RESEARCH REPORT
SWINE HEALTH

Title: Comparative analysis: The pathogenesis of disease induced in piglets by group A, B and C rotaviruses, singularly or concurrently – **NPB #11-043**

Investigator: Darin Madson

Institution: Iowa State University

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Industry Summary

Rotavirus is not a new pathogen; however, there is evidence of re-emergence as noted by the increased diagnoses at multiple diagnostic laboratories. New testing modalities (PCR) have been developed to detect different serogroups of rotavirus in diseased or diarrheic pigs; however, interpretation of test finding is difficult because the basic research comparing these different serogroups has not previously been done. With this research we compared fecal shedding and intestinal pathology of naïve pigs inoculated with serogroups A, B, or C singularly and in all combinations of coinfections. This was accomplished by successfully cultivating serogroup C rotavirus in cell culture and purifying serogroup B rotavirus for experimental inoculation. Generated data from this research indicates all serogroups are able to cause clinical diarrhea and intestinal pathology by 24 hrs post-infection (hpi) with fecal shedding as early as 12 hpi. Serogroups B and C infection resulted in diffuse small intestinal pathology at 24 hpi, while serogroup A infection preferentially damaged the jejunum in the early phase of disease. Serogroup coinfections were not more severe than singular infections in this study, but serogroup C virus was more frequently detected in feces when in combination with other serogroups. Alternatively, serogroup B was less frequently detected when in combination with another serogroup. In summation, the current study shows that all rotavirus serogroups are capable of causing diarrhea and intestinal lesions when given alone or in different combinations.

Contact information
Darin Madson
Iowa State University, Veterinary Diagnostic Laboratory
1600 South 16th
Ames, IA 50011
madson@iastate.edu

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- Swine, rotavirus, neonatal pigs, serogroups A, B and C

Scientific abstract
Rotavirus is a common diarrheic pathogen in neonatal swine and can be detected using new diagnostic PCR methods. The objective of this study was to compare viral shedding and microscopic intestinal changes of rotavirus serogroups A, B, and C singularly or in all combinations in caesarian-derived, colostrum-deprived (CDCD) neonatal pigs. A second objective was to refine a possible in vitro culture of serogroups C and B. Forty-eight, one-day old CDCD piglets were randomly divided into eight groups with six pigs each. Piglets were gastrically inoculated with similar quantities of total virus. Fecal swabs were collected every 12 hrs and three piglets in each group were sacrificed at 24 or 72 hrs post infection (hpi) for microscopic intestinal evaluation of five predetermined sections. Viral shedding was detected in all groups by 24 hpi, and was generally sustained until necropsy excluding serogroup B infected pigs. Serogroup C virus was detected more frequently and in higher quantities compared to the coinfected serogroup in combinational groups. Significant differences in villous height were determined at 24 and 72 hpi between sham and inoculated groups. Serogroups B and C resulted in diffuse small intestinal atrophy; however serogroup A infection was more restricted to the jejunum at 24 hpi. Significant crypt depth differences were also apparent at 72 hpi. Serogroup C, but not B was adapted for in vitro cell culture using MA-104 cells. Results of this study, suggest serogroup C rotaviruses can be cultured in vitro, and serogroups B and C are able infect the entire small intestinal tract early in the course of infection as compared to the serogroup A virus used in this study.

Introduction

Clinical diarrhea associated with rotavirus infection is common in the swine industry. New diagnostic testing methods, PCR in particular, have been recently developed leading to increased detection of rotaviral infection within submitted samples to Veterinary Diagnostic Laboratory. Previous to PCR detection, diagnostic testing for rotaviral infection was based on antigen capture ELISA, immunohistochemistry, electron microscopy and tradition virus isolation; many of these tests are for identification of serogroup A rotavirus infection only. Detection of non-serogroup A rotaviral infections and/or co-infections has caused diagnostic and clinical confusion couple with consternations in multiple field situations.

Rotaviruses are one of 11 known enteropathogenic viruses associated with clinical diarrhea in swine. Rotaviruses have a non-enveloped RNA virion with 11 double-stranded RNA segments.[1] Rotaviral virions have three layers: an outer layer containing viral protein (VP)7 and VP4, a middle layer associated with VP6, and an inner layer containing VP2. Serogroups of rotavirus are based on antigenic differences in the VP6 proteins; the VP6 layer is antigenically conserved.[1] There are 7 known rotavirus serogroups, however, only 4 have been associated with diarrhea in swine: A, B, C, and E. Serogroup E has only been identified in the United Kingdom.[1] Serogroup A, B and C rotaviruses are further classified into G and P serotypes based on differences in the VP7 and VP4 proteins, respectively.

Mortality and morbidity associated with rotaviral infection vary depending on factors such as virus strain, pig immunity (acquired and passive) and environmental conditions.[4] It is widely accepted that infection is associated with moderate economic impact in the swine industry.[2] Group A rotaviruses have been most commonly associated with post-weaning diarrhea and are cultivatable to a degree using *in vitro* virus isolation methods. Alternatively, groups B and C are extremely difficult, if not impossible, to isolate in cell culture using known available techniques. Electrophoresis with silver staining has typically been used to differentiate serogroups A-C.[1,4,5]

After ingestion, rotavirus attaches to and invades mature, villus tip enterocytes. Infection results in enterocyte destruction and sloughing with contraction of villus cores.[5] The histopathologic picture is one of villus atrophy and fusion of adjacent, damaged villi. Lesions are more commonly seen within the jejunum and are often segmentally distributed within the intestinal tract. Loss of enterocytes results in

decreased absorptive capacity and clinical diarrhea. Serogroups A and C are known to be associated with more severe diarrhea and microscopic lesions as compared to serogroup B rotaviruses.[4] In addition, some serogroup A rotaviruses have an enterotoxin, NSP4. This enterotoxin also contributes to diarrhea by causing hypersecretion, an unusual mechanism for viral diarrhea.[3]

The Iowa State University Veterinary Diagnostic Laboratory (ISU-VDL) receives numerous questions from swine practitioners regarding interpretation of results when non-serogroup A viruses are detected or when combinations of different serogroups are detected. Simply, there has been a knowledge gap within the veterinary community in understanding the significance when groups B and C are detected or when there are combination of the groups detected by the PCR. The aim of recent research at the ISU-VDL was to study the pathogenesis of groups A, B, and C and all combinations in neonatal pigs. The specific objective was to determine the extent and magnitude of diarrhea, viral shedding, and intestinal destruction in pigs infected with groups A, B, and C singularly and in all combinations including coinfecting a subset of pigs with all three groups simultaneously.

Objectives

1. Compare viral titers and duration fecal shedding, and location and extent of microscopic lesions across mono-infected and co-infected challenge groups.
2. To define/refine group C rotaviral cell culture techniques for advancement of scientific knowledge which will be used in future research (i.e. serology and immunity testing)
3. To define/develop group B rotaviral cell culture techniques for advancement of scientific knowledge which will be used in future research (i.e. serology, real time PCR, and immunity testing)

Materials & Methods

Animals

Pregnant commercial cross-bred sows, known to be negative for porcine reproductive and respiratory syndrome virus (PRRS), where purchased and delivered to Iowa State University (ISU) approximately one week prior to excepted farrowing date. On day 113 of gestation, caesarian surgeries were performed on sows for piglet derivation. Piglets did not receive colostrum. Neonatal piglets used in this study were caesarian derived and colostrum deprived (CDCD piglets). All CDCD piglets included in this study were triaged at birth with navels clamped, cut, and sprayed with gentile iodine. Piglets also received an iron and antibiotic injection (Excede®, Pfizer) per labeled directions

Study design

Piglets were randomly divided into eight groups with six piglets each. Piglets were sham inoculated (negative controls) or challenged with rotavirus serogroups A, B, or C singularly and all possible combinations. Table 1 summarizes the study design. Study termination points were 24 and 72 hours post inoculation (hpi); one half the pigs in each group were euthanized and 24 hpi and the remaining piglets at 72 hpi.

Table 1. *Experimental design. One day-old CDCD piglets were randomized into eight treatment groups and sham inoculated or challenged with different rotaviral serogroups or all combinations of serogroups A, B, and C.*

Groups	*n*	Age	Inoculation
1	6	1 day	None (negative control)
2	6	1 day	Rotavirus group A
3	6	1 day	Rotavirus group B
4	6	1 day	Rotavirus group C
5	6	1 day	Rotavirus group A & B
6	6	1 day	Rotavirus group A & C
7	6	1 day	Rotavirus group B & C
8	6	1 days	Rotavirus group A, B, & C

Housing
Piglets were individually housed in new plastic totes and each group was kept in a separate temperature controlled room. Piglets were fed milk replacer (Esbilac; Pet-Ag, Hampshire, IL) three times a day (7 am, 12 pm, and 7 pm). All feedings were done by oral-gastric lavage using an 8 gauge French catheter.

Inoculation
Piglets were oral-gastrically inoculated with virus (es) according to Table 1 approximately 4 hrs post-surgery using an 8 gauge French catheter. Rotaviruses used in this experiment were procured from field investigations.

The serogroup A and C rotavirus isolates were cultivated in MA-104 cells. Plaque forming units (PFU) were estimated at 10^6 PFU/ml for serogroup A and 10^3 PFU/ml for serogroup C. Serogroup B rotavirus was obtained from feces through a series of purification and amplification steps. The serogroup A, B, C viruses were then serially diluted and tested by gel based PCRs. The rotavirus B and C dilutions were negative at 1:1000 while rotavirus A dilutions were negative at 1:1000000. Gel based PCR was used to confirm specificity of each inoculum sample. Real-time quantitative PCR confirmed a similar amount of rotavirus for the serogroup A and C inoculum. Finally, each rotavirus inoculum that was estimated to contain 1,000 PFU/ml and 10,000 genomic copies/mL was used.

Singularly infected groups (groups 2, 3, & 4) received 3 mL of inoculum orally. Dual infected groups (groups 5, 6, & 7) received 1.5 mL of each serogroup and group 8 (triple infection) challenged pigs received 1 mL of each serogroup. All pigs were given a similar total numbers of rotavirus genomic copies.

Sample collection
Rectal swabs were taken from all piglets prior to inoculation and every 12 hrs thereafter. Rectal swabs, serum, colonic contents, and tissues were collected at necropsy from all pigs. Tissue sections of fixed small intestine were used for microscopic evaluation.

Rotavirus PCR
Fecal swabs and contents at necropsy were assayed as previously described.[6]

Histopathology
Five sections of small intestine: 1) duodenum, 2) proximal jejunum, 3) mid jejunum, 4) distal jejunum, and 5) ileum were evaluated from villous length and crypt depth. The entire small intestine was extracted

from each pig, the mesentery cut away, and then folded back on itself several times (3 times). The same locations were cut from each pig. Five full length villi and crypts in each microscopic section of intestine were measured using a computerized image system (Olympus DP72 camera, cellSens Standard).

Statistics

Summary statistics (JMP® software version 10.0.0), were calculated for all groups to assess the overall quality of the data set. Mean villous length by intestine sections and group were subjected to an analysis of variance (ANOVA) with mean comparison using an all pairs Tukey-Kramer adjacent. The same was done for crypt length. Necropsy time point (24 hrs and 72 hrs) lesions were analyzed by ANVOA and compare using a student's T test. Significance difference were determined when *P* was less than or equal to 0.05.

Results

Clinical diarrhea was not present in the negative control pigs during the duration of the study. At 24 hpi, challenged pigs in all groups had clinical diarrhea. Diarrhea continued and did not resolve prior to termination in all challenged groups. Clinically, there was not difference in diarrhea between singular or coinfected groups.

Fecal swab detection of viral shedding for singularly infected groups is summarized in Table 2. First detection of fecal shedding of rotavirus A only infected pigs was at 24 hpi. Shedding continued until necropsy at 72 hpi in 2/3 pigs. Serogroup B infection was detected at 12 and 24 hpi in half the infected pig and not detected thereafter in this study. Serogroup C rotavirus shedding was detected in the majority of piglets at 12 hpi and then in all pigs until their designated necropsy. Viral titer in feces did not decline with time for serogroup C infected piglets. In comparison, fecal shedding in rotavirus co-infected groups was more sporadic and variable depending on the combination as shown in Table 2.

Table 2. *Fecal swab PCR shedding results of piglets inoculated singularly or with different combinations of rotaviral serogroups at given hours post infection (hpi).*

Group	Fecal Shedding					
	12 hpi‡	24 hpi	36 hpi	48 hpi	60 hpi	72 hpi
A	0*	100	100	66	66	66
B	50	50	0	0	30	0
C	83	100	100	100	100	100
A/B	0 / 0*	83 /100	100 / 0	100 / 0	100 / 0	100 / 0
A/C	67 / 83	100 / 44	30 / 100	33 / 67	33 / 67	33 / 67
B/C	0 / 50	17 / 100	0 / 100	0 / 67	0 / 67	0 / 67
A /B/C	0 / 0 / 0	0 / 17 / 100	0 / 0 / 100	0 / 0 / 67	0 / 0 / 67	0 / 0 / 67

*% positives for each target serogroup
‡ hours post infection; hpi

At necropsy, challenged pigs were variably dehydrated, thin, and had fecal staining of the peritoneum. Small intestinal segments were segmentally thin-walled and spiral colons were distended by large amounts of watery contents. Table 3 summarizes the colonic content PCR testing by necropsy time point. Control piglets were normal with no fecal staining and had formed feces within the colon. Rotaviruses were not detected in any of colonic contents from the control piglets.

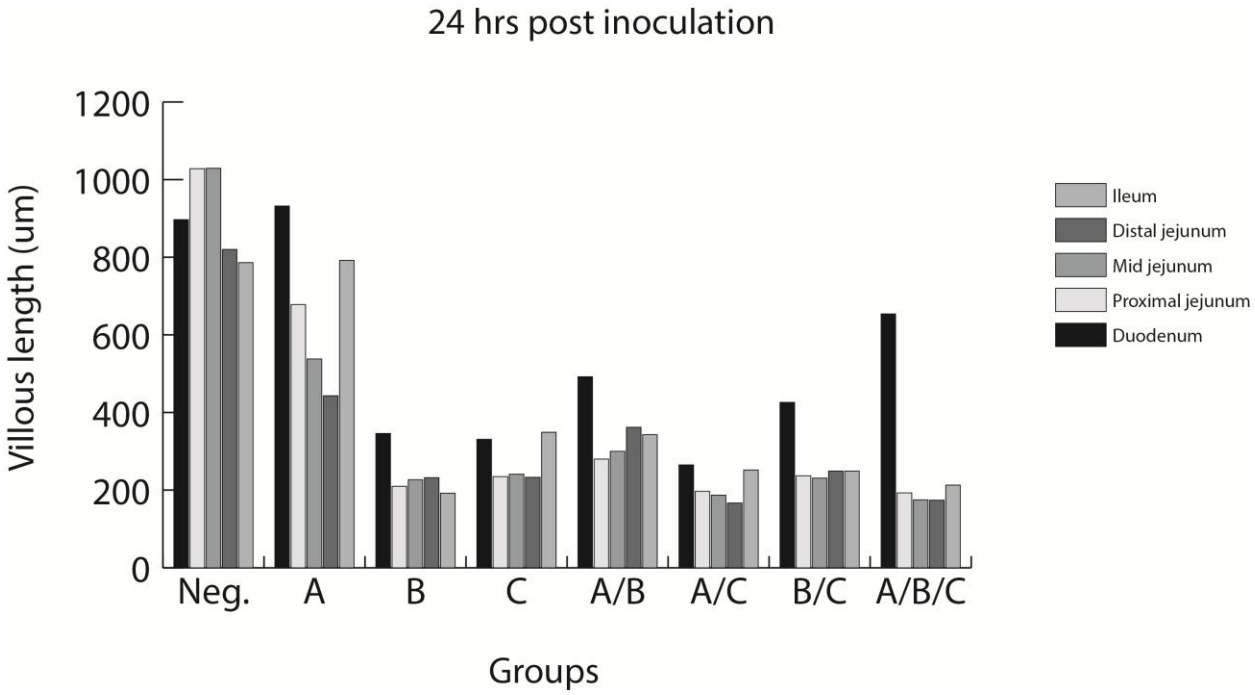
Table 3. *Colonic content PCR results at necropsy for sham and challenged piglets. Six piglets per group were challenged. Three random piglets per group were necropsied at 24 hpi and the remaining at 72 hpi.*

Group	Serogroup A		Serogroup B		Serogroup C	
	24 hpi‡	72 hpi	24 hpi	72 hpi	24 hr	72 hpi
Negative	0*	0	0	0	0	0
A	100	100	0	0	0	0
B	0	0	100	100	0	0
C	0	0	0	0	100	100
A/B	100	100	100	66	0	0
A/C	100	100	0	0	100	100
B/C	0	0	0	0	100	66
A /B/C	100	100	0	0	100	100

*% positives for each target serogroup
‡ hours post infection; hpi

Villous atrophy, as measured by villous length, for sham and inoculated groups is summarized in figures 1 (pigs necropsied at 24 hpi) and 2 (pigs necropsies at 72 hpi). Significant statistical differences were found between groups, and are summarized in the connecting letters report table below each figure.

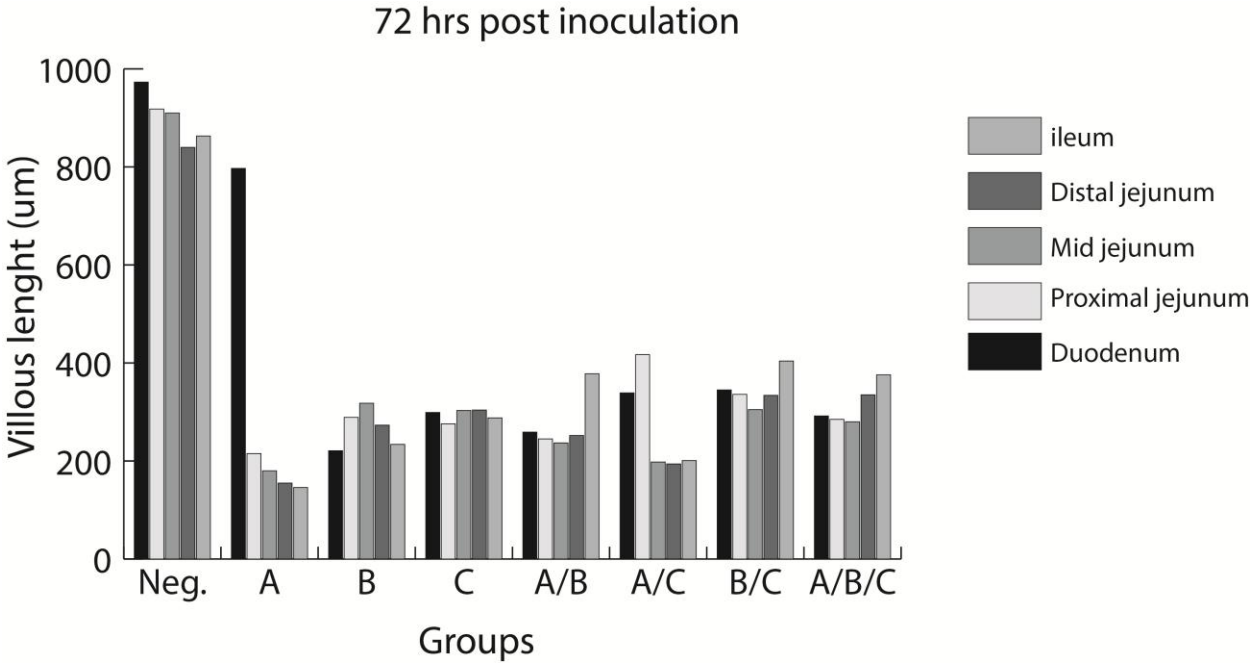
Figure 1. *Average full length villous height per small intestinal locations of negative control, piglets singularly inoculated or inoculation combination of rotavirus necropsied 24 hpi. The table below is a connecting letters reports of statistical significance (different letters correspond to a P≤ 0.05)*



Location	Group							
	Negative	A	B	C	A/B	A/C	B/C	A/B/C
Duodenum	A	A	B	B	A,B	B	A,B	A,B
Prox. Jejunum	A	A,B	C	C	B,C	C	C	C
Mid Jejunum	A	B	B	B	B	B	B	B

Distal Jejunum	A	A,B	B	B	B	B	B	B
Ileum	A	A	B	B	B	B	B	B

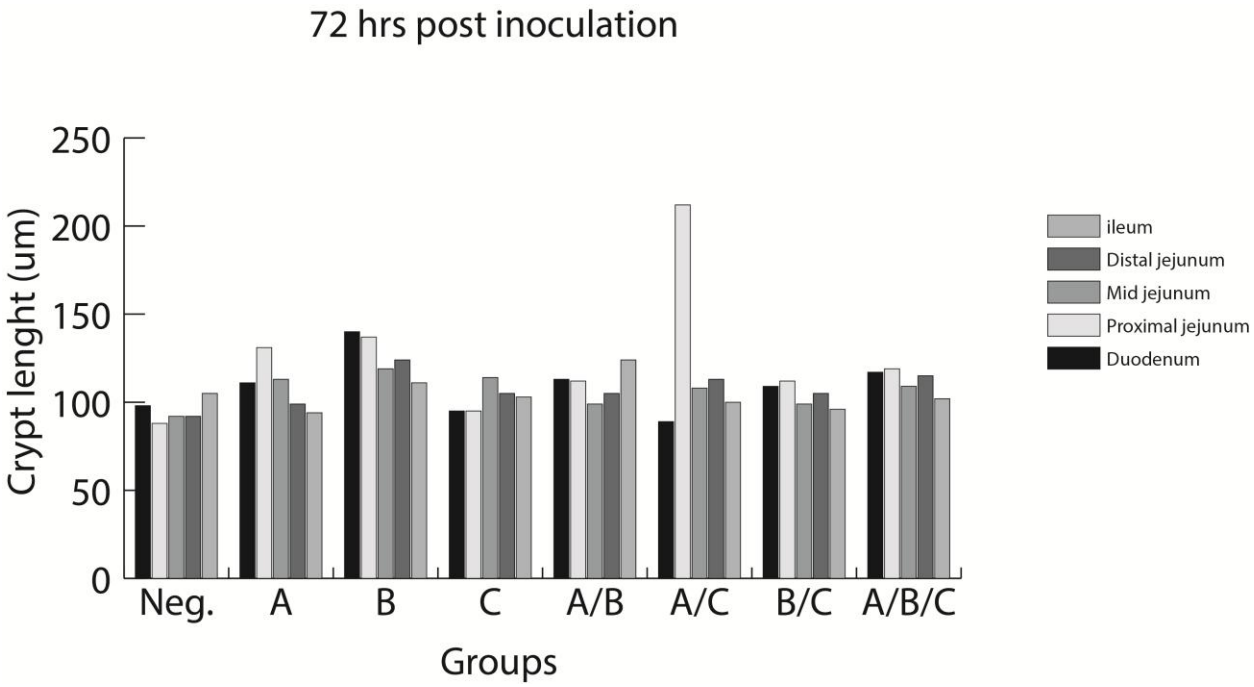
Figure 2. *Average full length villous height per small intestinal locations of negative control, piglets singularly inoculated or inoculation combination of rotavirus necropsied 72 hpi. The table below is a connecting letters reports of statistical significance (different letters correspond to a P≤ 0.05)*



Location	**Group**							
	Negative	A	B	C	A/B	A/C	B/C	A/B/C
Duodenum	A	A	B	B	B	B	B	B
Prox. Jejunum	A	B	B	B	B	B	B	B
Mid Jejunum	A	B	B	B	B	B	B	B
Distal Jejunum	A	B	B	B	B	B	B	B
Ileum	A	B	B	B	B	B	B	B

No significant statistical differences in crypt depth were found across all groups necropsied at 24 hpi. Differences were found by small intestinal location amongst examined groups at 72 hpi and are reported figure 3.

Figure 3. *Average crypt depth per small intestinal locations of negative control, piglets singularly inoculated or inoculation combination of rotavirus necropsied 72 hpi. The table below is a connecting letters reports of statistical significance (different letters correspond to a P≤ 0.05)*



Location	Group							
	Negative	A	B	C	A/B	A/C	B/C	A/B/C
Duodenum	A	A	A	A	A	A	A	A
Prox. Jejunum	B	A,B	A,B	B	A,B	A	A,B	A,B
Mid Jejunum	B	A,B	A	A	A,B	A,B	A,B	A,B
distal Jejunum	A	A	A	A	A	A	A	A
Ileum	A	A	A	A	A	A	A	A

Discussion

The aim of the present study was to add some clarity to concerns related to swine rotaviral infection; in particular, the significance of non-rotaviral serogroup A infection. Challenged neonatal CDCD piglets in this study developed diarrhea irrespective of serogroup; rotaviral serogroups A, B, and C can cause clinical diarrhea. Singularly infected piglets, with all tested serogroups, developed diarrhea by 24 hpi. Fecal shedding of virus can be detected by 12 hpi. An interesting result in this study was that serogroup A and C infection resulted in sustained viral fecal shedding until termination of the study, 72 hpi. Highest genomic copies were seen at 24-36 hpi in piglets infected with only serogroup A or C. Serogroup B infection(s) had a shorter detection period in feces, generally within the first 24 hpi.

Rotaviral infection in coinfected groups was not as consistent as in singular infected groups. Serogroup A detection in feces over time and in colonic contents predominated over serogroup B in the A/B challenged piglets. Alternatively, serogroup C detection was more frequent over time in A/C challenged pigs. Serogroup C predominated over serogroup B in this group as well. These results suggest that the serogroup C isolate used in this study was able out-compete the A and B viruses. A similar finding was also found in piglets inoculated with all three serogroups, serogroup C was detected more frequently than serogroups A and B. The reasoning for this is unknown, but maybe related to the particular virus isolate or competition for replication sites/receptors.

All rotavirus challenged piglets developed microscopic lesions of infection; atrophic enteritis. Significant difference in villous height were detected in inoculated piglets at 24 hpi in this study compared to the negative controls. Microscopic lesions were present in all examined sections of small intestine. An interesting finding was that serogroup A only infected piglets had significant longer villi at 24 hpi in duodenal and ileal sections compared to other inoculated groups. This finding suggests that the serogroup A rotavirus isolated used in this study preferential attacks enterocytes within the jejunum. In contrast, serogroups B and C isolates used were able to cause significant villous atrophy within all sections of small intestine at 24 hpi indicating a broader or more diffuse small intestinal infection. By 72 hpi, all sections of small intestine were significantly atrophied compared to negative control piglets. Villous regeneration, measured by crypt depth length, was not different at 24 hpi infection. However, by 72 hpi, crypt length began to lengthen within the jejunum compared to negative controls (figure 3).

In conclusion, clinical disease in CDCD piglets singularly infected with serogroup A, B, or C were similar; piglets developed diarrhea and at a similar time point post-infection. The serogroup C isolate used in this study appeared to out-compete other serogroups, and the serogroup B virus used was detected the least in coinfected groups. Microscopic intestinal lesions identified that serogroups B and C have a more diffuse small intestinal replication presence compared do the serogroup A virus used in this study at 24 hpi infection. Combinational infected groups were not different from singularly infected groups, but based on viral shedding, combinations containing A and C are predicted to be more detrimental to infected pigs and the population.

Objectives one and two were fulfilled. Objective 3; serogroup B virus cultivation in cell culture was not accomplished. Multiple cell lines were attempted with different growing condition; however, no protocol was successful for laboratory isolation.

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