

Summary of Methodology Used in Enterotoxigenic *Escherichia coli* (ETEC) Challenge Experiments in Weanling Pigs and Quantitative Assessment of Observed Variability

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Summary

Post-weaning diarrhea in pigs can be caused by the F4 or F18 strains of enterotoxigenic *Escherichia coli* (ETEC). To evaluate interventions for ETEC, experimental infection via a challenge model is critical. To our knowledge, there is a lack of explanation for the variability in responses observed across ETEC challenge studies. Our objective was to quantitatively summarize the responses and variability among recent ETEC challenge studies and develop a tool for sample size calculation. The most widely evaluated response criteria across ETEC challenge studies are growth performance, fecal consistency and bacterial shedding, intestinal morphology, and immune responses. Factors that contribute to the variability seen across studies include the type of ETEC studied, dose and timing of inoculation, and the number of replications. Generally, a reduction in average daily gain (ADG) and average daily feed intake (ADFI) are seen following an ETEC challenge, as well as a rapid increase in diarrhea. Fecal bacterial shedding is a common indicator of ETEC infection, but the responses seen across the literature are not consistent due to differences in bacterial enumeration procedures. Emphasis should also be placed on the piglet's immune response to ETEC, which is commonly assessed by quantifying levels of immunoglobulins and pro-inflammatory cytokines. Again, there is variability in these responses across published work. Small intestinal morphology is drastically altered following infection with ETEC and appears to be a less variable response criterion to evaluate. While there is a large degree of variability across ETEC challenge experiments, we have provided a quantitative summary of these studies, and a Microsoft Excel-based tool was created to help calculate sample sizes for future studies.

Introduction

One of the most common etiological agents prompting post-weaning diarrhea (PWD) in pigs is enterotoxigenic *Escherichia coli* (ETEC). These bacteria are typically categorized by their fimbriae, with the most common fimbriae types associated with PWD

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being F4 or F18. Due to concerns with antibiotics and pharmacological zinc oxide, the demand for alternative strategies to combat PWD is growing in need. There are many proposed solutions; however, to effectively evaluate any intervention for ETEC, it is important to appropriately replicate ETEC infection by purposefully inoculating pigs with the pathogen to induce an experimental infection, known as an *in vivo* challenge model. Utilizing an ETEC challenge model in post-weaning pigs has been demonstrated on numerous accounts, but with immense variability. To our knowledge, there has been no attempt to quantify this variability to help refine and standardize ETEC infection studies. Therefore, our objective was to review recent ETEC challenge experiments and provide quantitative measurements of the responses and variability among the most widely evaluated response criteria. Using this information, a second aim of this work was to generate a sample size calculation tool that accounts for this variability and that can assist researchers with designing future ETEC challenge studies.

Procedures

A literature search was conducted utilizing PubMed (www.pubmed.ncbi.nlm.nih.gov), Web of Science (www.webofscience.com), and Scopus (www.scopus.com). Search terms included a combination of the following: weanling pig OR nursery pig AND *Escherichia coli* OR ETEC F4 OR ETEC F18 OR ETEC K88 OR challenge. To narrow the search so that response criteria could be identified, a subset of search terms were included: growth OR immune OR fecal. Bibliographies from papers generated from the search were also scanned to identify relevant literature. Articles were restricted to peer-reviewed, *in vivo* studies, with English as the primary language and published between the years of 2010 and 2022. This time frame was selected to account for the vast changes in genetic composition, nutrition, health, and management seen in the swine industry over recent years. Additionally, these parameters allowed for enough data to be extracted without overlapping previously written reviews. Studies utilizing a lipopolysaccharide (LPS) challenge were not included. Once relevant papers were located and organized, the means and measure of variability were extracted for each response criterion where quantitative values were provided (i.e., data presented as figures or charts with no numeric measure were not utilized). For each response criterion, the mean and standard deviation (SD) were presented by the type of ETEC utilized for the challenge, then chronologically according to the publishing date for each reference. Additionally, for each response the percent change between the control and treatment groups was calculated to provide an estimated magnitude of effect. The data from these tables were then organized in a literature database within a Microsoft Excel spreadsheet, and two sample size calculators were developed.

Results and Discussion

Growth performance

Our findings indicate that there is a high degree of variability surrounding growth performance responses to both F4 and F18 ETEC. Based on the studies reviewed, both a larger dose and a greater effect size were needed to observe a significant difference in ADG when pigs were inoculated with F18 ETEC compared to F4. A final inoculum concentration of 2×10^9 CFU and a difference in ADG between treatment groups of approximately 21% was needed to detect statistical significance following an F18 challenge. Conversely, in F4 challenge studies, a dosage of 1×10^9 CFU and a difference in ADG of 16% yielded statistical significance. Table 1 summarizes the responses seen and

variability surrounding ADG, ADFI, and G:F. The means and standard deviations (SD) represent the period post-inoculation from each study. Additionally, a percent change column was included for each response to understand the difference that was observed between the control and treatment groups for each study.

Fecal consistency and bacterial shedding

Diarrhea can last anywhere from 1 to 5 days following ETEC infection,³ and in research settings, evaluation of fecal consistency as an indicator of diarrhea is a widely used tool to determine the effectiveness of the challenge model.⁴ Generally, visual fecal scoring and analysis of fecal dry matter (DM) are utilized to assess fecal consistency; however, there is substantial variability in the methodologies used to evaluate these response criteria. This difference in experimental procedures may seem small, but greatly contributes to the variability seen within literature as it relates to fecal consistency. Table 2 displays the ETEC challenge studies that evaluated fecal consistency and their respective responses. Unfortunately, nearly all fecal scoring data are presented in the form of figures and statistical information such as standard error of the mean is not provided, making it difficult to quantify the variability in fecal consistency responses seen within the literature. The ordinal nature of the data makes sample size calculation difficult, thus, fecal DM may be a more suitable response to evaluate. The body of literature strongly favors a worsening in fecal consistency when the ETEC challenge is induced, and this response was reported as early as 6 hours post-inoculation (hpi),⁵ and lasted as long as 25 days post-inoculation (dpi).⁶

In newly weaned piglets, infection with pathogenic bacteria such as ETEC causes an increase in the fecal shedding. In ETEC challenge studies, fecal bacterial shedding has been evaluated on several accounts, but with much variability in the methodologies used. Because *E. coli* shedding can occur even in healthy pigs, the most reliable evaluation of bacterial shedding as an indicator of ETEC infection should be specific to F4 or F18 strains. Typically, a 3- to 4-day measurement period post-inoculation is required for proper adherence, colonization, and toxin production within the small intestine.² Regardless of the methodology used, the literature suggests that if ETEC infection is carried out effectively, the rate and number of bacteria shed in feces will increase. Perhaps the greatest difficulty in interpreting responses in fecal bacterial shedding stems from the presentation of data. This often includes fecal bacterial shedding scores, log₁₀ CFU/g, and raw cycle threshold (CT) values, depending on the method of bacterial enumeration used. Directly comparing the means and variability across studies is not justified and could lead to inaccurate interpretation of results. Table 3 provides a summary of the challenge models which quantified bacterial shedding and the response

³ Sun, Y., and S.W. Kim. 2017. Intestinal challenge with enterotoxigenic *Escherichia coli* in pigs and nutritional intervention to prevent postweaning diarrhea. *Anim. Nutr.* 3(4):322-330. doi:10.1016/j.aninu.2017.10.001.

⁴ Luise, D., C. Lauridsen, P. Bosi, and P. Trevisi. 2019. Methodology and application of *Escherichia coli* F4 and F18 encoding infection models in post-weaning pigs. *J. Anim. Sci. Biotechnol.* 10(1):1-20. doi: 10.1186/s40104-019-0352-7

⁵ Lei, X. J., and I. H. Kim. 2020. Evaluation of coated zinc oxide in young pigs challenged with enterotoxigenic *Escherichia coli* K88. *Anim. Feed Sci. Technol.* 262:114399. doi: 10.1016/j.anifeedsci.2020.114399

⁶ Sun, Y., M. E. Duarte, and S. W. Kim. 2021. Dietary inclusion of multispecies probiotics to reduce the severity of post-weaning diarrhea caused by *Escherichia coli* F18+ in pigs. *Anim. Nutr.* 7(2):326-333. doi: 10.1016/j.aninu.2020.08.012

observed. Despite being unable to provide quantitative values for the variation in fecal bacterial shedding responses within these papers, Table 3 displays information that may be useful in the planning of future challenge work.

Intestinal morphology

Evaluation of small intestinal morphology can be a beneficial measurement to assess following an ETEC challenge, especially when nutritional intervention is expected to alleviate the detrimental effects that bacterial adhesion and toxin release can have on the intestinal mucosa. Table 3 summarizes the studies included in this review that evaluated changes in small intestinal morphology. Our summary indicates that an ETEC infection, regardless of inoculum dose, timing, or other factors, impairs villi height; however, very few authors reported a significant response in CD post-inoculation. This is supported by Al Masri et al.,⁷ who reviewed piglet small intestinal morphology surrounding weaning and suggested there was no clear evidence that CD changes immediately post-weaning or upon pathogenic infection. Including small intestinal morphology as an outcome variable appears to be a valuable tool to help assess the impacts of ETEC on the weaning pig. Because morphological changes can occur around weaning even in clinically healthy pigs, the use of non-challenged control pigs would be beneficial when assessing small intestinal morphology following an ETEC challenge to determine if changes in morphology are attributed to ETEC.

Conclusions

In summary, there is considerable variation in piglet responses to ETEC F4 and F18 infection during challenge experiments. We quantitatively summarized the responses and variability in growth performance and intestinal morphology from ETEC challenge models and generated a tool to allow for more accurate and simplified sample size calculations for future work. Additional responses, such as immunoglobulins and pro-inflammatory cytokines, were also evaluated, but were not included in this report. Further research to standardize infection models and reduce between-experiment variability is needed to more effectively investigate nutritional or management strategies to mitigate PWD caused by ETEC.

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⁷ Al Masri, S., H. Hünigen, A. Al Aiyan, J. Rieger, J. Zentek, K. Richardson, and J. Plendl. 2015. Influence of age at weaning and feeding regimes on the postnatal morphology of the porcine small intestine. *J. Swine. Health Prod.* 23(4):186-203.

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Table 1. Summary of variability in growth performance responses following ETEC challenge in weanling pigs¹

Reference ²	Description of challenge model				ADG, g/d		ADFI, g/d		G:F	
	ETEC type	Inoculum dose (CFUs)	Inoculation time (dpw) ³	Study length (dpi) ⁴	Mean ± SD	% Change ⁵	Mean ± SD	% Change ⁵	Mean ± SD	% Change ⁵
Liu et al., 2010	F4	3 × 10 ¹¹	7	7	265 ± 19.0 [*]	-16	392 ± 76.8 [*]	-7	0.67 ± 0.21 ⁺	-11
Nyachoti et al., 2011	F4	1 × 10 ¹⁰	8	7	161 ± 96.0	+79	230 ± 99.0	+50	0.72 ± 0.39	+20
Trevisi et al., 2011	F4	2 × 10 ¹⁰	7	5-7	138 ± 63.7 [*]	-39	285 ± 36.1 [*]	-16	-	-
Lee et al., 2012	F4	5 × 10 ⁹	14	14	449 ± 39.2 [*]	-31	647 ± 203.3	-16	0.69 ± 0.09	-18
Wu et al., 2012	F4	5 × 10 ⁹	13	6	363 ± 18.1 [*]	+18	595 ± 27.7 ⁺	+6	0.62 ± 0.03 [*]	+13
Almeida et al., 2013	F4	9 × 10 ¹⁰	6	5	143 ± 180.2 [*]	-42	591 ± 545.9	-12	0.27 ± 0.14	-32
Gao et al., 2013	F4	1 × 10 ⁹	1, 3, 5, 7	11	100 ± 48.9 [*]	-29	280 ± 73.5	-13	0.37 ± 0.05 [*]	-16
Khafipour et al., 2014	F4	6 × 10 ¹⁰	8 – 10	10	245 ± 69.8	-16	333 ± 36.7	-12	0.73 ± 0.22	-4
Yang et al., 2014	F4	1 × 10 ⁹	15	3	323 ± 147.0	-9	374 ± 36.7	-2	0.80 ± 0.47	-11
Li et al., 2015	F4	2 × 10 ¹⁰	14	27	400 ± 60.9	+8	598 ± 76.7 [*]	+3	0.67 ± 0.05 [*]	+5
Trevisi et al., 2015	F4	2 × 10 ⁸	7	14	201 ± 231.5	-5	-	-	-	-
Chen et al., 2016	F4	5 × 10 ¹⁰	7	7	198 ± 73.5	+8	351 ± 83.1	+24	0.56 ± 0.15 [*]	+19
Han et al., 2016	F4	3 × 10 ⁸	0	7	142 ± 51.4 [*]	-73	-	-	-	-
Lee et al., 2017	F4	3 × 10 ¹⁰	7	14	142 ± 59.4 [*]	-	-	-	-	-
Lei et al., 2017	F4	5 × 10 ¹⁰	8 – 10	14	222 ± 8.9 [*]	+44	298 ± 35.8 [*]	+34	0.74 ± 0.07	+6
Pan et al., 2017	F4	1 × 10 ¹¹	9	3	320 ± 45.8 [*]	-21	391 ± 53.6 [*]	-20	0.82 ± 0.05	-1
Koo et al., 2019	F4	5 × 10 ¹⁰	10	3	376 ± 150.7	-33	590 ± 182.2 ⁺	-22	0.67 ± 0.20 ⁺	+7
Lopez-Colom et al., 2019	F4	2 × 10 ⁹	7	8	117 ± 256.0	-9	211 ± 129.5	-4	-	-
Luise et al., 2019	F4	2 × 10 ⁵	7	13	125 ± 94.2	+24	313 ± 148.0	+18	0.33 ± 0.35	-33
Choi et al., 2020	F4	5 × 10 ⁷	7	5	306 ± 149.4 ⁺	-46	559 ± 105.3	-15	-	-

continued

Table 1. Summary of variability in growth performance responses following ETEC challenge in weanling pigs¹

Reference ²	Description of challenge model				ADG, g/d		ADFI, g/d		G:F	
	ETEC type	Inoculum dose (CFUs)	Inoculation time (dpw) ³	Study length (dpi) ⁴	Mean ± SD	% Change ⁵	Mean ± SD	% Change ⁵	Mean ± SD	% Change ⁵
Lei et al., 2020	F4	5 × 10 ⁹	22	2	428 ± 47.0*	-16	643 ± 40.3	-6	0.66 ± 0.08*	-10
Wang et al., 2020	F4	1 × 10 ¹¹	15	5	334 ± 40.3	+20	830 ± 57.7*	+21	0.40 ± 0.29	-4
Xu et al., 2020	F4	1 × 10 ¹⁰	7	14	444 ± 83.8*	-27	729 ± 104.1*	-22	0.61 ± 0.05	-8
Yu et al., 2021	F4	1 × 10 ¹²	19	2	390 ± 36.6	-8	486 ± 19.6*	-8	-	-
Kim et al., 2019	F18	3 × 10 ¹⁰	7	11	214 ± 141.3*	-59	437 ± 201.9*	-36	0.46 ± 0.27	-33
Li et al., 2019	F18	2 × 10 ¹⁰	7	7	250 ± 94.8*	-41	682 ± 94.9*	-31	0.62 ± 0.18	-20
Wojnicki et al., 2019	F18	9 × 10 ¹⁰	13	10	505 ± 159.0	-10	1,282 ± 785.0	+3	0.39 ± 0.20*	-15
Becker et al., 2020	F18	1 × 10 ¹⁰	7	10	238 ± 31.6*	-51	335 ± 31.6*	-33	0.63 ± 0.19	-33
Duarte et al., 2020	F18	2 × 10 ⁹	13	13	355 ± 116*	-21	465 ± 82.0	-5	0.76 ± 0.16*	-17
Hong et al., 2021	F18	2 × 10 ⁹	7	13	335 ± 86.6*	+78	416 ± 96.7*	+70	0.82 ± 0.10	+1
Sun et al., 2021	F18	6 × 10 ⁹	7	12	435 ± 333.8	-3	664 ± 449.7	+0.39	0.65 ± 0.14	-5
Chang et al., 2022	F18	3 × 10 ¹⁰	7	14	251 ± 57.2*	-50	393 ± 31.7	-3	0.64 ± 0.12*	-49
F4 Average	-	9 × 10 ¹⁰	-	-	260 ± 86.3	-	436 ± 104.8	-	0.62 ± 0.17	-
F18 Average	-	3 × 10 ¹⁰	-	-	323 ± 127.5	-	584 ± 328.5	-	0.62 ± 0.17	-

*Indicates that a statistically significant difference was observed between the control and treatment groups ($P < 0.05$).

†Indicates that a marginally significant difference was observed between the control and treatment groups ($0.05 < P < 0.10$).

¹Mean ± SD represent the period reported immediately post-inoculation from each study.

²See previous pages for full list of references.

³Time of inoculation with enterotoxigenic *Escherichia coli* (ETEC), days post-weaning (dpw).

⁴Length of time data were collected following inoculation with ETEC, days post-inoculation (dpi).

⁵The calculated percent change between the control and treatment groups.

Table 2. Summary of ETEC challenge studies evaluating fecal consistency as a response criterion

Reference ¹	Description of challenge model						Fecal consistency response ⁵	
	ETEC type	Inoculum dose (CFUs)	Inoculation time (dpw) ²	Number of replicates	Method ³	Collection Time (dpi) ⁴	Pre-inoculation	Post-inoculation
Heo et al., 2010	F4	1 × 10 ⁸	3 – 5	12	FS	0 – 14	-	↑
Liu et al., 2010	F4	3 × 10 ¹¹	7	6	FS	0 – 7	-	↑
Nyachoti et al., 2011	F4	1 × 10 ¹⁰	8	9	FS	0 – 5	-	ND
Trevisi et al., 2011	F4	2 × 10 ¹⁰	7	6	FS	1 – 7	-	ND
Lee et al., 2012	F4	5 × 10 ⁹	14	6	FS	0 – 14	-	↑
Almeida et al., 2013	F4	9 × 10 ¹⁰	6	8	FS	0 – 5	-	ND
Gao et al., 2013	F4	1 × 10 ⁹	1, 3, 5, 7	6	FS	0 – 11	-	↑
Khafipour et al., 2014	F4	6 × 10 ¹⁰	8 – 10	5	FS	2, 4, 7	-	↑
Li et al., 2015	F4	2 × 10 ¹⁰	14	7	FS	7, 14, 21, 28	-	↓
Trevisi et al., 2015	F4	2 × 10 ⁸	7	10	FS	-12 hpi – 6	ND	↑
Chen et al., 2016	F4	5 × 10 ¹⁰	7	9	FS	1 – 5	-	↓
Han et al., 2016	F4	3 × 10 ⁸	0	6	FS	1 – 7	-	↓
Rhouma et al., 2016	F4	1 × 10 ⁹	7	12	FS	-3 – 35	ND	↑
Lee et al., 2017	F4	3 × 10 ¹⁰	7	8	FS	0 – 14	-	↑
Lei et al., 2017	F4	5 × 10 ¹⁰	7	5	FS	-7 – 21	↓	↓
Pan et al., 2017	F4	1 × 10 ¹¹	9	6	FS	-9 – 12	ND	↑
Choi et al., 2020	F4	5 × 10 ⁷	7	6	FS	0 – 54 hpi ¹	-	↑
Wang et al., 2020	F4	1 × 10 ¹¹	15	6	FS	0 – 6	-	↑
Lei et al., 2020	F4	5 × 10 ⁹	22	5	FS	-21 – 3	ND	↑
Kim et al., 2019	F18	3 × 10 ¹⁰	7	12	FS	0 – 11	-	↑
Li et al., 2019	F18	2 × 10 ¹⁰	7	10	FS	-7 – 7	ND	↑
Wojnicki et al., 2019	F18	9 × 10 ¹⁰	13	12 – 13	FS	-13 – 10	ND	↑
Becker et al., 2020	F18	1 × 10 ¹⁰	7	8 – 10	FS	0 – 10	-	↑
Duarte et al., 2020	F18	2 × 10 ⁹	7	8	FS	-3 – 20	ND	↑
Smith et al., 2020	F18	9 × 10 ¹⁰	10	18	DM	7	-	↑
He et al., 2021	F18	6 × 10 ¹⁰	7	12	FS	0 – 21	-	↑
Sun et al., 2021	F18	6 × 10 ⁹	7	8	FS	-11 – 25	ND	↑
Chang et al., 2022	F18	3 × 10 ¹⁰	4	9	FS	0 – 14	ND	ND

¹Hours post-inoculation (hpi).

²See previous pages for full list of references.

³Time of inoculation with enterotoxigenic *Escherichia coli* (ETEC), days post-weaning (dpw).

⁴Method for evaluation of fecal consistency: FS = fecal scoring; DM = fecal dry matter (DM) analysis.

⁵Length of time data were collected following inoculation with ETEC, days post-inoculation (dpi).

⁶Fecal consistency response following challenge with ETEC: ↑ indicates an increase in diarrhea; ↓ indicates a decrease in diarrhea; ND indicates no statistical difference in diarrhea.

Table 3. Summary of variability in small intestinal morphology responses after ETEC infection¹

Location:	Ileum			Duodenum			Jejunum		
	Villus height, m	Crypt depth, m	VH:CD	Villus height, m	Crypt depth, m	VH:CD	Villus height, m	Crypt depth, m	VH:CD
Reference ²									
Trevisi et al., 2011	217 ± 34*	198 ± 23*	1.12 ± 0.19*	276 ± 34*	240 ± 23*	1.19 ± 0.19*	255 ± 34*	192 ± 23*	1.34 ± 0.19*
Nyachoti et al., 2012	403 ± 30*	278 ± 54	1.45 ± 0.30*	-	-	-	-	-	-
Wu et al., 2012	390 ± 35*	215 ± 16	1.84 ± 0.11*	435 ± 26	223 ± 13	1.93 ± 0.59	434 ± 45	226 ± 17*	1.94 ± 0.02*
Almeida et al., 2013	300 ± 26	223 ± 24*	1.36 ± 0.23	374 ± 60	266 ± 110	1.54 ± 0.74	-	-	-
Yang et al., 2014	565 ± 108	300 ± 54	1.90 ± 0.39	438 ± 78*	359 ± 56*	1.29 ± 0.34*	475 ± 88*	275 ± 27*	1.86 ± 0.54*
Pan et al., 2017	341 ± 33*	223 ± 22	1.54 ± 0.15*	426 ± 50*	244 ± 32	1.76 ± 0.27*	408 ± 41*	229 ± 26	1.79 ± 0.22*
Kim et al., 2019	365 ± 43	215 ± 22	1.70 ± 0.13	441 ± 68	307 ± 66	1.44 ± 0.14	395 ± 116*	241 ± 82	1.64 ± 0.30
Becker et al., 2020	265 ± 39*	178 ± 31 ⁺	1.51 ± 0.29*	-	-	-	-	-	-
Choi et al., 2020	-	-	-	-	-	-	449 ± 95*	262 ± 42	1.98 ± 0.56
Duarte et al., 2020	-	-	-	-	-	-	395 ± 72*	275 ± 30*	1.48 ± 0.25*
Koo et al., 2020	394 ± 53	208 ± 27	1.91 ± 0.25	456 ± 62*	247 ± 34	1.87 ± 0.28*	444 ± 57	208 ± 25*	2.69 ± 0.25*
Lei et al., 2020	379 ± 64*	226 ± 35	1.70 ± 0.34*	475 ± 71*	224 ± 42	1.43 ± 0.45*	344 ± 69	225 ± 28	1.51 ± 0.47
Sun et al., 2021	314 ± 82	258 ± 52*	1.22 ± 0.17	-	-	-	314 ± 56*	231 ± 24*	1.80 ± 0.20*
Chang et al., 2022	351 ± 31*	159 ± 27 ⁺	2.28 ± 0.44*	-	-	-	-	-	-
Average	364 ± 52	224 ± 34	1.62 ± 0.26	420 ± 57	267 ± 39	1.53 ± 0.33	389 ± 70	237 ± 33	1.80 ± 0.31

¹Indicates that a statistically significant difference was observed between the control and treatment groups ($P < 0.05$). ETEC = enterotoxigenic *Escherichia coli*.

²Means and SD represent the period reported immediately post-inoculation for each study.

³See previous pages for full list of references.