

Antibody response of young pigs to autogenous *Haemophilus parasuis* vaccine

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Introduction

Infection of immune-naïve pigs by *Haemophilus parasuis* has become one of the most significant swine diseases in the past few years. Good, homologous protection against some strains of *H parasuis* in pigs vaccinated with an autogenous vaccine has been reported,^{1,2} and the early vaccination of young pigs with an autogenous *H parasuis* vaccine has been adopted in many high health status pig farms. However, due to a lack of an appropriate serological test, an evaluation of the antigenicity of the autogenous vaccine and the antibody response in vaccinated pigs is seldom done on most pig farms. The published data about the serologic profile of pigs vaccinated against *H parasuis* is very limited. In 1991, Miniats et al had indicated that their attempts to detect the presence of specific antibodies against *H parasuis* strains in the sera of the vaccinated or exposed pigs by the passive hemagglutination test or by ELISA were unsuccessful.

The antigens employed in Miniats' ELISA serology were either supernatants from boiled bacteria or dialyzed hot phenol water extracts of the *H parasuis*.³ Although Tadjine et al recently described a protocol to screen for mouse monoclonal antibodies against *H parasuis* by ELISA using whole cell suspension, boiled cell suspension, and sonicated cell suspension as the coated antigens,⁴ a standardized ELISA that can be used to evaluate the antibody response in vaccinated pigs still needs to be developed.

The aim of this study was to use an indirect enzyme-linked immunosorbent assay (ELISA) developed at MVP Laboratories (Omaha, NE) to evaluate the antigenicity of four different *H parasuis* field strains used in autogenous vaccines through the determination of specific antibody titer in individual vaccinated pigs. This protocol may also be used to monitor the time of infection with *H parasuis* in commercial pig farms and help swine veterinarians determine the optimal time of vaccination.

Materials and methods

Test vaccines: Five autogenous vaccines, each containing one of the five *H parasuis* isolates (isolate #6204803, serovar 7; #6204536, serovar 13; #6205360, serovar 2; #6204553, serovar 4; and #6200077, sero-nontypeable) were prepared at MVP Laboratories. Each *H parasuis* culture was inactivated with formalin and adjuvanted with 12% Emulsigen (MVP Laboratories) and 4% Rehydrigel.

A standardized vaccination study: Twenty five 18-day-old pigs, testing seronegative for *H parasuis*, were purchased from a high health status pig farm with no history of *H parasuis* infection. These pigs were weaned at 18 days of age and moved to the University of Nebraska Veterinary Research facility in Lincoln, Nebraska. The pigs were individually identified with a unique ear number. The pigs were allowed to acclimate to the new surroundings for 14 days and then were randomly assigned to one of the five groups. The test vaccines and group assignments were: 1) no vaccine for the untreated controls; 2) bacterin prepared from *H parasuis* serovar 7, isolate 6204803; 3) bacterin prepared from *H parasuis* serovar 13, isolate 6204536; 4) bacterin prepared from *H parasuis* serovar 2, isolate 6205360; and 5) bacterin prepared from *H parasuis* serovar 4, isolate 6204553. On day 0, a blood sample was collected from all pigs and each pig from groups 2, 3, 4, and 5 was vaccinated with a 2.0 ml dose of one of the four autogenous vaccines, subcutaneously. They were vaccinated again at day 21. On day 42, day 63, and day 84, all pigs were bled again. The serum was separated by centrifugation and stored at -20°C before use.

A field vaccination study: Ten 14-day old pigs were selected from a commercial pig farm that had outbreaks of *H parasuis* infection. An autogenous vaccine was prepared using a sero-nontypeable strain of *H parasuis* (#6200077) isolated from the same farm. Each pig was individually identified with a unique ear tag number. On day 0, a blood sample was collected from all of the ten pigs and all of the pigs were vaccinated with a 2 ml

dose of the autogenous *H parasuis* vaccine subcutaneously. They were vaccinated again on day 14. On day 14 and day 35, all of the ten pigs were bled again. The serum was stored at -20°C before use.

Enzyme-linked immunosorbent assay (ELISA): Antibodies directed against seven commonly seen serotypes of *H parasuis* (serovar 2, 4, 5, 7, 12, 13 and 14) in the US⁵ were detected by use of an indirect ELISA. Antibodies against the vaccine strain were also detected using the plate coated with the soluble proteins obtained from the vaccine strain. Briefly, each of the seven standard strains as well as the five field isolates of *H parasuis* were grown on Frey Chocolate agar plates and harvested into sterile PBS (pH 7.2). The washed bacterial cells were treated with CellLytic reagent (Sigma Chemical Co.) and the soluble protein antigens obtained from each isolate or standard strains were adjusted to 1 mg/ml using sterile PBS. A negative control antigen was made in the same way. Each well of a 96-well plate (Immulon 2, Dynatech) was either coated with 2 µg of the mixed soluble antigens obtained from *H parasuis* isolates as positive antigens (PA) or 2 µg of the negative control antigen (NA). Some plates were coated with the soluble proteins obtained from a single field isolate as the homologous antigens. Both the test sera and control sera were first diluted to 1:200 using a dilution buffer (PBS Tween buffer with 0.5% BSA) and two fold serial dilutions were made from there. A rabbit antiserum against all seven commonly seen serovars (serovar 2, 4, 5, 7, 12, 13, 14) of *H parasuis* was used as the positive control and a pig serum from an unexposed pig was used as a negative control. Each well of the plate was blocked with 50 µl of a blocking agent (PBS buffer with 1% BSA) for 30 minutes. After washing with PBS-Tween buffer 3 times, each diluted test serum sample and control serum sample was dispensed into two wells of the PA and two wells of NA in an amount of 50 µl per well. The plate was incubated at 37°C for 90 minutes. After washing with PBS-Tween buffer, 50 µl of alkaline phosphatase labeled goat anti-pig IgG (KPL Laboratories), diluted at 1:100, was added to each well of the plate containing pig serum. Fifty microliters of alkaline phosphatase labeled goat anti-rabbit IgG (KPL Laboratories), diluted at 1:200, was added to each well of the plate containing rabbit serum. The plate was incubated at 37°C for 90 minutes. After washing, 100 µl of a chromagen containing phosphatase substrate (Sigma Chemical) was added to each well of the plate and incubated at room temperature for thirty minutes. The optical density (OD) was measured at 405 nm. As the OD reading of the positive control serum (1:200)

reached around 1.50, the color reaction was stopped by adding 50 µl of a 5N sodium hydroxide to each well and the plate was read at 405 nm. The average OD of the NA for each test sample was subtracted from the average OD of PA of the same test sample and was used for evaluation of its antibody titer. All of the serum samples showing larger than 50% of the OD value of the positive control were considered sero-positive (S/P > 0.5). The antibody titer was reported as the reciprocal of the last positive dilution. For the assay to be valid, the adjusted OD readings of the positive control serum must be around 1.50 and the negative control must be around 0.20 at 1:200 dilution. Any serum sample that had a titer equal to or less than 400 was considered to be sero-negative against *H parasuis* in this assay system.

Demonstration of an acceptable antigenicity of the vaccine strains: In order to evaluate the antigenicity of the *H parasuis* strain used in an autogenous vaccine, an arbitrary criterion was set as follows: If on day 42 at least 80% of the pigs from a vaccinated group could demonstrate at least a four fold increase in antibody titer over the pre-vaccinated serum samples, the antigenicity of that *H parasuis* strain used to test in that group would be acceptable. The negative control pigs should remain sero-negative throughout the whole test period.

Statistical method: Two-tailed Student's t test was conducted to determine the statistical significance of the serological data obtained from a standardized vaccination study and a field vaccination study. *P* values below 0.05 were considered statistically significant.

Results

Serologic evaluation for a standardized vaccination study: A summary of the titer of each of the 25 pigs is shown in Table 1 and Figure 1. In order to calculate the increase in antibody titer from day 0 to day 42 (conversion factor), pigs showing titers of < 200 were considered to be 200. If a pig was found to have a titer < 200 on day 0 and 800 on day 42, it would have a conversion factor of 4. It was found that all pigs were sero-negative against *H parasuis* on day 0. Each of the pigs in the non-vaccinated group (group 1) remained sero-negative throughout the study. At least 4 out of 5 pigs (80%) in each vaccinated groups sero-converted by day 42, exhibiting at least a four-fold increase in titer over day 0. On day 35, a pig from group 2 developed an umbilical hernia and was sold on day 42. Either on day 63 (six weeks after second vaccination) or day 84 (nine weeks after second vaccination), 13 out of 19

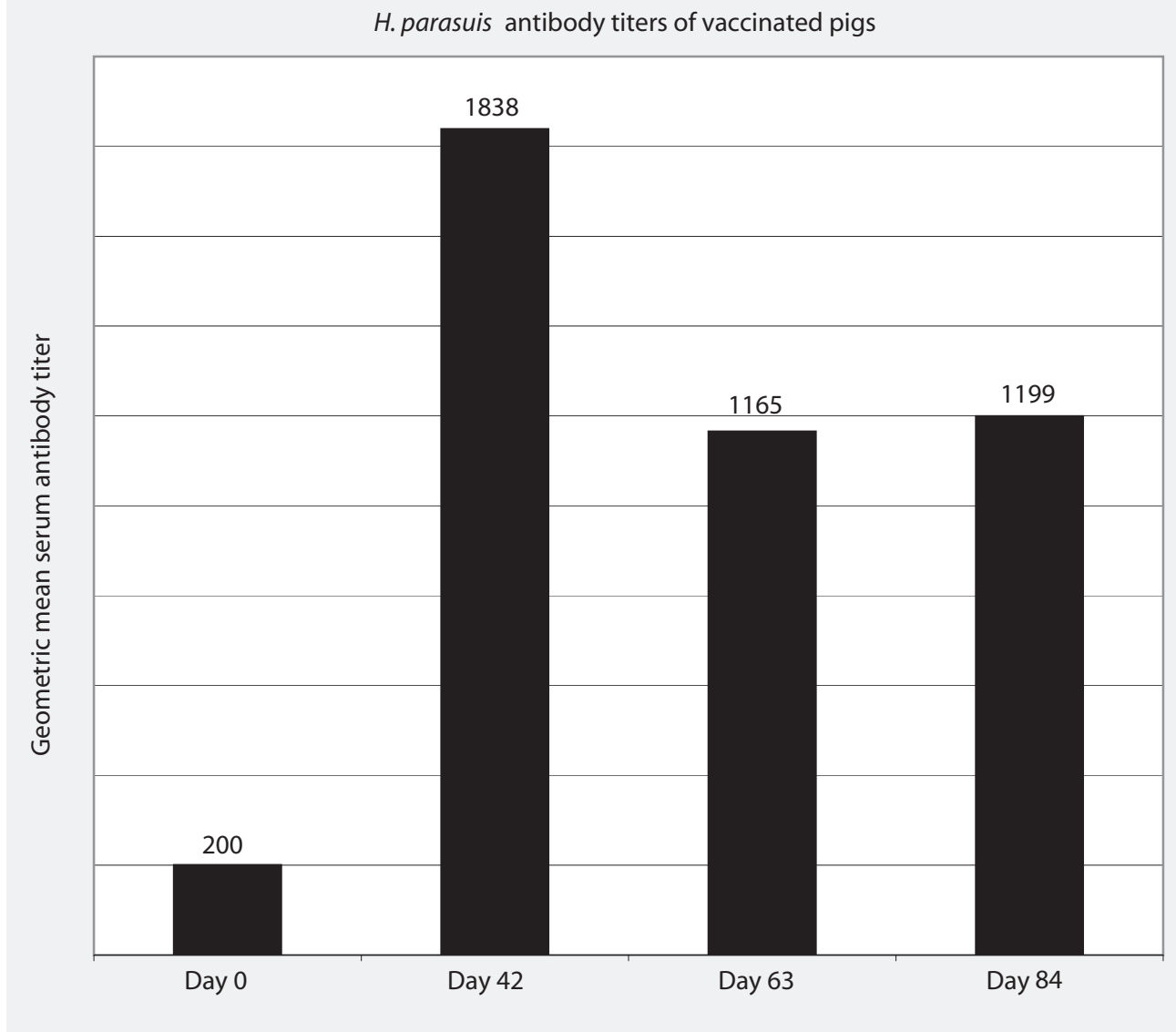
Table 1: Serum antibody titers and conversion factors for each pig in a standardized vaccination study using ELISA plates coated with homologous antigens.

Group	Pig ID	Day 0 titer	Day 42 titer	CF	Day 63 titer	CF	Day 84 titer	CF
1	1	< 200	< 200	1	< 200	1	< 200	1
1	2	< 200	< 200	1	< 200	1	< 200	1
1	3	< 200	< 200	1	< 200	1	< 200	1
1	4	< 200	< 200	1	< 200	1	< 200	1
1	5	< 200	< 200	1	< 200	1	< 200	1
2	6	< 200	6400	32	6400	32	800	4
2	7	< 200	6400	32	6400	32	6400	32
2	8	< 200	200	1	< 200	1	< 200	1
2	9	< 200	6400	32	400	2	400	2
2	10	< 200	6400	32	200	1	200	1
3	11	< 200	3200	16	400	2	400	2
3	12	< 200	6400	32	800	4	800	4
3	13	< 200	6400	32	ND		ND	
3	14	< 200	400	2	800	4	6400	32
3	15	< 200	6400	32	6400	32	6400	32
4	16	< 200	6400	32	6400	32	6400	32
4	17	< 200	6400	32	6400	32	6400	32
4	18	< 200	6400	32	3200	16	3200	16
4	19	< 200	200	1	< 200	1	400	2
4	20	< 200	6400	32	6400	32	6400	32
5	21	< 200	6400	32	6400	32	6400	32
5	22	< 200	6400	32	6400	32	6400	32
5	23	< 200	6400	32	6400	32	6400	32
5	24	< 200	200	1	400	2	400	2
5	25	< 200	6400	32	6400	32	6400	32
Geometric mean		< 200	1838		1165		1199	

CF = Conversion factor

ND = Not done

Figure 1: Geometric mean antibody titers of pigs vaccinated with autogenous *H parasuis* vaccines in a standardized vaccination study.



vaccinated pigs (68.4%) still showed at least a four-fold increase in titers over day 0. This serologic study indicated that the antigenicity of the four autogenous vaccine strains of *H parasuis* was acceptable.

Serologic evaluation for a field vaccination study:

The ELISA titers for anti- *H parasuis* antibodies are shown in Table 3. Three out of ten 2-week old pigs were found to be sero-positive and had high antibody titer against *H parasuis* before vaccination. Two weeks after the first vaccination, ELISA titers for all ten pigs remained unchanged on day 14. Three weeks after two vaccinations, eighty percent of the ten vaccinated pigs reached a high ELISA titer of 6400.

Discussion

In a standardized vaccination study, the geometric mean of anti-*H parasuis* antibody titers of 20 vaccinated pigs showed a significant ($P < 0.01$) increase from day 0 to day 42, day 63, and day 84 (Table 1). The level of specific antibodies against *H parasuis* increased significantly ($P < 0.01$) on day 42 in vaccinated pigs as compared with non-vaccinated control pigs and this tendency persisted for a minimum of 9 weeks after two vaccinations. Although the antibody titers of the vaccinated pigs obtained from the ELISA using homologous antigens were significantly ($P < 0.05$) larger than the titer of the same serum obtained from the ELISA

Table 2: Determination of serum antibody titers using ELISA plates coated with either homologous or heterologous antigens in a standardized vaccination study.

Pig ID	Homologous antigen		Heterologous antigen	
	Titer		Titer	
	Day 0	Day 42	Day 0	Day 42
6	<200	6400	<200	1600
7	<200	6400	<200	3200
8	<200	200	<200	400
9	<200	6400	<200	800
10	<200	6400	<200	800
21	<200	6400	<200	6400
22	<200	6400	<200	6400
23	<200	6400	<200	800
24	<200	200	<200	200
25	<200	6400	<200	6400
Geometric mean	<200	3200	<200	1493

Table 3: Antibody titers and conversion factors for young pigs vaccinated with an autogenous *H parasuis* vaccine at a commercial pig farm.

Pig ID	Day 0	Day 14		Day 35	
	(2 weeks old)	(4 weeks old)		(7 weeks old)	
	Titer	Titer	CF	Titer	CF
1	200	200	1	6400	32
2	6400	6400	1	6400	1
3	6400	6400	1	6400	1
4	6400	6400	1	6400	1
5	200	200	1	6400	32
6	200	200	1	6400	32
7	200	200	1	200	1
8	200	200	1	6400	32
9	200	200	1	6400	32
10	200	200	1	200	1
Geometric mean	566	566		3200	

using heterologous antigens (Table 2), antibody titers obtained from plates coated with either homologous or heterologous antigens all indicated that 80% of the vaccinated pigs sero-converted by day 42. Due to a high complexity in the antigenic structures of *H parasuis* field strains, ELISA plates coated with homologous antigens significantly increase the sensitivity of the assay system. However, ELISA plates coated with soluble antigens extracted from 7 commonly seen *H parasuis* serovars (heterologous ELISA) have been tested in this study and demonstrate the ability to show the same trend of antibody increase in vaccinated pigs as the homologous ELISA.

In a field vaccination study, the geometric mean of anti-*H parasuis* titer increased significantly ($P < 0.05$) on day 35 in pigs after 2 vaccinations as compared with the same pigs before vaccination. In this study, three out of ten test pigs were sero-positive against *H parasuis* before vaccination on day 0 when they were 2 weeks old. These 3 pigs might carry maternal antibodies from vaccinated sows or infected sows. Even after one or two vaccinations, all three pigs still showed the same titer of 6400 on day 14 and day 35. It appears that serum antibody titers in these 3 pigs were not interfered by maternal antibodies and their titers did not drop down during the five weeks period after first vaccination. Among the 7 pigs that showed sero-negative before vaccination, a sero-conversion could be seen in 5 pigs on day 35, while none of the 7 pigs became sero-positive on day 14. One vaccination may not be enough to induce sero-conversion in young pigs.

In the standardized vaccination study, four vaccinated pigs did not show any sero-conversion on day 42 (3 weeks after second vaccination). Among them, only one pig

became sero-positive on day 63 and day 84. The other 3 pigs did not show any sero-conversion from day 42 through day 84. Two out of the ten pigs in a field vaccination study also did not show sero-conversion from day 14 through day 35. This may imply that perhaps 20% of the young pigs will not respond to the vaccination with *H parasuis* vaccines. Further study is needed to search for the causes of the inability to have antibody response to *H parasuis* vaccines in some percentage of younger pigs.

In this study, it has been demonstrated that ELISA using soluble proteins obtained from homologous *H parasuis* strains as coated antigens can be used for the investigation of antibody titers in young pigs after two vaccinations. This serologic study can also be used for the evaluation of the antigenicity of bacterial isolates used in *H parasuis* vaccines.

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