IMMUNOGLOBULIN G RESPONSE OF STREPTOCOCCUS SUIS BACTERIN – VACCINATED PIGS

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Abstract

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.

***Key words*:** *Streptococcus suis, immunoglobulin G*

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. 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.

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.

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All 30 pigs were challenged with a 1.0 ml intravenous dose containing 107 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 7 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	Temperature Range
0	< 103.0oF
1	103.0 to 103.9oF
2	104.0 to 104.9oF
3	=> 105.0oF

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 107 CFU of washed, formalin-inactivated *S. suis* for 3 months. The hyperimmune sera was aliquoted and stored at -20^{o}C. Non-reactive sera from 10 SPF pigs were also stored at - 20^{o}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 (4oC) Lysis Buffer (0.1M Tris pH 8.0, 0.8 mg EDTA, 1 mg lysozyme) for 5 minutes. Approximately 240 µl 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 µl, H2O: qs. 7 ml) and heated to 100oC for 1 minute. The SDS-PAGE (7.5%) gels were prepared and 20 µl of the heated sample was loaded on each well of 2 gels and 10 µl 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 l 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 5oC 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 50 µl (6.25 ug/well) of the diluted antigen. The plates were air dried overnight at room temperature. Plates were then filled with 50 µl 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 µl 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 µl 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 µl 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 µl 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 107 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 µl 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) µl 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 x 107 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. A band of 59 kD was frequently recognized by most of the pig sera including pre-vaccinates, vaccinates, and survivors after challenge.

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. or with heat extracted antigens of *S. suis* serotype 2. 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.

ELISA procedures using either 35-50 kD proteins of *S. suis* serotype 2 or heatextract 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 nonvaccinated 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.

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 Vaccinates	Clinical Cumulative Signs	Necropsy Lesions	Histopathology Lesions	Score
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	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: 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

	Dilution of Pig Serum										
	1:800		1:1600		1:3200		1:6400		1:12800		Antibody Titer
Group	OD	S/N	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
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

	Dilution of Pig Serum										
	1:800		1:1600		1:3200		1:6400		1:12800		Antibody Titer
Group	OD	S/N	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

Continuing table 3

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
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 *S. suis* 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.

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