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Synergism between porcine reproductive and respiratory syndrome virus (PRRSV) and *Salmonella choleraesuis* in swine

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Abstract

Porcine reproductive and respiratory syndrome virus (PRRSV) and *Salmonella choleraesuis* are two leading causes of economic loss in the swine industry. While respiratory disease is common in both *S. choleraesuis* and PRRSV infections, the factors that contribute to its development remain largely undefined. We investigated the interaction of PRRSV, *S. choleraesuis*, and stress in 5-week-old swine. All combinations of three factors (inoculation with *S. choleraesuis* on Day 0, PRRSV on Day 3, and treatment with dexamethasone on Days 3–7) were used to produce eight treatment groups in two independent trials. Fecal samples, tonsil and nasal swabs, serum samples and postmortem tissues were collected for bacteriologic and virologic examinations. No clinical signs were observed in pigs inoculated with only PRRSV or only *S. choleraesuis*. In contrast, pigs which were dually infected with *S. choleraesuis* and PRRSV exhibited unthriftiness, rough hair coats, dyspnea, and diarrhea. The pigs which received all three treatment factors were the most severely affected and 43% (three of seven) of the animals in this group died. Individuals in this group shed significantly higher quantities of *S. choleraesuis* in feces and had significantly higher serum PRRSV

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titers compared to other treatments ($p \leq 0.05$). In addition, *S. choleraesuis* and PRRSV were shed longer and by more pigs in this group than other groups and *S. choleraesuis* was recovered from more tissues in this group on Day 21 post inoculation. These results suggested that PRRSV, *S. choleraesuis*, and dexamethasone acted synergistically to produce a syndrome similar to that observed in the field. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Porcine reproductive and respiratory syndrome (PRRS); Salmonella choleraesuis; Interaction; Pig-bacteria; Pig-viruses

1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) virus (PRRSV) and *Salmonella choleraesuis* are important components of the porcine respiratory disease complex (PRDC). The recognition of *S. choleraesuis* as an important and common cause of swine respiratory disease and the emergence of PRRS as a new swine disease have both occurred only relatively recently.

At present, the pathogenesis of PRRS is not yet fully understood. The reproductive component of the disease syndrome can be routinely reproduced under experimental conditions (Terpstra et al., 1991; Collins et al., 1991; Christianson et al., 1992; Plana et al., 1992). Although respiratory disease is a major component of the syndrome in field cases (Keffaber, 1990), clinical signs of respiratory disease have not been consistently reproduced under experimental conditions. Histopathological lesions consistent with those seen in field cases of PRRS have been reproduced experimentally in gnotobiotic pigs (Collins et al., 1991, 1992) and in Cesarean derived, colostrum deprived pigs (Pol et al., 1991). It has been postulated that the lack of clinical signs of pneumonia may be due to the absence of bacterial pathogens (Collins et al., 1992).

Subclinically infected pigs are considered the most common source of new *S. choleraesuis* infections. A carrier state was demonstrated in which pigs experimentally inoculated with *S. choleraesuis* remained persistently infected for at least 12 weeks (Gray et al., 1995). While respiratory disease is a common sequela of acute *S. choleraesuis* infection, the factors that contribute to its development remain largely undefined. A variety of stressors, including the presence of viral disease, have been suggested to potentiate or exacerbate clinical outbreaks of salmonellosis (Schwartz, 1991). Exacerbation of infectious diseases, including *S. choleraesuis*, has been attributed to concurrent infection with PRRSV. *Salmonella choleraesuis* was one of several common secondary infections reportedly associated with herds chronically infected with PRRSV (Joo and Dee, 1993). Increases in the incidence and severity of salmonellosis and other diseases in association with PRRSV infections have been recognized in Britain (Done and Paton, 1995). Investigators demonstrated a positive association between the seroprevalence of PRRSV and infection with porcine respiratory corona virus or porcine influenza virus (Groschup et al., 1993). Stevenson et al. (1993) suggested that concurrent PRRSV infections were responsible for increased nursery mortality due to *S. choleraesuis* septicemia. However, few controlled studies have shown an interaction between PRRSV and a secondary infection. Galina et al. (1994) inoculated 13-day-old pigs with

Streptococcus suis seven days after inoculation with PRRSV. The dually infected pigs developed suppurative meningitis, mononuclear perivascular cuffing in the brain, while non-inoculated and *S. suis*-only inoculated pigs did not. Shimizu et al. (1994) reported increased severity of *Mycoplasma hyorhinis* pneumonia five days after PRRSV. However, only two pigs were used in each group.

Other researchers, however, have been unable to demonstrate significant interactions between PRRSV and secondary bacterial infections such as *Haemophilus parasuis, S. suis, S. choleraesuis*, or *Pasteurella multocida* (Cooper et al., 1995). In fact, mortality was greater in pigs singly infected with *H. parasuis* or *S. suis* compared to pigs inoculated with PRRSV prior to bacterial inoculation. The authors noted that unidentified stressors and virulence factors present in field conditions might be necessary for disease expression.

The objective of this study was to characterize the interaction of PRRSV, *Salmonella choleraesuis*, and stress in the production of disease in young swine.

2. Materials and methods

2.1. Experimental design

Two replicate trials were conducted. In each trial, 5-week-old pigs from four litters were randomly divided into eight treatment groups. Each treatment group consisted of a different combination of three factors (Table 1): inoculation with *S. choleraesuis* at a concentration of 10^6 colony forming units (CFU) on Day 0 (S), inoculation with PRRSV at a rate of 10^3 50% tissue culture infectious doses ($TCID_{50}$) on Day 3 (P), and treatment with dexamethasone at a rate of 2 mg/kg on Days 3–7 (D). The absence of a factor was designated by N. Dexamethasone was used as an experimental proxy for stress. Isolation rooms were used and strict biosecurity measures maintained, to ensure no cross contamination between groups.

Table 1
Description of eight treatment groups derived from combinations of three factors: *S. choleraesuis* (S), porcine reproductive and respiratory syndrome virus (P), and dexamethasone (D)

Group ID	Treatments			
	Intranasal inoculation of *S. choleraesuis* on Day 0	Intranasal inoculation of PRRSV on Day 3	Intramuscular injection of dexamethasone on days 3–7	Pigs in group on Day 0
NNN	−	−	−	7
NND	−	−	+	7
SNN	+	−	−	7
SND	+	−	+	6
NPN	−	+	−	7
NPD	−	+	+	6
SPN	+	+	−	7
SPD	+	+	+	7

2.2. Animals

The sows from which the pigs were derived originated from a herd known to be free from PRRSV infection through repeated serological and virological monitoring over a period of 3 years. The dams and pigs were handled so as to reduce the likelihood of concurrent subclinical infections. The sows were moved into farrowing crates in an isolation ward 2–5 days prior to farrowing. The sows were given intramuscular (IM) injections of ceftiofur sodium at a rate of 4.0 mg/kg (Naxcel®, Upjohn Company, Kalamazoo, MI) once daily for 7 days starting 1–5 days prior to farrowing. One or more fecal samples from each sow were collected pre- and post-farrowing and cultured to determine that the sow was not shedding *Salmonella* spp.

Pigs were given 100 mg iron dextran (The Butler Company, Dublin, OH) IM at 1 and 10 days of age and 10 mg ceftiofur sodium IM once daily for 7 days starting 7 days prior to weaning. Pigs were individually identified by randomly assigning numbered ear tags. At 12–15 days of age the pigs were weaned and moved to nursery decks in an isolation barn at the National Animal Disease Center, Ames, IA. During the week prior to weaning, pigs were given a starter feed (Momentum® 5/10, Master Mix Feeds, Fort Wayne, IN) containing 75 mg/lb apramycin in creep feeders placed in the farrowing crate. Post-weaning pigs were fed ad libitum a corn-soybean based diet containing no antibiotics.

One week prior to the start of the experiment, pigs were moved into pens in isolation rooms and grouped according to treatment group (Table 1). Four separate isolation buildings were used with treatment groups placed in separate rooms or pens with concrete floors within buildings: the control group (NNN) and dexamethasone treatment (NND) groups in one building; the PRRSV-inoculated (NPN) and PRRSV/dexamethasone (NPD) groups in a second building, the *Salmonella* (SNN) and *Salmonella*/dexamethasone (SND) groups in a third facility, and the *Salmonella*/PRRSV (SPN) and the *Salmonella*/PRRSV/dexamethasone (SPD) group in the fourth. Moribund animals were euthanized and necropsied immediately. All protocols were carried out in accordance with institutional animal care and use guidelines and approval.

2.3. Bacteria

S. choleraesuis var Kunzendorf 3246 (Kelly et al., 1992) cultures were prepared as previously described (Gray et al., 1996a,b). This strain is resistant to streptomycin. The inoculum was adjusted to a final concentration of 10^6 CFU/ml in PBS. Final concentrations were confirmed by plate counts. Pigs were inoculated intranasally with 1.0 ml of the suspension by alternating drops into each naris during inspiration.

2.4. Virus

Porcine reproductive and respiratory syndrome virus isolate ISU-P was used to inoculate pigs. The isolate originated from clinically affected pigs from a herd experiencing an acute outbreak of PRRS. The challenge virus cultures were prepared as previously described (Yoon et al., 1996). Pigs were inoculated intranasally by infusing 0.5 ml of a solution containing approximately 10^3 $TCID_{50}$ PRRSV per ml into each naris.

2.5. Sample collection and clinical evaluation

A single investigator evaluated the health status of each pig once daily over the course of the experiment for general appearance and presence of dyspnea, coughing, depression, chills, vomiting, and/or diarrhea. Each pig was also given a clinical score from 0 to 3 for dyspnea, coughing, chills, vomiting, and diarrhea. A score of 0 indicated absence of a clinical sign while a score of 3 indicated severe clinical signs.

Using minimal restraint, the rectal temperature of each pig was recorded once daily from Day 0 through Day 14 and on Day 21. A separate electronic thermometer was used for similarly inoculated groups of pigs. The precision of the thermometers was assessed at the start of the trials and found to be within 0.5°C.

Body weight of each pig was determined on Day 0 and Day 21 of the trials. Average daily gain (ADG) was calculated by dividing the difference between weights on Day 0 and Day 21 by 21.

Blood samples were drawn from the anterior vena cava using a single use collection system on days 0, 3, 7, 10, 14, 17, and 21. Serum was harvested by centrifugation and stored at −70°C until needed.

Fecal samples were collected from pigs in the SNN, SND, SPN, and SPD treatment groups using a separate sterile fecal loop for each pig. Approximately 1.0 g of feces was collected from the rectum and suspended in 5.0 ml of GN-Hajna broth (GN; Difco, Detroit, MI) and used for quantitative bacteriological determination of *S. choleraesuis* shedding in the feces (Gray et al., 1995). The exact quantity of feces collected was determined by subtracting the pre-sample weight of the tube containing medium from the weight of the tube and medium containing the sample.

Tonsil, nasal, and rectal swabs for bacterial culture were collected from all pigs prior to the start of the trials. Thereafter, tonsil, nasal, and rectal swabs were taken from pigs in groups NNN, NND, NPN, and NPD on days 0, 3, 7, 10, 14, and 21. Tonsil and nasal swabs were taken from pigs in groups SNN, SND, SPN, and SPD on days 0, 3, 7, 10, 14, and 21. The swabs were subjected to qualitative bacteriological determination of *S. choleraesuis*.

The pigs were euthanized by intravenous injection of a solution containing tiletamine HCl and zolazepam HCl (Telazole®, Fort Dodge Laboratories, Fort Dodge, IA), ketamine HCl (Ketaset®, Fort Dodge Laboratories, Fort Dodge, IA), and xylazine (Rompun®, Miles, Shawnee Mission, KS) followed by exsanguination and necropsy using sterile techniques. Gross pathologic changes were recorded. Tonsil, lung, liver, spleen, middle ileum, ileocolic junction, cecum, cecal contents, colon, and mesenteric, brachial, ileocolic, and colonic lymph nodes were aseptically collected. Samples of all tissues collected from *S. choleraesuis*-inoculated pigs and ileocolic junction samples from non-*S. choleraesuis*-inoculated pigs were qualitatively assayed for the presence of *S. choleraesuis* as described below.

2.6. PRRSV Isolation

Virus isolation was done using 1-day old porcine alveolar macrophages (PAM) cultures on the same day of sample collection. Porcine alveolar macrophages were harvested from 4- to 6-week-old pigs by lung lavage as previously described (Yoon et al., 1996) and

stored frozen at −80°C until centrifuged and resuspended in RPMI-1640 (Sigma, St. Louis, MO) supplemented with 10% fetal calf serum (Sigma, St. Louis, MO), 10 mM HEPES (Sigma, St. Louis, MO) and an antibiotic-antimycotic mixture (RPMI growth media). The antibiotic-antimycotic mixture consisted of 100 IU/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamicin, and 25 μg/ml amphotericin B. The cells were then seeded in 48-well plates (Corning Costar, Cambridge, MA) at a concentration of 10^6 cells/well and incubated in RPMI growth medium for 24 h at 37°C in a humidified 5% CO_2 atmosphere. Culture medium was removed and 200 μl of each serum sample was inoculated into duplicate wells containing PAMs. Inoculated cells were incubated for 60 min at 37°C and 500 μl of fresh RPMI growth medium was added to each well. The cells were further incubated for five days at 37°C and subjected to one cycle of freeze-thawing at −70°C/37°C. The presence of PRRSV in the cells was confirmed by subinoculating 100 μl of each culture medium to 24 h-old MARC-145 cell (Kim et al., 1993) monolayers prepared in 96-well plate, incubating the cells for 48 h at 37°C, and staining fixed cell monolayers with optimally diluted PRRSV-specific monoclonal antibody (SDOW17) conjugated with FITC (D. Benfield, South Dakota State University).

2.7. PRRSV titration

Levels of PRRSV in serum samples were determined using a microtitration infectivity assay. For this, 1-day-old confluent MARC-145 cell monolayers were prepared in 96-well plates. Serum samples were serially diluted 10-fold in serum-free Eagle's minimum essential media (MEM) supplemented with the antibiotic-antimycotic mixture. Triplicate wells containing MARC-145 cells were inoculated with 100 μl of each undiluted and diluted sample. Inoculated cells were incubated for 60 min at 37°C and 100 μl of Eagle's MEM supplemented with 10% FCS, L-glutamin, 10 mM HEPES and the antibiotic-antimycotic mixture were added to each well. The cells were further incubated at 37°C and examined for cytopathic effect (CPE) for up to 7 days post inoculation. At the end of the 7-day incubation period, the cells were fixed in cold acetone : methanol (70 : 30) mixture for 5 min. The presence of PRRSV in the cells was confirmed by fluorescent antibody (FA) staining with SDOW17 direct conjugate. Virus titers were calculated using the Reed-Muench method based on the number of wells showing CPE and/or positive by the FA test and expressed as $TCID_{50}$/ml.

2.8. PRRSV serology

Serum samples were completely randomized and then assayed by a commercially available ELISA (HerdChek: PRRS®, IDEXX Laboratories, Westbrook, ME) following the procedure described by the manufacturer. Samples were considered positive if the calculated sample to positive (S/P) ratio was 0.4 or greater.

2.9. Bacteriology for *S. choleraesuis*

All tonsil swabs, nasal swabs, rectal swabs, feces, and postmortem tissues were cultured for *S. choleraesuis* as previously described (Gray et al., 1996b). Quantitative

bacteriology was conducted using the 5-tube most probable number (MPN) method (Wood and Rose, 1992) with GN, Rappaport-Vassiliadis broth (Difco, Detroit, MI) and Brilliant Green Sulfadiazene agar (Difco, Detroit, MI) with streptomycin added at 30 μg/ml as described previously (Gray et al., 1996a). Results were reported as the mean value of the respective group for each sample day.

2.10. Statistical analysis

Changes in body weight, differences in *S. choleraesuis* numbers for each sampling day, and differences in PRRSV titers for each sampling day were evaluated by analysis of variance (Proc GLM, SAS System for Windows 6.11, SAS Institute, Cary, NC). Duncan's Multiple Range Test was used to determine the significance of differences among treatment means. Likelihood ratio chi square tests were used to test the independence of association of proportions of pigs which were positive for an etiologic agent (*S. choleraesuis* or PRRSV) across treatment groups for each sample day (Proc FREQ, SAS System for Windows 6.11, SAS Institute, Cary, NC). Bonferroni's method was used to prevent Type I errors.

Regression analyses on the proportion of pigs with fevers within treatment groups over time were conducted using Proc Reg (SAS System for Windows 6.11, SAS Institute, Cary, NC). The full model for the different treatment groups was tested against the reduced model for lack of fit for each of the groups. In the reduced model, the individual groups making up the full model were combined into one group. An F statistic was calculated using the sum of the individual error sum of squares of the groups in the full model in the numerator and the error sum of squares of the reduced model in the denominator. A significant lack of fit indicated that the proportion of pigs with fever were different between the groups. If a significant lack of fit was determined when all groups were considered, subsets of groups were also compared.

3. Results

3.1. Clinical findings

Pigs which were given no treatment (NNN) a single treatment (NND, SNN) or a dual treatment of dexamethasone and an infectious agent (SND, NPD) did not demonstrate overt clinical signs. Pigs which were dually infected with *S. choleraesuis* and PRRSV (SPN) exhibited notable clinical signs of disease, i.e. unthriftiness, rough hair coats, dyspnea, and diarrhea. Pigs that received all three factors (SPD) were the most severely affected. Three of the seven SPD pigs died or were euthanized when moribund. Compared to other treatment groups, the death loss was statistically significant (Fischer's exact test, $p = 0.01$).

Fever response was determined by first calculating the clinically normal range among all pigs in the experiment. The 97.5 percentile temperature of all pigs on Day 0 was found to be 40.06°C. Temperatures greater than this were indicative of fever. Regression plots of the percentage of pigs within treatment groups that had fevers are presented in Fig. 1.

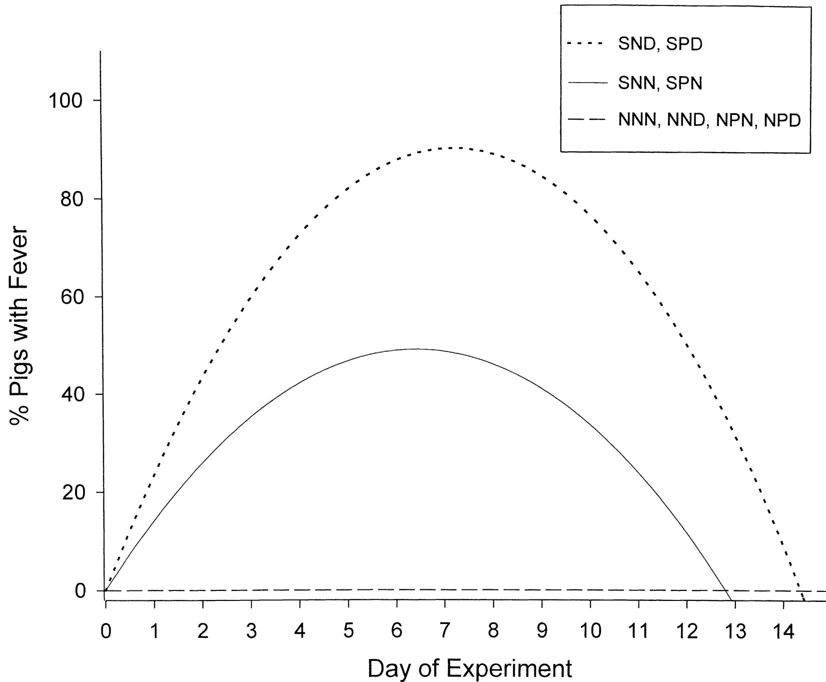

Fig. 1. Regression plots of percentage of pigs within treatment groups with rectal temperatures exceeding 40.06°C (104.1°F). Treatment groups represented by a single regression line were not significantly different when plotted singly. Regression equations: ········Estimated percent of pigs with fever = 25.0924(t)1.7482(t^2). —— Estimated percent of pigs with fever = 15.3238(t)1.1983(t^2). – – – Estimated percent of pigs with fever = 0.

None of the pigs in the NNN or NND groups had fevers during the observation period. The percentage of pigs with fevers in groups SNN and SPN were not significantly different ($p = 0.948$) from each other, nor were groups SND and SPD different ($p = 0.364$) from each other. However, there was a significant lack of fit ($p < 0.001$) when a reduced model combining these four groups was attempted. Thus, the proportion of pigs with fevers in groups SNN and SPN was significantly different from the proportion of pigs with fevers in groups SND and SPD. Half of the NPD and 28% of the NPN pigs had fevers on Day 5. One NPN pig had fever on Day 6. Inoculation with PRRSV appeared to result in some pigs having fever; however, the phenomenon occurred within 48 h and did not provide enough data points to estimate the function mathematically or statistically.

Average daily gain was affected by treatment (Table 2). Pigs given dexamethasone in combination with PRRSV, *S. choleraesuis*, or both had the lowest ADG values that were significantly lower than the values of NNN or NND groups. However, this analysis is incomplete. The three SPD pigs that died during the experiment weighed less at the time of their death than their initial body weight, i.e. had lost weight over the course of the experiment. They were not included in the calculation of the ADG since they did not

Table 2
Average daily gain (ADG) of pigs surviving till Day 21[a]

Treatment	n	ADG (kg)
NNN	7	0.39 ± 0.12a
NND	7	0.39 ± 0.08a
SNN	7	0.32 ± 0.05a,b
SPN	7	0.28 ± 0.14b,c
NPN	7	0.26 ± 0.11b,c
SPD[b]	4	0.26 ± 0.09b,c
SND	6	0.25 ± 0.07b,c
NPD	6	0.18 ± 0.07c

[a] Values are means ± standard deviation of the ADG. Mean ADG values with the same letters are not significantly different ($p > 0.05$) using Duncan's Multiple Range Test.
[b] SPD treatment mean excludes three pigs that died on days 10, 12, and 17.

survive till Day 21. Therefore, the values for the SPD group are biased upward by the exclusion of the most severely affected pigs.

3.2. Bacteriology

Salmonella spp. were not isolated from any of the samples collected prior to inoculation with *S. choleraesuis* on Day 0. Significant differences between treatments ($p = 0.0099$) were shown by analysis of variance for repeated measures of quantitative recovery of *S. choleraesuis* from fecal samples over time. Mean levels of *S. choleraesuis* shedding in feces among treatment groups are summarized in Table 3. The $\log_{10}$ MPN/g feces of the SPD group was significantly greater ($p < 0.05$, Duncan's Multiple Range Test) than the SNN group on Day 7, all groups on Day 10, the SPN and SNN groups on Day 14, and the SNN group on Day 21.

The proportion of pigs within treatment groups from which *S. choleraesuis* was isolated from at least one fecal sample, tonsil swab, or nasal swab are presented in Table 4. A greater proportion ($p \leq 0.008$, Bonferroni's Method) of pigs in the SPD group had

Table 3
Quantitative recovery of *S. choleraesuis* from fecal samples[a]

Treatment	n	Days of Experiment				
		3	7	10	14	21
SNN	7	1.5 ± 1.13a	2.2 ± 1.17a	2.0 ± 1.42a	1.3 ± 1.47a	0.0 ± 0.00a
SND	6	1.8 ± 1.26a	2.8 ± 0.75a,b	2.0 ± 1.84a	2.7 ± 0.90a,b	1.2 ± 1.53a,b
SPN	7	2.2 ± 1.75a	2.9 ± 1.01a,b	2.4 ± 1.44a	1.5 ± 1.66a	1.3 ± 1.89a,b
SPD	7[b]	2.1 ± 0.72a	3.6 ± 1.00b	4.4 ± 1.24b	4.4 ± 1.87b	2.3 ± 0.67b

[a] Values are means ± standard deviations of the $\log_{10}$ most probable number per gram of feces for individual pig samples. Mean values within a column with the same superscript are not significantly different ($p > 0.05$) using Duncan's Multiple Range Test.
[b] Three pigs in SPD treatment died during the experiment (days 10, 12, and 17).

Table 4
Percent of pigs within treatment groups with at least one fecal sample, tonsil swab, or nasal swab positive for *S. choleraesuis*[a]

Treatment	*n*	Day of experiment				
		3	7	10	14	21
SNN	7	71a	86a	86a	57a	0b
SND	6	83a	100a	100a	100a	67a
SPN	7	71a	100a	100a	71a	57a
SPD	7[b]	100a	100a	100a	100a	100a

[a] Treatments within a column with the same superscript are not significantly different ($p > 0.008$) using Bonferroni's Method for pairwise comparisons of proportions.

[b] Three pigs in SPD treatment died during the experiment (days 10, 12, and 17).

positive fecal samples on Day 21, positive tonsil swabs on Day 14, and positive nasal swabs on Day 7, 10, and 14 when compared to the SNN group.

Salmonella choleraesuis was recovered from all tissue types sampled postmortem but not from all pigs. The ileocolic junctions of pigs not inoculated with *S. choleraesuis* were negative for *Salmonella spp*. Significant differences were seen among treatment groups in the proportion of pigs that were *S. choleraesuis* positive for particular tissues (Table 5). Those tissues included mediastinal lymph node, cecal contents, middle ileum, and lung. Although the proportions of positive pigs varied among these four tissues, the order of the

Table 5
Percent of postmortem tissue samples from pigs surviving till Day 21 within treatment groups positive for *S. choleraesuis*[a]

Tissue	Treatments				*P*-value[b]
	SPD ($n = 4$)	SPN ($n = 7$)	SND ($n = 6$)	SNN ($n = 7$)	
Bronchial lymph nodes	50a	57a	83a	14a	0.071
Cecal contents	100a	43a,b	50a,b	0b	0.002
Cecum	100a	71a	50a	43a	0.139
Colon	75a	57a	67a	43a	0.120
Colonic lymph nodes	100a	57a	67a	43a	0.172
Ileocolic junction	100a	43a	67a	57a	0.172
Ileocolic lymph nodes	100a	86a	100a	100a	0.462
Liver	75a	71a	33a	14a	0.077
Lung	100a	57a	83a	0b	0.000
Mediastinal lymph nodes	100a	57a	67a	0b	0.001
Middle ileum	100a	29a,b	67a	0b	0.001
Spleen	25a	29a	17a	0a	0.341
Tonsil	75a	57a	50a	14a	0.173
Among all tissues	85a	55b	62b	23c	0.000

[a] Treatments within a row with the same superscript are not significantly different ($p > 0.008$) using Bonferroni's Method for pairwise comparisons of proportions.

[b] Likelihood Ratio χ^2.

Table 6
Percent of pigs within treatment groups from which PRRSV was recovered from serum samples[a]

Treatment		Days of experiment					
	n	3	7	10	14	17	21
NPN	7	0a	100a	100a	100a	86a	43a,b
NPD	6	0a	100a	100a	100a	83a	17b
SPN	7	0a	100a	100a	100a	86a	57a,b
SPD[b]	7[†]	0a	100a	100a	100a	100a	100a

[a] Treatments within a column with the same superscript are not significantly different ($p > 0.008$) using Bonferroni's Method for pairwise comparisons of proportions.
[b] Three pigs in SPD treatment died during the experiment (days 10, 12, and 17).

treatment groups remained constant. The SPD group always had the highest proportion while tissues from the SNN pigs were sometimes negative for *S. choleraesuis*. The SND and SPN groups were intermediate and were never significantly different from each other. When all tissues sampled post mortem were considered, a similar pattern was seen. Group SPD had a significantly greater ($p \leq 0.008$, Bonferroni's Method) proportion of positive tissues than other groups. The SND and SPN groups were intermediate and similar to each other. The SNN group had the lowest proportion of positive tissues.

3.3. Virology

Serum samples collected on Day 3 were negative by both PRRSV ELISA and virus isolation confirming the pigs had not been previously exposed to PRRSV. Virus was isolated from the serum of all of the PRRSV inoculated pigs on days 7, 10, and 14 (Table 6). On Day 21, a significantly greater proportion of pigs in the SPD group (100%) was virus positive compared to the NPD group (17%). The proportions of PRRSV positive pigs in the NPN and SPN groups were intermediate to the other two groups but not significantly different from them. The mean serum PRRSV titers were similar for all PRRSV inoculated groups on days 10 and 14 (Table 7). The mean titer of the SPD group was significantly higher than the titers of the NPN and NPD groups on Day 17 and the

Table 7
Serum PRRSV titers[a]

Treatment	*n*	Day of experiment					
		3	7	10	14	17	21
NPN	7	0.0 ± 0.00a	2.7 ± 0.33a	2.3 ± 0.39a	1.8 ± 0.42a	1.0 ± 0.81b	0.4 ± 0.50b
NPD	6	0.0 ± 0.00a	3.3 ± 0.26b	2.6 ± 0.49a	2.1 ± 0.38a	1.0 ± 0.68b	0.1 ± 0.24b
SPN	7	0.0 ± 0.00a	2.8 ± 0.36a	2.6 ± 0.45a	2.5 ± 0.88a	1.2 ± 0.64a,b	0.4 ± 0.48b
SPD[b]	7	0.0 ± 0.00a	2.7 ± 0.43a	2.5 ± 0.62a	2.2 ± 0.43a	1.9 ± 0.50a	1.1 ± 0.59a

[a] Values are means ± standard deviation of the $\log_{10}$ ($TCID_{50}$/ml + 1) for individual pig samples. Mean values within a column with the same superscript are not significantly different ($p > 0.05$) using Duncan's Multiple Range Test.
[b] Three pigs in SPD treatment died during the experiment (days 10, 12, and 17).

titers of the NPN, NPD and SPN groups on Day 21. On Day 7, the mean titer of the NPD group was significantly higher than the other groups.

4. Discussion

The challenge doses of PRRSV and *S. choleraesuis* were selected so that single factors would not produce severe clinical disease. This allowed interactions among the factors to be detected more effectively. This strategy was successful and pigs infected with only *S. choleraesuis* or only PRRSV showed no signs, while dually infected animals showed severe clinical signs. In particular, pigs that were dually infected and given dexamethasone as a proxy for stress were the most severely affected with three of the seven pigs dying. The observed clinical results support observations in the field in which PRRSV often appears to have its most profound influence on pigs when acting in concert with other disease agents.

Average daily gain was used as an objective measure of treatment effect. However, the relatively small number of animals per treatment group made it difficult to document biologically significant differences. Dexamethasone in combination with PRRSV, *S. choleraesuis*, or both had the most profound effect on ADG. Body weights of all the SPD pigs were not taken at the times of death of the three SPD pigs; therefore, the weights of the dead pigs could not be included in the analysis for ADG. The pigs that died had actually lost weight during the trial, and therefore, would probably have lowered the ADG of the SPD group if they had survived. As a result, the values for the SPD group were biased upward by the exclusion of the most severely affected pigs. Overall, the trends suggest that growth performance was most severely affected by pathogens in conjunction with stress.

Treatment differences were also seen in the proportion of pigs that had fevers. The results indicated that the presence of fever was primarily a result of *S. choleraesuis* infection that was exacerbated by either PRRSV or dexamethasone. Inoculation with *S. choleraesuis* resulted in a higher proportion of pigs with fevers over a longer period of time. In contrast, PRRSV alone produced a relatively high proportion of pigs with fevers but only for a short period of time. Although the anti-inflammatory affects of dexamethasone might be expected to moderate rather than contribute to a fever response, it seemed to increase the fever response in *S. choleraesuis* infected pigs to a greater degree than concurrent infection with PRRSV. This may be due to the enhanced level of *S. choleraesuis* infection in these pigs.

Concurrent infection with *S. choleraesuis* and treatment with dexamethasone appeared to also increase the duration and virus titer of PRRSV infection. Significant differences in the proportion of PRRSV positive pigs were only evident on Day 21. Differences in virus titers were present on days 17 and 21. Future studies should extend beyond 21 days in order to confirm these observations and more completely determine the effect of concurrent infection and dexamethasone treatment on viremia and duration of infection.

Dexamethasone and concurrent infection with PRRSV appeared to have an additive effect on the duration and level of *S. choleraesuis* fecal shedding. The level and duration of shedding of *S. choleraesuis* for the SPN and SND groups tended to be less than SPD

and greater than SNN pigs. Extrapolated into field conditions, the prolonged and elevated shedding of *S. choleraesuis* seen in SPD pigs would be expected to provide higher and longer levels of exposure of *S. choleraesuis* to herdmates, thereby providing a mechanism for more severe disease. Other research has shown higher infectious doses of *S. choleraesuis* results in more severe clinical disease (Gray et al., 1996b). These results in combination with evidence of more widely systemic infections at necropsy also suggest pigs concurrently infected with PRRSV and subjected to stress were less resistant to spread of *S. choleraesuis* and may be more susceptible to overwhelming infections with the bacteria. This mechanism would support clinical observations that a variety of stressors, including other viral infections, exacerbate clinical outbreaks of salmonellosis (Schwartz, 1991). It would also support suggestions that PRRSV was associated with increasing the clinical effects of salmonellosis in the field (Stevenson et al., 1993).

The potential for PRRSV to modulate the host immune system, at least locally, is supported by the fact that the virus replicates preferentially in macrophages and results in their destruction (Wensvoort et al., 1991; Pol and Wagenaar, 1992). A significant decrease in the proportion of PAMs has been observed in pigs following PRRSV infection. This may have major consequences on defense mechanisms and susceptibility to secondary infections. Under experimental conditions, the proportion of alveolar macrophages in lung lavage decreased from >95% of total cells collected to approximately 50% by Day 7 post challenge (Molitor et al., 1992; Zhou et al., 1992). In addition, alterations in the function of pulmonary macrophages were also observed after PRRS virus infection. The production of inflammatory cytokines by alveolar macrophages, such as interleukin-1 and tumor necrosis factor, was enhanced and nonspecific bactericidal activity of the cells was suppressed (Zhou et al., 1992). In addition to PAMs, pulmonary intravascular macrophages (PIMs) are susceptible to PRRSV infection (Thanawongnuwech et al., 1997). PIMs play an important role in the clearance of bacteria and particulates from blood (Winkler, 1988). Thanawongnuwech et al. (1997) found that intravascular macrophages are susceptible to PRRSV infection and infection was detrimental to their bactericidal activity. In a follow-up study, it was demonstrated that PRRSV infection impaired the ability of intravascular macrophages to clear blood-borne particles (Bautista et al., 1999). Since PIMs play a critical role in the clearance of foreign bodies from the blood stream, decreased function may make pigs more susceptible to bacterial infection, septicemia, and clinical disease. This would provide a partial explanation for the observed synergism between PRRSV and *S. choleraesuis*.

The typical clinical presentation of *S. choleraesuis* infection can include septicemia as well as enterocolitis (Wilcock and Schwartz, 1992). However, the occurrence of pneumonia associated with *S. choleraesuis* infection in swine has increased in recent years and is also considered a common clinical manifestation of infection (Turk et al., 1992). Experimental studies have demonstrated *S. choleraesuis* pneumonia using intranasal inoculation (Baskerville and Dow, 1973; Gray et al., 1995). In addition to inducing pneumonia, studies have demonstrated that the respiratory exposure is an important route of infection in swine and can result in systemic infection and enterocolitis as well as a carrier state with *S. choleraesuis* and *Salmonella typhimurium* (Fedorka-Cray et al., 1995; Gray et al., 1995). It is likely that lowered numbers of PAMs and decreased PIMs antimicrobial activity as a result of a PRRSV infection will serve to exacerbate

S. choleraesuis infection. This was demonstrated in this study with the SPN group showing decreased ADG, increased fecal shedding, and increased tissue dissemination when compared to the SNN group.

Dexamethasone was used in this experiment as a proxy for stress. Stress is a factor that is elusive to measure but is believed to play an important role in PRRSV infections, as well as many other infections including *S. choleraesuis*. Because of its anti-inflammatory effects, dexamethasone might be expected to moderate some of the clinical affects of infection. However, the treatment clearly potentiated both the PRRSV and the *Salmonella* infections, as it was designed to do. We observed the most profound effects of each individual pathogen when it was combined with the dexamethasone and the most severe clinical effects when all three factors were combined. The higher febrile response observed when *Salmonella* was combined with dexamethasone is somewhat contradictory when considered with the decreased monocyte activity expected by this anti-inflammatory agent. However, the treatment combination also resulted in a more disseminated *Salmonella* infection that would provide increased levels of pyrogenic endotoxin.

5. Conclusions

In conclusion, our observations strongly support the concept that interactions between PRRSV and other infectious agents are central to understanding their pathogenesis and explaining clinical outbreaks in the field. Treatment differences were seen in growth rates, levels and duration of *S. choleraesuis* shedding, distribution of *S. choleraesuis* in tissues, degree of clinical disease, and mortality. The data suggest that differences in the severity of systemic and respiratory disease attributed to PRRSV can be explained in part by the interactions of PRRSV-infected swine with their environment and other infectious agents.

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