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Murine model of Hirschsprung-associated enterocolitis. I: Phenotypic characterization with development of a histopathologic grading system

Zhi Cheng^{a,1}, Deepti Dhall^{b,1}, Lifu Zhao^a, Hanlin L. Wang^b, Terence M. Doherty^c, Catherine Bresee^d, and Philip K. Frykman^{a,*}

^aDivision of Pediatric Surgery and The Pediatric Surgery Laboratory, Department of Surgery, Cedars-Sinai Medical Center, Los Angeles, CA 90048, USA

^bDepartment of Pathology, Cedars-Sinai Medical Center, Los Angeles, CA 90048, USA

^cDepartment of Biomedical Sciences, Cedars-Sinai Medical Center, Los Angeles, CA 90048, USA

^dThe Samuel Oschin Comprehensive Cancer Institute, Cedars-Sinai Medical Center, Los Angeles, CA 90048, USA

Abstract

Purpose—The aim of the study was to characterize enterocolitis in the *Ednrb*-null (*Ednrb*^{-/-}) mouse with aganglioneurosis of the colon and to develop and validate a semiquantitative histopathologic grading system to assess enterocolitis.

Methods—We isolated colon and ileal specimens of *Ednrb*^{-/-} and control mice (*Ednrb*^{+/+}) and performed histochemical staining (H&E) on tissue sections. After establishing inflammation grading criteria, 2 blinded pathologists independently assessed the severity and depth of inflammation of proximal colon segments on 2 separate occasions. Interclass correlations (ICCs) and coefficient of variation (CV) were calculated to determine interrater and intrarater agreement. We then prospectively applied the enterocolitis grading system to *Ednrb*^{-/-} mice that became clinically ill. A cohort of *Ednrb*^{-/-} mice were observed until they developed clinical illness, at which time they were euthanized and had multiple organ homogenates cultured for bacteria, and colon and small bowel were histopathologically graded for enterocolitis. Spearman's rank correlations comparing enterocolitis scores with level of bacteremia were performed.

Results—Intra- and interrater ICCs of the histologic scoring system were satisfactory (0.61 and 0.94, respectively), as were intra- and interrater CVs (18% and 9%, respectively). Of the *Ednrb*^{-/-} mice, 65% developed bacteremia. Those with bacteremia had significantly higher enterocolitis scores than those without bacteremia ($P < .01$). *Ednrb*^{-/-} mice that developed bacteremia showed a strong positive correlation between total enterocolitis scores and number of bacterial colony forming units in peritoneal lavage, liver, kidney, and aerobic spleen.

Conclusions—The Ednrb^{-/-} mouse with aganglionosis develops enterocolitis and has features similar to Hirschsprung-associated enterocolitis in humans. Our grading system is a reliable way to assess enterocolitis. By performing microsurgical pull-through, we can now perform controlled, hypothesis-driven, mechanistic studies to evaluate etiologic factors affecting enterocolitis in the Ednrb^{-/-} mouse.

Keywords

Hirschsprung's disease; Hirschsprung-associated enterocolitis; Aganglionic megacolon; Ednrb-B-null mice; Enteric nervous system; Enteric neuropathy; Histopathologic grading system

1. Background

The treatment of Hirschsprung disease (HD) is often straightforward and curative and frequently consists of a one-step minimally invasive pull-through surgery that excises the aganglionic section of colon. However, in 20% to 38% of patients, surgery does not resolve the clinical condition, and instead, these patients are subject to bouts of enterocolitis, called *Hirschsprung-associated enterocolitis* (HAEC).

Postoperative HAEC is in fact the single largest cause for hospitalization of infants and children with HD who have undergone pull-through corrective surgery. Hirschsprung-associated enterocolitis can be associated with severe infections of the small bowel and colon, and these can lead to life-threatening sepsis and death. Hirschsprung-associated enterocolitis occurs independent of pull-through technique and is present worldwide [1–3]. In severe cases, or chronic recurrent episodes affecting a child's growth, a diverting colostomy needs to be performed. Hirschsprung-associated enterocolitis is a well-recognized condition and moderately well characterized from a clinical perspective but not from a mechanistic perspective. Factors that increase risk of HAEC include delayed diagnosis of HD, trisomy 21, long segment HD (ie, aganglionosis proximal to the splenic flexure), and family history of HD [4–6]. Many possible etiologies and contributing factors have been proposed [7,8], but the molecular, cellular, and genetic causes of HAEC remain unknown.

Mechanistic studies have been hampered by the lack of a suitable animal model that mimics HAEC associated with aganglionosis. Several spontaneous and targeted gene mutations in mice and rats produce aganglionosis of the colon (and in some models, small bowel), including mice that fail to express either the endothelin receptor B [9] or its cognate ligand, endothelin-3 [10], and Sox10 [11]. However, most of the affected animals succumb by 7 to 8 weeks of age because of intestinal obstruction from aganglionic megacolon. Furthermore, these genotypes do not accurately mimic the clinical realities of children with HD (who invariably undergo pull-through operations within the first few months of life) and, more importantly, do not offer any opportunity to study postoperative enterocolitis.

We sought to create a murine model that not only mimics the major salient features of aganglionic colon but that also enables us to study the mechanisms mediating the enterocolitis that often occurs after surgical correction. We therefore developed a microsurgical pull-through operation and performed this operation successfully in Ednrb^{-/-} mice [12], which are characterized by short segment aganglionic megacolon that invariably

leads to death by around 4 to 6 weeks of age. Because our long-term objective is to study postoperative enterocolitis, we next sought to characterize relevant features and perform histopathologic analyses in animals without surgery and after surgery.

Here we report development and validation of a semiquantitative histopathologic scoring system to quantify intestinal inflammation that occurs in these mice before surgical correction. We found a clear relationship between bacteremia and the degree of intestinal inflammation and determined that bacterial species that are typically found in the gut infect multiple organs. These results further support the use of this model as a means of studying pathologic mechanisms that may cause or contribute to enterocolitis in the context of aganglionosis of the colon.

2. Materials and methods

2.1. Animals

Animal protocols were approved by the Institutional Animal Care and Use Committee at Cedars-Sinai Medical Center (Los Angeles, Calif). Mice (*Ednrb*^{tm1Ywa/J} on a hybrid C57BL/6J-129Sv background; JAX stock no. 003295 [9]) were purchased from Jackson Laboratory (Bar Harbor, ME) and housed at the animal facility of Cedars-Sinai Medical Center. The mice were maintained on a 12 hour/12 hour light-dark cycle at 25°C and received regular mouse chow (Laboratory Rodent Diet 5001, Purina Mills, St. Louis, MO [13]) and water ad libitum. A breeding colony of the *Ednrb*^{tm1Ywa/J} heterozygous mice was established and maintained at Cedars-Sinai Medical Center. The *Ednrb*^{tm1Ywa/J} is a targeted null mutation of the endothelin receptor B gene that segregates in an autosomal recessive manner. The homozygotes (we refer to these as *Ednrb*^{-/-} in this article) are easily identified by the white coat color and progressively enlarging abdomens because of aganglionosis leading to megacolon [9]. The wild-type and heterozygote littermates (we refer to these as *Ednrb*^{+/+} and *Ednrb*^{+/-}, respectively) are phenotypically normal and therefore indistinguishable from one another and required genotyping using a polymerase chain reaction-based assay.

2.1.1. Bacteremia studies—Breeding pairs of *Ednrb*^{+/-} male and female mice were divided into 3 groups: group 1 received regular mouse chow only, group 2 received liquid rodent diet (LD101, Purina Mills [13]) 4 days before pup weaning that was continued after weaning, and group 3 received triple antibiotic drinking water before delivery of pups that was continued through the nursing period and after weaning and liquid rodent diet 4 days before pup weaning that was continued after weaning. Group 3 pregnant females were monitored, and 3 to 4 days before delivery, triple antibiotic drinking water containing enrofloxacin, 0.25 mg/mL; metronidazole, 0.2 mg/mL; and vancomycin, 0.25 mg/mL, [14] was placed in the cages, removing regular water. The triple antibiotic drinking water was continually administered after the pups were weaned.

Ednrb^{-/-} pups in each group were monitored at least once per day monitoring for signs of clinical illness manifested by decreased activity, poor feeding, weight loss, and increasing abdominal distension. Each animal's body weight was measured every 2 to 3 days. When at least 2 of these clinical signs were observed, the animals were euthanized using isoflurane

anesthesia in a desiccator jar followed by exsanguination, peritoneal lavage, and organ harvest under sterile conditions.

Heparinized blood; peritoneal lavage; and tissue homogenates of the entire spleen, both kidneys, and entire liver were prepared in cold sterile Dulbecco's phosphate buffered saline, and serial dilutions of each were plated on blood agar plates and incubated under aerobic and anaerobic conditions at 37°C [15]. Bacterial colonies were counted after 48 hours. Colony forming units (CFUs) were calculated per organ using the formula $CFU \text{ (per } 200 \mu\text{L of total organ homogenate)} = (N/D) \times (V1/V2)$, where N is the counted bacterial colonies; D, dilution; V1, total volume of organ's homogenization (200 μL); and V2, total volume inoculated on blood agar plate (20 μL). Representative colonies from aerobic and anaerobic plates were identified and speciated by the Cedars-Sinai Medical Center clinical microbiology laboratory.

2.1.2. Histologic preparation—The entire colon (including anus) and 2 cm of terminal ileum was excised, gently washed with cold phosphate buffered saline, and fixed with 10% neutral buffered formalin at room temperature overnight. The specimens were cut into 4 segments (1 cm in length) and recorded as anus and rectum, 1; distal colon, 2; proximal colon, 3; and terminal ileum, 4 (Fig. 1). All specimens were embedded in paraffin, and the anorectal segment was sectioned in a sagittal orientation, and all other segments were sectioned in a transverse orientation. Each segment was sampled at 3 regions and serially sectioned using 6- μm sections and stained with H&E.

2.2. Histologic grading system development

Together with our colleagues in the Department of Pathology (DD & HW), a semiquantitative histologic scoring system was developed to evaluate the severity and depth of inflammation in intestinal specimens of Ednrb^{-/-} mice and wild-type controls. Initially, all segments from Ednrb^{-/-} animals were evaluated, and we found considerable variability in the length of aganglionic segment and degree of megacolon in segments 1 and 2. We chose segment 3 to score because it was consistently in the ganglionic region of the colon of Ednrb^{-/-} mice and could also be compared to Ednrb^{+/+} animals (control) without megacolon. Each pathologist scored the sampled regions of the proximal colon (segment 3) for inflammation and depth, twice in a blinded fashion for each animal, creating 4 sets of scores per animal on a cohort of 23 Ednrb^{-/-} mice (Table 1) The highest score from the sampled regions in each segment was recorded.

2.2.1. Validation of histologic methods—Interclass correlations (ICCs) were calculated for the scores generated from the cohort mentioned above and are measures of the strength of a linear relationship between 2 measures and ranges from -1 to +1. The more nonzero a correlation is, the stronger the association. Interclass correlation was calculated according to the method of Bartko [16]. The coefficient of variation (CV) was generated and is a measure of relative variation and is reported on a percentage scale.

2.2.2. Statistical analysis—The data collected from the study animals on various diet and antibiotic conditions were compared using Student's *t* test, 2-tailed, with Welch's

correction because of unequal variances in the groups. Histopathologic score data were analyzed via the Wilcoxon's rank-sum test and Spearman's rank correlations [17]. Data are presented as means $\pm$ SD. Statistical analysis was performed using SAS computer software (SAS Institutes Inc, Cary, NC).

3. Results

3.1. Development of histopathologic grading system for enterocolitis

Enterocolitis has previously been described in multiple studies of the piebald-lethal mouse strain (with an *Ednrb* gene mutation) [18–20], so it was not surprising that the *Ednrb*^{−/−} mice showed histologic evidence of enterocolitis. We developed a grading system that evaluated both the severity and depth of inflammation, both of which are combined to give a total enterocolitis score (Table 1 and Fig. 2). This grading system was then validated prospectively on a cohort of 23 *Ednrb*^{−/−} mice (group 1) using ICCs and CV calculations. On the basis of the data from this cohort, we report that this methodology (ie., the total score) has an intra- and interrater ICC of 0.61 and 0.94, respectively, with an intra- and interrater CV of 18% and 9%, respectively (Table 2). Overall, the inflammation score was much more consistent than the depth score, with higher correlation and lower CV both within (intra-) and across (inter-) raters. Our enterocolitis grading system has a high degree of reproducibility and is validated as a reliable tool to grade enterocolitis for further studies.

3.2. Diet and antibiotic effects on *Ednrb*^{−/−} mouse survival

To assess the effect of diet and enteral antibiotics on survival of *Ednrb*^{−/−} male and female mice, the 3 cohorts of mice were observed under different conditions: group 1, regular mouse chow (control); group 2, liquid rodent diet (LD); and group 3, liquid rodent diet and antibiotic drinking water (LD + Abx). The end point was clinical illness, at which point, the animals were euthanized and tissues collected as described in Methods. Animals that died before tissue collection had survival time recorded; however, tissues were not collected nor analyzed further. *Ednrb*^{−/−} mice in group 1 had a mean survival time of 26.9 days. Weaning the *Ednrb*^{−/−} pups to liquid diet (group 2) prolonged mean survival 3 days compared to regular solid mouse chow ($P = .18$), the addition of triple antibiotic drinking water with liquid diet (group 3) prolonged mean survival an additional 6 days ($P = .10$); however, the liquid diet and triple antibiotic drinking water significantly prolonged mean survival of 9 days (to 36.4 days) compared to control ($P = .009$) (Fig. 3). These data suggest that many *Ednrb*^{−/−} mice are dying through a bacterially mediated process, a mechanism that is partially ameliorated by enteral antibiotics.

3.3. Enterocolitis and bacteremia

To evaluate the potential role of enterocolitis as a major cause of mortality in the *Ednrb*^{−/−} mice, we observed a cohort of 20 mice in groups 2 ($n = 18$) and 3 ($n = 2$) until they appeared clinically ill. At such time, blood, peritoneal lavage, kidney, liver, and spleen were collected for bacterial cultures, and colon and ileum were harvested for histologic preparation and scoring. Both raters had high agreement in pathologic scoring for depth ($r_s = 0.91$; $P < .01$), inflammation ($r_s = 0.90$; $P < .01$), and total score ($r_s = 0.91$; $P < .01$) using Spearman's rank correlation.

The animals with bacteremia had significantly higher total enterocolitis scores (3.35 ± 1.72) than those without bacteremia (0.64 ± 1.18) (Wilcoxon's rank-sum test, $P < .01$) (Table 3). No animals had any positive bacterial cultures from blood samples, and the animals reported as negative for bacteremia had no growth of the bacterial cultures, either aerobic or anaerobic at 48 hours. Those that were reported as positive for bacteremia had bacterial growth on cultures in at least one of the tissues or fluid tested. There was significant bacterial growth found in 13 of 20 Ednrb^{-/-} mice in the cohort; however, there was no significant difference in the level of bacterial CFUs between the anaerobic and aerobic cultures in those animals with bacteremia (Wilcoxon's ranksum test, $P = .72$) (Table 4). The intraperitoneal lavage had the highest number of both anaerobic and aerobic bacterial cultures, whereas the liver had the lowest. Aerobic and anaerobic cultures from 4 animals were speciated, and the most common organism cultured was *Enterococcus* sp (3/4), with *Bacillus* sp, *Enterobacter cloacae*, and *Clostridium* sp, each cultured from one of the animals. Not surprisingly, these are all gut-associated organisms.

Within those Ednrb^{-/-} mice that developed bacteremia, Spearman's rank correlations were performed comparing mean total enterocolitis scores to the level of bacteremia in each tissue (Table 5). Both anaerobic and aerobic bacterial cultures of the liver had the highest correlation with average total enterocolitis scores. Only anaerobic cultures of the spleen did not demonstrate any significant correlation with total enterocolitis scores.

4. Discussion

We report development and validation of a semiquantitative histopathologic grading system to assess the severity of intestinal inflammation (enterocolitis) in Ednrb^{-/-} mice. We prospectively tested this grading system on a cohort of 23 Ednrb^{-/-} mice and found excellent reproducibility and internal consistency, as evaluated by Pearson correlation coefficients, ICC, and CVs. The total score had an intra- and interrater ICC of 0.61 and 0.94, respectively, and an intra- and interrater CV of 18% and 9%, respectively. Our enterocolitis grading system, therefore, has a high degree of reproducibility and can be used to grade enterocolitis in further studies.

Of interest, we found gut flora had infected multiple organs in Ednrb^{-/-} mice and that the gut inflammation score was directly and linearly related to the extent of bacterial colonization. We also found strong positive correlation between total enterocolitis scores and all cultures of peritoneal lavage, liver, kidney, and aerobic spleen but significantly weaker with the anaerobic splenic culture (Table 5). In contrast, Caniano et al [21] found that 38% of spontaneously dying piebald-lethal mice had splenic cultures that were positive for enteric organisms (*Proteus mirabilis*, *Enterococcus* sp, *Bacteriodes* sp, *Serratia* sp) but found no histologic evidence of enterocolitis. They concluded that functional distal obstruction of the piebald-lethal mice lead to loss of colonic mucosal barrier integrity, leading to bacterial translocation and systemic sepsis. However, Fujimoto et al [19,20] reported finding significant histologic evidence of enterocolitis in the ganglionic portion of the colon of 3- to 4-week-old piebald-lethal mice deemed clinically ill and reported acute splenitis, but no bacterial cultures were performed. The difference in the findings of the 2 published studies may be explained on differences in animal care and housing conditions at

different institutions (varying microbial milieus and possible pathogens) and/or genetic drift within each colony, so that the phenotype may have changed over successive generations from that of the colony founders. Nor is there an agreement between the 2 studies on the histopathologic definition for enterocolitis.

Our results are similar to those of Fujimoto et al [19,20] in the timing of onset, clinical illness, and histopathologic findings. The *Ednrb*^{-/-} mouse on a hybrid strain background appears to be more susceptible to the developing enterocolitis compared to the piebald-lethal inbred strain.

We noted a significant effect of diet and enteral antibiotics on mean survival time of the *Ednrb*^{-/-} mice. We speculate that the liquid diet may benefit the animals with aganglionosis mechanically compared to regular chow, thereby, slowing the progression of megacolon and marginally prolonging life. We hypothesize that enteral antibiotics may reduce the severity of enterocolitis or suppress the bacteremia that likely results from the enterocolitis, or both. Regardless of which mechanism(s) is responsible, these data strongly point to a bacterially mediated process that is partially ameliorated by enteral antibiotics.

Several genetic mutations in mice and rats result in abnormally ganglionated intestines. Among these are the *Ednrb*-null mouse [9] that we used in this study but also the endothelin-3 ligand-deficient mouse [10], the *Hoxb5* dominant negative conditional (*Cre-Lox*) transgenic genotype [22], *erbb2*/nestin-*Cre* conditional mutant mouse [23], the *Dom* spontaneous mutant mouse [24], a conditional $\beta 1$ integrin knockout mouse [25], trisomy 16 mice [26,27], and mice deficient in the *c-ret* proto-oncogene [28]. In addition, the endothelin B receptor-deficient rat (spotting lethal rat) [29–31] and the *fmc/fmc* (familial megacecum and colon) rat [32] also display a phenotype that mimics HD. The histopathologic scoring system we developed can now potentially be applied in studies of aganglionic or hypoganglionic megacolon in these genotypes. Furthermore, studies analyzing histopathologic abnormalities after corrective pull-through surgery in these mice [12] can now be conducted.

Ultimately, we wish to determine what mechanism(s) cause or contribute to enterocolitis despite technically successful corrective surgery in the context of aganglionosis (eg., in post pull-through HAEC). Our data do not currently allow us to determine whether bacterial overgrowth worsened intestinal inflammation in *Ednrb*^{-/-} mice, or conversely, whether acute intestinal inflammation somehow allowed bacterial overgrowth that in turn led to bacteremia and colonization of distant organs. Alternatively, it is also conceivable that some as yet unidentified molecular or immunologic defect contributed to both intestinal inflammation and the susceptibility to systemic bacterial infection, sepsis, and eventual death. We recognize that these possibilities are not mutually exclusive. Nevertheless, the histopathologic enterocolitis scoring system that we report here can now be used in conjunction with our previously reported surgical pull-through [12] to directly test the validity of these and other potential causes of enterocolitis and bacterial infection. Such controlled mechanistic studies could then lead to improved understanding of these sometimes devastating diseases, which in turn could drive development of improved therapies.

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Murine Megacolon

Fig. 1. Murine colon and ileal segments studied. Segment 1, anus and rectum; segment 2, distal colon; segment 3, proximal colon; and segment 4, terminal ileum. Each segment was cut 1 cm long.

Fig. 2. Histologic examples of enterocolitis scores (H&E stain at 200 $\times$). A, Total score 0 (normal). B, Total score 3 (mild inflammatory infiltrate in the submucosa, inflammation 1, depth 2). Arrows show neutrophils. C, Total score 5 (mild inflammatory infiltrate at the serosal surface only, inflammation 1, depth 4). Arrow shows inflammatory cells at the serosa. D, Total score 7 (transmural necrosis, depth 4, inflammation 3).

Fig. 3.

Survival of *Ednrb*^{-/-} mice with aganglionosis under different dietary conditions. Three cohorts of *Ednrb*^{-/-} mice fed: regular solid mouse chow (control), liquid rodent diet (LD), or liquid rodent diet with antibiotic drinking water containing vancomycin, enrofloxacin, and metronidazole. There was no statistically significant difference in survival between control and LD groups or between LD and LD + Abx ($P = .10$). There was a statistically significant difference between the control and LD + Abx ($P = .009$).

Table 1

Murine enterocolitis grading system

Feature graded	Grade	Description
Severity of inflammation	0	No inflammation, rare neutrophil
	1	Mild inflammatory infiltrates, no necrosis
	2	Moderate to marked inflammatory infiltrates and mucosal necrosis
	3	Transmural necrosis
Depth of inflammation	0	None
	1	Mucosa
	2	Submucosa
	3	Muscularis propria
	4	Subserosa and serosa
Score range	0–7	

Severity of inflammation grade added to the depth of inflammation grade yields the total enterocolitis score.

Table 2

Validation of enterocolitis grading system

Intrarater corr (<i>r</i>)			Interrater corr (<i>r</i>)	
	Rater DD	Rater HW		
Inflammation	0.877	0.970	Inflammation	0.960
Depth	0.337	0.307	Depth	0.935
Total score	0.679	0.645	Total score	0.948
Intrarater ICC			Interrater ICC	
	Rater DD	Rater HW		
Inflammation	0.877	0.968	Inflammation	0.959
Depth	0.245	0.268	Depth	0.923
Total score	0.604	0.613	Total score	0.939
Intrarater CV			Interrater CV	
	Rater DD	Rater HW		
Inflammation	7%	6%	Inflammation	9%
Depth	20%	37%	Depth	9%
Total score	15%	20%	Total score	9%

The correlation (*r*), ICC, and CV were performed enterocolitis scores of 2 pathologists (DD and HW). This methodology (using the total score) has an intra- and interrater ICC of 0.61 and 0.94, respectively, with an intra- and interrater CV of 18%, and 9%, respectively.

Table 3

Comparison of enterocolitis in the presence or absence of bacteremia

	Means $\pm$ SD across mice with and without bacteremia	
	No bacteremia (n = 7)	Bacteremia (n = 13)
	Mean $\pm$ SD	Mean $\pm$ SD
Enterocolitis scores		
Depth	0.43 $\pm$ 0.79	2.50 $\pm$ 1.41 *
Inflammation	0.21 $\pm$ 0.39	0.85 $\pm$ 0.38 *
Total	0.64 $\pm$ 1.18	3.35 $\pm$ 1.72 *

Mean enterocolitis scores $\pm$ SD are reported. Enterocolitis scores were tested across groups using a Wilcoxon's rank-sum test.

* $P < .05$, indicates groups are significantly different.

Table 4

Aerobic and anaerobic bacterial CFU per organ

Bacteremia (n = 13)	
Mean $\pm$ SD	
Aerobic cultures	
IP lavage	4.25 $\pm$ 2.20
Kidney	3.70 $\pm$ 1.63
Liver	2.94 $\pm$ 1.44
Spleen	3.24 $\pm$ 1.57
Anaerobic cultures	
IP lavage	4.25 $\pm$ 2.19
Kidney	3.88 $\pm$ 1.65
Liver	2.88 $\pm$ 1.53
Spleen	2.97 $\pm$ 1.73

IP indicates intraperitoneal. Data are reported as mean log₁₀ CFU $\pm$ SD in mice with bacteremia and mean log₁₀ CFU $\pm$ SD across mice with bacteremia.

Table 5

Spearman's rank correlations of level of bacteremia ($\log_{10}$ CFU) to total enterocolitis score

Spearman's rank correlations (r_s)		
		Total enterocolitis score
Aerobic	IP lavage	0.67*
	Kidney	0.60*
	Liver	0.78*
	Spleen	0.60*
Anaerobic	IP lavage	0.67*
	Kidney	0.50*
	Liver	0.72*
	Spleen	0.39

Both anaerobic and aerobic bacterial cultures of the liver had the highest correlation with average total enterocolitis scores. Only anaerobic cultures of the spleen did not demonstrate any significant correlation with total enterocolitis scores.

* $P < .05$, significantly nonzero.