Effect of competitive exclusion treatment on colonization of early-weaned pigs by *Salmonella* serovar Choleraesuis

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Summary

Objective: To assess the potential of two porcine-derived competitive exclusion cultures to enhance colonization resistance of early-weaned pigs to *Salmonella* serovar Choleraesuis.

Methods: Litters from three and two sows, respectively, were treated at birth and again at weaning with either of two porcine-derived competitive exclusion cultures, one less diverse than the other. Another litter was treated as above with the least diverse competitive exclusion culture that had been supplemented with *Bacteroides thetaiotaomicron*, a bacterium implicated in effecting gut cell development. Three other litters served as placebo-treated or untreated controls. All piglets were challenged at 15 days of age (1 day postweaning) with 10^6–10^7 colony-forming units (CFU) of *Salmonella* Choleraesuis. Rectal swabs (collected daily post challenge) and specimens (collected at necropsy 7–9 days postchallenge) were cultured for *Salmonella* Choleraesuis to assess the incidence of fecal shedding and colonization status of each piglet. Statistical analysis was not performed in this preliminary study due to the confounding of treatment effects with litter effects.

Results: There were fewer pigs shedding in litters that received the competition exclusion culture. There were fewer piglets with salmonellae culture-positive tonsils within the litters treated with either competitive exclusion culture. *Salmonella* Choleraesuis was recovered from the ileocolic lymph nodes of all 32 of the placebo-treated or nontreated piglets and from most but not all of the piglets treated with either of the competitive exclusion cultures. While the incidence of shedding of *Salmonella* Choleraesuis was less for the litter treated with the least diverse competitive exclusion culture that had been spiked with *Bacteroides thetaiotaomicron* than for the controls, no other benefits were observed with this treatment.

Implications: Competitive exclusion treatment of baby piglets may enhance colonization resistance to *Salmonella* Choleraesuis, which may reduce the potential for horizontal transmission of the host-adapted pathogen. However, in the absence of statistical analysis, our interpretation should be considered preliminary.

*S*almonella infections cause economic losses to the swine industry (Schwartz; *Proc Ann Meet US Anim Health Assoc*, 1990;94:443–449). In the United States, infections by *Salmonella* serovar Choleraesuis, the etiologic agent of swine paratyphoid, account for the vast majority (up to 90%) of diagnosed cases of swine salmonellosis.[1] Enterocolitis caused by *Salmonella* serovar Typhimurium ranks second as a cause of salmonellosis.[1] Whereas *Salmonella* Typhimurium is an important cause of foodborne disease,[2–4] human cases of *Salmonella* Choleraesuis infections are rare and the importance of foodborne transmission of this serotype has not been established.

Diseased or asymptomatic carriers may play a critical role in the spread of swine infections, and stressful events such as transportation or deprivation of feed or water may predispose animals to increased fecal shedding.[5–8] It is likely that pigs will experience these sorts of stresses during weaning, placing them at an enhanced risk for infection. Consequently, producers need strategies to reduce the potential for horizontal transmission at weaning by decreasing the pathogen load presented to the environment by shedding animals and/or increasing the resistance of uninfected pigs to infection.

One such strategy is that of competitive exclusion—excluding enteric pathogens from the alimentary tract by preferentially colonizing it with commensal or beneficial bacteria indigenous to a particular animal species. The aim of such technology is to facilitate the natural succession of a healthy gut microflora,[9] which otherwise may take up to a week or longer to become established in the very young animal.[10–11] Mechanisms proposed to describe how competitive exclusion cultures might exclude enteropathogens from the gut include:

- competition for nutrients or attachment sites within the gut,[9]
- production of antibacterial substances or conditions (e.g., volatile fatty acids, bacteriocins, or anaerobiosis),[9] and
- immunostimulation.[9]

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While none of these proposals are solely adequate to explain how competitive exclusion works, such treatment has been shown to facilitate the establishment of a mature anaerobic flora[12] that readily colonizes the gut epithelium.[13]

Regardless of the mode of action, competitive exclusion cultures have been used extensively outside the United States to enhance colonization resistance of avian species to *Salmonella*[14] and the effectiveness of this technology has been well documented.[15–20] In the United States, however, competitive exclusion cultures are classified as drugs by the Center for Veterinary Medicine (CVM)(CVM Update; February 21, 1997. FDA, Center for Veterinary Medicine, Office of Management and Communications, Rockville, MD). Only recently has an avian-derived competitive exclusion product of known microbial composition, trade named PREEMPT™, been approved by the United States Food and Drug Administration (Federal Register; 1998;63(88):25163). Drawing upon experiences gained during the development of PREEMPT™, we have developed two porcine-derived competitive exclusion cultures and we herein report preliminary results from pilot studies designed to evaluate the effectiveness of these cultures in preventing *Salmonella* Choleraesuis colonization of early weaned pigs.

Materials and methods

Continuous-flow cultures

Porcine-derived continuous-flow culture number 1 (pCF1) was propagated from cecal contents collected from a 6-week-old healthy pig. To obtain cultures less diverse than pCF1, two subsequent continuous-flow cultures were propagated; pCF2 from a 10^6 dilution of pCF1 and pCF3 from a 10^8 dilution of pCF2. All three cultures were maintained via continuous-flow culture as described for avian cultures.[21] Because of the limited number of animals at our disposal, pCF2 was not tested.

Study design

In three separate experiments, piglets from three litters were treated with pCF1 and piglets from two litters were treated with pCF3 (Figure 1). Treatments were administered via oral gavage of 5 mL suspensions (equivalent to $2–9 \times 10^9$ CFU) within 12 hours of farrowing (day 1), and then again within 1 hour postweaning (day 14).

Piglets from a sixth litter were treated with pCF3 that included 10^8 colony forming units (CFU) of *Bacteroides thetaiotaomicron*, an organism implicated in effecting gut cell differentiation.[22] *Bacteroides thetaiotaomicron* (an avian isolate from the Food Animal Protection Research Laboratory culture collection), cultured overnight at 37°C in Viande Levure (VL) broth,[23] was spiked (1% vol:vol) into the pCF3 culture immediately prior to dosing. The dose strengths of the consortiums and the pure culture of *B. thetaiotaomicron* were determined by viable cell count on *Brucella* blood agar (Anaerobe Systems, San Jose, California) incubated in an anaerobic chamber at 37°C.

Three other litters served as control groups, one for each of the three experiments. The control litters used in experiments one and three were treated with sterile VL medium.[23] The control litter in experiment two was farrowed at a commercial production facility and thus received no treatment; however, after weaning these piglets were reared at the Food Animal Protection Research Lab.

Salmonella challenge

One day postweaning, all piglets were challenged orally with a novobiocin (NO)- and nalidixic acid (NA)-resistant strain of *Salmonella* Choleraesuis that had been serially cultured two consecutive times (at 24 hour intervals at 37°C) in tryptic soy broth (Difco, Detroit, Michigan) containing NO (25 µg per mL) and NA (20 µg per mL) (Figure 1). This NO-NA-resistant mutant was propagated from a pig isolate of *Salmonella* Choleraesuis var. kunzendorf 3246pp, generously provided to us by Dr. P. Fedorka-Cray (Athens, Georgia). The challenge doses of *Salmonella* Choleraesuis were determined via viable cell count following overnight incubation (37°C) on brilliant green agar (Oxoid, Unipath LTD., Basinstoke, Hampshire, United Kingdom) supplemented with 25 µg NO per mL and 20 µg NA per mL (BGANO/NA).

Housing and nutrition

All piglets other than the untreated piglets in experiment two were farrowed at the Food Animal Protection Research Lab facilities in 1.5 m × 2.1 m Sampson farrowing crates (Hog Slat Inc., Newton Grove, North Carolina). Crates were kept in pens separated by concrete walls. Piglets were weaned into adjacent, concrete floored pens approximately 6 m^2, separated from each other by concrete walls.

All animals were cared for according to standard swine husbandry practices and were fed a typical phase 1 weaning diet formulated to meet or exceed NRC requirements(NRC; *Nutrient Requirements of Swine,* 9th Edition;1988).

Sampling

Beginning at weaning (day 14), rectal swabs were collected daily from each piglet for qualitative cultivation of salmonellae until the piglets were euthanized on day 22. Wild-type salmonellae were not detected in rectal swabs collected from each piglet for the 2 consecutive days immediately prior to *Salmonella* Choleraesuis challenge. These prechallenge swabs were cultured via preenrichment in GN-Hajna (GN) broth (Difco), further enrichment in Rappaport-Vassiliadis broth (Difco), and selective differentiation on BGA plates containing NO (BGANO) as previously described.[24]

Approximately 1 week post *Salmonella* Choleraesuis challenge, piglets were euthanized by injection with sodium pentobarbital, and ileocolic lymph nodes and cecal contents were collected during necropsy. In some of the experiments, the tonsils (all groups in all experiments but the controls in experiment 3), ileocolic junction (experiment 1), and colon (experiments 2 and 3) were collected. Resource limitations did not permit us to collect all of these samples for each experiment.

Rectal swabs, tissues, and cecal contents collected from piglets post challenge were cultured for *Salmonella* Choleraesuis as above, except selective differentiation was accomplished using BGANO/NA, which facilitated our recovery of the challenge organism. Also, serial tenfold dilutions ($10^1–10^4$) of the cecal contents were spread directly onto

Figure 1
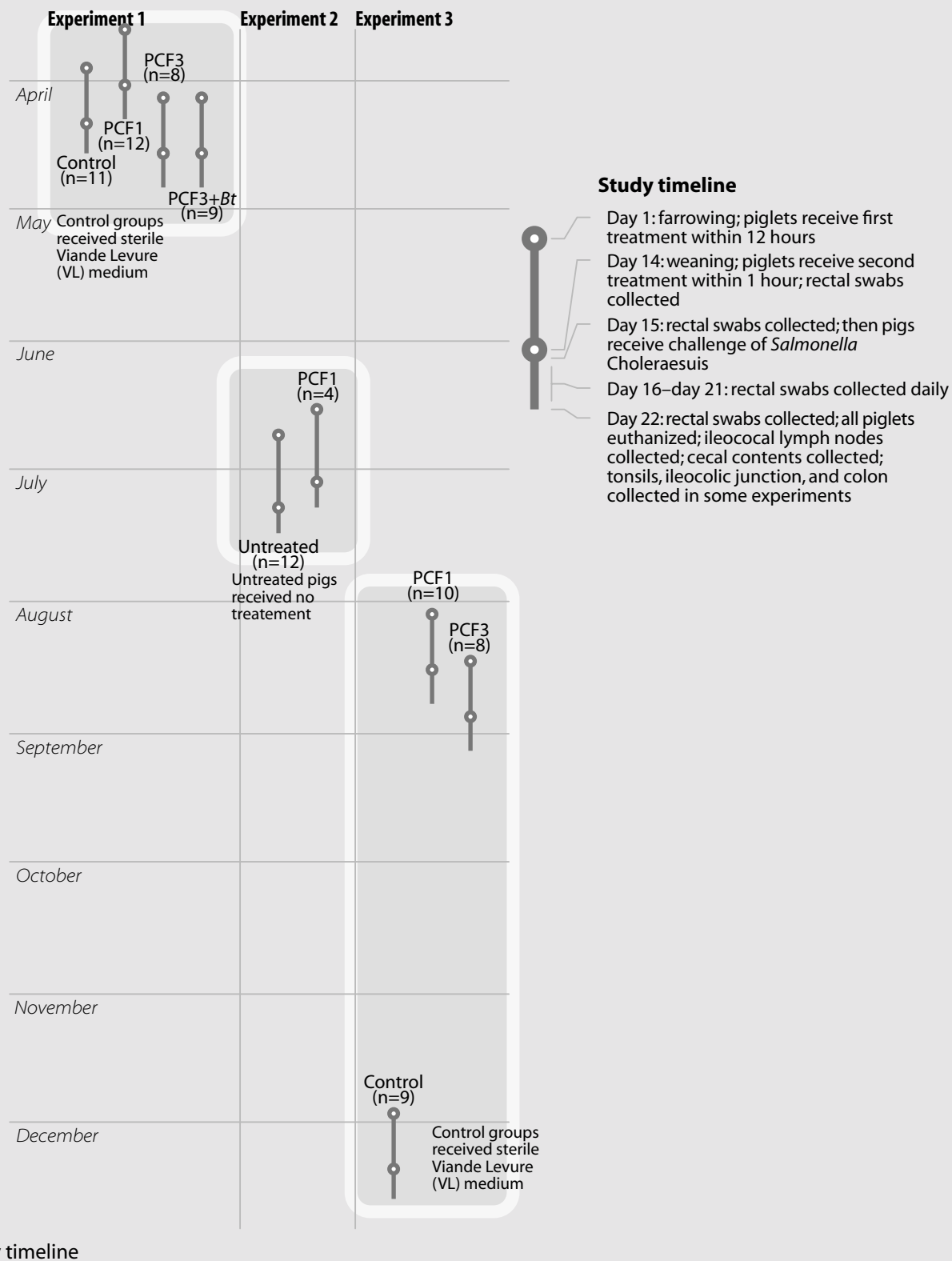
BGANO/NA plates for quantitative cultivation of the challenge organism. Plates were examined for colonies exhibiting typical salmonellae morphology and suspect colonies were confirmed via serum agglutination using *Salmonella* Antiserum Poly A I-IV and Group C1, Factors 5 and 6 (Difco). Several representative colonies were also sent to National Veterinary Services Laboratory, Ames, Iowa for serotyping and all were confirmed as *Salmonella* Choleraesuis var. kunzendorf.

Effect of competitive exclusion culture treatment on colonization of pigs
by the host-adapted pathogen *Salmonella* Choleraesuis

Treatment	Experiment 1				Experiment 2	
	Control	pCF1	pCF3	pCF3+*Bt*	Untreated	pCF1
Challenge dose of *Salmonella* Choleraesuis given (CFU)	4.0×10^6	3.2×10^6	9.0×10^6	9.0×10^6	1.5×10^7	1.5×10^7
No. of piglets detected to be shedding at least once post challenge (n)	10 (11)	7 (12)	6 (8)	9 (9)	11 (12)	4 (4)
Proportion of rectal swabs culturing positive for *Salmonella* Choleraesuis during the 7 days immediately post challenge	55%	20%	23%	30%	44%	32%
Tissue distribution:						
tonsil	10/11 (91%)	6/12 (50%)	8/8 (100%)	9/9 (100%)	9/12 (75%)	3/4 (75%)
ileocolic lymph nodes	11/11 (100%)	10/12 (83%)	8/8 (100%)	9/9 (100%)	12/12 (100%)	4/4 (100%)
ileocolic junction	7/11 (64%)	9/12 (75%)	6/8 (75%)	8/9 (89%)	not done	not done
colon	not done	not done	not done	not done	4/12 (33%)	2/4 (50%)
Cecal contents:						
Culture positive	10/11 (91%)	4/12 (33%)	6/8 (75%)	9/9 (100%)	11/12 (92%)	3/4 (75%)
(Log_{10} CFU)	2.4	0.9	2	3.1	3.1	2.2

Bt   *Bacteroides thetaiotaomicron*

Statistical analysis

Within each experiment, treatment and challenge were applied to individual piglets. Our treatments were by necessity restricted to administration within litter (because we could not exclude the possibility of horizontal transfer of beneficial microbes between suckling littermates). Consequently, our design confounds possible treatment effects with possible litter effects (due to the potential of maternal influence), and thus our results are reported descriptively by experiment and interpreted empirically. Therefore, the results are solely suggestive. Cultures showing the greatest potential benefit were further tested in replicate experiments. For the purpose of overall treatment comparisons, we also present combined data from animals receiving like treatments.

Results

Fewer piglets shed *Salmonella* Choleraesuis and lower proportions of rectal swabs were cultured positive for *Salmonella* Choleraesuis from those piglets given either competitive exclusion culture (pCF1 or pCF3) (Table 1). Fewer of the pCF1- or pCF3-treated piglets had cecal contents that cultured positive for *Salmonella* Choleraesuis. Recovery of *Salmonella* Choleraesuis from the colonic portion of the gut was proportionately less than from the cecal portion, regardless of treatment. Fewer of the pCF1- and pCF3-treated piglets had *Salmonella*

Choleraesuis culture-positive tonsils.

The ileocolic lymph nodes of all but five of the piglets cultured positive for *Salmonella* Choleraesuis; those cultured negative were within litters treated with pCF1 or pCF3. *Salmonella* Choleraesuis was cultured from both the tonsils and ileocolic junction of two of these pigs (among those treated with pCF1), and two of the other piglets (among those treated with pCF3) cultured negative for *Salmonella* Choleraesuis throughout the experiment. The incidence of shedding in control piglets was greater than that by piglets treated both with pCF3 and *B. thetaiotaomicron*, but obvious differences were not observed otherwise.

Discussion

Competitive exclusion technology has traditionally been targeted to the very young and naive gut ecosystem[9] and numerous studies have shown that such technology facilitates colonization of the chick, poult, and suckling pig with a healthy gut microflora capable of excluding *Salmonella* (Nisbet, et al; *Proc 2nd Intl Symp Epid and Control of Salmonella in Pork*, 1997;176–177. Fedorka-Cray, et al; *Proc 2nd Intl Symp Epid and Control of Salmonella in Pork*, 1997;164–166).[12–13,15–21] Our present results suggest, but do not prove, that a protective effect of early pCF1 or pCF3 treatment is present even after weaning. Our observation that fewer pigs shed *Salmonella*

	Experiment 3			Combined analysis			
	Control	pCF1	pCF3	Control and untreated	pCF1	pCF3	pCF3+*Bt*
	5.4×10^6	7.0×10^7	1.0×10^7	8.9×10^6	3.1×10^7	9.5×10^6	9.0×10^6
	9 (9)	5 (10)	4 (8)	30 (32)	16 (26)	10 (16)	9 (9)
	57%	9%	7%	51%	18%	15%	30%
	not done	4/10 (40%)	0/8 (0%)	19/23 (83%)	13/26 (50%)	8/16 (50%)	9/9 (100%)
	9/9 (100%)	10/10 (100%)	5/8 (63%)	32/32 (100%)	24/26 (92%)	13/16 (81%)	9/9 (100%)
	not done	not done	not done	7/11 (64%)	9/12 (75%)	6/8 (75%)	8/9 (89%)
	2/9 (22%)	1/10 (10%)	0/8 (0%)	6/21 (29%)	3/14 (21%)	0/8 (0%)	not done
	9/9 (100%)	9/10 (90%)	6/8 (75%)	30/32 (94%)	16/26 (62%)	12/16 (75%)	9/9 (100%)
	3.7	2.9	2.8	3	1.9	2.4	3.1

Bt   *Bacteroides thetaiotaomicron*

enhanced resistance to colonization by *Salmonella* Choleraesuis within the gut lumen. This possibility is further supported by our observation that concentrations of the pathogen were lower in cecal contents cultured from the treated piglets. Why *Salmonella* Choleraesuis was recovered less frequently from the colon than from the cecum, regardless of treatment, was indiscernible from our data.

Acquisition of a mature gut flora provides the host a measure of protection against infections by enteric pathogens,[9,26–27] but the mechanism by which this occurs is unclear. Recently, Bry, et al.,[22] suggested that certain members of the host's resident flora, specifically *B. thetaiotaomicron*, may promote differentiation of the host epithelial tissue within certain regions of the gut. Aside from the proposed effect on gut cell development, increased gut populations of *B. thetaiotaomicron* have also been associated with stress in humans.[28] Our observations that treatment of pigs with both pCF3 and *B. thetaiotaomicron* appeared to negate some of the beneficial effects of pCF3, although preliminary, are consistent with these earlier findings.

Our high recovery of *Salmonella* Choleraesuis from ileocolic lymph nodes supports earlier conclusions[24,29–30] that qualitative culture of ileocolic lymph nodes is the best indicator of *Salmonella* Choleraesuis colonization in pigs. Since our challenge organism was cultured from the ileocolic lymph nodes of all control and most of the treated piglets, we conclude that our oral dose of $\geq 10^6$ CFU *Salmonella* Choleraesuis was sufficient to cause colonization regardless of any potential maternal influence that may have been expressed by the sows. The fact that most of the treated pigs were colonized within their lymph tissue, and are thus potential carriers, reveals a limitation of the present study (i.e., we could not assess the beneficial effects of competitive exclusion beyond 1 week post inoculation).

While our work addresses infections caused by *Salmonella* Choleraesuis, which primarily causes postweaning disease in pigs,[1] other salmonellae serotypes are frequently found in pigs during the finisher phase,[31–32] which suggests that other critical control points exist beyond the weaning period. Future studies to evaluate the long-term persistence of any protective effect of competitive exclusion may be warranted.

It is not surprising that the invasiveness of this host-adapted pathogen was not mitigated by treatment with either pCF1 or pCF3 since the challenge concentrations used in our studies were likely too rigorous to

Choleraesuis in the pCF1- or pCF3-treated groups compared to controls was relatively consistent across experiments. Studies that recover salmonellae from rectal swabs have been criticized because this pathogen is shed intermittently.[25] Unlike most other studies, in which swabs are typically collected infrequently, our results are based on swabs collected consecutively over a period of several days, which enhanced our ability to detect intermittent shedders over the 1-week time span immediately postchallenge.

Our observation in experiment one that the proportion of *Salmonella* Choleraesuis culture-positive tonsils was less for the litter treated with pCF1 compared to controls suggests that recurrent oral or nasal exposure to the pathogen was reduced as a consequence of the reduced fecal shedding by these pigs. The incidence of *Salmonella* Choleraesuis shedding by piglets treated with pCF3 (which was presumed to be less diverse based on the dilution process) was comparable to that observed with pCF1, thus suggesting that the protective bacteria in the consortium were present in high numbers ($\geq 10^8$ CFU per mL). A beneficial effect of pCF1 treatment was not readily evident in experiment two, although this may have been due to the small number of treated pigs used in that experiment.

Our observations that fewer pCF1- or pCF3-treated piglets had *Salmonella* Choleraesuis culture-positive cecal contents suggests an

test for such an effect. For instance, natural challenge concentrations are considered to range between 10^4–10^6 CFU,[29–30,33] and at such concentrations relatively few pigs become long-term carriers.[29] Moreover, Gray et al.,[30] showed that establishing the carrier state in pigs is dose dependent, with short-term carriers arising from an intranasal inoculation of 10^6 CFU *Salmonella* Choleraesuis. Most of these short-term carriers, however, ceased shedding the pathogen by 1 week post inoculation. Because competitive exclusion may reduce the degree and frequency of salmonellae shedding, it is reasonable to hypothesize that, under natural conditions of infection, horizontal transmission would be reduced. This may play a particularly important role in stemming the spread of *Salmonella* Choleraesuis, since this host-adapted pathogen is thought to be spread primarily from pig to pig.[1] In support of our hypothesis, preliminary results have shown that pCF1 treatment reduced transmission of *Salmonella* Choleraesuis from experimentally infected pigs to naive pigs in the short term (1 week postweaning) (Anderson, et al; *Second World Congr on Anaerobic Bacteria and Infections*, October,1998;153), and additional experiments are planned to determine the long-term benefits of either pCF1 or pCF3 treatment.

Implications:

- Administering the competitive exclusion cultures pCF1 and pCF3 to newborn piglets may decrease the short-term prevalence of fecal shedding of *Salmonella* Choleraesuis.

- Such treatment may reduce the potential for horizontal transmission of this host-adapted pathogen.

- The proportion of pigs with cecal contents culturing positive for *Salmonella* Choleraesuis as well as the cecal concentrations of *Salmonella* Choleraesuis may be reduced by the competitive exclusion cultures.

- While the results of this pilot study are encouraging, definitive conclusions await further studies supported by statistical analysis.

Acknowledgements

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