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# Immunization of Suckling Pigs Against Enterotoxigenic *Escherichia coli*-Induced Diarrheal Disease by Vaccinating Dams with Purified K99 or 987P Pili: Antibody Production in Response to Vaccination

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Pilus-specific antibody levels measured by enzyme-linked immunosorbent assays in serum and colostrum of pregnant swine (dams) were shown to increase after parenteral vaccination with pili. Pilus-specific antibody levels in dams were correlated with protection of their suckling offspring against fatal diarrhea caused by enterotoxigenic *Escherichia coli* possessing the same pilus as the vaccine.

Adhesion of enterotoxigenic *Escherichia coli* (ETEC) to the small intestines of pigs is mediated by specific pili. ETEC that possess either K88, K99, or 987P pili can adhere to the mucosal surfaces of porcine small intestines (4, 5, 8, 11). Adhesion by ETEC facilitates their colonization of small intestines, thus fulfilling one step in the pathogenesis of ETEC-induced diarrheal disease.

The use of pili as vaccines to prevent ETEC-induced diarrheal disease in farm animals has been well documented (1, 6, 9, 10). Suckling offspring from dams that were vaccinated parenterally with purified pili were protected against severe (fatal) diarrheal disease when challenged with ETEC producing the same pilus as in the vaccine (homologous pilus). However, protection against disease by ETEC producing a different pilus (heterologous pilus) did not occur. It was assumed that protection by pilus vaccines was obtained by consumption of maternal colostrum containing antibodies specific for the vaccine pilus. The precise mechanism of protection is not known but is believed to be by interference with ETEC adhesion.

We previously reported on the protection of offspring from dams that had been vaccinated parenterally with either K99, 987P, or saline (9). We now report on the correlation between pilus-specific antibody levels in maternal serum and colostrum samples, obtained in that experiment, and protection.

Pregnant swine (gilts) were bled just before each of two vaccinations (vaccinations were subcutaneous and 11 days apart) with K99, 987P, or saline and just before challenge of the offspring with ETEC (5 to 17 days after the second vaccination). At the time of collection of the third blood sample, colostrum was also collected. Blood samples were allowed to clot for 16 to 24 h, and the sera were separated by centrifugation. Sera were filtered through 0.45-μm-pore size filters and stored at −70°C. The colostrum samples were centrifuged at 95,000 × *g* for 1 h at 4°C, and the fluid whey was filtered through 0.45-μm filters and stored at −70°C.

Pilus-specific antibody titers in serum and colostrum were determined using enzyme-linked immunosorbent assays (ELISA). For anti-987P, 100 μl of purified 987P (10; manuscript in preparation) (100 μg/ml in phosphate-buffered saline [PBS]: 0.01 M sodium phosphate, pH 7.3; 0.85% sodium chloride) was added per well to polyvinyl chloride microtiter plates (Cooke) and incubated at 37°C for 2 h and then overnight at 4°C. The coated wells were washed three times with PBS (3 min per wash) and then filled with 1% bovine serum albumin in PBS. After 30 min at 37°C, the wells were washed three times with PBS-Tween 20 (0.05% Tween 20). Serial twofold dilutions of test serum or colostrum were prepared in PBS-Tween 20, and 100 μl was added per well. Each dilution was run in duplicate. Serum controls run daily included: (i) pig serum obtained from an animal that had not been vaccinated with pili and that did not cause piliated (K99 or 987P) ETEC to agglutinate (normal pig serum), and (ii) a serum sample known to have anti-987P antibodies (standard anti-987P serum). The normal pig serum was used at a dilution of 1:100 and served as a negative control. Twenty wells per day always contained normal pig serum. The standard anti-987P serum was titrated daily in the ELISA and served as a standard to correct for minor daily variations and as a check on the validity of each day's tests. After 2 h of incubation at 37°C, the wells were washed three times with PBS-Tween 20. Rabbit anti-pig serum

(Miles) with alkaline phosphatase (Sigma) conjugated to it (2) was diluted 1:2,000 in PBS-Tween 20, and 100 μl was added to each well. The plates were incubated for 2 h at 37°C, then washed four times with PBS-Tween 20, and 100 μl of the substrate *p*-nitrophenyl phosphate (Sigma; 1 mg/ml in 0.05 M $Na_2CO_3$; 1 mM $MgCl_2$, pH 9.8) was added to each well. After 30 min at room temperature, the reactions were stopped by the addition of 150 μl of 0.33 N NaOH. Samples were read spectrophotometrically at 400 nm by using a Gilford Stasar II spectrophotometer fitted with a 200-μl aspirating microcuvette. The anti-K99 ELISA was the same with the following exceptions: (i) polystyrene microtiter plates were used (Cooke), (ii) purified K99 (3) was used instead of 987P, and (iii) the standard anti-K99 serum was used instead of the standard anti-987P serum. The endpoint of a serum or colostrum titration was the reciprocal of the highest dilution yielding an absorbancy at 400 nm of 0.1 higher than the mean negative control (normal pig serum) reading. A difference of 0.1 unit of absorbancy at 400 nm over the normal pig serum is greater than 2 standard deviations from the mean negative control values. Endpoint values were multiplied by the number necessary to adjust the standard anti-987P serum endpoint to the same value as was established during preliminary tests (six titrations of standard anti-987P serum). Thus, endpoints determined on different days could be directly compared.

The data published by Morgan et al. (9) showed a correlation between vaccination with pili and protection against ETEC-induced diarrheal disease by strains possessing the homologous pilus (disease was measured as deaths). Only 1 pig of 116 (0.86%) died in litters that were expected to be protected, whereas 54 pigs of 223 (24.2%) died in litters that were not expected to be protected. Table 1 is a summary of the maternal serum and colostrum responses to vaccination. Vaccination with K99 caused increases in both serum and colostrum antibodies against K99 but not against 987P. Conversely, vaccination with 987P caused increases in serum and colostrum antibodies against 987P but not against K99. In one dam, after two vaccinations with 987P, high titers of anti-K99 antibodies were also detected in both serum (2,560) and colostrum (1,280). The reason for this unexpected response to K99 is not known.

It can be concluded that parenteral vaccination of pregnant gilts with pili leads to the production of pilus-specific antibodies in both serum and colostrum. Since transplacental immunity does not normally occur in pigs, the correlation between high titers of antibodies in maternal colostrum against the pilus used in the vaccine and protection of offspring leads to the conclusion that protection was a result of consumption of pilus-specific antibodies in colostrum.

In a comparison of antibodies produced against K99 or 987P in response to the first and second vaccinations with K99 or 987P, respectively (i.e., a comparison of sera from the second and third bleedings), it was found that a statistically significant increase in pilus-specific antibody titers resulted from the second vaccination. The differences were statistically significant at $P \leq 0.0001$ for K99 and at $P \leq 0.001$ for 987P by Student's *t* test. However, this statistical result is misleading. Nine of 16 pigs vaccinated with K99 and 10 of 17 pigs vaccinated with 987P showed either a twofold rise, no rise, or a twofold decrease in antibody titers specific for the pilus in the vaccine after the second vaccination (Table 2).

The observation that greater than 50% of all the pigs vaccinated in this experiment with pili had a secondary immune response after a single injection is consistent with the notion that these animals had been previously exposed to the pilus antigens. This is not surprising since the animals used were conventionally reared. ETEC are widely distributed, and thus the animals would be occasionally exposed to these antigens. In support of this, it was observed that, of those

TABLE 1. *Summary of the pilus-specific serum and colostrum response to vaccination as measured by ELISA*

| Vaccine | No. of gilts | Geometric mean of anti-987P titers (range) | | | | Geometric mean of anti-K99 titers (range) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 1st bleeding | 2nd bleeding | 3rd bleeding | Colostrum | 1st bleeding | 2nd bleeding | 3rd bleeding | Colostrum |
| K99 | 15 | <3.36 (<1–8) | <3.83 (<1–16) | <3.51 (<1–8) | <3.83 (<1–16) | <4.58 (<1–64) | 299.8 (80–5,120) | 3,910.2 (320–20,480) | 11,188.3 (2,560–81,920) |
| 987 | 16 | <4.38 (<1–8) | <96.21 (<4–1,024) | 266.6 (64–1,024) | 400.8 (64–2,048) | <4.2 (<2–32) | <3.84 (<2–8) | <5.68 (<1–2,560) | <6.37 (<1–1,280) |
| Saline | 15 | <3.17 (<2–4) | <3.99 (<2–64) | <3.48 (<2–16) | <4.31 (<2–64) | <4.19 (<1–8) | <3.32 (<1–4) | <4.75 (<1–32) | <5.65 (<1–64) |

TABLE 2. *Serum antibody response of individual gilts to vaccination with pili as measured by ELISA*

| Vaccine | Gilt no. | Serum antibody titer to vaccine pilus[a] | | |
|---|---|---|---|---|
| | | 1st bleeding | 2nd bleeding | 3rd bleeding |
| K99 | 1 | 2 | 320 | 1,280 |
| | 2 | 2 | 1,280 | 10,240 |
| | 3 | 8 | 2,560 | 5,120 |
| | 4 | 2 | 2,560 | 5,120 |
| | 5 | 8 | 5,120 | 5,120 |
| | 6 | 64 | 5,120 | 5,120 |
| | 19 | 16 | 2,560 | 5,120 |
| | 20 | 8 | 640 | 1,280 |
| | 21 | <4 | 2,560 | ND |
| | 22 | <4 | 10,240 | 20,480 |
| | 23 | <4 | 2,560 | 5,120 |
| | 35 | <1 | 80 | 2,560 |
| | 36 | 1 | 640 | 320 |
| | 37 | ND[b] | 160 | 1,280 |
| | 38 | <8 | 1,280 | 20,480 |
| | 39 | <8 | 1,280 | 5,120 |
| 987P | 7 | <4 | 256 | 256 |
| | 8 | <4 | <4 | 256 |
| | 9 | <4 | 4 | 256 |
| | 10 | <4 | 256 | 512 |
| | 11 | 4 | 128 | 512 |
| | 12 | <1 | 32 | 64 |
| | 24 | <4 | 64 | 128 |
| | 25 | ND | 64 | 128 |
| | 26 | <4 | 64 | 256 |
| | 27 | <4 | 32 | 64 |
| | 28 | 8 | 256 | 256 |
| | 29 | <4 | 128 | 512 |
| | 40 | 4 | 128 | 1,024 |
| | 41 | <8 | 1,024 | 512 |
| | 42 | <8 | 1,024 | 512 |
| | 43 | <4 | 128 | 64 |
| | 44 | 2 | 256 | 1,024 |

[a] Serum antibody titers for K99 vaccine are anti-K99, and those for 987P vaccine are anti-987P.
[b] Not determined.

pigs showing a secondary immune response after the first vaccination, five vaccinated with K99 and one vaccinated with 987P had a pilus-specific serum antibody titer of 1:8 or greater against the pilus in the vaccine before the first injection. Natural exposure to these pilus antigens is likely to be by the oral route. Oral exposure of pregnant pigs to live *E. coli* present on the same farm is the basis for an effective vaccination procedure to protect offspring from ETEC-induced diarrheal disease (7). It is therefore likely that the reason for observing the secondary immune response in greater than 50% of the pigs in this experiment after a single parenteral vaccination was a result of previous oral "vaccinations" with piliated *E. coli* possessing K99 or 987P.

The nature of the protective antibody in the colostrum samples collected in this experiment is not known. Based upon the vast amount of literature on colostrum, and the assumption that repeated oral exposures to pilus antigens occurs on farms, it would be predicted that immunoglobulin A was the protective class of antibodies. However, in preliminary experiments using ELISAs that detect class-specific antibodies, it appears as if immunoglobulin G is the major protective antibody. Resolution of this question is being pursued.

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