An investigation into the association between *cpb2*-encoding *Clostridium perfringens* type A and diarrhea in neonatal piglets

Abdolvahab Farzan, Jasmina Kircanski, Josepha DeLay, Glenn Soltes, J. Glenn Songer, Robert Friendship, John F. Prescott

Abstract

To investigate the possible role of *cpb2*-positive type A *Clostridium perfringens* in neonatal diarrheal illness in pigs, the jejunum and colon of matched normal and diarrheic piglets from 10 farms with a history of neonatal diarrhea were examined grossly and by histopathology, and tested for *C. perfringens*, for *C. perfringens* beta2 (CPB2) toxin, as well as for *Clostridium difficile* toxins, *Salmonella*, enterotoxigenic *Escherichia coli*, rotavirus, transmissible gastroenteritis (TGE) virus, and coccidia. *Clostridium perfringens* isolates were tested using a multiplex real-time polymerase chain reaction (PCR) to determine the presence of *cpa*, consensus and atypical *cpb2*, and other virulence-associated genes. The numbers of *C. perfringens* in the intestinal contents were lower in diarrheic piglets ($\log_{10}$ 5.4 CFU/g) compared with normal piglets ($\log_{10}$ 6.5 CFU/g) ($P < 0.05$). The consensus *cpb2* was present in 93% of isolates in each group, but atypical *cpb2* was less common (56% healthy, 32% diarrheic piglets isolates, respectively, $P < 0.05$). The presence of CPB2 toxin in the intestinal contents of normal and diarrheic piglets did not differ significantly. *Clostridium difficile* toxins and rotavirus were each detected in 7 of the 21 (33%) diarrheic piglets. Rotavirus, *C. difficile* toxins, *Salmonella*, or enterotoxigenic *E. coli* were concurrently recovered in different combinations in 4 diarrheic piglets. The cause of diarrhea in 8 of the 21 (38%) piglets on 6 farms remained unknown. The etiological diagnosis of diarrhea could not be determined in any of the piglets on 2 of the farms. This study demonstrated that the number of *cpb2*-positive type A *C. perfringens* in the intestinal contents was not a useful approach for making a diagnosis of type A *C. perfringens* enteritis in piglets. Further work is required to confirm whether *cpb2*-carrying type A *C. perfringens* have a pathogenic role in enteric infection in neonatal swine.

Résumé

Dans le but d'étudier le rôle possible de Clostridium perfringens *type A possédant le gène* cpb2 *dans les cas de diarrhée néonatale chez les porcs, le jéjunum et le côlon de porcelets provenant de 10 fermes avec une histoire de diarrhée néonatale et pairés en fonction qu'ils aient ou non de la diarrhée ont été examinés macroscopiquement et en histopathologie, et testés pour* C. perfringens, *la toxine bêta2 de* C. perfringens *(CBP2), ainsi que pour les toxines de* Clostridium difficile, Salmonella, Escherichia coli *entérotoxinogène, rotavirus, le virus de la gastro-entérite transmissible (TGE) et les coccidies. Les isolats de* C. perfringens *ont été testés par réaction d'amplification en chaîne par la polymérase (PCR) multiplex pour déterminer la présence de* cpa, *de* cpb2 *consensus et atypiques, ainsi que d'autres gènes associés à la virulence. Le nombre de* C. perfringens *dans le contenu intestinal des porcelets diarrhéiques étaient plus faible ($\log_{10}$ 5,4 UFC/g) que dans celui des porcelets en santé ($\log_{10}$ 6,5 UFC/g) (*$P < 0,05$*). Le* cpb2 *consensus était présente chez 93 % des isolats dans chaque groupe, mais le* cpb2 *atypique était moins fréquent (56 % des isolats de porcelets en santé, et 32 % des isolats provenant de porcelets diarrhéiques, respectivement,* $P < 0,05$*). La présence de la toxine CPB2 dans le contenu intestinal de porcelets avec ou sans diarrhée ne différait pas de manière significative. Les toxines de* C. difficile *et les rotavirus ont chacun été détectés chez 7 des 21 (33 %) des porcelets diarrhéiques. Des rotavirus, les toxines de* C. difficile, Salmonella *ou des* E. coli *enterotoxinogènes ont été retrouvés de manière concomitante en différentes combinaisons chez 4 porcelets diarrhéiques. Chez 8 de 21 (38 %) porcelets provenant de 6 fermes, la cause de la diarrhée est demeurée inconnue. Le diagnostic étiologique de la diarrhée n'a pu être déterminé chez aucun des porcelets de 2 fermes. Cette étude démontre que le nombre d'isolats de* C. perfringens *de type A positifs pour* cpb2 *dans le contenu intestinal n'était pas une approche utile pour établir un diagnostic d'entérite à* C. perfringens *type A chez les porcelets. Des études supplémentaires sont nécessaires pour confirmer si les isolats de* C. perfringens *de type A porteurs de* cpb2 *ont un rôle pathogène dans les infections entériques chez les porcelets nouveau-nés.*

(Traduit par Docteur Serge Messier)

Department of Population Medicine (Farzan, Friendship), Department of Pathobiology (Kircanski, Soltes, Prescott), Animal Health Laboratory, University of Guelph, Guelph, Ontario N1G 2W1 (DeLay); and Department of Veterinary Microbiology and Preventive Medicine, Iowa State University, Ames, Iowa 50011-1250, USA (Songer).

Address all correspondence to Dr. John F. Prescott; telephone: 519-824-4120 ext. 54716; fax: 519-824-5930; e-mail: prescott@uoguelph.ca

Received November 26, 2011. Accepted February 16, 2012.

Introduction

Mortality and morbidity among newborn piglets through enteric disease is a substantial problem in the swine industry. Type A *Clostridium perfringens* with other microbial pathogens, including enterotoxigenic *E. coli* (ETEC), *Salmonella* spp., rotavirus, transmissible gastroenteritis virus (TGE), and *Isospora suis,* have been associated with diarrhea in suckling piglets (1).

Neonatal enteritis attributed to *C. perfringens* type A infection associated with pre-weaning mortality has been reported previously (1,2). Disease is typically seen in piglets within 48 h of birth and is associated with creamy or pasty feces lasting several days. Necropsy shows flaccid, thin-walled, gaseous, or watery small intestine and pasty colonic contents (3,4). Microscopically, intestinal sections may have superficial villous tip necrosis, with fibrin and neutrophils, or appear normal or perhaps slightly congested (4).

The *cpb2* gene and its expressed protein, a 27.6 kD toxin (CPB2 toxin), was first described in an isolate from a piglet with necrotic enteritis (5). Subsequently, *cpb2* was demonstrated by polymerase chain reaction (PCR) in isolates from a variety of animals with diarrheic illness or fatal enteritis (6). The CPB2 toxin is encoded by either a "consensus" gene or by an "atypical" gene with 80.4% similarity between the 2 proteins (7,8). Type A, consensus *cpb2*-positive and CPB2 toxin-expressing isolates from swine form a homogenous genetic cluster (9).

The association between *cpb2*-carrying type A *C. perfringens* and neonatal diarrhea in piglets remains unclear. Some studies have reported an association between the presence of the consensus *cpb2* gene and type A-associated diarrheal illness of neonatal piglets compared with healthy controls (10–13). In one report, the presence of *cpb* gene in isolates from piglets with type C *C. perfringens* infection was a confounding variable that makes it difficult to make an association between *cpb2* and diarrhea in piglets (10). Reports of this association have not, however, been designed to systematically investigate differences, if any, between healthy and diarrheic neonatal piglets. A proportion of *C. perfringens* recovered from healthy piglets also carry *cpb2* (6). Attributing a pathogenic role to type A *C. perfringens* in neonatal enteritis of piglets is potentially problematic because of the marked colonization of the stomach and small intestine of neonatal piglets by *C. perfringens* that occurs within the first hours of life (14). In addition, the gross and histopathological changes described in piglets diagnosed with type A *C. perfringens* are strikingly variable (2–4). Therefore, the diagnosis of type A *C. perfringens*-associated porcine neonatal diarrhea requires the isolation of large numbers of *cpb2*-positive bacteria and the exclusion of other causes (4,15).

The objective of this study was to detect and quantify *cpb2*-positive type A *C. perfringens* in piglets with diarrhea compared with unaffected cohort piglets and to determine its association with neonatal diarrheal illness in pigs.

Materials and methods

Study farms and sample collection

Ten farms with a consistent history of diarrheal illness attributed to type A *C. perfringens* neonatal diarrhea were identified. From each farm, 2 to 4 neonatal diarrheic piglets with liquid or watery feces and 1 to 2 healthy, non-diarrheic, piglets from a different litter were selected. In total, 46 piglets were selected (36 with diarrhea and 12 without diarrhea), of which 44 piglets were aged 1 to 4 d and 4 pigs were aged between 6 to 13 d. The piglets were submitted to Animal Health Laboratory, University of Guelph. Live animals were euthanized, necropsied, and intestinal samples were collected for histopathology and microbiologic testing (outline follows). Samples from small intestine and colon were stored at −70°C to be tested for the presence of *C. perfringens* CPB2 toxin by enzyme-linked immunosorbent assay (ELISA). Approval of this study was obtained from the Animal Care Committee, University of Guelph, in accordance with guidelines of the Canadian Committee on Animal Care.

Histopathology and microbiological analysis

Intestinal samples were collected immediately following euthanasia for histopathologic examination and for microbiological testing. For histopathology, samples were collected from 32 piglets (21 with diarrhea and 11 healthy) from duodenum, jejunum, ileum, and colon, and fixed in 10% neutral buffered formalin for 24 h. Following histologic processing, hematoxylin and eosin-stained slides were examined by light microscopy. For cases in which histologic lesions of atrophic enteritis were identified, immunohistochemistry (IHC) was carried out for TGE viral antigen using anti-porcine coronavirus monoclonal antibody (Custom Monoclonals International, Sacramento, California, USA). For each of the 32 piglets, mucosal scrapings from fresh samples of ileum and spiral colon were cultured on trypticase soy agar containing 5% sheep blood and MacConkey's agar (Difco, Detroit, Michigan, USA), and *E. coli* isolates were serotyped using polyvalent 'O,K' antiserum pool to detect the most common serotypes of enterotoxigenic *E. coli*, as well as antisera against F4 (K88) and F5 (K99) fimbriae. The ELISA for *C. difficile* toxins A and B, and rotavirus A latex agglutination (RLA) testing were carried out on colonic content. For those RLA-negative cases with histologic evidence of atrophic enteritis and negative TGE IHC results, further testing for porcine rotavirus was carried out by Rotavirus A/C RT-PCR and a conventional gel based PCR for Rotavirus B RT-PCR (Animal Health Laboratory, University of Guelph). The presence of intestinal coccidia was evaluated histologically and, in some animals, by sucrose wet mount examination of colonic content. Samples from the small intestine and colon were taken from all 48 piglets to quantify *C. perfringens*, to genotype the *C. perfringens* isolates, and to test for CPB2 toxin. Histopathological examination and microbial identification was done on 21 piglets with diarrhea and 11 normal cohorts from the 10 farms (on 9 farms 2 piglets with diarrhea and 1 normal piglet each, and on 1 farm 3 piglets with diarrhea and 2 normal piglets).

Clostridium perfringens quantification

Clostridium perfringens was recovered quantitatively from fresh colonic and jejunal contents obtained at necropsy from piglets. Samples were delivered to the laboratory at 4°C and then immediately weighed and serially diluted in phosphate-buffered saline (PBS), pH 7.2. A 100 μL aliquot of each serial dilution was plated onto Shahidi-Ferguson Perfringens (SFP) medium (Difco) containing cycloserine and 5% egg yolk, and incubated anaerobically

Table I. Primers and probes used for fluorogenic (*Taq*Man) polymerase chain reaction (PCR)

Target gene	Nucleic acid sequences of primers and probes (5′–3′) with probe dyes	Product length (bp)
cpa	AAGAACTAGTAGCTTACATATCAACTAGTGGTG (54°C) TTTCCTGGGTTGTCCATTTCC (51°C) TEX-TTGGAATCAAAACAAAGGATGGAAAAACTCAAG-BHQ2 (55°C)	124
cpb	TGGAGCGTGAAAGAAACTGTTATTA (51°C) GGTATCAAAAGCTAGCCTGGAATAGA (52°C) FAM-CTTAATTGGAATGGTGCTAACTGGGTAGGACAA-BHQ1 (57°C)	85
cpb2	TATTTCAAAGTTTACTGTAATTTTTATGTTTTCA (49°C) CCATTACCTTTCTATAAGCGTCGATT (65°C) CalFluor Orange 560-TGCACTTGCTTTCATTGGACTTATTGCTCC-BHQ-1 (58°C)	127
cpe	AGCTGCTGCTACAGAAAGATTAAATTT (52°C) TGAGTTAGAAGAACGCCAATCATATAA (50°C) FAM-ACTGATGCATTAAACTCAAATCCAGCT-BHQ1 (53°C)	88
etx	TTTGATAAGGTTACTATAAATCCACAAGGA (50°C) AGAGAGCTTTTCCAACATAAACATCTTC (52°C) CalFluor Orange 560-TAATCCTAAAGTTGAATTAGATGGAGAACCA-BHQ-1 (52°C)	121
iap	GCATTAAAGCTCACACCTATTCCA (51°C) GAGATGTGAGAGTTAATCCAAATTCTTG (51°C) FAM-CTAACTTAATTGTATATAGAAGGTCTGGTCC-BHQ1 (51°C)	85
netB	GGCGGTAATATATCTGTTGAAGG (53°C) ACCGTCCTTAGTCTCAAC (51°C) CalFluor Orange 560-ACTGCTGGTGCTGGAATAAATGCTTCA-BHQ1 (56°C)	168
Atypic cpb2	GTAGTAGACCATTGGATGTGGG (54°C) GAAAGTTTCTCCTGAACCTAGA (51°C) CalFluor Orange 560-GAGCTTCTCAATGGGTATATGGTG-BHQ1 (55°C)	114
tpeL	GGAATTAGCAGCTAGAATTGG (51°C) CCTGCAATTGGCACTA (49°C) FAM-TGACAGCAGCTATTAGTTCT-BHQ1 (51°C)	120

at 37°C overnight. Colonies with the characteristic *C. perfringens* morphology and lecithinase activity were counted and the $\log_{10}$ number of colony-forming units (CFU) per gram of intestinal content determined.

Genotyping by multiplex real-time PCR

This method and primers were described by Albini et al (16) except that 3 new primers were designed for atypical *cpb2*, *netB*, and *tpeL* (Table I). The lysed bacteria were subjected to *Taq*man real-time PCR (Roche LC480II instrument; Roche Canada, Mississauga, Ontario). A volume of 1 × Quanta PCR Master mix and primers were loaded onto a 384-well PCR plate (Roche Canada) and 2 μL of the lysed bacterial samples and reference strains were subjected to PCR in a final volume of 15 μL. The control isolates with known toxin genotype profiles were treated exactly the same way as the untested bacterial isolates both in the lysis and PCR stages. The resulting PCR curves for the reference isolates were checked for consistency with the known genotype control strains and the template controls were checked for absence of amplification. A software package (Roche LightCycler 480 SW 1.5) was used to analyze the data to present the results with *P*-values. When available, 4 isolates were typed per intestinal sample.

ELISA for *C. perfringens* CPB2 toxin

The ELISA plates (96-well, MaxiSorp C: Nunc, Roskilde, Denmark) were coated with 100 μL per well of capture antibody (rabbit polyclonal anti-CPB2 antibodies in carbonate/bicarbonate buffer, pH 9.6). After 2 h at 37°C, incubation was continued overnight at 4°C. Plates were washed twice using washing buffer (PBS, 0.05% Tween 20, 0.5% fish skin gelatin) and once using PBS, pH 7.4, which was followed by blocking with the blocking buffer (PBS, 0.05% Tween 20, 2% bovine serum albumen (BSA) for 2 h at 37°C. Plates were washed 3 times with the washing buffer, and 100 μL per well of purified recombinant CPB2 (used as a positive control) and processed intestinal content samples from neonatal piglets were added into the plate. Washing and the addition of the antigen were done on ice. The washing buffer was used as a negative control. Incubation at 4°C overnight was followed by 5 washings on ice using cold washing buffer, after which

Table II. Colony-forming unit (CFU) of *C. perfringens* and etiological diagnosis in 36 diarrheic and 12 healthy piglets on from 10 farms

Farm	Pig	Age (day)	Status	Log_{10} of colony-forming unit (CFU/g)		Etiological diagnosis
				Small intestine	Colon	
1	1	4	D	5.2	6.3	Unknown
	2	4	D	4.2	6.0	Rota
	3	4	H	7.7	8.9	Unknown
2	1	1	D	7.0	7.5	Rota
	2	1	D	6.6	7.1	Unknown
	3	1	D	7.6	8.2	*C. difficile*
	4	1	H	7.3	8.4	Unknown
	5	3	H	6.4	8.3	Unknown
3	1	1	D	5.8	7.5	Unknown
	2	1	D	5.7	7.5	Unknown
	3	2	H	5.1	7.8	Unknown
4	1	3	D	6.9	7.0	*C. difficile*
	2	3	D	ND	ND	Unknown
	3	3	H	7.8	7.2	Unknown
5	1	3	D	6.8	7.5	*C. difficile*
	2	4	H	5.9	7.0	Unknown
	3	1	D	1.9	7.0	*C. difficile*
	4	3	H	6.5	7.9	NT
	5	3	D	7.7	7.8	NT
	6	1	D	7.3	8.1	NT
6	1	1	D	3.0	6.8	Unknown
	2	6	H	2.2	5.4	Unknown
	3	3	D	2.0	7.0	*C. difficile*
	4	8	D	1.8	7.1	NT
	5	10	D	1.8	5.0	NT
	6	13	D	2.0	7.0	NT
7	1	3	H	ND	5.2	Unknown
	2	1	D	3.5	5.0	Unknown
	3	3	D	ND	8.2	Unknown
	4	3	D	ND	7.1	NT
	5	3	D	ND	5.9	NT
	6	2	D	6.5	6.3	NT
8	1	2	D	ND	5.4	Rota
	2	2	D	ND	3.8	Rota/*Salmonella*
	3	2	D	ND	5.2	NT
	4	2	D	3.7	ND	NT
	5	2	D	4.3	5.1	NT
	6	2	H	ND	4.6	Unknown
9	1	3	D	1.0	2.0	Rota
	2	3	D	2.3	7.2	Rota
	3	4	H	7.7	8.0	ETEC
	4	4	D	ND	6.0	NT
	5	3	D	7.3	7.5	NT
10	1	1	H	4.0	4.0	NT
	2	1	D	3.6	6.0	*C. difficile*
	3	4	D	2.0	3.0	*C. difficile*/Rota
	4	3	D	2.0	4.0	NT
	5	3	D	2.3	ND	NT

D — Diarrhea; H — Healthy; ND — Not detected; NT — Not tested for *C. difficile,* rotavirus, *Salmonella,* enterotoxigenic *E. coli* (ETEC), transmissible gastroenteritis (TGE), and coccidia.

Table III. Mean of $\log_{10}$ colony-forming unit (CFU) and standard error of the *C. perfringens* in intestinal contents of 12 normal piglets and 36 piglets with diarrhea

		$\log_{10}$ of CFU/g (SE)			
	Number of positive samples	Normal pigs	Pigs with diarrhea	Total	*P*-value
Jejunum	38	6.1 (0.58)	4.4 (0.42)	4.8 (0.37)	0.038
Colon	46	6.9 (0.48)	6.3 (0.26)	6.5 (0.23)	0.28
Total	84	6.5 (0.37)	5.4 (0.27)	5.7 (0.22)	0.035

SE — standard error.

Table IV. Regression analysis for $\log_{10}$ of colony-forming units (CFU) of *C. perfringens* in the intestinal contents of normal piglets and piglets with diarrhea with farm as random effect

Tissue	Parameter	Coefficient	Standard error	95% confidence interval	*P*-value
Group	Normal	0.8	0.40	0.02, 1.6	0.04
	Diarrhea	Reference			
Sample	Colon	1.7	0.36	3.9, 5.3	< 0.001
	Small intestine	Reference			

the detecting antibody (mouse monoclonal anti-CPB2 antibody, labeled with horseradish peroxidise, mAb-HRP) was applied (100 µL per well). The plate was then incubated 1 h at room temperature and washed 3 times with the washing buffer. An hour after addition of the chromogenic substrate (100 µL per well, incubation at room temperature), the reaction was stopped [0.5% sodium dodecyl sulfate (SDS), 50 µL per well] and the optical density was measured in an ELISA spectrophotometer at 405 and 490 nm.

Data analysis

Data were entered into a spreadsheet (Microsoft Excel 2003; Microsoft Corporation, Redmond, Washington, USA) and imported into another program for analysis (Stata 11 Intercooled for Windows XP; StataCorp LP, College Station, Texas, USA). A mixed multivariable analyzing method was used to investigate the association between $\log_{10}$ of *C. perfringens* numbers as continuous variable and neonatal diarrhea. In addition, a logistic regression method was applied in order to find relationships between categorical parameters including *C. perfringens* genes, CPB2 toxin, *C. difficile* toxin, enterotoxigenic *E. coli*, rotavirus, and histopathologic findings with diarrhea in piglets. In both methods, farm was included as random effect in order to take clustering within piglets on each farm into consideration. In addition, for each model, other variables were tested for any possible confounding.

Results

Quantification of intestinal *C. perfringens*

Ninety-seven (48 jejunal, 49 colonic) samples from 48 piglets (36 diarrheal, 12 normal) were examined. Type A *C. perfringens* was not isolated from 2 out of 24 (8.3%) and 11 out of 72 (15.1%) of intestinal samples from normal and diseased pigs, respectively; it was present in either the jejunum or colon contents of all 48 piglets but not in both sites from 3 piglets with diarrhea (Table II). The mean number ($\log_{10}$ CFU/g) of *C. perfringens* is shown in Table III; larger numbers of *C. perfringens* were isolated from the colon than from the jejunum ($P < 0.001$). In regression analysis with farm as the random effect, the $\log_{10}$ colony-forming units (CFU/g) of *C. perfringens* in the intestinal contents of healthy piglets was 0.8 higher than that in the diarrheic piglets ($P = 0.02$) (Table IV). Moreover, the $\log_{10}$ CFU of *C. perfringens* increased by 1.7 in the colonic contents compared to the small intestine ($P < 0.001$). Age of piglets was not significantly associated with $\log_{10}$ CFU of *C. perfringens*.

Genotyping by multiplex RT PCR

Genotyping was done on 1 to 4 isolates per jejunal and colonic samples so that 201 isolates (43 isolates healthy, 158 diarrheic piglets) were genotyped. Results are shown in Figures 1A and 1B, respectively. Of the 9 genes used in the PCR, only *cpa*, consensus *cpb2*, and atypical *cpb2* were identified. No significant difference in distribution of the *cpa* and *cpb2* was observed between isolates in healthy and diarrheic piglets. The consensus *cpb2* was present in 93% of isolates in both groups. A greater proportion of isolates from healthy pigs carried the atypical *cpb2* gene ($P < 0.05$).

Clostridium perfringens CPB2 toxin

Some samples were unusable because of "noise" attributed to intestinal protease activity. Overall, 32% of 50 samples (from 8 normal, 24 diarrheic piglets) tested positive for CPB2; a piglet was defined as positive if CPB2 could be detected by ELISA in either the small intestinal or colonic contents. Three of 8 normal (37.5%) and 9 of 24 diarrheal (37.5%) and piglets were positive for CPB2. The positive proportion did not differ significantly in samples collected from normal and diarrheic piglets (Figure 2). The numbers

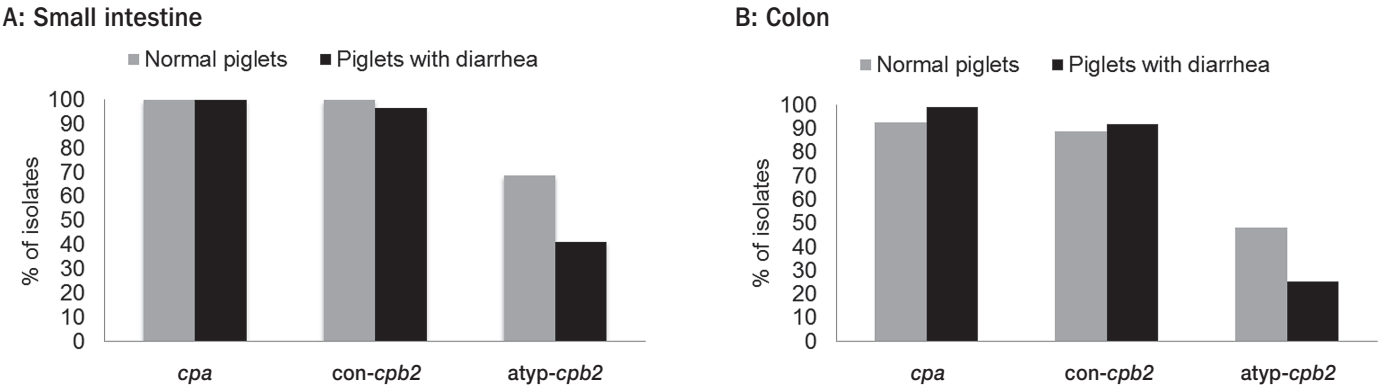

Figure 1. Distribution of *cpa*, consensus *cpb2*, and atypical *cpb2* genes among *C. perfringens* isolates recovered from jejunum (A) and colon (B) of normal piglets and piglets with diarrhea.

of *C. perfringens* were higher in CPB2 toxin-positive piglets than in piglets that tested negative ($\log_{10}$ 6.8 *versus* 5.1) ($P = 0.02$).

Other infectious agents

The final etiologic diagnosis of 21 piglets with diarrhea on 10 farms is shown in Figure 3. The etiology of the diarrhea in 8 of the 21 (38%) of piglets remained unknown. On 2 farms, the etiology of diarrhea could not be determined (Table II).

The intestine of 11 normal and 21 diarrheal piglets from the 10 farms were examined for histopathologic changes, and by isolation procedures for *Salmonella* spp. and enterotoxigenic *E. coli*. All 21 piglets with diarrhea and 2 normal piglets were tested for *C. difficile* toxins; these were detected in the intestinal contents of 7 of 21 (33%) diarrheic piglets from 4 farms but were not identified in the 2 healthy piglets tested. Twelve diarrheic piglets with histologic evidence of atrophic enteritis but negative RLA and TGE IHC results were tested by PCR for rotavirus. Rotavirus (groups A and C) was detected in 7 (33.3%) piglets on 4 farms. Rotavirus group C, *Salmonella* (Orion Var.15+34+), *C. difficile* toxins, or K88-positive enterotoxigenic *E. coli* were identified in different combinations in 4 piglets with diarrhea from 2 farms. Tissues from 8 diarrheic piglets examined immunohistochemically for TGE virus antigen were negative. Coccidia were not identified in any piglets using sucrose wet mount examination of feces or histologic examination of intestine. The ETEC or *Salmonella* spp. were not isolated from the small intestine or colon of any of the clinically normal (non-diarrheic) piglets. The $\log_{10}$ mean CFU/g of *C. perfringens* in the jejunum of diarrheic piglets that were positive for *C. difficile* toxins was slightly higher than in *C. difficile*-negative piglets (7.3 *versus* 6.7) ($P = 0.07$) but it did not differ based on rotavirus status.

Histopathological changes

Histopathological findings in diarrheic (21) and normal (11) piglets are shown in Figure 4. Atrophic enteritis was the most frequent diagnosis in diarrheal (62%) and normal (36%) piglets. Other findings in diarrheal and normal piglets included neutrophilic enteritis (19% *versus* 18%), neutrophilic colitis (24% *versus* 18%), and erosive colitis (10% *versus* 9%), respectively. There was no significant difference in the presence of these histologic findings between diarrheic and normal piglets except that mesocolonic edema was more frequent in

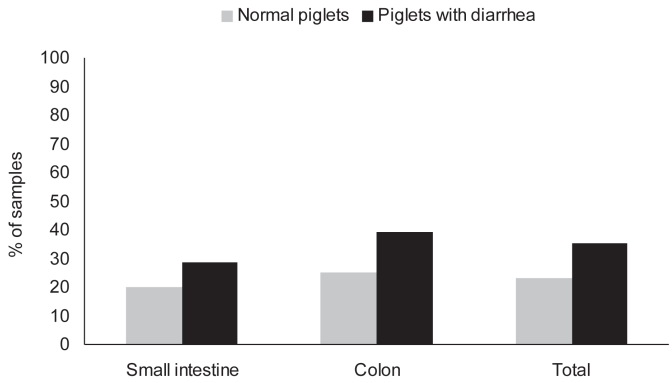

Figure 2. Proportion of intestinal contents of normal piglets and piglets with diarrhea testing positive for *cpb2* toxin using ELISA.

normal piglets (74% *versus* 33%) ($P = 0.03$). Of the diarrheic piglets with histologic evidence of neutrophilic colitis, one tested positive for *C. difficile* toxins A and B by ELISA. No histopathological changes were present in 14% and 9% of diarrheic and normal piglets, respectively. There was no correlation between histological changes observed and the presence of different toxin genes in *C. perfringens* isolates recovered from the intestinal contents ($P > 0.05$). No association was seen between histopathology findings and presence of other infectious agents at the level of $P < 0.05$. Other than atrophic enteritis, which seemed to be associated with a lower CFU of *C. perfringens* in jejunal contents ($P = 0.06$), no correlation between the CFU in the intestinal contents and histological changes was observed.

Discussion

If *C. perfringens* type A cause diarrhea in neonatal piglets, as is widely believed, most aspects of the disease are unclear and the diagnosis is equivocal, made only by exclusion of other enteric pathogens together with the detection of large numbers of *cpb2*-positive *C. perfringens* in the small intestine (4). Type A *C. perfringens* was recovered in large numbers from the small and large intestines of both diarrheic and normal piglets in this study, with lower numbers in diarrheic animals. In the absence of examination of control piglets, some of the piglets in this study, designated as having diarrhea of

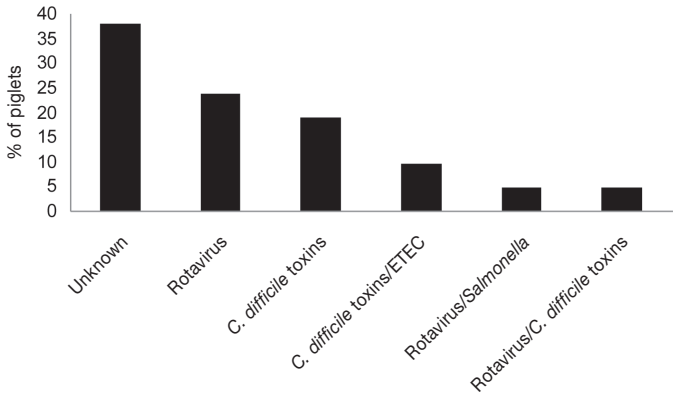

Figure 3. Etiological diagnosis of cause of diarrhea in piglets on 10 farms with a history of neonatal diarrheal illness.

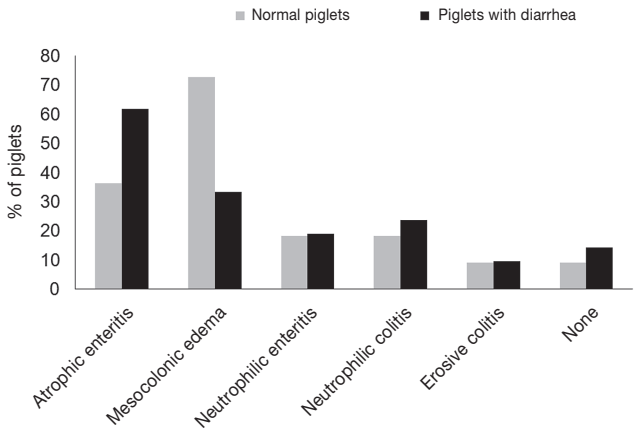

Figure 4. Histopathological changes in 11 normal piglets and 21 piglets with diarrhea.

"unknown" etiology, might well have been assigned a diagnosis of type A *C. perfringens* enteritis based on the presence of "large numbers" of *cpb2*-positive *C. perfringens* and the exclusion of other causes (4). Most piglets had evidence of inflammation of some type.

Clostridium perfringens rapidly colonize the gastrointestinal lumen after birth and are present throughout the life of animals (14). The finding of large numbers of *C. perfringens* in piglets reported here are consistent with the report by Smith and Jones (14) on the development of the intestinal microflora of healthy piglets. In those studies intestinal colonization of the healthy newborn piglet by *C. perfringens* occurred initially in the stomach and small intestine, and followed that of *E. coli*. *Clostridium perfringens* were first found in these sites 12 h after birth, with numbers reaching $\log_{10}$ 8.2 in stomach and 8.3 in the mid-small intestine. By 7 d after birth, numbers had fallen markedly, to $\log_{10}$ 2.0 in the stomach and $\log_{10}$ 2.7 in the mid-small intestine, whereas numbers in the large intestine were higher ($\log_{10}$ 6.4). We report here that healthy neonatal piglets were commonly colonized with large numbers of *C. perfringens*, interestingly in numbers exceeding those observed in diarrheal piglets. It was more common to find these bacteria in the colon than the jejunum, also consistent with the earlier findings of Smith and Jones (14). These finding considerably complicate a diagnosis of type A *C. perfringens* enteritis, since the presence of large numbers of these bacteria in the stomach and small intestine is part of the normal intestinal colonization.

This is the first study measuring CPB2 toxin in the intestinal tract. The CPB2 was detected in only about 1/3 of samples with no significant difference between healthy and diarrheic piglets, although only just over half of the samples could be tested because of signal "noise" and sample size was low. The CPB2-positive pigs had a higher number of *C. perfringens* in jejunum, but the toxin was not detected in the 2/3 of piglets with a high number of *C. perfringens*. Although the consensus *cpb2* gene is almost always expressed in porcine origin *C. perfringens* (7), its expression and the factors affecting its expression *in vivo* have not been investigated. Further studies are required to determine whether it is involved in neonatal enteritis in piglets; as noted by others, *cpb2* may be a marker for virulence of porcine isolates rather than being directly involved in intestinal disease (4). One interesting feature of this study, that has not been described in detail previously, was the recognition that atypical *cpb2* was also present in a proportion of isolates, and, interestingly, a significantly higher proportion of isolates from normal piglets carried this gene. The atypical gene is widespread and moderately common in isolates from different animals (6). We did not identify other toxin genes in the isolates.

The lack of a specific association between the presence of *cpb2*-positive *C. perfringens* (17) and neonatal diarrhea contrasts with previous studies (10–12) but is consistent with a report of the high prevalence of *cpb2*-positive isolates in piglets dying of mostly identified causes other than necrotic enteritis (and type A *C. perfringens* infection) (18). Garmory and others (11) found *cpb2* in 82% of 33 isolates from piglets with diarrhea and it was absent in 7 control piglets. Bueschel and others (12) detected *cpb2* in more than 90% of 381 isolates from porcine neonatal cases of enteritis but only in about 10% of isolates from 11 "normal pigs." Waters et al (13) confirmed that 29 isolates from diarrheic pigs carried *cpb2*, but this gene was not found in 6 isolates from "healthy pigs." The studies of these authors (11–13) were not, however, designed rigorously to address the hypothesis of an association between *cpb2* and diarrheal illness in neonatal piglets and, by contrast to the current study, the relative numbers of healthy to diarrheic pigs were low, and the provenance of healthy pigs was unclear.

One possible explanation for the lack of observed association between *cpb2*-positive *C. perfringens* and diarrhea in piglets in the present study is that the normal and diarrheal piglets were selected during an outbreak of neonatal diarrhea. It is possible that *cpb2*-positive *C. perfringens* spread between piglets and that normal appearing piglets were infected but did not develop the diarrhea because of the lack of other disease contributing parameters (19). However, 62% of 21 diarrheic piglets had microbiological diagnoses other than type A *C. perfringens* and the etiology of diarrhea in other diarrheic piglets remained unknown. As noted, it is possible that, in the absence of recognition of rotavirus or *C. difficile* infection, diarrhea in these piglets might be erroneously attributed to type A *C. perfringens* on the basis of numbers of bacteria isolated and their possession of *cpb2*.

There have been reports of the reproduction of enteric disease by *C. perfringens* type A in colostrum-deprived, hysterectomy-derived,

neonatal piglets (20) or in colostrum-deprived neonates (21–23). In one study, all piglets died or were euthanized *in extremis* within 72 h (20), whereas in the other study mortality was about 10% but morbidity was 55% in infected animals (21). A general loss of condition was common with diarrhea in about half of the affected animals (21). Small intestinal contents described as fluid with flecks of blood and necrotic debris (20) are not characteristic of descriptions of *C. perfringens* type A-associated enteritis, although the diffuse hyperemia and mild enteritis observed in some instances are more consistent with field descriptions (21). Because of the difficulty of rearing colostrum-deprived piglets, including their tendency to develop septicemia, reproduction of disease in hysterectomy-derived gnotobiotic piglets may be required to convincingly demonstrate a role for type A *C. perfringens* in neonatal piglet enteric disease.

Clostridium difficile toxins were detected in 7 scouring piglets and enterotoxigenic *E. coli* were detected in 2 of these. *Clostridium difficile* plays an important role in neonatal diarrhea in piglets (4). Rotavirus was also detected in 7 piglets with histopathological changes suggesting rotaviral infection. The co-incidence of rotavirus with *C. difficile* toxins in 2 piglets with diarrhea and with *Salmonella* in 1 diarrheal piglet indicates that the secondary infection with other infectious agents may cause more serious disease. Therefore, it is possible that other pathogens including *C. difficile* and rotavirus might form a multiple cause of the enteritis in neonatal piglets.

The etiologic diagnosis found in this study may not be extrapolatable to the larger population of Ontario swine farms since only a small number of farms were included in this study and the farms were not randomly selected. A future study with a larger sample size needs to be done to determine the causes of diarrhea in neonatal piglets and its associated risk factors.

In conclusion, this study highlights the complexity of diagnosis of porcine neonatal diarrhea associated with *cpb2*-positive type A *C. perfringens* (4). We were unable to distinguish between healthy and diarrheic piglets on the basis of bacterial numbers in the intestine, the presence of consensus *cpb2* in *C. perfringens* isolates, the expression of CPB2 in the intestine of piglets, and between diarrheal piglets with known or unknown causes of diarrhea. No association was observed between histopathological findings and the presence of CPB2. This study adds urgency to the previously identified need for more definitive diagnostic criteria (4). Based on the current study, exclusion of other causative agents (4) is not an adequate diagnostic criterion for diagnosis of the disease. An alternative suggestion is that, in the absence of other criteria, the presence of large numbers of *cpb2*-positive type A *C. perfringens* in the intestine of neonatal piglets should be regarded as normal commensals.

Acknowledgments

The authors gratefully acknowledge the Ontario Ministry of Agriculture Food and Rural Affairs (OMAFRA) — University of Guelph Research Program, and the OMAFRA Animal Health Strategic Initiative for funding this project. We also acknowledge funding from the Natural Sciences and Engineering Council of Canada. The authors thank Drs. Charbonneau, Scorgie, and Templeton for recruiting the farms, as well as Vivian Nicholson and Yanlong Pei for laboratory assistance.

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