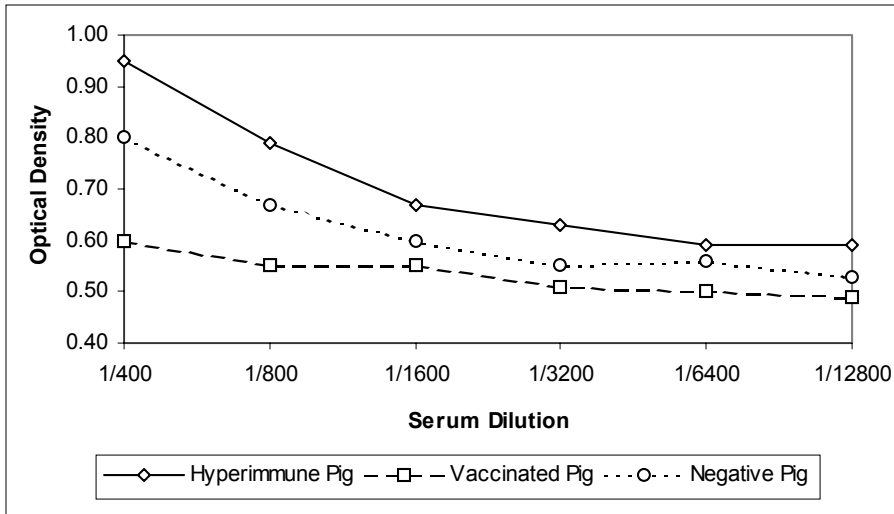


FIG. 5. Serial dilutions of pig sera from *S. suis* Bacterin-vaccinated pigs, and *S. suis* hyperimmune pig were compared to those from *S. suis*-negative pig by ELISA using washed whole cells of *S. suis*.

TABLE 1. Scores of clinical signs, necropsy lesions, histopathology and mortality after challenge of both vaccinates and nonvaccinated pigs with virulent *S. suis* serotype 2.

Test Animals	Clinical Signs	Necropsy Lesions	Histopathology Lesions	Cumulative Score
Vaccinates				
1	5	1	0	6
2	2	0	0	2
3	3	0	0	3
4	4	0	0	4
5	0	0	0	0
6	1	0	1	2
7	6	0	2	8
8	4	0	1	5
9	7	0	2	9
10	6	0	0	6
11	0	0	0	0
12	0	0	0	0
13	5	0	0	5
14	2	0	0	2
15	3	2	0	5
16	1	0	0	1
17	0	0	0	0
18	7	0	0	7
19	11	0	0	11
20	2	0	0	2
			Mean cumulative score: ^a	3.9
Controls:				
21	41	3	0	44
22	64	3	2	69
23	69	3	2	74
24	28	0	0	28
25	33	2	0	35
26	52	3	0	55
27	59	3	0	62
28	51	1	2	54
29	36	2	0	38
30	50	1	0	51
			Mean Cumulative Score: ^a	51

^a P <0.05, one-tailed Student's t-Test

TABLE 2. Absorbance and antibody titers obtained with pooled sera from *S. Suis* Bacterin-vaccinated and non-vaccinated pigs by ELISA using a specific fraction (35-50kD) of *S. suis* serotype 2 as antigens

Group	Dilution of Pig Serum										Titer ^c
	1:800		1:1600		1:3200		1:6400		1:12,800		
	OD ^a	S/N ^b	OD	S/N	OD	S/N	OD	S/N	OD	S/N	
Vaccinates											
Pre-vaccination	0.29	1.0	0.26	0.9	0.20	1.0	0.27	1.0	0.26	0.9	0
After 1st vaccination	0.49	1.8	0.33	1.2	0.32	1.1	0.29	1.0	0.26	0.9	800
After 2nd vaccination	0.57	2.0	0.39	1.4	0.34	1.2	0.29	1.0	0.26	0.9	800
Post-challenge	1.14	4.1	0.75	2.7	0.53	1.9	0.35	1.3	0.29	1.0	3200
Controls											
Pre-challenge	0.27	1.0	0.30	1.1	0.25	0.9	0.23	0.8	0.22	0.8	0
Post-challenge	1.42	5.1	0.80	2.9	0.53	1.9	0.48	1.7	0.40	1.4	6400
Positive control ^d	0.96	3.4	0.54	1.9	0.44	1.6	0.35	1.3	0.31	1.1	3200
Negative control ^e	0.28		0.27		0.26		0.22		0.27		0

^a Optical density at 405 nm

^b OD test / OD negative control

^c Reciprocal of the lowest serum dilution having a S/N of 1.5 or larger.

^d Hyperimmune pig serum

^e Normal pig serum

TABLE 3. Absorbance and antibody titers obtained with pooled sera from *S. Suis* Bacterin-vaccinated and non-vaccinated pigs by ELISA using heat-extract antigens of *S. suis* serotype 2

Group	Dilution of Pig Serum										Titer ^c
	1:800		1:1600		1:3200		1:6400		1:12,800		
	OD ^a	S/N ^b	OD	S/N	OD	S/N	OD	S/N	OD	S/N	
Vaccinates											
Pre-vaccination	0.36	0.6	0.36	0.6	0.40	0.7	0.41	0.7	0.42	0.7	0
After 1st vaccination	0.70	1.1	0.58	0.9	0.53	0.9	0.48	0.8	0.47	0.8	0
After 2nd vaccination	1.06	1.7	0.79	1.3	0.67	1.1	0.58	0.9	0.57	0.9	800
Post-challenge	0.94	1.5	0.79	1.3	0.65	1.0	0.55	0.9	0.54	0.9	800
Controls											
Pre-challenge	0.62	1.0	0.47	0.8	0.42	0.7	0.42	0.7	0.45	0.7	0
Post-challenge	0.99	1.6	0.78	1.3	0.63	1.0	0.55	0.9	0.55	1.9	800
Positive control ^d	1.19	1.9	0.92	1.5	0.67	1.1	0.56	0.9	0.53	0.9	1600
Negative control ^e	0.62		0.52		0.43		0.43		0.45		0

^a Optical density at 405 nm

^b OD test / OD negative control

^c Reciprocal of the lowest serum dilution having a S/N of 1.5 or larger.

^d Hyperimmune pig serum

^e Normal pig serum

TABLE 4. Antibody titers obtained with pooled sera from baby pigs at 2 - 15 days of age by ELISA using 35 to 50 kDa proteins of *S. suis* as antigens

Age of Pig (Days)	Titers ^a of Pooled Sera from 8 Baby Pigs Farrowed by Unvaccinated Sow	Titers of Pooled Sera from 8 Baby Pigs Farrowed by Vaccinated Sow
2	0	800
5	0	800
8	0	800
11	0	0
15	0	0
Unvaccinated Sow	0	NA
Vaccinated Sow	NA	1600

^a Reciprocal of the lowest serum dilution having a S/N of 1.5 or larger.

Immunoglobulin G response of Streptococcus suis bacterin-vaccinated pigs

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ABSTRACT

Thirty (30) specific pathogen free pigs known to be free of Streptococcus suis infection were randomly selected from four litters at 10 to 12 days of age and allocated to 20 vaccinates and 10 controls. Each of the vaccinates was injected intramuscularly with a 0.85 ml dose of a S. suis bacterin at 10 or 11 days of age, followed by a 1.7 ml dose three weeks later at 31 or 32 days of age. All of the animals were challenged with S. suis 16 days following the second vaccination. The vaccinates were protected and the controls showed clinical signs and necropsy lesions. An indirect enzyme linked immunosorbant assay using 35 to 50-kDa proteins from S. suis for coating plates was standardized for the detection of specific antibodies following vaccination and challenge. Western blot analysis identified the special proteins between 35 and 50 kDa that were recognized by all sera from pigs vaccinated twice and also by sera from pigs surviving challenge. These 35 to 50-kDa proteins were not recognized by sera from prevaccinates and negative controls.

Keywords: Streptococcus suis, IgG response, ELISA

INTRODUCTION

Streptococcus suis has become one of the major pig infections in today's modern pig operations using medicated early weaning (MEW), segregated early weaning (SEW) or any other high health technologies to eliminate most of the pathogens from pig herds¹. This is because pigs are colonized with S. suis during parturition or between 5 and 10 days of age, which is far earlier than the actual weaning day in the above early weaning systems.

Colonized piglets will transmit S. suis to other nursery pigs and cause outbreak of clinical disease as maternal antibody declines². For this reason, to vaccinate pigs with a S. suis bacterin at an appropriate age could be one method of keeping this disease under control. Attempts to control this disease with vaccines have been made by several researchers^{3,4,5}. However, in the absence of a reliable serological method of detecting the antibody response, it is difficult to evaluate the immune response and the level of protection induced in vaccinated pigs.

The aim of this study was to identify certain bacterial protein fractions that can only be detected by sera from vaccinated pigs which survived virulent challenge, and to develop an enzyme linked immunosorbant assay (ELISA) to measure specific immunoglobulin G (IgG) levels against these proteins in vaccinated or challenged pigs.

MATERIALS AND METHODS

Test bacterin

Streptococcus Suis Bacterin, Serial LS-101, MVP Laboratories, Inc., is an inactivated bacterin adjuvanted with Emulsigen (an oil-in-water adjuvant) and aluminum hydroxide.

Challenge strain

S. suis, Serotype 2 (MVP Reference Strain 7837).

Efficacy test and serum collection

Thirty (30) S. suis free specific pathogen free (SPF) pigs from four litters were randomly assigned to a test group (20 pigs) and a control group (10 pigs) when they were 10-11 days of age. Each of the 20 pigs from the test group was injected intramuscularly with a 0.85 ml dose of test bacterin at 10 or 11 days of age, followed by a 1.7 ml dose three weeks later at 31 or 32 days of age. Concurrently, each of the ten control pigs was injected with similar volumes of a sham vaccine which contained only the growth media plus adjuvant.

All 30 pigs were challenged with a 1.0 ml intravenous dose containing 10^7 CFU of virulent S. suis 16 days after the second vaccination. Clinical signs were recorded daily.

Serum samples were taken prior to each vaccination and prior to challenge. Serum samples were also taken at 10 days after challenge or just prior to death by euthanasia.

The challenged pigs were temperatured and observed daily for clinical signs of S. suis infection. Observations were made for seven to 10 days following challenge.

Clinical signs such as dyspnea, lameness (swollen joints), meningitis, depression and anorexia were evaluated daily on a 3 point scale as follows:

Score/Day	Degree of Dysfunction
0	Normal
1	Mild
2	Moderate
3	Severe

Necropsy observations and histopathology results were scored in a similar fashion. Fever was scored daily as follows:

Score/Day	Body Temperature Range
0	< 103.0°F
1	103.0 to 103.9°F
2	104.0 to 104.9°F
3	=> 105.0°F

Death attributed to S. suis infection received a score of 15.

The cumulative scores of vaccinated animals and control animals were compared using the one-tailed Student's t test.

Production of hyperimmune pig serum

Prior to immunization, a young pig was bled and its sera was tested by slide agglutination to confirm the absence of S. suis antibodies. The sero-negative pig was immunized with two doses of Streptococcus Suis Bacterin Serial LS-101, followed by weekly intravenous injections of 10^7 CFU of washed, formalin-inactivated S. suis for 3 months. The hyperimmune serum was aliquoted and stored at -20°C . Non-reactive sera from 10 SPF pigs were also stored at -20°C for use as a negative control.

SDS-PAGE and Western blotting

Ten (10) ml of the formalin-killed S. suis whole culture was centrifuged at $1000 \times g$ for 10 minutes at room temperature. The culture was lysed by resuspending the pellet in 1 ml of cold (4°C) lysis buffer (0.1M Tris pH 8.0, 0.8 mg EDTA, 1 mg lysozyme) for 5 minutes. Approximately 240 ul of the lysed whole cells were mixed with 120 ul of gel loading buffer (DTT: 0.11 gm, 20% sodium dodecyl sulfate (SDS): 0.71 ml, 1M Tris pH 6.8: 0.57 ml, glycerol: 1.07 ml, 1% Bromophenol Blue: 4 ul, H_2O : qs. 7 ml) and heated to 100°C for 1 minute. The SDS-polyacrylamide gel electrophoresis (PAGE) 7.5% gels were prepared and 20 ul of the heated sample was loaded on each well of 2 gels. Ten (10) ul of the molecular weight marker was also loaded on the outside well of each gel. After electrophoresis and electroblotting, blots were probed with pig serum.

ELISA procedure using special fractions of S. suis as antigen

A formalin inactivated culture of S. suis was concentrated 10 fold and an SDS-PAGE was performed as described above. After electrophoresis, the gel was removed and placed in a staining box for silver staining. As soon as the bands started to appear, the stain reaction was stopped by adding 5% citrate solution., The area between 35 and 50

kDa of the gel band of interest was excised with a scalpel and fragmented using a tissue grinder. One (1.0) ml of extraction buffer (0.5% SDS in 125 mM Tris-HCl, pH 6.8) was added and the suspension was incubated overnight at 5°C.

The suspension was centrifuged at 1000 x g for 10 minutes and the supernatant was transferred to a fresh centrifuge tube containing 1 ml of cold precipitation buffer (90% acetone, 5% acetic acid, and 5% trimethylamine). The tube was put on ice for 30 minutes and then centrifuged at 5000 X g at 5°C for 10 minutes. The supernatant was removed and the pellet was resuspended with an appropriate amount of phosphate buffered saline (PBS), pH 7.2, to make an antigen solution containing 0.125 mg protein per ml. Each well of the 96-well microtiter plates (Dynatech Immulon-2) was coated with 50 ul (6.25 ug/well) of the diluted antigen. The plates were air dried overnight at room temperature. The plates were then filled with 50 ul per well of a blocking agent (1% bovine serum albumen [BSA] in PBS pH 7.2) and incubated at room temperature for 30 minutes. After washing 3 times with 150 ul per well of PBS-Tween 20, the antigen-coated plates were incubated at room temperature for 90 minutes with 50 ul of diluted test serum. Hyperimmune pig serum was used as a positive control and serum from non-exposed pigs was used as a negative control. Each plate contained positive and negative control samples diluted in the same manner as test serum samples. Serial dilutions of test sera were made from 1:400 to 1:12,800. After three additional washes with PBS-Tween 20, 50 ul of alkaline phosphatase conjugated rabbit anti-porcine IgG (Sigma Chemical Co.) diluted 1:3000 was added to each well. Plates were incubated at room temperature for 90 minutes. After three washes with PBS-Tween, 100 ul of alkaline phosphatase substrate solution (Sigma Chemical Co.) was added to each well. The plates were allowed to react at room temperature until the optical density (OD) of the negative control was between 0.20 and 0.30 at 405 nm. Then 50 ul of 5N NaOH was added to stop the color reaction. Results were reported as titers which were

defined as the reciprocal of the lowest serum dilution having a ratio (OD test / OD negative control) of 1.5 or larger.

ELISA procedure using washed whole cells of S. suis as antigen

One (1.0) ml of a whole culture containing 10^7 CFU per ml of S. suis was added to 9 ml of sterile PBS and mixed well by vortexing. After centrifugation at $1000 \times g$ for 10 minutes, the pellet was saved and resuspended in 10 ml PBS. More PBS was added to adjust the cell suspension to an OD of 0.10 at 405 nm. A 1:100 dilution of this cell suspension was made in PBS. Each well of a 96-well microtiter plate was coated with 100 μ l of the cell suspension. The plate was centrifuged at $1000 \times g$ for 20 minutes. The supernatant was removed and the plate was allowed to air dry before the ELISA was started. The ELISA procedure was the same as described above.

ELISA procedure using heat extracted antigens of S. suis as antigen

The confluent growth from overnight cultures of S. suis serotype 2 was grown on a sheep blood agar plate and was harvested with sterile 0.85% NaCl solution and heated at 121°C for 2 hours. Cells were pelleted by centrifugation and the supernatant was adjusted to 6.25 μ g of protein per ml with PBS. Fifty (50) μ l of the diluted antigen extract was used to coat each well of a 96-well plate and air dried. The ELISA procedure was the same as described above.

Serological study of baby pigs from vaccinated and nonvaccinated sows using ELISA

A total of 16 baby pigs, 8 from a vaccinated sow and 8 from a nonvaccinated sow, were randomly selected from their siblings for a S. suis serological study after receiving colostrum by nursing. All of the 16 baby pigs were bled at 2, 5, 8 and 11 days of age. The sows were bled at parturition. The antibody titer profiles of each of the 16 pigs and 2 sows were determined by using the ELISA procedure described above.

RESULTS

Efficacy test

The pigs from the vaccinated group were protected from a virulent challenge with 1×10^7 CFU of *S. suis* serotype 2 while the control pigs from the non-vaccinated group showed clinical signs and necropsy lesions. The mean cumulative scores of clinical signs, necropsy lesions, histopathology and mortality was 3.9 for vaccinates and 51.0 for the controls ($P < 0.05$) (Table 1).

Western blots

Western blot analysis of the cellular proteins from homologous *S. suis* serotype 2 with pig sera obtained from prevaccinates and vaccinates identified protein bands between 35 and 50 kDa which were recognized by all sera from pigs vaccinated twice and also by sera from pigs surviving virulent challenge. These bands were not recognized by sera from pre-vaccinates and negative controls (Figures 1 & 2). A band of 59 kDa was frequently recognized by most of the pig sera including pre-vaccinates, vaccinates, and survivors after challenge (Figure 2).

Development of ELISA

Serial dilutions of pig sera from negative control pigs, *S. suis* bacterin-vaccinated pigs, and *S. suis*-hyperimmune pigs were assayed using 3 different antigens of *S. suis* serotype 2 as described above. At a serum dilution of 1:800, absorbance values from *S. suis*-vaccinated and *S. suis*-negative pigs were widely separated when 96-well microtiter plates were coated either with 35 to 50 kDa proteins of *S. suis* serotype 2 (Figure 3) or with heat extracted antigens of *S. suis* serotype 2 (Figure 4). Absorbance values from *S. suis*-vaccinated and *S. suis*-negative pigs were quite similar to each other when plates were coated with washed whole cells of *S. suis* serotype 2 (Figure 5).

ELISA procedures using either 35 to 50 kDa proteins of *S. suis* serotype 2 or heat-extract antigens of *S. suis* serotype 2 for coating plates were compared in their detections

of antibody response in the same group of pigs, before and after immunization, or before and after virulent challenge. Comparison was also made between the vaccinated and non-vaccinated groups of pigs (Tables 2 & 3). At a serum dilution of 1:800, background readings (absorbance value from S. suis-negative pigs) were higher when heat extract antigens were used as coated antigens, and low when special fractions (35 to 50 kDa proteins) were used as coated antigens. IgG antibody titers increased from 0 (prevaccination) to 800 (postvaccination) and 3200 (post challenge) when 35 to 50 kDa proteins of S. suis serotype 2 were used as antigens.

Sensitivity and specificity

Sensitivity of ELISA using 35 to 50 kDa proteins of S. suis serotype 2 as coated antigens was established with sera from 64 pigs which were obtained from infected herds showing clinical signs of S. suis infection and at least one positive culture for S. suis serotype 2. Sera from 2 of the 64 pigs had a S/N (signal to noise) ratio below the 1.5 cutoff value, giving a sensitivity of 97%. Specificity of the ELISA was also determined with sera from another group of 64 pigs, which were obtained from SPF pig herds and conventional pig herds showing no clinical signs and no positive culture for S. suis serotype 2. Sera from 6 of this group of 64 S. suis serotype 2-negative pigs had a S/N ratio above the 1.5 cutoff value, giving a specificity of 91%.

Serological study of baby pigs

Antibody titer of the unvaccinated sow was 0 while the titer of the vaccinated sow was 1600. Piglets from the vaccinated sow had titers of 800 at 2 days, 5 days, and 8 days, and 0 at 11 days and 15 days. Piglets from the unvaccinated sow had titers of 0 at 2 days, 5 days, 8 days, 11 days and 15 days (Table 4).

DISCUSSION

Previous experiments have shown that IgG and immunoglobulin M directed against surface components of S. suis serotype 2 are important in protection of pigs that had been

inoculated with live and inactivated cultures of S. suis serotype 2^{3,6}. It has also been reported that rabbit IgG generated against such cell surface components could passively protect mice against the challenge with reference S. suis serotype 2 strain^{7,8}. While one report indicates that mice injected with cell proteins of 33, 128 and 136 kDa were protected against challenge with the homologous S. suis strain⁸, another paper indicates rabbit serum against 78 and 94 kDa proteins can protect mice against challenge⁷. However, no experiment has been done to identify such bacterial cell surface components by sera from pigs surviving virulent challenge.

In this experiment, we found that 35 to 50 kDa proteins were recognized by all sera from pigs vaccinated twice with Streptococcus Suis Bacterin and also by sera from pigs surviving virulent challenge. These 35 to 50 kDa proteins were not recognized by sera from prevaccinates and negative controls. These data suggest that the 35 to 50 kDa proteins of S. suis serotype 2 may play a role in stimulating protective antibodies in pigs. In a separate experiment, we also found that such 35 to 50 kDa proteins could be identified by sera either from mice immunized with a subunit vaccine containing 35 to 50 kDa proteins or from mice immunized with a S. suis serotype 2 bacterin using Western blot analysis and an indirect ELISA⁹. This further confirms the usefulness of mice for studying S. suis infection in pigs as described before by other authors^{10,11}. This may indicate the feasibility of developing a standardized in-vivo serological test to compare the antibody titers induced by two or more S. suis bacterins. Further experiments using mice as an animal model for pigs will be needed.

Test results in this experiment indicate that it is difficult to get reliable results with an indirect ELISA using formalinized whole culture of S. suis serotype 2 for coating of plates. This is consistent with a previous report³.

In order to confirm a strong correlation between protection of pigs vaccinated twice with S. suis bacterin and the presence of antibody titers of 800 or higher against the specific

proteins (35 to 50 kDa) of S. suis serotype 2, an indirect ELISA using 35 to 50 kDa proteins for coating of plates was developed. The present study has shown an acceptable sensitivity and specificity of the ELISA, using 35 to 50 kDa proteins of S. suis for coating plates. In the present study, a heat extract antigen of a whole cell preparation of S. suis serotype 2 was also used to develop an indirect ELISA. Test results showed that the ELISA using 35 to 50 kDa proteins for coating plates gave lower background readings than that using heat extract antigens for coating plates.

Due to the present lack of knowledge about the specific immunogenic components of S. suis serotype 2, it is still premature to adopt such an ELISA for a routine S. suis serology study. However, data from the present study show that a specific antibody response against S. suis can be measured by an indirect ELISA. Further use of such ELISA for determining the protection levels in pigs after immunization will need further study.

It is known that pigs are colonized with S. suis before 15 days of age and that such colonization is largely responsible for S. suis disease¹. Therefore, it may be necessary to immunize sows with S. suis bacterin to provide maternal protection for young pigs on farms adopting the SEW system. However, there are no reported data about the level of maternal antibody titers in young pigs shortly after birth and before 15 days of age. In this experiment we found the titer of antibodies against 33-50 kDa proteins of S. suis serotype 2 was 0 for an unvaccinated sow and its offspring, 1600 for a vaccinated sow when pigs were farrowed, 800 when pigs from a vaccinated sow were 2 days, 5 days and 8 days of age, and 0 when pigs from a vaccinated sow were 11 days and 15 days of age. In order to study the maternal protection of young pigs on farms using a SEW system, further experiments are needed.

ACKNOWLEDGMENTS

We thank J. McGonigle for reviewing the manuscript and B. Brotherton for assistance with the manuscript preparation.

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Figure 1 Western blot of lysed *S. suis* serotype 2 probed with negative pig serum (lanes 1,2), pre vaccinated pig serum (lanes 4,5), and vaccinated pig serum (lanes 7,8). Lanes 3 and 6 are low range molecular weight markers (106 kDa, 80 kDa, 50 kDa, 35 kDa, 28 kDa and 20 kDa). Lane 9 is a high range marker (205 kDa, 117 kDa, 80 kDa and 50 kDa).

Figure 2 Western blot of lysed S. suis serotype 2 probed with serum from unvaccinated control pig (lane 1), post challenge control pig (lane 3), pre vaccinated pig (lane 4), twice vaccinated pig (lane 6), and vaccinated pigs after challenge (lane 8). Lane 2 is high range marker and lane 9 is low range marker.

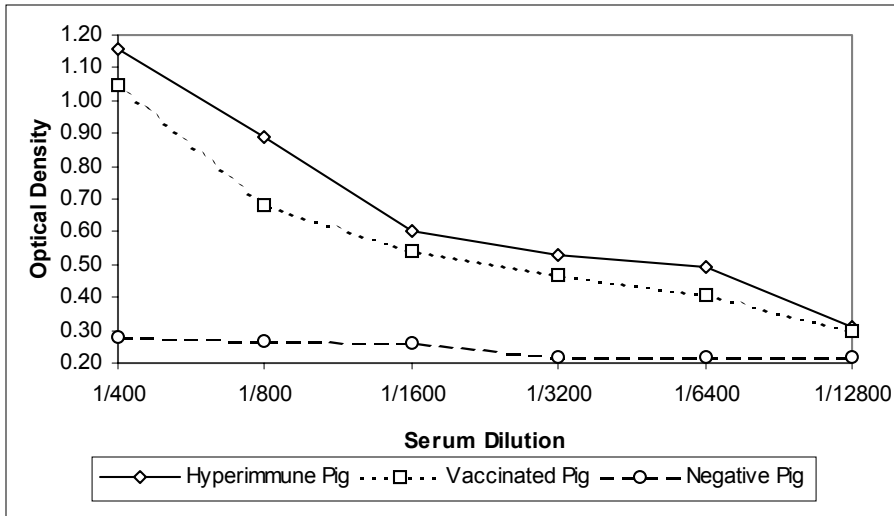


Figure 3 Serial dilutions of pig sera from *S. suis* bacterin-vaccinated pigs, and *S. suis* hyperimmune pig were compared to those from *S. suis*-negative pig by ELISA using special fractions of *S. suis* as antigens.

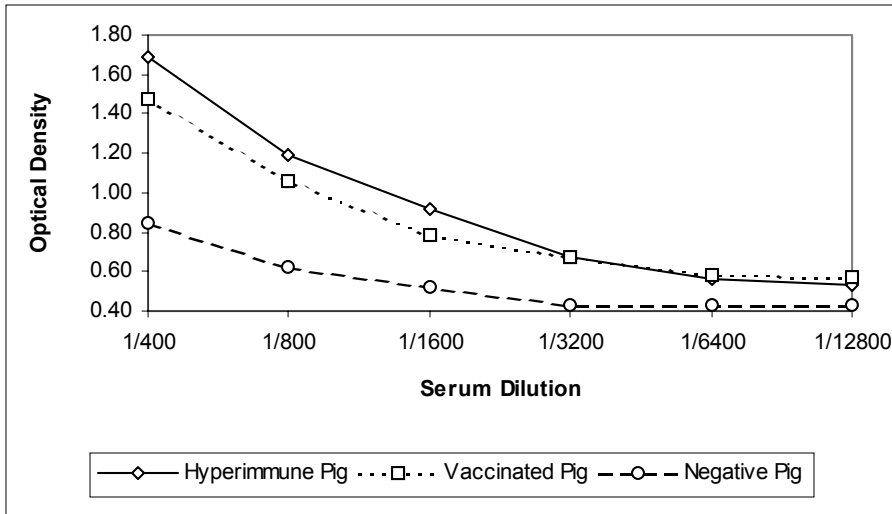


Figure 4 Serial dilutions of pig sera from *S. Suis* Bacterin-vaccinated pigs, and *S. suis* hyperimmune pig were compared to those from *S. suis*-negative pig by ELISA using heat-extracted antigens of *S. suis*.

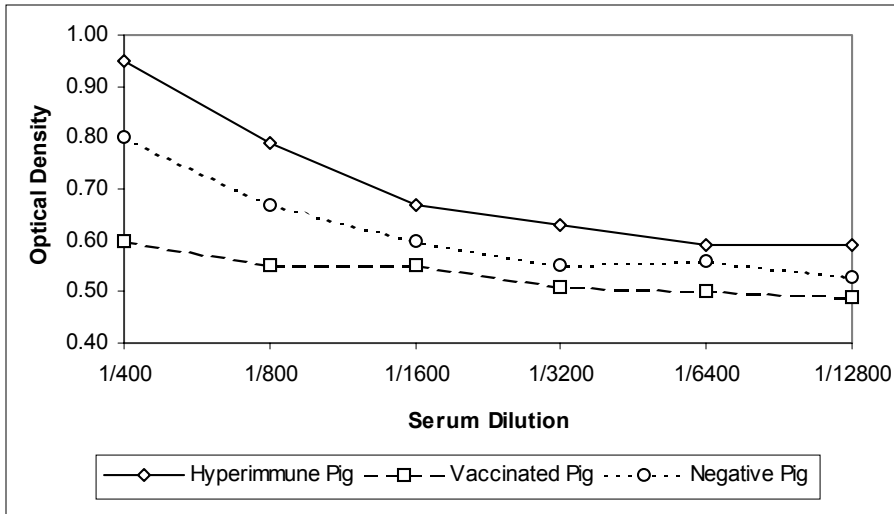


Figure 5 Serial dilutions of pig sera from *S. Suis* Bacterin-vaccinated pigs, and *S. suis* hyperimmune pig were compared to those from *S. suis*-negative pig by ELISA using washed whole cells of *S. suis*.

Table 1 Scores of clinical signs, necropsy lesions, histopathology and mortality after challenge of both vaccinates and nonvaccinated pigs with virulent *S. suis* serotype 2.

Test Animals	Clinical Signs	Necropsy Lesions	Histopathology Lesions	Cumulative Score
Vaccinates				
1	5	1	0	6
2	2	0	0	2
3	3	0	0	3
4	4	0	0	4
5	0	0	0	0
6	1	0	1	2
7	6	0	2	8
8	4	0	1	5
9	7	0	2	9
10	6	0	0	6
11	0	0	0	0
12	0	0	0	0
13	5	0	0	5
14	2	0	0	2
15	3	2	0	5
16	1	0	0	1
17	0	0	0	0
18	7	0	0	7
19	11	0	0	11
20	2	0	0	2
			Mean cumulative score: ^a	3.9
Controls:				
21	41	3	0	44
22	64	3	2	69
23	69	3	2	74
24	28	0	0	28
25	33	2	0	35
26	52	3	0	55
27	59	3	0	62
28	51	1	2	54
29	36	2	0	38
30	50	1	0	51
			Mean Cumulative Score: ^a	51

^a P <0.05, one-tailed Student's t-Test

Table 2 Absorbance and antibody titers obtained with pooled sera from *S. Suis* Bacterin-vaccinated and non-vaccinated pigs by ELISA using a specific fraction (35-50kD) of *S. suis* serotype 2 as antigens

Group	Dilution of Pig Serum										Titer ^c
	1:800		1:1600		1:3200		1:6400		1:12,800		
	OD ^a	S/N ^b	OD	S/N	OD	S/N	OD	S/N	OD	S/N	
Vaccinates											
Pre-vaccination	0.29	1.0	0.26	0.9	0.20	1.0	0.27	1.0	0.26	0.9	0
After 1st vaccination	0.49	1.8	0.33	1.2	0.32	1.1	0.29	1.0	0.26	0.9	800
After 2nd vaccination	0.57	2.0	0.39	1.4	0.34	1.2	0.29	1.0	0.26	0.9	800
Post-challenge	1.14	4.1	0.75	2.7	0.53	1.9	0.35	1.3	0.29	1.0	3200
Controls											
Pre-challenge	0.27	1.0	0.30	1.1	0.25	0.9	0.23	0.8	0.22	0.8	0
Post-challenge	1.42	5.1	0.80	2.9	0.53	1.9	0.48	1.7	0.40	1.4	6400
Positive control ^d	0.96	3.4	0.54	1.9	0.44	1.6	0.35	1.3	0.31	1.1	3200
Negative control ^e	0.28		0.27		0.26		0.22		0.27		0

^a Optical density at 405 nm

^b OD test / OD negative control

^c Reciprocal of the lowest serum dilution having a S/N of 1.5 or larger.

^d Hyperimmune pig serum

^e Normal pig serum

Table 3 Absorbance and antibody titers obtained with pooled sera from *S. Suis* Bacterin-vaccinated and non-vaccinated pigs by ELISA using heat-extract antigens of *S. suis* serotype 2

Group	Dilution of Pig Serum										Titer ^c
	1:800		1:1600		1:3200		1:6400		1:12,800		
	OD ^a	S/N ^b	OD	S/N	OD	S/N	OD	S/N	OD	S/N	
Vaccinates											
Pre-vaccination	0.36	0.6	0.36	0.6	0.40	0.7	0.41	0.7	0.42	0.7	0
After 1st vaccination	0.70	1.1	0.58	0.9	0.53	0.9	0.48	0.8	0.47	0.8	0
After 2nd vaccination	1.06	1.7	0.79	1.3	0.67	1.1	0.58	0.9	0.57	0.9	800
Post-challenge	0.94	1.5	0.79	1.3	0.65	1.0	0.55	0.9	0.54	0.9	800
Controls											
Pre-challenge	0.62	1.0	0.47	0.8	0.42	0.7	0.42	0.7	0.45	0.7	0
Post-challenge	0.99	1.6	0.78	1.3	0.63	1.0	0.55	0.9	0.55	1.9	800
Positive control ^d	1.19	1.9	0.92	1.5	0.67	1.1	0.56	0.9	0.53	0.9	1600
Negative control ^e	0.62		0.52		0.43		0.43		0.45		0

^a Optical density at 405 nm

^b OD test / OD negative control

^c Reciprocal of the lowest serum dilution having a S/N of 1.5 or larger.

^d Hyperimmune pig serum

^e Normal pig serum

Table 4 Antibody titers obtained with pooled sera from baby pigs at 2 - 15 days of age by ELISA using 35 to 50 kDa proteins of S. suis as antigens

Age of Pig (Days)	Titers ^a of Pooled Sera from 8 Baby Pigs Farrowed by Unvaccinated Sow	Titers of Pooled Sera from 8 Baby Pigs Farrowed by Vaccinated Sow
2	0	800
5	0	800
8	0	800
11	0	0
15	0	0
Unvaccinated Sow	0	NA
Vaccinated Sow	NA	1600

^a Reciprocal of the lowest serum dilution having a S/N of 1.5 or larger.